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# „Application of DNA/RNA- based technologies in monitoring food safety and quality“

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**Habilitation**

# Application of DNA/RNA-based technologies in monitoring food safety and quality

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# List of publications

This work “habilitation work” is based on the following publications, referred to by their respective roman numeral in the text:

I. **Abdulmawjood, A.** and M. Bülte (2001) Snail species identification by RFLP-PCR and designing of species-specific oligonucleotide primers. J. Food Sci. **66,**1287-1293

II. **Abdulmawjood, A.** and M. Bülte (2001) Identification of ostrich meat by restriction fragment length polymorphism (RFLP) analysis of *cytochrome b* gene. J. Food Sci. **66,** 1688-1691

III. **Abdulmawjood, A.**, S. Roth and M. Bülte (2002) Two methods for construction of internal amplification controls for the detection of *Escherichia coli* O157 by polymerase chain reaction. Mol. Cell. Prob. **16,** 335-339

IV. **Abdulmawjood, A.**, H. Schönenbrücher and M. Bülte (2003) Development of a polymerase chain reaction system for the detection of dog and cat meat in meat mixtures and animal feed. J. Food Sci. **68,** 1757-1761

V. **Abdulmawjood, A.**, M. Bülte, N. Cook, S. Roth, H. Schönenbrücher and J. Hoorfar (2003) Towards an international standard for PCR-based detection of *E. coli* O157, Part 1: Assay development and multicenter validation. J. Microbiol. Meth. **55,** 775-786

VI. **Abdulmawjood, A.**, M. Bülte, S. Roth , H. Schönenbrücher, N. Cook, A. E. Heuvelink and J. Hoorfar (2004) Development, validation, and standardization of polymerase chain reaction-based detection of *E. coli* O157. J. AOAC Int. **87,** 596-603

VII. **Abdulmawjood, A.**, S. Roth, H. Schönenbrücher, M. Bülte, N. Cook, M. D’Agostino, B. Malorny, K. Jordan, S. Pelkonnen and J. Hoorfar (2004) Toward an international standard for PCR-based detection of foodborne *Escherichia coli* O157: validation of the PCR-based method in a multicenter interlaboratory trial. J. AOAC Int. **87,** 856-60

VIII. Malorny, B., N. Cook, M. D´Agostino, D. De Medici, L. Croci, **A. Abdulmawjood**, P. Fach, R. Karpiskova, T. Aymerich, K. Kwaitek, T. Kuchta and J. Hoorfar (2004) Multicenter collaborative trial validation for a PCR-based method for detection of Salmonella in chicken and pig samples. J. AOAC Int. **87,** 861-866

IX. Hoorfar, J., N. Cook, B. Malorny, M. Wagner, D. De Medici, **A. Abdulmawjood** and P. Fach (2004) Making internal amplification control mandatory for diagnostic PCR. J. Appl. Microbiol. **96,** 221-222 and in J. clin. Microbiol. **41,** 5835

X. **Abdulmawjood, A.**, H. Schönenbrücher and M. Bülte (2005) Novel molecular method for detection of bovine-specific central nervous system tissues as bovine spongiform encephalopathy risk material in meat and meat products. J. Mol. Diagn. **7,** 368-374

XI. Bülte M., H. Schönenbrücher and **A. Abdulmawjood** (2005) [From farm to fork-*Mycobacterium avium* ssp. *paratuberculosis* (MAP) as zoonotic agent?] Berl. Münch. Tierarztl. Wochenschr. **118,** 377-85 (Review)

XII. **Abdulmawjood, A.**, Schönenbrücher H. and M. Bülte (2006) Collaborative trial for validation of a real time RT-PCR system for detection of central nervous system tissues as BSE risk material in meat and meat products: Part 1. J. AOAC Int. **89,** 1335-1340

XIII. Schönenbrücher, H., **A. Abdulmawjood**, K. A. Göbel and M. Bülte (2007) Detection of central nervous system tissues in meat products: Validation and standardization of a real-time PCR-based detection system. Vet. Microbiol. **123,** 336-345

XIV. Schönenbrücher, H., **A. Abdulmawjood** and M. Bülte (2008) A new triplex-Real time-PCR-assay for detection of *Mycobacterium avium* ssp. *paratuberculosis* in bovine faeces. Appl. Environm. Microbiol. **74,** 2751-2758

XV. Glanemann, B., H. Schönenbrücher, N. Bridger, **A. Abdulmawjood,** R. Neiger and, M. Bülte (2008) Detection of Mycobacterium avium subspecies paratuberculosis-specific DNA by PCR in intestinal biopsies of dogs. J. Vet. Intern. Med. **22,**1090-1094

# Summary

DNA/RNA-based methods for food analysis have emerged from the laboratory and are becoming integrated into regular analytical usage. Problems remain in the standardisation of procedures between laboratories, and the often highly variable nature of template DNA preparation. Quality control is essential in the food industry and efficient quality assurance is becoming increasingly important. Food analytical researchers are increasingly asking for efficient control methods, in particular through up to date quality sensors, firstly to satisfy the consumer and regulatory requirements and secondly to improve the production feasibility, quality sorting, automation and reduction of production cost and production time. Therefore, all three drivers of the quality control, consumers, authorities and food producers, have great interest in the development of new sensing systems which are beyond the existing on-line technologies. The molecular based analytical technique offers the advantages of versatility and extreme sensitivity.

This work is based on three main issues. These include: DNA based methods for detection of food-borne pathogens, detection of risk material in food chain and identification of various animal species in food and feed matrices. The first issue was done under cooperation with an European network-research project (Food PCR). The aim of this project was to validate and standardize the use of diagnostic PCR for the detection of foodborne pathogens. The plan of Food-PCR was to devise noncommercial and nonpatented, standardized PCR-based detection methods for five major pathogens: *E. coli* O157, *Salmonella* spp., *Campylobacter* spp., enterohemorrhagic *Listeria monocytogenes* and *Yersinia enterocolitica*. The role of the Institute of Veterinary Food Science, Giessen in this project was task leader for development and validation of a specific PCR assay for detection of *E. coli* O157 to provide a diagnostic method which will be suitable for routine adoption and future proposal as a standard. This goal was preceeded in three phases. In phase 1, researchers working in expert laboratories have developed and selected promising candidate PCR-based methods, and tested them for efficiency and selectivity against comprehensive collections of reference strain DNA. The final selected PCR assays were optimized, and taken forward into phases 2 and 3. In phase 2, an interlaboratory trial was conducted to confirm the selectivity of the PCR assays. This phase will itself be conducted in two steps: in the first all reagents will be supplied by the originating laboratory, while in the second the participants will use their

own. This provided a thorough evaluation of the efficiency and robustness of the PCR assays.

In phase 3, the complete procedure comprising sample pre-treatment and the PCR assay (PCR-based method) will be subjected to interlaboratory trials, to provide validated PCR-based pathogen detection protocols.

The second issue was the detection of BSE risk material in food chain. For an efficient consumer's protection, European legislation prohibited several bovine tissues encompassing mainly central nervous system tissues from food chain. A quantitative real-time RT-PCR was designed to identify BSE risk material in meat and meat products. This was based on an mRNA assay that used bovine, ovine, and caprine glial fibrillary acidic protein (GFAP) encoding gene sequences as a marker. The real time RT-PCR assay included a housekeeping gene as an endogenous control. The quantitative real-time RT-PCR detection of GFAP mRNA appeared to be useful as a routine diagnostic test for the detection of illegal use of CNS tissues in meat and meat products. The stability of the specific region of GFAP mRNA allows the detection of CNS tissues also after meat processing steps. This region was protected by an international patent.

The third issue is the identification of various animal species in food. Authentication of food starts at the species level of the used meat as raw material for other productions. Food authentication linked to problems of adulteration. Adulteration usually involves substituting high quality raw materials with cheaper ones. For prevention of mislabelling or undeclared admixture, either deliberate or accidental, PCR-RFLP, species specific primer and PCR sequencing have been adopted for the differentiation of snails, ostrich dog and cat species in food and feed matrices. These DNA based methods being highly sensitive, reproducible, rapid, simple and not expensive could be used even after heat treatment.

# 1. DNA based methods for detection of food-borne pathogens (publication V, VI, VII VIII, XI XIV and XV)

## I. European project (Food-PCR):

Food-PCR is a 3-year EU-funded research project, which aims to validate and standardize the use of diagnostic PCR for the detection of foodborne pathogens. The aim of Food-PCR was to devise noncommercial and nonpatented, standardized PCR-based detection methods for five major pathogens: *E. coli* O157, *Salmonella* spp., thermophilic *Campylobacter* spp., enterohemorrhagic *Listeria monocytogenes* and *Yersinia enterocolitica*. The methods will focus on four sample types from primary food production: poultry-carcass rinse, pig-carcass swab, cattle swab and milk.

The project comprises 6 work packages and 20 tasks. The tasks include production of certified DNA material, preparation of a thermocycler validation guideline, and performance of PCR ring-trials. Another important area is automated detection, including ELISA-format and real-time PCRs. The project also has a work package devoted to sample pretreatment. Here, methods will be developed based on current ISO pre-enrichment procedures. These procedures will be adapted where necessary to allow a subsequent PCR assay to proceed efficiently enrichment and/or detection (Malorny et al., 2003).

The development of the standardized methods will proceed in three phases. In Phase 1, researchers working in expert laboratories have prepared defined DNA material, selected promising candidate PCR-based methods, and tested them for efficiency and selectivity against comprehensive collections of reference strain DNA. The final selected PCR assays (amplification and detection) were optimized, and taken forward into Phases 2 and 3 **(publication V, VI, VIII and XIV)**. In Phase 2, an interlaboratory trial was conducted to confirm the selectivity of the PCR assays. This phase will itself be conducted in two steps: in the first all reagents will be supplied by the originating laboratory, while in the second the participants will use their own. This was provided a thorough evaluation of the efficiency and robustness of the PCR assays.

In Phase 3, the complete procedure comprising sample pretreatment and the PCR assay (PCR-based method) will be subjected to interlaboratory trials, to provide validated PCR-based pathogen detection protocols **(publication VI, VII and VIII)**..

The role of the Institute of Veterinary Food Science in the Food PCR project was as a task leader for developing and validation of a specific PCR assay for detection of *E. coli* O157 to provide a diagnostic method which will be suitable for routine adoption and future proposal as a standard. The assay is fully selective, and contains an internal amplification control.

The successful outcome of FOOD-PCR was encouraged by the implementation of sensitive and cost-effective methods for detecting foodborne pathogens by diagnostic laboratories, and impel the metamorphosis of PCR from the scientist's esoteric research technique into a routine and facile diagnostic tool.

At the European level, standardization of analytical methods is developed within CEN Technical Committee 275 (TC 275). Within the TC 275, the Working Group 6 (WG 6) deals specifically with microbial contamination in food and feeding stuffs. An ad hoc expert group within the WG 6, called task group 3 (TAG 3), entitled "PCR for the detection of foodborne pathogens in food and animal feeding stuffs", was established with the aim to elaborate European standards for the analysis of microbial contaminants by PCR. In the first instance, four different standards will be elaborated in collaboration with the FOOD-PCR project: (i) polymerase chain reaction (PCR) for the detection of foodborne pathogens-general method specific requirements (prEN ISO/DIS 22174:2002), (ii) requirements for sample preparation for qualitative detection, (iii) performance criteria for thermal cyclers, and (iv) requirements for amplification and detection for qualitative detection. In addition, TAG 3 intends to elaborate separate standards for the detection of different pathogenic microorganisms (Malorny et al., 2003).

## II. Criteria for a standardized food diagnostic PCR

A food standardized PCR-based method for the detection of foodborne pathogens should optimally fulfill the following criteria:

### i. Analytical and diagnostic accuracy

The PCR-based method should have a high degree of an analytical and diagnostic accuracy. Analytical accuracy includes selectivity, defined as a measure of the degree of response from target and nontarget microorganisms using pure strains, and detection limit. Consequently, a selective PCR-based method comprises inclusivity

(detection of the target-pathogen from a wide range of strains) and exclusivity (lack of response from a relevant range of closely related but nontarget strains). Diagnostic accuracy takes into account the target and nontarget microorganisms in the presence of a biological matrix and comprises the terms specificity and sensitivity. Diagnostic specificity is defined as a measure of the degree to which the method is affected by nontarget components present in a biological matrix, which may result in false-positive responses. Diagnostic sensitivity is defined as a measure of the degree to detect the target-pathogen in the biological matrix, which may result in false-negative responses. A high degree of diagnostic accuracy means therefore, to detect, true and precisely the target microorganism in the presence of a biological matrix without interference from nontarget components. Therefore, diagnostic accuracy is used to evaluate the closeness of agreement between results of a PCR-based method and the accepted reference traditional method.

### ii. Detection limit

Secondly, the PCR-based method should have a low (good) detection limit. International standards derived from traditional detection methods require a detection threshold of one cell per 25 g of sample. The theoretical detection limit of one microbial cell per PCR reaction can usually be translated in practice into $10^3$–$10^4$ cells per ml of pre-enriched sample provided a small volume of initial matrix is used in the PCR reaction. Therefore, a food PCR assay, usually preceded by an enrichment step for a primary multiplication of bacterial cells, should itself detect at least 10–100 copies of the bacterial target DNA in the reaction. Furthermore, the detection limit must be determined in relation to a detection probability calculation. Thus, the expected relative frequency of a positive PCR response at various concentrations of target nucleic acids or cells must be established (Knutsson et al., 2002).

### iii. Robustness

The method should be tolerant towards a range of physical and chemical parameters. The most critical parameters usually are quality of template DNA (physical integrity of the chromosome, absence or presence of PCR inhibitors), batch differences in purity of the reagents, pipetting errors, accuracy of temperatures reached during PCR, adequacy of time duration of each PCR step, and rates of change ("ramping rates") between the different temperatures required during amplification. However, a

recent ring-trial clearly demonstrated the significance of variation in thermocycler performance on the outcome of a diagnostic PCR (Saunders et al., 2001). Thus, a high robustness of the method is a good indication of high interlaboratory reproducibility. Nevertheless, the instruments used (thermocycler, pipettes, etc.) must be checked routinely for good performance. In addition, reagents (e.g. microtubes, nucleotides, polymerase enzyme, water) must be of a molecular biology grade.

### iv. Amplification controls

The robustness of the PCR can be monitored by the use of positive and reagent (negative) test control reactions. For this purpose, the availability of certified DNA reference materials from a reference institution could be very helpful. The presence of PCR inhibitors should be monitored by the use of an appropriate internal amplification control in each reaction (see Quality safety).

### v. Contamination

The PCR-based method should have a minimal carry-over contamination risk. To minimize this risk, practices such as the use of separate working areas, decreasing the number of pipetting steps and the use of filter tips must be adhered to strictly. Carry-over contamination can also be prevented by incorporation of uracil- *N*-glycosylase (UNG) in the reactions, which renders all amplicons inactive for further amplification (Kitchin and Bootmann, 1993). Homogenous DNA-based technologies, also known as real-time PCR, where the amplification is continuously monitored by fluorescence within the reaction microtubes, can also reduce the risk of carry-over contamination (Foy and Parkes, 2001).

### vi. Flexibility with respect to various sample matrices

A major bottleneck in diagnostic PCR and, in particular, the detection limit of the overall method is the pre-PCR processing step and the lack of flexibility regarding the applicability to various sample matrices. Therefore, standardized PCR-based methods should include general methods of sample preparation aiming to concentrate target microorganisms and overcome the effects of PCR-inhibitory substances as well as reduce the heterogenicty of biological samples to homogeneous PCR compatible samples in order to ensure variations between various sample matrices. However, many sample preparation techniques are presently too complicated, time consuming and unreliable. A future challenge for diagnostic PCR is to integrate pre-PCR processing in an automated manner.

### vii. Acceptance by end-users

If any benefits are to be reaped from a standardized method, these will arise from its wide dissemination, acceptance and adoption. Besides, the necessity that the diagnostic PCR-based method is validated, it is important that neither the method nor the necessary reagents (e.g. primers, control DNA) are restricted for public use or are patented. However, the patent rights on *Taq* polymerase and UNG enzymes compel the end-users to buy a license for commercial use. Nevertheless, the basic patents for *Taq* polymerase expired in Europe in 2005, with a consequent decrease in the cost of consumables.

Another important issue is that the method should be presented in a clear fashion (e.g. in its first publication) and be accompanied by easily accessible and user-friendly protocols for its application and interpretation.

### viii. Other requirements

Other criteria for wide applicability of a standardized PCR are simplicity (user-friendliness) of the diagnostic method, high speed of result production, cost effectiveness and the possibility for automation. The possibility to adapt a PCR for quantitative analysis would also be an obvious advantage (only if enrichment is not necessary) (Malorny et al., 2003).

## III. Validation and demonstration of a good performance by ring trial

Validation plays an important role within the standardization, demonstrating that the new method can generate results that are comparable if not better to those obtained by the current reference method. Moreover, it aims at confirming the specificity and reproducibility of the method when used by different laboratories. A common procedure to obtain validation data is an interlaboratory study, called a “ring-trial”, in which the performance of the method is tested using identical material in several laboratories, under control of a supervising laboratory. In this process, it is important to simulate real-life conditions; for example, the use of naturally contaminated samples should be strongly considered. Validation of a method consists of two stages: (i) a study where the diagnostic PCR-based method is compared with the current reference method (in our case, traditional media-based detection of foodborne pathogens) using artificial and naturally contaminated food samples **(publication V)**, and (ii) an interlaboratory study **(publication VII, VIII).**

## i. Limits to harmonization

There are several factors that can affect the efforts for harmonization of PCR:
the quality of all PCR components, including the template DNA derived from sample preparation prior to PCR the reaction conditions the detection system used the equipment used the environment (temperature, humidity, chemical and microbiological cleanliness) personnel practice. All these factors should be taken into account in the development of a harmonized PCR

However, it is practically impossible to compare all available types of reagents, buffer systems and equipment with regard to their effect on amplification efficiency. Thus, the development of a paradigm protocol for testing published PCR primer sets against the criteria for a standardized PCR-based method is of importance. The comparison of primer sets should be performed with a single buffer and polymerase system, all other components and equipment also remain constant. A list of reference strains must first be defined for testing the selectivity of primer sets: this should include the epidemiologically most important strains of the target organism, and a panel of strains belonging to related species or serotypes which should not be detected by the primers.

## ii. Future perspectives and developments

Diagnostic PCR is very young compared to traditional detection methods. However, its generally high ratings with respect to convenience and specificity should hopefully establish it in diagnostic laboratories, as a routine reference method alongside traditional detection techniques, within the next 10 years. Real-time PCR enables automated direct readout of the results in a quantitative format, increasing the speed of each reaction, and therefore lowering the overall cost per reaction. The potential for carry-over contamination is greatly reduced, as real-time PCR avoids multiple pipetting steps. Efforts towards standardization therefore should not be restricted to conventional PCR methodology but should also take into account developments such as real-time PCR.

Quantitative analysis of foodborne pathogens is a major issue that has to be elaborated in the future, and is closely related with the use of real-time PCR. Quantitative PCR has become available in several different real-time formats (Hoorfar et al., 2000, Nogva et al., 2000, Wolffs et al., 2001, Foy and Parkes, 2001,

Hein et al., 2001). However, the implementation of quantitative PCR is currently hampered by the lack of ability to distinguish between viable and dead cells, and the lack of sample preparation methods that do not involve prior multiplication of bacterial cells by enrichment. In addition, it is important, for appropriate risk management, to know if viable or dead cells are present in food. However, all PCR-based techniques detect DNA, which survives cell death. Decontamination of food is often aimed at inactivating pathogen cells (Bolder, 1997), rather than removing the organisms altogether, but detection of DNA will give no indication of the effectiveness of a decontamination procedure. One approach to distinguish viable bacterial cells from dead cells by PCR-based methodology is the use of mRNA rather than DNA as the initial template for amplification (Klein and Juneja, 1997, Sheridan et al., 1998, Szabo and Mackey, 1999).

The direct application (i.e. in the absence of an enrichment step) of PCR to foods will require effective methods for the separation of bacterial cells from food matrices. These methods ideally must possess the lowest possible number of steps, the ability to completely remove PCR inhibitors, and a high potential for conversion to an automated format. Considerable research has been conducted with a view to identifying suitable approaches for such methods (Lantz et al., 2000).

## IV. Quality safety (internal amplification control) (publication III, IX and XIV)

The explosive increase since the beginning of 1990's in the number of publications reporting PCR-based methods for detection or molecular typing of foodborne pathogens has attracted the attention of end-user laboratories.

However, the well recognized difficulties in reproducing published tests due to variation in performance of PCR thermal cyclers, in efficiency of different DNA polymerases, personnel and the presence of PCR inhibitors in the sample matrix can hamper implementation in laboratories, particularly those with extensive quality assurance programs. Lack of reproducible methods often forces testing laboratories to spend substantial resources on adaptation of the published tests. It is thus necessary to have internationally validated, open- formula PCR-based methods available in which the target gene, performance characteristics and validation criteria are known (Jones et al., 2000) and which follow the ISO criteria for validation of

alternative microbiological methods (Anonymous, 2002, Belotserkovskii and Johnston, 2003). A major drawback of most published PCRs, surprisingly even to date, is that they do not contain an internal amplification control (IAC). In contrast to a (external) positive control, an IAC is a non-target DNA sequence present in the very same sample tube, which is co-amplified simultaneously with the target sequence. In a PCR without an IAC, a negative response (no band or signal) could mean that there was no target sequence present in the reaction. But, it could also mean that the reaction was inhibited, due to malfunction of thermal cycler, incorrect PCR mixture, poor DNA polymerase activity, or not least the presence of inhibitory substances in the sample matrix (Sachadyn and Kur, 1998). Conversely, in a PCR with an IAC, a control signal should always be produced even though there is no target sequence present. This can reveal failure of a PCR reaction.

The European Standardization Committee (CEN), in collaboration with International Standard Organization (ISO) has proposed a general guideline for PCR testing of food-borne pathogens that requires presence of IAC in the reaction mixture (Anonymous. 2002a). However, CEN has left the design of the IAC open.

## i. Amplification strategy

Whilst some design approaches such as cloning require substantial technical skills, others can be done using basic PCR methodology. There are two main strategies for use of an IAC in a diagnostic PCR assay **(publication III).** Their difference lies in whether the IAC is to be used competitively or non-competitively.

### a. Competitive IAC

By using the composite primer technique the target and the IAC are amplified with one common set of primers and under the same conditions and in the same PCR tube. In this strategy, there is always some competition between target DNA and IAC, and the amount of IAC is critical to the detection limit **(publication III)**. One has to consider that simultaneous amplification of two different DNA fragments flanked by the same primer sites can result in either inhibition or enhancement of one or both products depending on the molar ratio, the length, the sequence and the secondary structure of those DNA fragments. The competition by IAC can, however, lower the amplification efficiency of PCR and thereby result in a lower detection limit. Thus, the most critical parameter to consider is the concentration of the IAC itself. The

lowest reproducible IAC DNA concentration must be determined carefully; otherwise too many IAC DNA will compete with the target DNA product and abolish the target signal. This will by itself cause a false negative result. In addition, if used at high concentration, the IAC might not detect weak inhibition, which could cause false-negative results if the target is present in extremely low concentrations. The calculation of detection probability would provide an indication of the detection limit of final PCR method in diagnostic samples **(publication III).** The second critical parameter is the size of the IAC. Increasing the size of one target relative to another should, in theory, drive the reaction kinetics towards the smaller target PCR product. However, some authors have mentioned that regardless of the size of IAC, competition with the target sequence in PCR could be observed; **publication III** reported that IAC size of less than 500 bp does not influence the native PCR sensitivity. Nevertheless, one can recommend that the size of the IAC should be larger than the target sequence, to ensure the competitive edge of the latter.

Owing to competition, if the target DNA is amplified but the IAC is not, it is assumed that the target DNA is present in a proportionally greater amount. When this occurs, the positive result is valid because the IAC amplification is unnecessary. If neither the IAC nor the target DNA is amplified, it is assumed that inhibition of the PCR has occurred and the test for that sample is not valid. However, the drawback of this approach could be a lower detection limit due to the competition by IAC.

### b. Non-competitive IAC

Here, the target and IAC are amplified using a different primer set for each. This requires a PCR in which two reactions with different kinetics proceed simultaneously. The kinetics of each reaction are not influenced by a competition for the primers. The IAC primer set targets a synthetic DNA (e.g. IAC plasmid DNA) or another gene (e.g. encoding ribosomal RNA), which is present in any microorganism and in higher copy number than the principal target gene. Of course, if no target bacteria are present, there will be no amplicon from IAC. In this approach, PCR amplification of the IAC must be limited by a controlled concentration of the IAC specific primers in order to limit the competition of the target- and the IAC-specific reaction for oligonucleotides and DNA polymerase. The disadvantage is that amplification of non-competitive sequences may not accurately reflect amplification of the primary target due to differences in the primer sequences. Therefore nucleotide composition and size of the IAC have to be carefully considered. Taking these

criteria into account, using IAC in a non-competitive PCR requires the development of two PCR reactions, optimized to work by the same PCR conditions, which may become sub-efficient for one or both reactions. One way to overcome this problem is to optimize the assay only for the target and let the IAC reaction follow that; optimization of IAC part of the PCR reaction is not senso stricto part of the assay development, as long as the IAC amplicon is detected. In addition, it is important to limit the production of IAC amplicon by keeping the concentration of its primers to a suboptimal (minimal) level. The main advantage of this method is that it can be used for many different assays in the same laboratory. The most popular approach is use of primers specific to conserved sequences of 16S and 23S ribosomal DNA. Another approach is to add, to the PCR mix, a microorganism, which is usually not found in the sample type to be tested. The latter approach could also be done in competitive PCR, but with addition of IAC plasmid in the vector microorganism. However, few end-use laboratories have permission to work with recombinant microorganisms (Hoorfar et al., 2004).

### ii. Production of IAC

The simplest approach is to produce PCR products, which differ in size and hence can be easily visualized separately from the native product by an agarose gel electrophoresis. The most common approach is the composite primer technique, using the same primer as for the target DNA (Rosenstraus et al., 1998). This can be achieved by addition of a completely non-relevant DNA to the PCR mixture; e.g. a fish virus for a PCR-based detection of *Salmonella enterica* in pig fecal samples (Jensen and Hoorfar, 2002).

Another approach is production of a modified target IAC (so-called mimic) by deleting, inserting or modifying sequences between the recognition primer sites as in **publication III**. Via PCR mutagenesis it is possible to exchange only a short stretch of DNA, whose sequence differs from the target probe only in a few nucleotides. Based on this difference, a hybridization probe can be designed which detects the IAC specifically. These methods may also be applied to construction of IAC for quantitative PCR. Since many of these methods require a cloning step, it is strongly recommended to check any new chimeric sequence for the possible presence of undesired mutational changes. Therefore, it would be necessary to determine the sequence of the IAC, before design of hybridization probes, e.g. for use in fluorescence-based real-time PCR assays. In addition, the IAC amplicon size will be

smaller than the target amplicon, which may favour the amplification of the IAC over that of the target amplicon.

However, this method is rather time-consuming and laborious intensive, because of the digestion, ligation, cloning, and purification steps. In order to have the chance of similar amplification conditions between target and IAC DNA, both sequences should not be too heterologous. Modifying distinct nucleotides of DNA often requires complicated PCR mutagenesis methodology (Cubero et al., 2002). Such IACs are useful when sequence-dependent detection (hybridization) methods are applied for the confirmation of the specific PCR products. A size-dependent discrimination (gel electrophoresis) of target and IAC DNA will then not be possible. However, homologous target and IAC sequences can form heteroduplex PCR products, which often leads to a reduced detection limit for the desired target pathogen. Addition of the recognition primer sites could also be constructed by, e.g. overlap-extension PCR (Rosenstraus et al., 1998). The addition method is easier than the modification method, as IACs are synthesised in one step PCR reaction. The primers used in this reaction possess 5' over-hanging ends, which are identical to the primers used in the diagnostic reaction, whereas their 3' ends are complementary to a predetermined DNA sequence (pUC19 for example) of defined length and sequence. The advantage of the methodology is that the possibility of heteroduplex formation during PCR due to sequence similarity of the target DNA is avoided (Malorny et al., 2003). Another methodology allows the construction of an IAC with completely designed nucleotide sequence (Rådström et al., 2003, Siebert and Larrick, 1992, Stöcher et al., 2003). Several pairs of partially overlapping oligonucleotides that contain the entire IAC sequence are annealed and extended with *E. coli* DNA polymerase I. An alternative method is the artificial construction of the complete IAC in one run, which could be easily obtained commercially. The latter method is currently limited to construction of IACs up to 100 bp. However, restriction of the length of the PCR target to less than 150 bp is an aspect, which should be considered to achieve optimal PCR efficiency (Anonymous, 2002a).

### iii. Cloning versus non-cloning

One of the ways of constructing a competitive IAC is to insert the (modified) target sequence in a plasmid. This has several benefits, such as better control of stability, size and copy number. In addition the plasmid can be safely stored in convenient quantities for long periods in minimum degradation tubes (Hoorfar and Cook, 2003).

However, the modified target sequence could be obtained completely artificial from a commercial source as well. Usually, IAC DNA is embedded in a vector system. Here, linearized (loop-free), recombinant plasmid DNA, including the IAC sequence, serves as template in the PCR reaction. The advantage of cloned IAC DNA is that it allows simple storage of a recombinant plasmid DNA within bacterial cells, which guarantees the continuous availability and quality of the IAC. Care must however be taken to minimise laboratory contamination with plasmid DNA, as it can be quite persistent. It is also possible to use purified PCR products of the IAC with flanking sequences of plasmid DNA (Rådström et al., 2003). The direct use of a single-stranded oligonucleotide as IAC template might be a new strategy. The advantage of using artificial oligonucleotides as IACs is easy access of synthesis, as well as guaranteed consistent quality assurance worldwide, which would be an added advantage in regard to standardization of diagnostic PCR.

### iv. Storage loss of IAC

The safest IAC storage method seems to be as part of a recombinant plasmid in an *E. coli* strain, which can be kept as a glycerine culture, lyophilized, or frozen as a "micro-bank". However, free DNA should be stored undiluted in an alkaline buffer such as 0.1 M TE (pH 8.0), which stabilizes it. We have experienced substantial unintentional loss of IAC signal during storage at low concentrations or in distilled water (Müller et al., 1998). Therefore, addition of EDTA is important to chelate ions that can function as cofactors of DNA degrading enzymes. Carrier nucleic acid can be also used to aid stabilization. A usually overlooked factor is the storage of DNA in appropriate plastic tubes. DNA can bind to polypropylene and interact with the tube walls inducing conformational changes, which influence the amplification efficiency and accuracy, and non-the-least detection limit (Brightwell et al., 1998). Incidentally, many PCR assays claim detection limits of one DNA target copy number, although some DNA could have been, unintentionally, absorbed to the tube. It has been shown that polyallomer tubes are more suitable for storage as they do not show adsorption and denaturation of DNA (Hoorfar and Cook, 2003). Alternatively, DNA can be freeze-dried.

### v. Quantification of IAC

There is a requirement to titrate the internal control DNA template prior to use in the PCR assay. For further use it is possible to prepare ready-to-go mixtures containing

every reagent, including final concentration of IAC template and primers. They can be aliquoted and stored frozen for several months until use (Jensen and Hoorfar, 2002). The titration and calculation of the correct IAC copies used in the PCR assay is important for the exact function of the IAC. With low concentrations (approx. 20-40 copies per reaction), IAC molecules are distributed among replicate amplifications according to Poisson's law. Rosenstrauss et al. (1998) describe a method for the titration of IAC molecules based on this type of distribution, calculating the average number of molecules in a given volume of solution and the probability that no molecule exists in a particular sample of given volume. The advantage of the method is the independence of fluorometric or spectrophotometric measurement, which each have a different reproducibility (Malorny et al., 2003a). However, it must be noticed that residual reagents from the purification of plasmids or PCR products might interfere with both fluorometric and spectrophotometric measurements. Also, the method of Rosenstrauss et al. (1998) can be laborious and time-consuming. It has been shown that the concentration of PCR products (after purification by gel filtration to separate nucleotides, salt and primers) can be correctly measured by absorbance at 260 nm according to standard procedures using spectrophotometric measurements that correspond well to the empirical detection limit of the IAC. A more accurate approach is the use of quantitative real-time PCR, although this requires costly instruments.

### vi. Detection of IAC amplicon

Amplicons are usually detected and identified according to their size. Restriction fragment analysis allows unambiguous confirmation of specificity of the amplification. However, this conventional method uses ethidum bromide that requires strict and constraining regulations in many countries. The use of this reagent is facing increased precaution measures and restrictions in most laboratories. Moreover, gel electrophoresis requires additional time- and labour-intensive processing of amplicons, and increases the risk of contaminating the laboratory environment with the carry-over products. Development of the so-called ELISA-PCR assays has helped to avoid the use of gel electrophoresis (Fach et al., 2002, Hoorfar et al., 2000). Closed-tube fluorogenic PCR methods based on addition of SybrGreen and analysis of melting curves can in some situations be more helpful than gel electrophoresis. This system can be based on the measurement of the increasing fluorescence due to the incorporation of the SybrGreen I dye during the

synthesis of amplicons. By using an IAC that melts at a different temperature than the target amplicons, it is possible to separate the target signal from IAC signal. Specific identification of melting peaks permits the IAC and target DNA to be co-amplified in the same tube and still be distinguished. However, this would only be the case when the melting curve of the IAC amplicon is quite different from the target sequence. There are a number of more specific real-time PCR techniques, which use fluorescence labelled hybridization probes. These are designed to bind adjacent to one another on the amplicon (Courtney et al., 1999).

Hybridisation probes, such as Fluorescence Resonance Energy Transfer (FRET), labelled by fluorescent dyes can be used to design real-time PCR detection.

Different dye-labelled probes can bind specifically to the target and IAC sequence making it possible to perform duplex detection of the target and IAC. The 5'-nuclease PCR based on the use of a fluorogenic probe (TaqMan probe) that hybridises within the target sequence bound by PCR primers can differentiate between the wavelengths emitted by different dyes, making it possible to perform duplex detection of the target and IAC. Usually, the probes specific of the target are 5'-labelled with the fluorescent reporter dye 6-carboxyfluorescein (FAM) or fluoresceine (Fluo), those targeting the IAC with a different fluorescent reporter dye like VIC, JOE or TET. Thus, using two probes labelled with distinct reporter dyes, allows simultaneous detection of the target gene and the IAC in a duplex reaction **(publication XIV).** The specificity of the probes ensures that no signal is generated by non-target amplicons. An interesting development in real-time PCR is generation of multiple IACs for a panel of PCR assays with a single DNA fragment. In routine laboratories with many real-time PCRs, a single IAC and IAC probe is generated rapidly by a multiple primer composite technique and is used for many assays.

## vii. Concluding remarks

Construction of IACs can be performed in several ways, at the choice and discretion of the user. However, we recommend the competitive method to avoid the risk of undesired interactions of multiple primers, and to have both PCR reactions (the target-specific and the IAC-specific) working with the same primer set and under identical PCR conditions regardless of the strategy.

On the other hand, the competitive method can require more optimization work in order to achieve a sensitive detection limit. The overlap extension technique is simple and effective, and creates IAC DNA with the same primer-binding sequence

as the target DNA. The cloning of IAC into a plasmid provides an unlimited amount of IAC. Using the same primers for IAC and target is an advantage, because multiple sets of primers might interfere with the amplification of one or both of the target genes, due to the differences in primer sequence. Differences in size, internal sequences of the amplified products, and the relative amounts of the two targets might also interfere with the amplification. A PCR-based method cannot be given diagnostic status, no matter how limited the application, before it includes, as a minimum, an IAC, a processing-positive control, a processing-negative control, and a reagent control (blank), as suggested by the MicroVal protocol (Belotserkovskii and Johnston, 2003, Jones et al., 2000) and draft standard document (Anonymous, 2002a). However, an IAC is only an indicator of PCR failure, and does not in itself have any counter-effect against inhibitory factors. The detection limit and thereby the diagnostic sensitivity of a PCR assay, particularly on sub-clinical samples with low target pathogens, depends also on an effective sample treatment procedure (Malorny et al., 2003). Even then, an IAC will not show whether the purified DNA can be readily amplified. When the DNA to be amplified is derived from very complex matrices using harsh extraction protocols, the amplification of a sequence of a housekeeping gene or 16S ribosomal gene, i.e. a sequence that is definitely present in the DNA, should be performed as one of the positive controls in every setup to check the integrity of the purified DNA. Here it must be emphasized that inclusion of an IAC should not be a replacement for good laboratory practice, such as proper treatment and storage of samples on arrival. Finally, the support of editorial boards and reviewers is important if we are going to maintain the credibility of PCR as a useful tool for laboratory diagnostics. We have proposed that publication guidelines should be extended to require inclusion of IAC in any PCR intended for diagnostic use, either as a detection or sub-typing tool (Lübeck et al., 2003). This should certainly bring home to developmental scientists the importance of an IAC.

# 2. Detection of risk material in food chain (publication X, XII and XIII)

## I. What is the BSE risk material?

To contain the epidemic and for the protection of consumers, in addition to the ban on feeding meat and bone meal, a general BSE analysis programme was introduced in the EU, which, for instance in Germany, includes every slaughtered animal aged 48 months or more. The available test methods are applied to one specific brain-stem region, called the obex. The immuno-chemical detection of PrPsc assumes a relatively high molecular concentration, however. Because there are so far no exact dose effect ratios, there is an unknown residual risk for the end user. According to EU Regulations: PrPc is a glycosylated protein, with a molecular weight of approx. 30 kD, found as a structural element mainly in nerve tissue. Its function is to a great extent unclear. Taking account of the PrPsc-intake and transport paths in the body, a ban on the processing of risk materials in foodstuffs was issued by the European Union (EU) (Anonymous, 2000). In accordance with this, spinal cord, brain, lymph nodes, eyes, and intestines amongst other organs must no longer enter the food chain; these results in a reduction of the residual risk mentioned above **(**Weyandt, 2001).

## II. Detection of BSE risk materials

Several phenotypic methods for detection of BSE risk material (CNS tissues) have been developed, including ELISA, HPLC, Western-Blot and immunohistochemical methods. However, these methods allowed neither differentiation of CNS tissues of banned species from CNS tissues of other animal species nor an exact quantification of the detected CNS. In addition, a test system should also allow an effective control of heat treated samples. In the **publication X,** a real-time RT-PCR method based on a species specific GFAP mRNA-region was developed for the detection of bovine, ovine and caprine CNS tissues in raw and heat treated meat products. The relative quantitative technique was evaluated in CNS and other tissues of various animal origins.

## III. Isolation of mRNA

In veterinary research RNA extracted from fat or collagen rich tissues, or tissue sampled after long time at the slaughterhouse often has a lower yield and is of lesser

quality, and contains partly degraded RNA sub-fractions, especially the messenger RNA (mRNA) fraction (Pfaffi, 2003). Particular RNA extraction techniques can act more effectively on one specific tissue type compared to another one, and result in up to 10-fold variations in total RNA yield

In the **publication X**, a sensitive detection assay for bovine, ovine and caprine CNS tissues was developed using a mRNA-based quantitative real-time RT-PCR with the GFAP gene as marker. This protein is expressed at high levels in the astrocyte cells of the CNS tissues but not or only at very low levels in the cellular elements of other organs and peripheral nerves. GFAP protein has been previously used as a marker for the detection of CNS tissues. A detection was performed by Western blotting, (Lücker et al., 1999; 2000), ELISA (Schmit et al., 1999 and 2001) and by immunohistochemistry (Wenisch et al., 1999) The disadvantage of these GFAP detection methods is that non of these methods appeared to be species specific. A species specificity could be determined by gas chromatography spectrometric analysis of the fatty acid composition of meat components (Biedermann et al., 2002) or by PCR-RFLP analysis of GFAP mRNA (Seyboldt et al., 2003). However, the spectrometric analysis require expensive laboratory equipment and the latter shows cross reactions with other organs e.g. muscle or heart. In addition, the PCR-RFLP method used a conventional detection by non quantitative gel electrophoresis.

In the **publication X** partial gene sequences of bovine, ovine, caprine and porcine GFAP genes were analyzed to find conserved and variable regions. These regions were used for the selection of species specific oligonucleotide primers and a fluorogenic probe to amplify RT-PCR products which allow an amplification of species specific regions of mRNA of bovine, ovine and caprine origin but not of mRNA of porcine origin. The assay allowed the detection of CNS tissues and in parallel the identification of the species. An mRNA based analytical test to determine the presence of CNS tissues in meat should be sensitive and should reliably avoid false positive results of DNA contamination. Based on this consideration, we designed a MGB TaqMan® probe, which was selected in the junction of exon-exon region. The presented real-time RT-PCR amplified and detected only mRNA but not bovine or porcine DNA.

The sample preparation is one of the most critical aspects of mRNA assays because this might cause false-negative results. The RNA isolation method, using a combination of a mechanical step (Fast Prep®) and the RNeasy Lipid Tissue kit, as

performed in this study provided a good quality of total RNA. The high lysis efficiency of this method enables to get a sufficient amount of total RNA in about 1h. In comparison with classical RNA extraction protocols, it has been shown that the selected method increased the sensitivity of the detection of GFAP mRNA in meat products.

Most RNA preparations are contaminated with DNA and protein at very low levels. Even high quality commercially obtained RNA contain detectable amounts of DNA. While this is not a problem for some applications, the tremendous amplification power of kinetic PCR may result in even the smallest amount of DNA contamination to interfering with the desired "specific amplification". To confirm the absence of residual DNA either a "minus-RT" or "water control" should always be included in the experimental design. It may be necessary to treat the RNA sample with commercially available RNAse-free DNAse, to get rid of residual DNA (Pfaffi, 2003).

Anyhow, in the **publication X** a mRNA based analytical test to determine the presence of CNS tissues in meat should be sensitive and should reliably avoid false positive results of DNA contamination. Based on this consideration, a MGB TaqMan® probe was designed, which was selected in the splice junction of exon-exon region. The presented real-time RT-PCR amplified and detected only cDNA but not bovine or porcine DNA.

## IV. Housekeeping gene and relative-quantitative detection of CNS tissues

For the determination of expression level of GFAP mRNA in different organs a relative quantitative real-time PCR and $2^{-\Delta\Delta Ct}$ method was used which is included an endogenous control (18S rRNA gene) and a calibrator organ muscle **publication X**. The purpose of the endogenous control is to normalize the PCRs for the amount of the mRNA added to the reverse transcription reactions. The choice of calibrator for $2^{-\Delta\Delta Ct}$ method depends on the type of target gene. The present assay detected very low levels of GFAP in non neural organs.

In conclusion, quantitative real-time RT-PCR detection of GFAP appears to be useful as a routine diagnostic test for detection of the illegal use of bovine CNS tissue in meat and meat products. Bovine GFAP mRNA exhibits certain stability, facilitating the detection of CNS tissue in raw and heat treated sausages. The technique should be evaluated through a ring trial to confirm the ability of the test in

different laboratories. According to our knowledge, this is the first report of a real-time RT-PCR method for the species- and tissue-specific detection of bovine CNS tissue in meat and meat products.

## V. Ring trial as a validation of the CNS detection

The challenge is being addressed through development of a new diagnostic test and to encourage acceptance of this test to be used in the routine control by the respective authority and by food industry, it is recommended that the system be thoroughly evaluated through collaborative trial of its performance in several laboratories with variant real time detection systems.

In **publication XII** a collaborative trial has been conducted to evaluate a real time RT-PCR assay for detection of central nervous system (CNS) tissues in meat products (e. g. sausages). The collaborative trial was designed according to the recommendations in CEN standard (CEN/TC 275/WG 6/TAG 3N 0119) which included the general requirements of real time PCR (Anonymous, 2005). Twelve laboratories participated in the multi-centre trial. The assay was evaluated through a multi-centre trial involving 12 participating laboratories which received coded cDNA obtained from three different types of sausages. The participants used five different real time detection systems. The results obtained in this validation, revealed that this real time RT-PCR assay performed well in the different laboratories with a detection limit of at least 0.1% CNS in those test materials that contained strongly heat-treated samples (sausages cooked at 120°C) and the medium heat-treated samples (sausages cooked at 80 °C). Regarding the liver-sausages investigated, the detection limit was determined to be 0.2% of CNS. Neither the samples with no CNS additive nor the bovine DNA and the negative control containing 100% swine brain gave any positive signals. The results indicate that the real time RT-PCR assay was just as reproducible between laboratories, as repeatable within a laboratory and could reliably be used for detection of BSE risk material in meat and meat products and signifies that it may be used with confidence in any laboratory. To our knowledge, at present no other collaborative trial has validated a similar real time-RT PCR-based method for detection of CNS tissues in meat and meat products.

## VI. Assay protection by national and international patent

Title of the patent: “Species-specific and quantitative detection of BSE-risk material in processed foodstuff”. This real time-PCR system has been protected by an international patent (04802928.4-2402-DE 2004002723 on 13.12.2004). The Justus-Liebig-University Giessen, Germany is the holder of the patent for this procedure. Further information is available under:

http://www.hipo-online.de/deutsch/d_expose.cfm?expose_id=135

For the first time a real time-PCR test system makes a procedure available, which allowed the improving of the food quality insurance by controlling the existence of these high risk materials in sausages or other meat products as well as on slaughter animal carcasses.

The organizations that might be able to adopt the applying of this system are:

Food industry

The public authorities

Quality control laboratories

The consumer protection organisations

**Sample material that which could be tested with this assay:**

Raw meat

Meat products

Heat-treated meat products

Surfaces (Swab-technique)

**Advantages:**

Very sensitive detection of CNS-material (cut off 0,1%).

Species-specific CNS-detection of bovine, ovine and caprine CNS-tissue.

No cross reactivity with for example porcine or poultry CNS-Tissue. (By using a different assay it is possible to detect exclusivly porcine CNS-tissue).

No false positive results caused by blood or lymph node

# 3. Identification of various animal species in food (publications I, II and IV)

## I. Reasons for DNA based animal species identification

The verification of the authenticity of food starts at the species level. Prevention of mislabelling or undeclared admixture, either deliberate or accidental, is relevant for economic reasons as well as for public health and for respecting religious beliefs.

**Economic reasons:**

Incorrect labeling of the delicatessen e.g. ostrich meat or snails art. These products are usually imported as frizzed or canned products and, to lower the cost, might be incorrectly labeled

**Public health reasons:**

BSE problematic bovine origin meat and meat products. For human consumption of beef from BSE-infected cattle might cause the emergence of a juvenile form of Creutzfeldt Jakob's disease in human (Will, 1999). On the other hand in some European countries ban on feeding the ruminant material to other ruminants. European guidelines prohibit, with a few exception, the addition of any processed animal material to feedstuff unless the absece of ruminant proteins can be demonstrated (EEC Commision decision 94/381/EEC)

Animal-related allergies are exaggerated reactions of the body's immune system to certain animal proteins, also known as Animal species allergens. One source of these allergens is animal meat of different animal species (Fiocchi et al., 2000; Restani et al., 2004). In worst cases, allergic disorders can cause death. Food allergy is explained as a rejective reaction - a kind of defensive reaction. All sorts of food can trigger allergy: chicken egg, chicken meat, milk, beef, pork, fish and shellfish (cod, salmon, redfish, shrimp, scallop, clam, etc.) (Kazuhiko, 1992).

**Ethical and animal-protection reasons:**

Some religious beliefs prohibit consuming of meat from certain animal species. For example, Muslims and Jews may only eat certain animals and many other animal species are forbidden as pig, dog, cat etc.

The animal protection organization control. The game law enforcement authorities need to differentiate meat of protected species from that of unprotected and domestic animals.

Because of all these reasons, there is a need for a suitable nonmorphological technique to identify meat of different animal origin.

Conventional methods for species identification are based on immunoassays with antisera raised against food extracts or on the generation of species-specific protein electrophoresis patterns. In more modern methods, the species origin is identified by the detection of specific DNA sequences which has the following advantages:

The experimental behavior of DNA is predictable and does not depend on the species, which allows the use of universal assay formats.

DNA is relatively stable and less affected by a physiological condition of samples and environmental factors. The samples can even be tested after being heated up to 120°C. The diversity of DNA allows the differentiation of closely related species or even of subspecies or populations. The DNA is not tissue-specific and thus can be detected at any phase of animal development.

The most current methods are based on PCR, which offers the advantages of versatility and extreme sensitivity. In the following sections we elaborate the various methods used for meat species identification and review similar methods for the analysis of dairy products, feedstuff, fish, plants and cell lines.

# II. DNA techniques for animal species identification in meat products

## i. DNA isolation procedures

Several methods for lysis of tissue and subsequent purifying of DNA can be found in the standard books on molecular biology methodology. A common procedure for animal tissues is a lysis by proteinase K and sodium dodecyl sulphate (SDS), followed by removal of the proteins by phenol/chloroform organic and precipitation of DNA by alcohol. Adaptations of these methods to isolation of DNA from food for PCR assays have been reported.

Several commercial kits are now available for fast purification of double-stranded DNA, which exploit the specific binding of DNA to proprietary resins under special conditions.

## ii. Molecular marker technologies for meat species identification (publication I, II and VI)

The most DNA-based assays in food inspection are based on PCR amplification, which is driven by the extension of two primers in opposite orientations. Thus one primer generates the substrate for the other and vice versa, which results in a chain reaction. Since this may be initiated by only a small amount of template DNA, PCR is inherently sensitive, while the requirement of the binding of two primes ensures a high specificity. The main drawback of PCR is that the occurrence of false-positives caused by contamination of the reagents is difficult to control (see PCR validation part).

For differentiation of animal species, mitochondrial DNA is the obvious choice because it has accumulated about ten times as many mutations per generation as nuclear DNA and is present in thousands of copies per cell. So amplification of a mitochondrial DNA is relatively sensitive and generates a DNA fragment with several species-specific mutations. However, the maternally inherited mitochondrial DNA may give misleading results in case of species hybridisation, which can be detected by analysis of satellite DNA (Nijman et al., 1999, Nijman and Lenstra, 2001, Verkaar et al., 2003) or other nuclear markers (Nijman et al., 2003). Further, it should be checked that there is no interference by (co)amplification of copies of mitochondrial DNA integrated in the nuclear genome.

## iii. PCR-RFLP on mitochondrial DNA (publication I, II and IV)

A convenient strategy for the differentiation of a panel of known sequences is PCR-RFLP (restriction enzyme fragment length polymorphism): PCR, digestion with an enzyme having a recognition sequence that is either created or abolished by the mutation, and analysis of the cleavage pattern by agarose gel electrophoresis. This method is also suitable for the detection of admixtures with a sensitivity of about 5%. The most common target gene for the PCR-RFLP assays is the mitochondrial cytochrome *b* (Meyer et al., 1995; Wilson et al., 1995; Ram et al., 1996; Yoshizaki et al., 1997; Carrera et al., 1996, 1998, 1999; Cespedes et al., 1999). The primers described originally by Kocher et al. (1989) yield a 359-bp fragment in all species relevant for food inspection, which can be differentiated by restriction digestion **(publication I, II).** Amplification of a slightly longer fragment reduced interference by nuclear copies of the mitochondrial sequence and allowed PCR-

RFLP identification of several game species (Burgener and Hubner, 1998, Wolf et al., 1999, Verkaar et al. (2001) optimised the primers for beef-producing species. Murray et al. (1995) differentiated various ungulates by PCR-RFLP of the mitochondrial D-loop, while a similar assay for authenticitation of snails has been based on sequence variation in the mitochondrial 12S and 16S rRNA regions **(publication I).**

PCR-RFLP provides a universal format for species detection and, better than methods based on species-specific primers **(publication IV),** allows discrimination between substitution and admixture. However, there are a number of caveats:

The primers do not match completely the mitochondrial DNA of all species to be analysed (Meyer et al., 1995). In general, mismatches near the 3'-OH end of the primer have more influence than near the 5'-OH end and may cause a preferential amplification of one of the components in a sample of mixed origin. So for analysis of admixture, the effect of mismatches should be checked and, if necessary, primers should be designed that amplify all components of the mixture equally well.

It should also be verified that the diagnostic restriction sites are not variable within the species (Wolf et al., 1999). For instance, the restriction site reported to discriminate wild boar and domestic pigs (Meyer et al., 1995) reflects intraspecies polymorphism (Kijas et al., 1998). Furthermore, a *Hintt* site described to be diagnostic for cattle is not present in the cytochrome *b* gene of related bovine species. So at least two diagnostic restriction sites should be used for an unambiguous identification **(publication I, II and IV)**.

Failure of restriction digestion for technical reasons may lead to a wrong assignment. So material from the appropriate reference animals should be analysed in the same experiment and again, identification should be based on at least two sites.

With the appropriate controls PCR-RFLP is, however, a fast and convenient method of species detection and requires substantial investments in equipment only for high-throughput implementations or for quantification of the restriction patterns.

### iii. Species-specific PCR on mitochondrial DNA (publication I and IV)

Special mitochondrial PCRs without subsequent restriction enzyme digestion have been designed for the differentiation of ostrich, **(publication II**) or for a sensitive

detection of porcine material (Montiel-Sosa et al., 2000). Dedicated methods for detecting bovine mitochondrial DNA have been developed for the inspection of feeding stuff. In general, these approaches require a careful check of the amplification of traces of DNA from the reagents, which in PCR-RFLP assays with generic primers are suppressed by the amplification of the sample DNA. Matsunaga et al. (1999) described a competitive multiplex PCR with one generic primer and six primers specific for cattle, sheep, goat, pig, horse and chicken, respectively, which generates amplicons of different lengths for the six respective species.

### iv. **PCR-sequencing** (publication II)

The most straightforward method for detection of species-specific mutation is sequencing. Originally this was termed FINS for forensic informative nucleotide sequencing (Bartlett and Davidson, 1992). It has been applied to the identification of whale and dolphin products (Baker et al., 1996), exotic meat species (Forrest and Carnegie, 1994) or of remains of endangered mammals (Hsieh et al., 2001). A BLAST search in the Genbank will identify immediately the species or the most related species for which a homologous sequence is available. However, sequencing is not suitable for analysing samples of mixed species composition **(publication II).**

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# Snail Species Identification by RFLP-PCR and Designing of Species-Specific Oligonucleotide Primers

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**ABSTRACT: To enforce labeling regulations in the authentication of snail species it might be of importance to use nonmorphological methods. Snail meat of 2 species was analyzed by restriction fragment length polymorphism (RFLP) of the genes encoding mitochondrial 12S rRNA and 16S rRNA. Digestion of the polymerase chain reaction (PCR) products of 12S rRNA with the endonucleases *Afi* III, *Dra* I and *Sty* I, and the 16S rRNA PCR product with the endonucleases *Nsp* I, *Sfu* I and *Taq* I, yielded specific banding patterns characteristic for *Helix pomatia* and *Helix lucorum*. The meat of both species could additionally be differentiated with species-specific oligonucleotide primers based on 16S rRNA gene sequences characteristic for both species.**

**Keywords: snail species identification, mitochondrial DNA, polymerase chain reaction, *Helix pomatia, Helix lucorum***



## Introduction

COOKED SNAIL MEAT (ESCARGOT) IS HIGHLY APPRECIATED IN France, Italy, Germany, and Austria. It is also gaining popularity in the United States, particularly in areas of French, Italian, and German concentration. In China, snails are considered as a treasured delicacy. In France, "Escargots a la Bourguignonne" is a standard menu item in "haute cuisine" restaurants and bistros. During Christmas and New Year, Parisians consume an estimated 20 metric tons of snails. On a national basis, France shows a consumption of 35,000 metric tons annually. Europe exports about 600 metric tons to the United States per year (Littel 1999).

Nutritionally, snails are an excellent food. The meat has low levels of cholesterol or fat (0.5 to 0.8%) and contains vitamins A and C, plus 1.5% minerals (zinc, copper, manganese, potassium, calcium, and iodine). As for protein (a total of 12 to 16%), the meat contains 9 of 10 essential amino acids important for humans. Of the known snail species, 116 are considered edible. The most popular edible snail is *Helix pomatia* (the Bourgogne or Burgundy snail). Another less valuable edible snail consumed in Europe is *Helix lucorum* (escargot turc or Turkish snail) from northern Italy and Turkey (Thomas 2000).

Snails are usually imported as canned products and, to increase profit, the cans might be incorrectly labeled. Snail species are usually identified and differentiated by morphological characteristics. However, specific snail species are unrecognizable after cooking and canning. Because of that there is a need for a suitable technique to identify canned snail meat, as there is for fish, crustaceans, and caviar (Carrera and others 1996,1998,1999). Bracchi (1988) attempted to differentiate between *Helix* and *Achatina* snail meat by SDS-PAGE electrophoresis of whole muscle or with actomyosin obtained from raw and autoclaved muscles. The described method showed differences between the whole muscle and actomyosin of the 2 species. The differences were clearer when using actomyosin, which can only be reliably analyzed in raw and frozen samples. Hence, this method is not very suitable for identifying mislabeled cooked and canned products.

The mitochondrial genome has become very popular for evolutionary and population genetics studies because of the ease of isolating it from the nuclear genome, the high number of copies inside the cell, and the small size and rapid accumulation of mutations (Moritz and others 1987; Sotelo and others 1993; Unseld and others 1995).

According to Carney and others (1997), Rigaa and others (1997) and Yoshizaki and others (1997), mitochondrial DNA (mtDNA) analysis requires the isolation of the mtDNA molecule and digestion of the mtDNA with a variety of restriction endonucleases. The resulting fragment patterns are then examined for polymorphisms within and among the populations investigated. Although conventional mtDNA methods are powerful for detecting variations in restriction fragment length, intensive DNA analysis would be difficult, especially on very small samples, such as fish eggs, which offer only a very small amount of DNA. To overcome this problem, the use of the polymerase chain reaction (PCR) method, which can amplify DNA sequences more than 10-million fold (Saiki and others 1988), appears to be useful.

Kocher and others (1989) used a universal primers to amplify the conserved region of the 12S-, the 16S rRNA and the *cytochrome b* genes in more than 100 animal species, including mammals, birds, amphibians, fishes, and some invertebrates.

PCR of mtDNA using species-specific primer pairs has been developed for the identification of 3 commercial Russian sturgeon (source of caviar) species (Chow and others 1993). In addition, a combination of PCR amplification and RFLP analysis has been used in species and stock identification studies (Wilson and others 1995; Ram and others 1996; Yoshizaki and others 1997; Carrera and others 1998, 1999; Cespedes and others 1999).

Our study was undertaken to investigate PCR methods for the identification and differentiation of *H. pomatia* and *H. lucorum.* This was based on PCR-RFLP of 3 conserved mito-

chondrial DNA regions in the 12S rRNA, the 16S Rrna, and in the *cytochrome b* gene. In addition, species-specific primers from the variant region of the 16S rRNA gene were designed and evaluated.

## Materials and Methods

### DNA extraction

Total cellular DNA was isolated from muscle samples of *H. pomatia* and *H. lucorum* with the Genome-DNA Isolation Kit (Merck, Darmstadt, Germany) according to the manufacturer's instructions. Briefly, 300 mg of muscle tissue was homogenized in 500 μL double distilled water (DDW) containing 40 μL of proteinase K (14.8 mg/mL), (Roche, Mannheim, Germany). The samples were incubated for 1 h at 55 °C with shaking (60 rpm). Of this suspension, 300 μL was incubated with 600 μL lysis buffer (Merck) for 10 min at 68 °C. The deproteination was performed by adding 900 μL chloroform, which was mixed in gently followed by centrifugation at 9000 × *g* for 2 min. The upper (aqueous) layer containing the DNA was transferred to a clean 2-mL tube. To precipitate the DNA, 100 μL buffer 2 (Merck) and 900 μL DDW were added, mixed gently, and followed by centrifugation at 9000 x*g* for 5 min. The pellets were resuspended in 300 μL resuspension buffer (Merck). Then the DNA was precipitated with 750 μL 96% ethanol; the pellets were washed with 70% ethanol and resuspended in 50 to 100 μL of sterile DDW.

The DNA was obtained from 49 snail muscle samples including 1 fresh *Helix lucorum* (kindly obtained from Mr. Wolf, Gastropoda, Pfaffen-Schwabenheim, Germany) and 48 canned samples of *Helix pomatia* (n = 25) and *Helix lucorum* (n = 23) (Gastropoda) as reference. All these samples were morphologically identified by specially trained people.

### Amplification of the mitochondrial subunits using universal primers

The oligonucleotide primers (12SAI/12SBI) and (16SAR/16SBR) used to amplify the 12S rRNA and the 16S rRNA genes of snails, respectively (Borgo and others 1996). The 12S rRNA primers (Simon and others 1991), correspond to regions 14588-14563 and 14234-14214 of the *Drosophila yakuba* mtDNA sequence (Clary and Wolstenholme, 1985). The 16S rRNA primers (Simon and others 1991), correspond to regions 13398-13378 and 12910-12888 of the *Drosophila yakuba* mtDNA sequence. The oligonucleotide primers Cytb-I/Cytb-II were used to amplify a *cytochrome b* gene subunit. These primers were designed by Bartlett and Davidson (1991) for the amplification of a conserved region of the *cytochrome b* gene in a tuna-fish species. The DNA was amplified by PCR. The reaction mixture (50 μL) contained 1 μL primer 1 (10 pmol/μL), 1 μL primer 2 (10 pmol/μL), 1 μL dNTP (10 mmol, Roche), 5 μL 10 × thermophilic-buffer (PE Applied Biosystem, Weiterstadt, Germany), 0.2 μL *Taq* DNA polymerase (5 U/μL; PE Applied Biosystem) and 39.3 μL DDW. Finally, 2.5 μL DNA preparation was added to each reaction tube. The PCR was carried out in a thermal cycler (PE GeneAmp PCR system 9600; PE Applied Biosystem) with the following program: 1 × 3 min precycle at 93 °C, 35 × 30 s denaturing at 93 °C, 30 s annealing at 52 °C and 45 s extension at 72 °C followed by a final extension incubation of 72 °C for 5 min. The presence of PCR products was determined by electrophoresis of 10 μL of the reaction product in a 2% agarose gel (Appligene, Heidelberg, Germany), with Tris acetate-electrophoresis buffer TAE (0.04 mol/L Tris, 0.001 mol/L EDTA, pH 7.8) and a 50 bp DNA ladder (Roche) as molecular marker.

### Cleanup and sequencing of the PCR products

PCR product (18 μL) of the 12S rRNA, the 16S rRNA and the *cytochrome b* genes of *H. pomatia* and *H. lucorum,* respectively, was mixed into 2 μL 5 mol/L sodium perchlorate ($NaClO_4$) (Sigma-Aldrich Fine Chemicals, Deisenhofen, Germany) and 10 μL isopropanol for 30 min at room temperature and centrifuged for 15 min at 9000 x*g* The sediment was washed twice with 50 μL of 70% ethanol, centrifuged for 5 min at 9000 x*g* and dried. Finally the pellet was resuspended in 10 μL DDW and sequenced directly using an Applied Biosystem, Inc., 373A DNA Sequencer. The protocols followed the manufacturer's description (PE Applied Biosystem) for "*Taq* cycle-sequencing" with fluorescent dye-labeled dideoxynucleotides.

The sequence data were further studied with the computer program Seg Man, DNASTAR Inc., Wis., U.S.A., Lasergene.

### Design of universal and species-specific primers

Based on the sequence analysis of this study, universal and species-specific primers for each species were designed using the OLIGO program, (National Biosciences Inc., Plymouth, Minn., U.S.A.) Primer Analysis Software, Vs 4.0, 1991. The sequence of the universal primers UNI12S-I and UNI12S-II was based on a conserved region of the 12S rRNA gene and the universal primers UNI16S-I and UNI16S-II on a

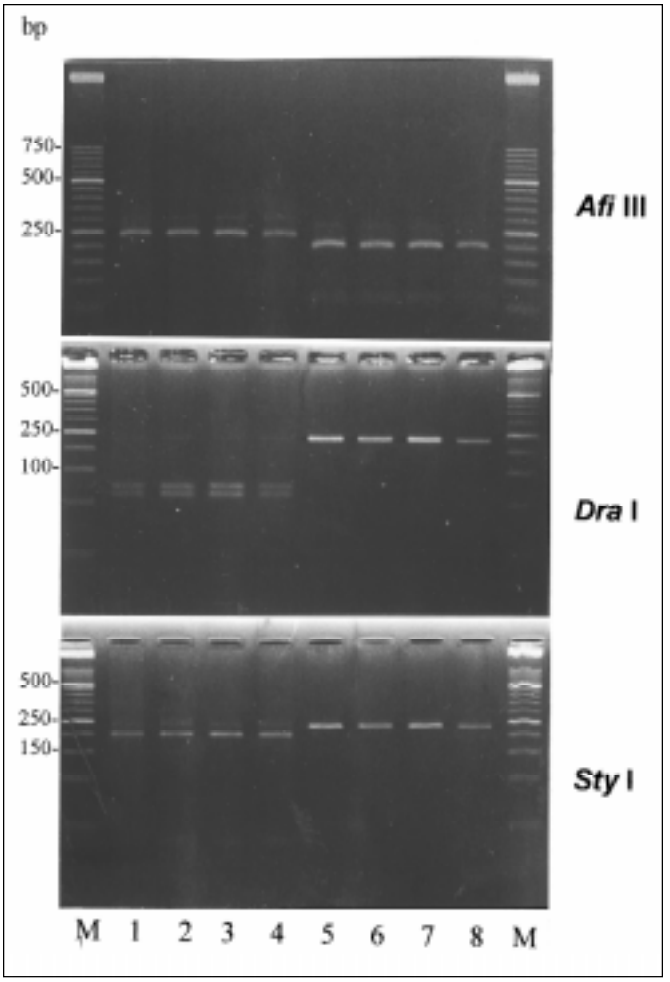


**Figure 1—Restriction profiles of the 12S rRNA PCR products obtained from *H. pomatia* (1-4) and *H. lucorum* (5-8) digested with *Afi* III, *Dra* I and *Sty* I. (M) DNA molecular weight marker XIII 50 bp ladder (Roche).**

conserved region of the 16S rRNA gene of both species.

The species-specific primers Pom-sp and Luc-sp were selected from a variable region of the 16S rRNA gene of *H. pomatia* and *H. lucorum,* respectively. The reaction mixture and thermal cycler programs were used as described above. All primers used in this study are summarized in Table 1.

### Restriction site analysis and enzymatic digestion of PCR products

Restriction maps of 12S rRNA, 16S rRNA, and *cytochrome b* gene sequences from *H. pomatia* and *H. lucorum* were compared by using the computer program Meg Align, DNASTAR. Using computer program Clone-Manager (Version 4.1, Scientific Educational Software, Durham, N.C., U.S.A.) for the 12S rRNA gene sequence of *H. pomatia* and *H. lucorum*, the restriction enzymes *Afi* III, *Dra* I and *Sty* I (Roche) were selected as suitable candidates for the identification of both species. When comparing the restriction maps of the 16S rRNA gene, the restriction enzymes *Nsp* I, *Sfu* I and *Taq* I (Roche) were selected. The PCR products were subjected to restriction digestion with the selected endonucleases without further purification. All reactions were performed in 30-μL volumes using digestion conditions specified by the manufacturer. The DNA fragments were separated by electrophoresis in 2% agarose gels.

### Nucleotide sequence accession numbers

The 12S rRNA, the 16S rRNA, and the *cytochrome b* genes for both species have been submitted to the GeneBank database under accession numbers: AF255654, AF255655, AF208296, AF208297, AF255652 and AF255652, respectively.

## Results and Discussion

THE AMPLIFICATION OF THE 12S RRNA AND THE 16S RRNA genes using the universal primers 12 SAI/12 SBI and 16 SAR/16 SBR produced DNA fragments of 380 bp and 450 bp for *H. pomatia* and *H. lucorum,* respectively (Table 1). In ad-

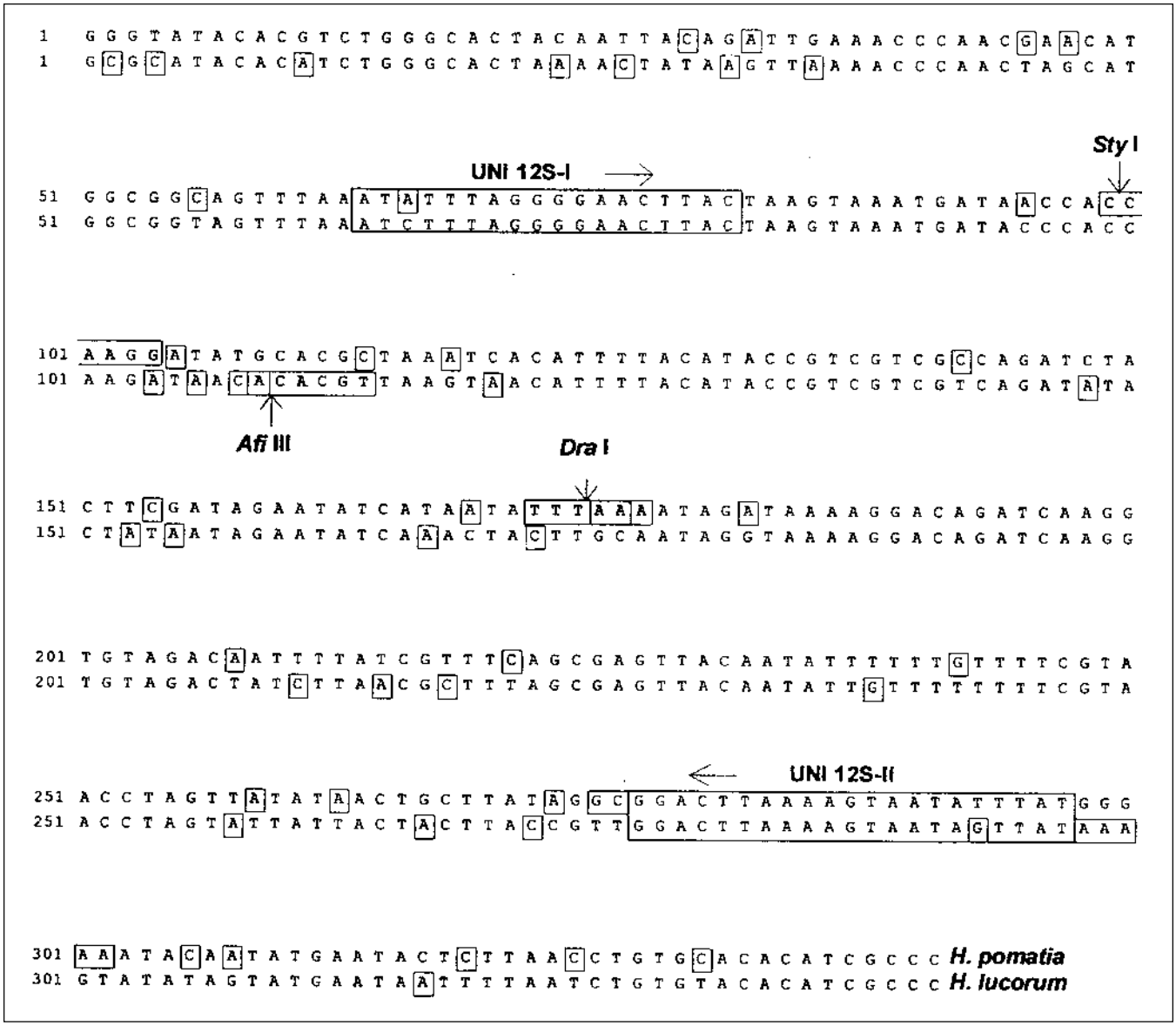


**Figure 2—DNA sequences from part of the 12S rRNA gene of *H. pomatia* aligned with *H. lucorum.* The positions of the oligonucleotide primers used for the following PCRs are marked; the shadow indicates the restriction sites.**

**Table 1—Oligonucleotide primers used in this study.**

| Target Gene | Primer | Primer sequence (5'-3') | Amplicon size | Reference |
|---|---|---|---|---|
| 12S rRNA | 12 SAI<br>12 SBI | AAACTAGGATTAGATACCCTATTAT<br>AAGAGCGACGGGCGATGTGT | 380 bp | Simon and others1991 |
| | UNI 12S-I<br>UNI 12S-II | ATCTTTAGGGGAACTTAC<br>ATAACTATTACTTTTAAGTCC | 232 bp | This study<br>This study |
| 16S rRNA | 16 SAR<br>16 SBR | CGCCTGTTTAACAAAAACAT<br>CCGGTCTGAACTCAGATCATGT | 450 bp | Simon and others1991 |
| | UNI 16S-I<br>UNI 16S-II | GTGCAAAGGTAGCATAATCAG<br>CCTATTAAAATTATGCTGTTATCC | 258 bp | This study<br>This study |
| | 16 SAR/<br>Pom-sp | CGCCTGTTTAACAAAAACAT<br>CGCCTGTTTAACAAAAACAT | ~200 bp | Simon and others1991<br>This study |
| | 16 SAR/<br>Luc-sp | CGAAAAAATGTGCTAAGACAG<br>GTCTTCTCGTCTTTTTTATTAGC | ~250 bp | Simon and others1991<br>This study |
| *Cyto-chrome b* | Cytb-I<br>Cytb-II | CCATCCAACATCTCAGCATGATGAAA<br>CCCCTCAGAATGATATTTGTCCTCA | 360 bp | Bartlett and Davidson 1991 |

dition, a DNA fragment of 360 bp could be amplified for the *cytochrome b* gene of *H. pomatia* and *H. lucorum* with the oligonucleotide primers Cytb-I and Cytb-II (Table 1). All 6 PCR products were sequenced.

Using the primers designed by Simon and others (1991),

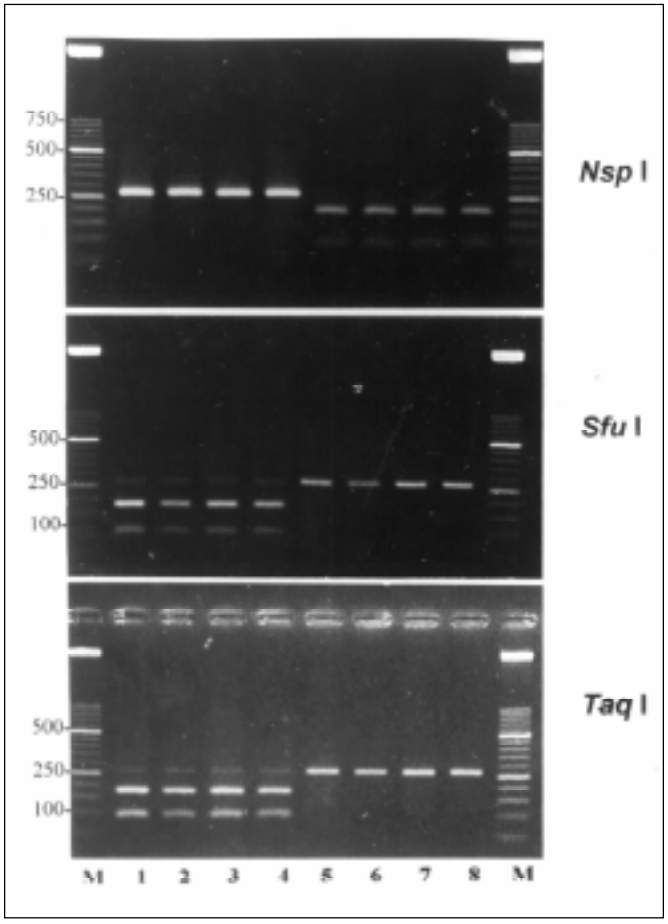


**Figure 3—Restriction profiles of the 16S rRNA PCR products obtained from *H. pomatia* (1-4) and *H. lucorum* (5-8) digested with *Nsp* I, *Sfu* I and *Taq* I. For marker see Figure 1.**

with a high-purified DNA we successfully amplified and sequenced the respective genes. Direct sequence analysis of the PCR products of the *cytochrome b* gene obtained with oligonucleotide primers designed by Kocher and others (1989), or with slightly modified ones, has been used to assess inter- and intraspecific differentiation of Atlantic cod *(Gadus mohrua)* populations (Carr and Marshall 1991; Pepin and Carr 1993), salmon species (McVeigh and others 1991; Hartley and others 1992; Lockwood and others 1993), and tuna species (Bartlett and Davidson 1991), showing interspecific variations that may be useful for their identification. However, the high cost of this technique and the need of individual sequences for detailed comparison make it inappropriate for the analysis of a large number of samples. As an alternative to sequencing, we have used PCR- RFLP analysis of a conserved region of the 12S- and 16S rRNA genes. The gene sequences were analyzed to find conserved and variable regions. This was used for the selection of oligonucleotide primers to amplify PCR products which show differences in their restriction sites and allow a differentiation of *H. pomatia* and *H. lucorum*. The selected primers UNI12S-I/UNI12S-II for the 12S rRNA subunit and UNI16S-I/UNI16S-II for the 16S rRNA subunit amplified a fragment of 232 bp and 258 bp, respectively (Table 1).

Figure 1 shows the results obtained after restriction analysis of the 12S rRNA-PCR products. Using the restriction enzyme *Afi* III, a single restriction site was found for *H. lucorum* yielding 2 fragments of 46 bp and 186 bp. Using the restriction enzyme *Dra* I, one restriction site was found in *H. pomatia* resulting in 2 fragments with a size of 110 bp and of 122 bp. The restriction enzyme *Sty* I digested the *H. pomatia* amplicon into 2 fragments of 36 bp and 186 bp. On the basis of these results, the enzymes *Afi* III, *Dra* I and *Sty* I were chosen for restriction site analysis of the 12S rRNA gene. The restriction map of the 12S rRNA subunit sequence is shown in Figure 2.

By restriction analysis of the 16S rRNA-PCR products a single restriction site was found in the *H. lucorum* sequence for the enzyme *Nsp* I yielding 2 fragments of 74 bp and 184 bp. In the *H. pomatia* sequence 1 restriction site was found for the enzyme *Sfu* I. The enzyme *Taq* I digested the amplicon of *H. pomatia* into 2 fragments of 86 bp and 172 bp. The results obtained by restriction analysis of the 16S rRNA gene are shown in Figure 3. The restriction maps of the 16S rRNA

subunit sequences (Figure 4) showed that the enzymes *Nsp* I, *Sfu* I, and *Taq* I are suitable for restriction analysis of the 16S rRNA-PCR products.

The *cytochrome b* gene sequence of both species showed no significant sequence variation useful for differentiation of *H. pomatia* and *H. lucorum* by restriction analysis or for the design of species-specific oligonucleotide primer (Figure 5).

All 49 individual samples of each species were correctly identified using PCR-RFLP of the 12S and the 16S rRNA genes. The results did not show intraspecific polymorphism for the endonucleases tested.

The PCR allows an amplification of specific regions of DNA, facilitating the detection of genetic differences between species or populations. When the PCR approach is used, it is important to establish which genes show variation for the species under study (Brown 1983). It should be

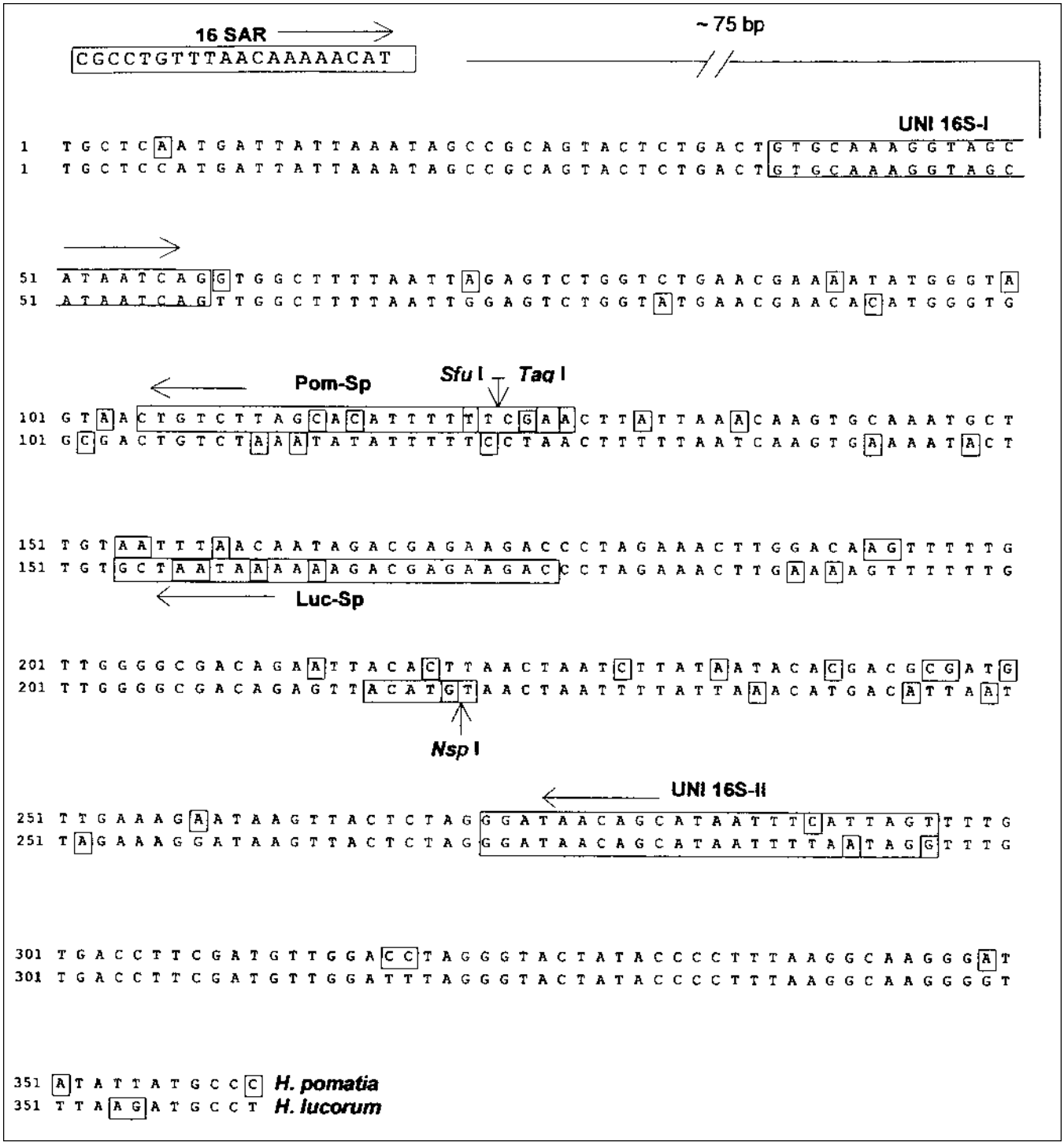


**Figure 4—DNA sequences from part of the 16S rRNA gene of *H. pomatia*, aligned with *H. lucorum*. The positions of the oligonucleotide primers used for the following PCRs, the *H. pomatia* specific primer Pom-sp and the *H. lucorum* specific primer Luc-sp are marked; the shadow indicates the restriction sites.**

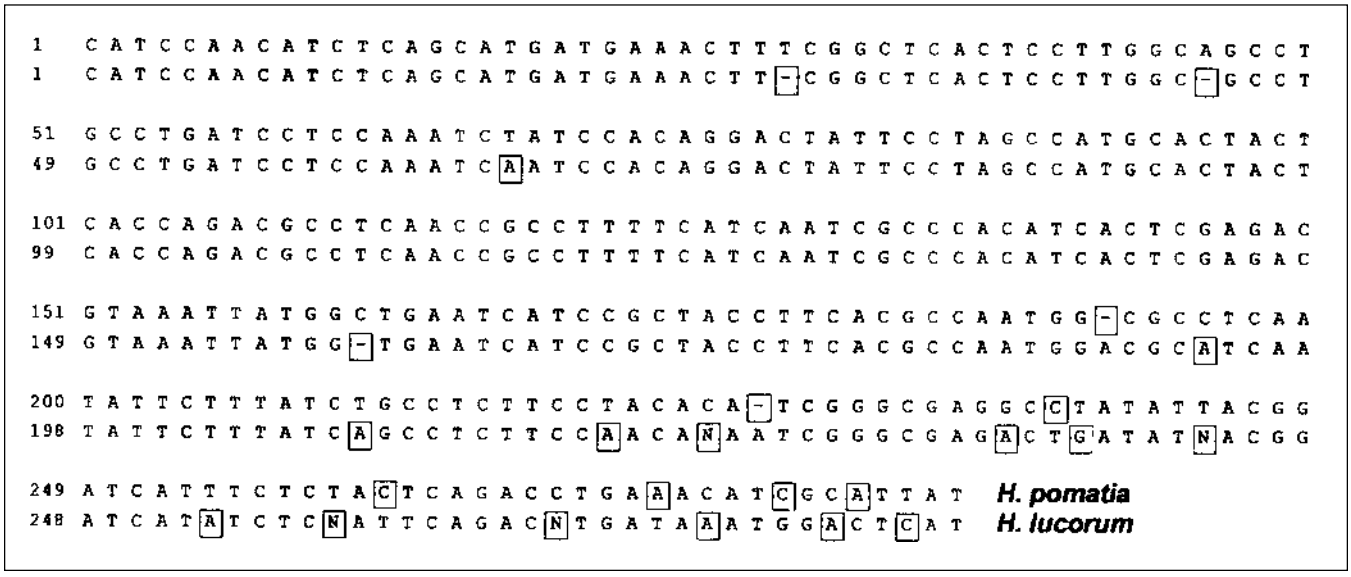


**Figure 5—DNA sequences from part of the *cytochrome b* gene of *H. pomatia,* aligned with *H. lucorum.***

stressed that, while it is likely that hypervariable sequences of the mtDNA molecule will prove more useful in studies of intraspecific variation (Ferguson and others 1995; Hall and Nawrocki 1995; Hansen and Loechcke 1996), relatively conserved sequences may be ideally suited for interspecific and intergeneric comparisons (Lockwood and others 1993). Also, Carrera and others (1999), in their review on the identification of fish eggs of Atlantic salmon and rainbow trout species, pointed out that the 16S rRNA markers could be considered as more specific than *cytochrome b* for discrimination of salmon and trout samples. However, further studies must be made on other regions of the *cytochrome b* gene.

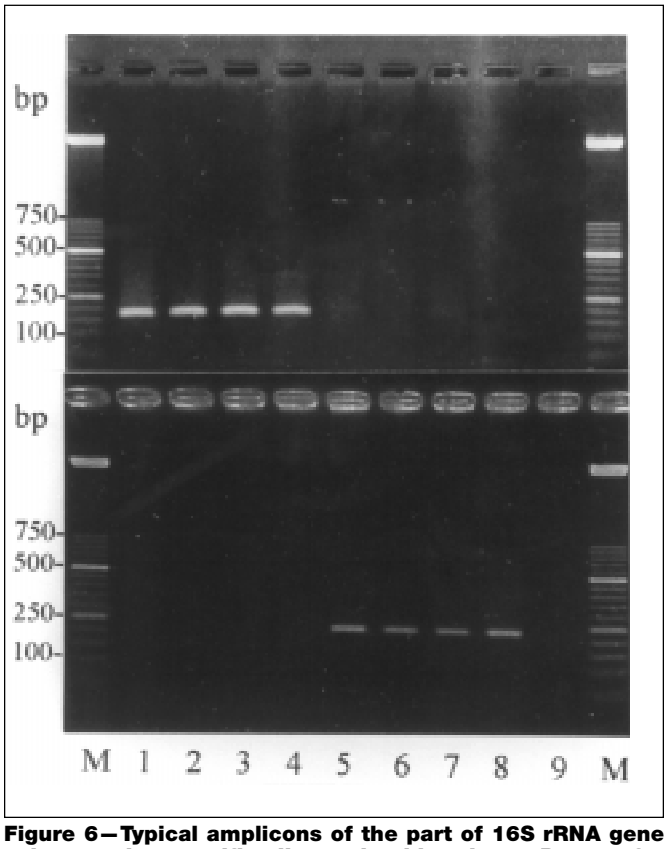


**Figure 6—Typical amplicons of the part of 16S rRNA gene using species-specific oligonucleotide primers Pom-sp for *H. pomatia* (A) and Luc-sp for the *H. lucorum* (B). The PCR was performed with DNA preparation of *H. pomatia* (1-4) and *H. lucorum* (4-8); Negative control (9). For marker see Figure 1.**

We analyzed the 16S rRNA gene sequence showing several unique nucleotide positions that could be used to identify each species. Using the primer combination 16SAR and Pom-Sp, a species-specific product of a size of approximately 200 bp could be amplified for all *H. pomatia* but not for the species *H. lucorum*. For this species a species-specific amplicon of approximately 250 bp could be amplified with all *H. lucorum* by using the primer combination 16 SAR and Luc-Sp (Figure 6). The designed species-specific primer Pom-sp for *H. pomatia* and Luc-sp for *H. lucorum* showed no cross-reaction between the 2 species. Desalle and Birstein 1996 used the intraspecific sequence variation to design a species-specific primer for the identification of black caviar.

## Conclusion

BOTH MITOCHONDRIAL MARKERS DESCRIBED IN THIS WORK (12S and 16S rRNA genes) were found appropriate to be for the correct discrimination between *H. pomatia* and *H. lucorum*. Therefore, they could be used with confidence in inspection programs in order to verify the correct labeling of *H. pomatia* and *H. lucorum* products. Moreover, availability of more than one genetic marker may be necessary as evidence in court cases to prove fraudulent substitution of species.

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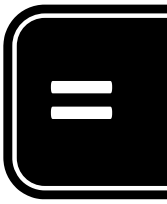

# Identification of Ostrich Meat by Restriction Fragment Length Polymorphism (RFLP) Analysis of *cytochrome b* Gene

A. ABDULMAWJOOD AND M. BÜLTE



**ABSTRACT: To enforce labeling regulations in the authentication of ostrich meat, it might be of importance to evaluate a method to identify the ostrich meat. A restriction site of the polymerase chain reaction (PCR) product has been used for the specific identification of ostrich meat. In the present study the part of the gene encoding *cytochrome b* was amplified and sequenced. The digestion of the PCR products using specific restriction enzymes *Hae* III, *Hinf* I, *Rsa* I, and *Tru* 9I were used to yield specific restriction profiles that allowed a direct identification of ostrich meat in raw and heat-treated samples from meat of other food animal species.**

**Keywords: *struthio camelus*, ostrich meat, mt DNA, cytochrome b, PCR-RLFP**

## Introduction

OSTRICH MEAT IS A NUTRITIONALLY excellent food. One hundred grams of cooked lean meat contains low levels of fat (2.8 g) and cholesterol (83 mg). As for protein, the total is 26.9%; and the meat contains 3.2 mg iron (TAES 2001). The species *Struthio camelus* (ostrich) belongs to the genus Struthio, family Struthionidae (ostriches), order Struthioniformes (ostriches, emus, cassowaries), and class Aves (birds) (Donegan 2001). Ostriches exist in several colors, each representing a different subspecies or group of subspecies. The African black ostrich (*Struthio camelus domesticus*) is a strain produced by selective breeding from blue and red species. The blue neck ostrich (*Struthio camelus molybdophanes*) belongs to one of three subspecies native to north, west or, south Africa. The African black ostrich is shorter, smaller, and has darker feathers than other species. It has little skin color. The blue neck ostrich has blue-gray skin on the neck, legs, and thighs (Jeffrey 2001).

In the 1990s, numerous cases of bovine spongiform encephalopathy (BSE) were observed in many European countries, and consumption of the relatively expensive ostrich meats increased; it has become of great importance as an alternative to other red meats, especially beef. Ostrich meats are usually imported as dressed cuts and, to raise profit, the products might be incorrectly labeled. Because of that, there is a need for a suitable nonmorphological technique to identify cut ostrich meat.

An identification of animal species could be performed by mitochondrial DNA (mtDNA) analysis. According to Carney and others (1997), Rigaa and others (1997), Yoshizaki and others (1997), and Abdulmawjood and Buelte (2001), mtDNA analysis requires the isolation of the mtDNA molecule and a digestion of the mtDNA with a variety of restriction endonucleases. The resulting fragment patterns are then examined for polymorphisms within and among populations examined.

A combination of PCR amplification of mtDNA and restriction fragment length polymorphism (RFLP) analysis has been used in species and stock identification studies (Wilson and others 1995; Carrera and others 1996, 1998, 1999; Ram and others 1996; Yoshizaki and others 1997; Cespedes and others 1999; Abdulmawjood and Buelte 2001).

The present study was designed to investigate PCR methods for identification of ostrich meat. This was based on PCR-RFLP of a conserved mitochondrial DNA region of the *cytochrome b* gene of ostrich and five other food animal species.

## Materials and Methods

### DNA extraction

Total cellular DNA was isolated from muscle samples of 22 fresh fillet pieces: 18 samples that were obtained from Albers GmbH (Duesseldorf, Germany), as well as 4 reference samples of African black ostrich (n = 2) and blue neck ostrich (n = 2). The reference samples were kindly supplied by Dr. Y. Hemberger, Ostrich Production (Namibia) (PTY) Ltd., Namibia. In addition, 8 samples were heat-treated, fried with cooking oil and spices (n = 2), or boiled at 100<abstract>ABSTRACT: To enforce labeling regulations in the authentication of ostrich meat, it might be of importance to evaluate a method to identify the ostrich meat. A restriction site of the polymerase chain reaction (PCR) product has been used for the specific identification of ostrich meat. In the present study the part of the gene encoding *cytochrome b* was amplified and sequenced. The digestion of the PCR products using specific restriction enzymes *Hae* III, *Hinf* I, *Rsa* I, and *Tru* 9I were used to yield specific restriction profiles that allowed a direct identification of ostrich meat in raw and heat-treated samples from meat of other food animal species.

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**Table 1—Predicted fragment sizes of the partial cytochrome b gene of different food animal species after PCR-RFLP analysis.**

| Species | Size of the DNA fragment (bp) after digestion with indicated restriction enzymes | | | | Sequence references |
|---|---|---|---|---|---|
| | *Hae* III | *Hinf* I | *Rsa* I | *Tru* 9I | |
| Ostrich | 227 | 233 | 205 | 298 | This study |
| | 132 | 126 | 149 | 615 | |
| Chicken | 159 | 188 | 210 | 292 | Desjardins and Morais 1990 |
| | 126 | 161 | 149 | 67 | |
| | 74 | 10 | | | |
| Turkey | 126 | 196 | 149 | 359 | Kornegay and others 1993 |
| | 103 | 161 | 109 | | |
| | 74 | | 101 | | |
| | 55 | | | | |
| Duck | 19 | 161 | 359 | 359 | Johnson and Sorenson 1998 |
| | 55 | 198 | | | |
| | 285 | | | | |
| Pig | 153 | 359 | 359 | 359 | Meyer and others 1995 |
| | 132 | | | | |
| | 74 | | | | |
| Cattle | 285 | 198 | 359 | 359 | Meyer and others 1995 |
| | 74 | 117 | | | |
| | | 44 | | | |
| Sheep | 159 | 198 | 359 | 244 | Meyer and others 1995 |
| | 126 | 161 | 115 | | |
| | 74 | | | | |
| Goat | 230 | 198 | 359 | 213 | Meyer and others 1995 |
| | 74 | 161 | 115 | | |
| | 55 | | 31 | | |

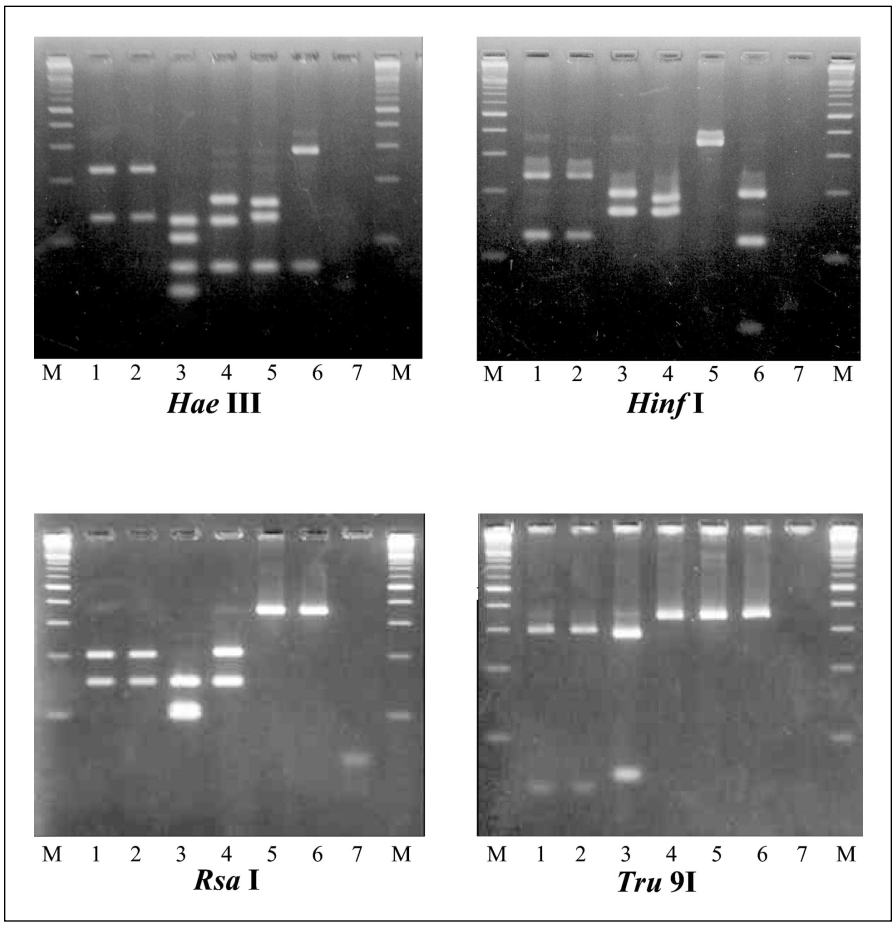


**Figure 1—Restriction profiles of the cytochrome b PCR products obtained from African black ostrich (1); blue neck ostrich (2); turkey (3); chicken (4); pig (5); beef cattle (6) and a negative control (7), after digesting with Hae III, Hinf I, Rsa I and Tru 91. (M) DNA molecular weight marker XIV 100 bp ladder (Roche).**

## Amplification of mitochondrial subunits

The oligonucleotide primer Cytb-I 5´-CCATCCAACATCTCAGCATGATGAAA-3´ and primer Cytb-II 5´-CCCCTCAGAATGATATTTGTCCTCA-3´ were used to amplify a *cytochrome b* gene subunit. These primers were designed by Kocher and others (1989) for the amplification of a conserved region of the *cytochrome b* gene. The reaction mixture (50 µL) contained 1 µL primer 1 (10 pmol/µL), 1 µL primer 2 (10 pmol/µL), 1 µL dNTP (10 mmol, Roche Diagnostic, Mannheim, Germany), 5 µL 10 × thermophilic-buffer (PE Applied Biosystem, Weiterstadt, Germany), 0.2 µL *Taq* DNA polymerase (5 U/µL, PE Applied Biosystem), and 39.3 µL double-distilled water. Finally, 2.5 µL DNA preparation was added to each reaction tube. The PCR was carried out in a thermal cycler (PE GeneAmp PCR system 9600; PE Applied Biosystem) with the following program: 1 × 3 min precycle at 93 °C, 35 × 30 s at 93 °C, 30 s at 52 °C, and 45 s at 72 °C, followed by a final extension incubation of 72 °C for 5 min. The presence of PCR products was determined by electrophoresis of 10 µL of the reaction product in a 2% agarose gel (Appligene, Heidelberg, Germany), with Tris acetate-electrophoresis buffer TAE (0.04 mol/L Tris, 0.001 mol/L EDTA, pH 7.8) and a 100-bp DNA ladder (Roche Diagnostic) as molecular marker.

## Cleanup and sequencing of the PCR products

The PCR product of the *cytochrome b* gene (18 µL) was mixed with 2 µL 5 mol/L sodium perchlorate ($NaClO_4$) (Sigma, Deisenhofen, Germany) and 10 µL isopropanol ($C_3H_8O$, Roth GmbH, Karlsruhe, Germany) for 30 minutes at room temperature and centrifuged for 15 minutes. The sediment was washed twice with 50 µL 70% ethanol ($C_2R_6O$, Roth GmbH, Karlsruhe, Germany), centrifuged for 5 min, and dried. Finally, the pellet was resuspended in 10 µL double-distilled water and directly sequenced using an Applied Biosystem, Inc. 373A DNA sequencer. The protocols followed the manufacturer's description (PE Applied Biosystem) for "*Taq* cycle-sequencing" with fluorescent dye-labeled dideoxynucleotides. The sequence data were further studied with the computer program Seg Man Lasergene (DNASTAR Inc., Madison, Wis., U.S.A.).

## Restriction site analysis and enzymatic digestion of PCR products

The *cytochrome b* gene sequence was

compared by using the computer program Meg Align (DNASTAR). Restriction maps were investigated for variant restriction enzymes using the computer program Clone-Manager (Version 4.1, Scientific Educational Software, (Durham, N.C., U.S.A.). The restriction enzymes *Hae* III, *Hinf* I, *Rsa* I, and *Tru* 9I (Roche) were selected as suitable candidates for the identification of ostrich meat. All reactions were performed in 30-µL volumes using digestion conditions specified by the manufacturer. The DNA fragments were separated by electrophoresis in 2% agarose gels.

### Nucleotide sequences accession number

The *cytochrome b* gene has been submitted to the GeneBank database under accession nr AY044050.

## Results and Discussion

THE AMPLIFICATION OF THE GENE encoding *cytochrome b* using the universal oligonucleotide primers Cytb-I and Cytb-II yielded a 359-bp amplicon. The PCR products of subspecies African black ostrich and blue neck ostrich were sequenced. Direct sequence analyses of the PCR products of the *cytochrome b* gene were performed with the oligonucleotide primers designed by Kocher and others (1989). This primer pair, or slightly modified primer pair, had already been used to amplify the *cytochrome b* gene in more than 100 animal species, including mammals, birds, amphibians, fish, and some invertebrates. In addition, these primers had been used to assess inter- and intraspecific differentiation of Atlantic cod (*Gadus mohrua*) populations (Carr and Marshall 1991; Pepin and Carr 1993), salmon species (McVeigh and others 1991; Hartley and others 1992; Lockwood and others 1993), tuna species (Bartlett and Davidson 1991), and snail species (Abdulmawjood and Buelte 2001). These authors showed interspecific variations, which might be useful for identifying the various species. However, the high cost of this technique and the need of individual sequences for detailed comparison make it inappropriate for the analysis of large numbers of samples. As an alternative to sequencing, the present study describes a PCR-RFLP analysis of a conserved region of the *cytochrome b* gene. Meyer and others (1995) used the same primers to amplify 10 different food animal species; the sequences were analyzed to find conserved and variable regions for the selection of restriction enzyme.

The sequence of the PCR products of the ostrich *cytochrome b* gene of the present investigation showed no differences between the African black and the blue neck ostrich in this region. A comparison of the sequence with other food animal species showed differences in their restriction sites and allowed an identification of ostrich meat. The results obtained after restriction analysis of the *cytochrome b*-PCR products of the ostrich *cytochrome b* compared with 5 different common meat animal species are shown in Figure 1. Using the restriction enzyme *Hae* III, a single restriction site was found in the ostrich *cytochrome b* yielding 2 fragments with a size of 132 bp and 227 bp. Using the restriction enzyme *Hinf* I, 1 restriction site was found resulting in 2 fragments with a size of 126 bp and 233 bp. The restriction enzyme *Rsa* I digested the PCR amplicon into 3 fragments with a size of 5 bp, 149 bp, and 206 bp. The restriction enzyme *Tru* 9I produced 2 fragments with a size of 61 bp and 298 bp. The *cytochrome b* gene restriction patterns of these enzymes with the other food animal species are shown in Table 1. On the basis of these results, the enzymes *Hae* III, *Hinf* I, *Rsa* I, and *Tru* 9I were chosen for differentiation of the ostrich *cytochrome b* gene from the *cytochrome b* gene of other food animal species.

Using PCR-RFLP analysis of the *cytochrome b* gene, all ostrich samples, including the heat-treated samples investigated in this study, were correctly identified. The results did not show any intraspecific polymorphism for the endonucleases tested.

PCR technology allows an amplification

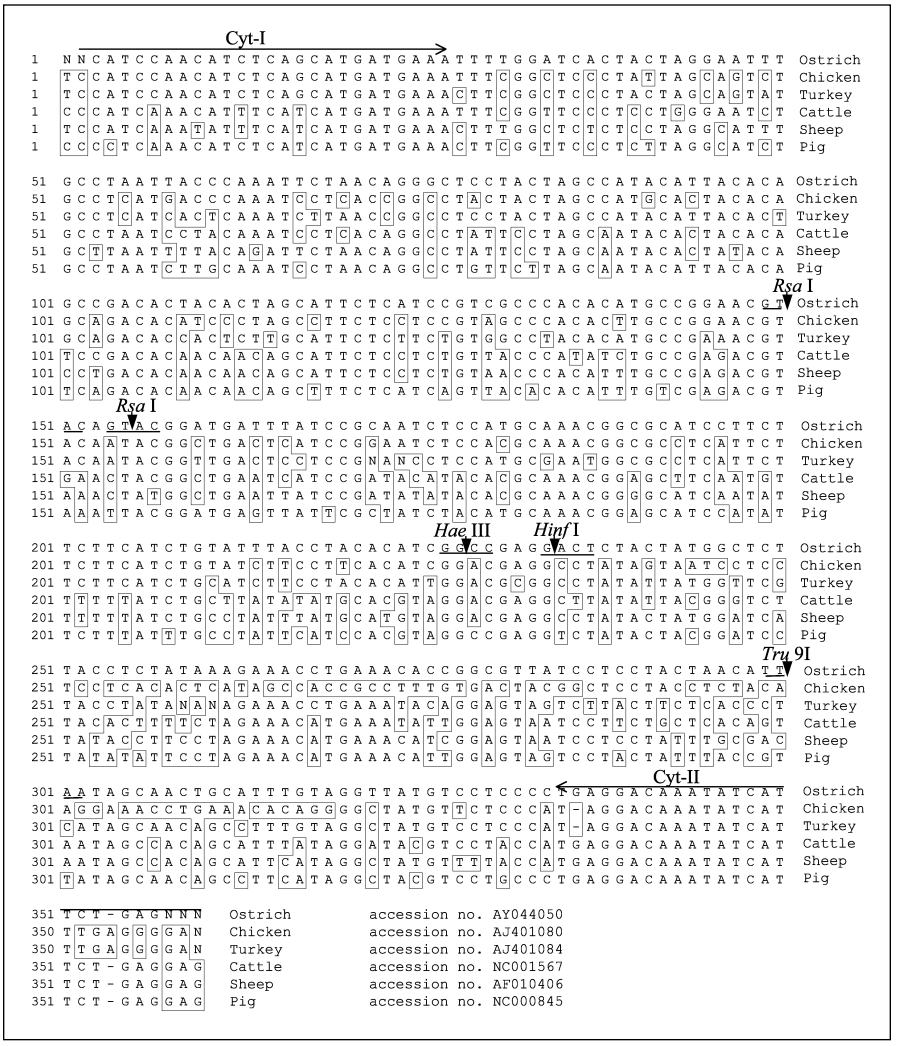


**Figure 2—DNA sequences from part of the cytochrome b gene of ostrich aligned with 5 other food animal species with the GeneBank database accession numbers. The restriction sites and the position of the different restriction enzymes are underlined. The boxes indicate the differences from ostrich sequence.**

of specific regions of DNA, facilitating the detection of genetic differences between species or populations. With this PCR approach, it is important to establish genes showing variations for the species under study (Brown 1983). It should be stressed that while it is probable that hypervariable sequences of the mtDNA molecule will prove more useful in studies of intraspecific variation (Ferguson and others 1995; Hall and Nawrocki 1995; Hansen and Loechcke 1996), relatively conserved sequences may be ideally suited for interspecific and intergeneric comparisons (Lockwood and others 1993). However, in this study we provide a simple, rapid, and universal method for identifying ostrich meat. Further studies must be done to design an ostrich species-specific primer and/or probe for the direct diagnosis and further analysis of the other mtDNA regions to provide more sequence data for the differentiation of the various ostrich breeds.

## Conclusions

THE USE OF PCR-RLFP ANALYSIS OF A conserved region of the cytochrome b gene provides a simpler, quicker, and cheaper alternative to sequencing for direct identification of ostrich species. This method could be used to identify inappropriately labeled ostrich meat. Morever, with this method even cooked meat could be successfully identified.

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# Two methods for construction of internal amplification controls for the detection of *Escherichia coli* O157 by polymerase chain reaction*

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For the detection of food born bacteria by polymerase chain reaction (PCR) in food products, an internal amplification control (IAC) is required in order to prevent false negative results that might be caused by PCR inhibitors. In the present study, two IACs were constructed using two different methods. These IACs were designed in a way that the same primer pair can be used to amplify the target DNA and coamplify the IAC. The first IAC with a size of approximately 200 bp was constructed by deleting a part of the amplicon of the original target DNA (500 bp) between the two primer sites to produce an IAC smaller than the target DNA. The second IAC with a size of approximately 600 bp was synthesized in a one step PCR reaction. The primers used in this reaction possessed 5′ overhanging ends, which were identical to the primers used in the diagnostic reaction, whereas their 3′ ends were complementary to the (pUC19) predetermined DNA sequence of defined length and sequence. The concentration of IACs appeared to be critical. Too much IAC DNA template would out-compete the target DNA template, thus giving a false negative result. However the use of an optimal IAC concentration increased the reliability of the PCR assays and appeared to be useful for food diagnostics. 

***KEYWORDS:*** internal amplification control, PCR, *Escherichia coli* O157, false negative results.

## INTRODUCTION

In food-PCR diagnostics, internal amplification controls (IACs) are required in order to prevent false negative results. The consequences of false negative results in the detection of a pathogenic micro organism may potentially be life threatening. In food samples it is important to take into account that components of the food sample or the competing micro flora may influence the effectiveness of the PCR, especially by reducing the detection limit and producing false negative results. Other PCR-inhibitors can be found in body fluids, soil, bacterial cells and non-target DNA.[1–5]

The presence of a PCR control product in the absence of the target PCR product allows a differentiation of true and false negative results. The simplest approach to develop internal amplification standards for PCR is to produce PCR products, which differ in size and hence can easily be visualized separately from the native product by agarose gel electrophoresis and ethidium bromide straining. This can be achieved by either deleting or inserting sequences between the recognition primer sites.

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The first strategy used in this study was to delete some of the intervening sequences between the two primer sites. The second strategy, performed according to Sachadyn and Kur[6] was to increase the size of the internal amplification control. The internal amplification controls were developed and tested for use in a PCR detection system for *E. coli* O157 by using *rfbE* gene.[7]

## MATERIAL AND METHODS

Both internal amplification controls were constructed by using the primer pair (GiO157-I and GiO157-II) designed by Abdulmawjood *et al.*[7] The small IAC was constructed by cutting an internal 295 bp DNA fragment (Fig. 1a). For this the original PCR amplicon (500 bp) was digested with the restriction enzyme *Hae*III cleaves at two sites within the amplicon producing three fragments of 295, 165 and 40 bp. After gel electrophoresis the two outer fragments (165 and 40 bp) were extracted from the gel using QiaexII Gel extraction kit (Qiagen, Darmstadt, Germany) and ligated using T4 DNA Ligase (Promega, Mannheim, Germany) to create an amplicon 295 bp smaller than the original amplicon. After gelelectrophoresis the shorter ligate was extracted, purified and cloned using the TOPO TA Cloning® Kit (Invetrogen, Holland) (Fig. 1a). The recombinant strain was stored as glycerine culture at −20°C until use. Plasmid DNA was isolated from the recombinant strain using the Qiagen Midi Plasmid kit (Qiagen) according to the manufacturer's protocol and used as a template in the subsequent PCR-reactions. A PCR product was produced using the corresponding primer-set and with conditions described by Abdulmawjood *et al.*[7] The PCR product was purified using QIAquick PCR purification kit (Qiagen) and serially diluted in TE buffer pH 7·6.

The large IAC was synthesized in one PCR reaction, using the commercial plasmid pUC19 as a template. The primers were identical to the primers used in the diagnostic reaction and possess 5′ overhanging ends (underlined), whereas their 3′ ends complementary to the predetermined (pUC19) DNA sequence of defined length and sequence (*italics*). The primer sequences were designed using the OLIGO Primer Analysis Software, ver. 4·0, 1991, USA. (Fig. 1b). The oligonucleotide primers, synthesized by MWG-Biotech (Ebersberg, Germany) had the sequence 5′ primer GiO157-PuC19-I-f 5′-*ATT GCG CTG AAG CCT TTG AGC GGG TGT TGG CGG GTG TC*-3′ (pUC 19 primer location 113–132) and the 3′primer GiO157-PuC19-I-r 5′-*CGA GTA CAT TGG CAT CGT G AGT GAG CGA GGA AGC GGA AGA GC*-3′ (pUC 19 primer location

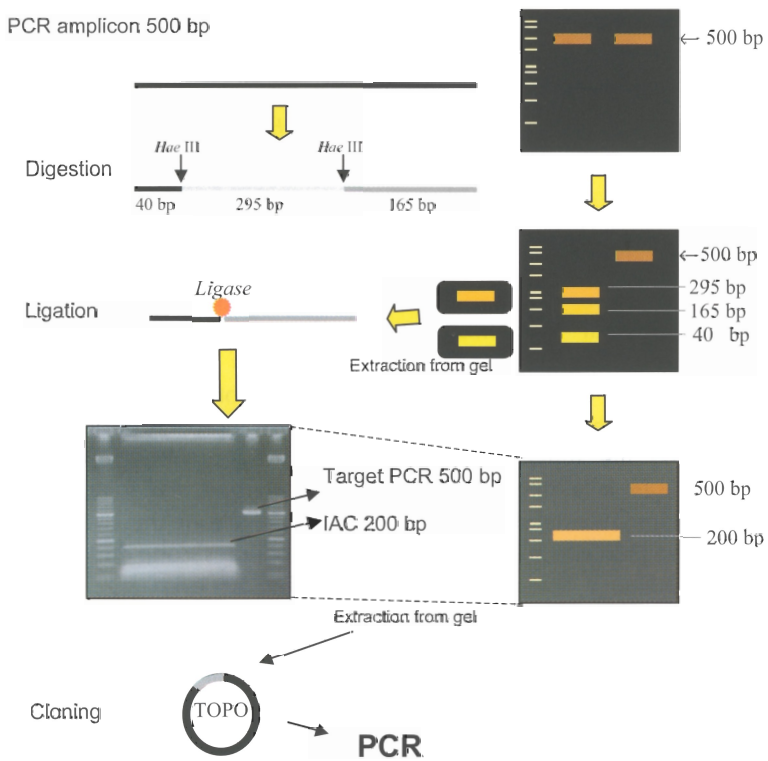


**Fig. 1a.** Scheme of the construction of the small internal control.

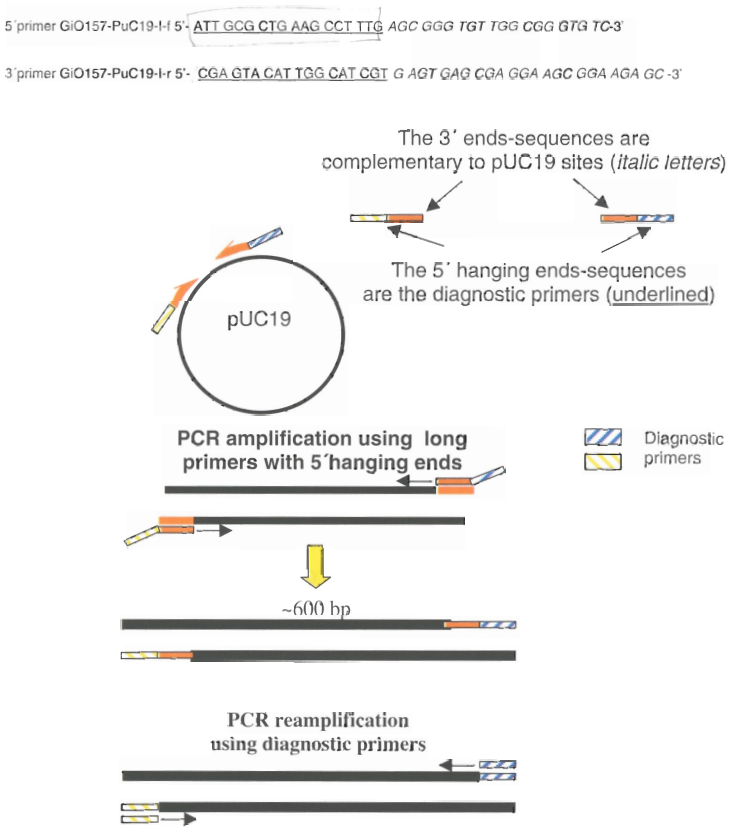


**Fig. 1b.** Scheme of the construction of the large internal control according to Sachadyn and Kur (1998).[6]

705–683). As the sequence of this IAC except for the primer sites, was not homologous to the PCR target product, the formation of heteroduplexes and non-specific PCR products should not occur. The PCR products of the IACs were purified using QIAquick PCR Purification Kit. The DNA concentration was estimated by using an UV spectrophotometer (DU 640 Backmann, USA).

The PCR reaction mixture for the simultaneous amplification of the IAC and the *E. coli* O157 target gene (25 µl) contained 1 µl 5´ primer (10 pmol/µl), 1 µl 3´ primer (10 pmol/µl), 1 µl dNTP (10 mM, Roche, Mannheim, Germany), 2·5 µl 10 × thermophilic-buffer (PE Applied Biosystem, Weiterstadt, Germany), 0·1 µl *Taq* DNA polymerase (5 U/µl, PE Applied Biosystem), 1 µl IAC and 19·4 µl aqua bidest. Finally 1·5 µl DNA preparation was added to each reaction tube. For DNA preparation the bacterial suspension was boiled for 10 min at 100°C then centrifuged (10,000 × *g*, 5 s) and subsequently cooled before use. The PCR was carried out in a thermal cycler (PE GeneAmp PCR system 9600; PE Applied Biosystem) with the following program: 1 × 3 min precycle at 93°C, 30 × 15 s at 93°C, 15 s at 60°C and 30 s at 72°C, followed by a final extension incubation of 72°C for 5 min. The presence

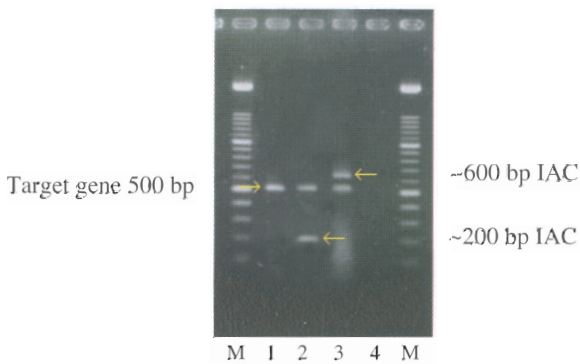


**Fig. 2.** The PCR product of the target gene with a size of 500 bp (1) and both co-amplified internal amplification controls (IAC) the small IAC ~ 200 bp (2); and the large IAC ~ 600 bp (3); negative control (4); M, 100 bp DNA ladder.

of PCR products was determined by electrophoresis of 10 µl of the reaction product in a 2% agarose gel (Appligene, Heidelberg, Germany), with Tris acetate-electrophoresis buffer TAE (0·04 mol $l^{-1}$ Tris, 0·001 mol $l^{-1}$ EDTA, pH 7·8) and a 100 l bp DNA ladder (Roche Diagnostic, Mannheim, Germany) as molecular marker.

## RESULTS AND DISCUSSION

However, the simultaneous amplification of two different DNA fragments flanked by the same primer sites resulted in either inhibition or enhancement of one or both products depending on the molar ratio of these DNA fragments. A PCR amplification of the two IACs used as template gave the predicted PCR products of 205 and 543 bp. The target PCR product had a size of 500 bp. All three PCR amplicons appeared without any non-specific bands (Fig. 2).

The influence of the detection limit in the presence of the IAC was investigated. A PCR using serial dilutions of *E. coli* O157 DNA with the primers GI O157 I and GI O157 II allowed a detection of $2{\cdot}89 \times 10^2$ (mean of 12 repeats SD $\pm$ 335·075) *E. coli* DNA copies per PCR reaction (Fig. 3a). To determine the effect of the IAC PCR products on the detection limit of the PCR serial dilutions of the small IAC were tested. The lowest two concentrations of IAC that gave a reliable PCR band visible by gel electrophoresis and ethidium bromide staining were used with a serial dilution of genomic *E. coli* O157 DNA. By using 5·23 fg of the IAC the detection limit was $5{\cdot}95 \times 10^2$ (SD $\pm$ 393·194) DNA copies/reaction (Fig. 3b). Increasing the concentration of the IAC 10 fold to 52·3 fg raised the detection limit to $1{\cdot}12 \times 10^4$ DNA copies/reaction (data not shown).

There is always some competition between target DNA and IAC and through the use of high concentrations of IAC the detection limit can be influenced by the amount of IAC template.

According to these results the lowest reproducible IAC template DNA concentration must be determined carefully otherwise too much IAC template DNA will compete with the target DNA product giving a false negative result.

To investigate the effect of the size of the IAC the two IACs of different sizes of 205 and 597 bp were checked and compared to test if they have a different influence on the detection limit of the target DNA. Increasing the size of the IAC PCR product should in theory drive the reaction kinetics towards the smaller target PCR product.[6] However, according to the present results, independent of the size of the IAC a comparable competition of the target gene PCR could be observed (data not shown). Corresponding to these results Brightwell *et al.*[8] mentioned that the size of the IAC PCR product does not influence the original PCR sensitivity. The most critical factor to consider when multiplexing with IACs, is the concentration of the IAC itself.[6,8] The lowest reproducible IAC template concentration must be determined carefully otherwise too much IAC template will compete with the native PCR giving false negative results. However, the present study demonstrated that an IAC could be incorporated into a PCR assay without significant loss of detection limit. Furthermore, it was required to titrate the internal control DNA template separately to the PCR assay. However, this has only to be done for one time to find out the optimal concentration of DNA. For further use it is possible to prepare master mixes containing the correct concentration of IAC template and primers. They can be aliquoted and stored frozen until use. The presented methods may also be applied to construction of internal amplification controls for quantitative PCR.

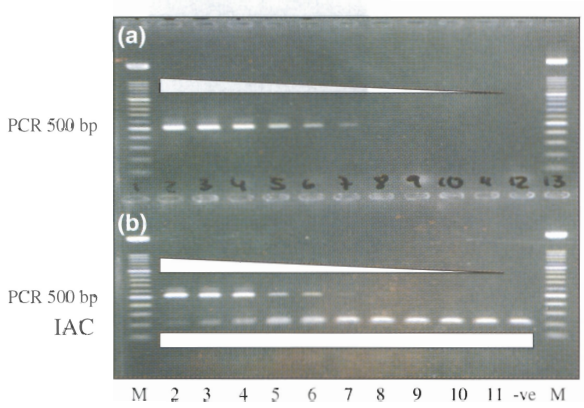


**Fig. 3.** Co-amplification of target PCR and small IAC. The amplification was performed with a semi tenfold dilution of target DNA without IAC (a) and with a defined amount (5·23 fg) of IAC (b). Lane 7 correspondent 560 copies; lane 8 correspondent 112; M, marker 100 DNA ladder.

## ACKNOWLEDGEMENT

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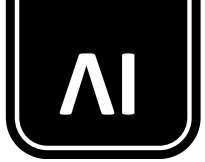

# Development of a Polymerase Chain Reaction System for the Detection of Dog and Cat Meat in Meat Mixtures and Animal Feed

A. ABDULMAWJOOD, H. SCHÖNENBRÜCHER, AND M. BÜLTE

**ABSTRACT: The identification of species origin of meat represents a considerable problem for food and animal feed analysis. In the present study a PCR-mediated method for the detection of dog and cat meat was developed. For this the *cytochrome b* gene sequence of both species was analyzed by restriction fragment length polymorphism (RFLP) analysis. The use of the restriction enzymes *Alu* I and *Hae* III yielded specific restriction profiles characteristic for each species. The meat of both species could additionally be differentiated with species-specific oligonucleotide primers based on specific parts of the *cytochrome b* gene sequences characteristic for dog and cat. The use of these oligonucleotide primers allowed a direct identification of dog and cat meat in meat mixtures even after heat treatment.**

**Keywords: PCR, species-specific primer, RLFP, dog meat, cat meat**

## Introduction

FOR ETHICAL AND MEDICAL REASONS, A BAN of dog and cat meat in food and in animal feed has been introduced in the European meat hygiene law. The identification of the species origin of meat presents a considerable problem for food analysts and game law enforcement authorities in many parts of the world. In many developing countries the food analyst is confronted with providing proof of fraudulent substitution of more expensive meats with cheaper meats. In addition the animal protection organizations need to differentiate meat of protected species from that of unprotected species and domestic animals.

The identification of the species origin of fresh meat could be achieved using methods such as immunodiffusion tests (Swart and Wilks 1982; Kangethe and others 1986), electrophoretic methods (Lundstrom 1981; Cota-Rivas and Vallejo-Córdoba 1997) and enzyme-linked immunosorbent assays (von Holst C and others 2000). However, the speciation of cooked meat is a more difficult task because the temperature and duration of heat may destroy or alter the species-specific epitopes.

The identification of the species origin of such heated meat remains with the detection of particular mitochondrial DNA (mtDNA) which retains its specificity during processing. The *cytochrome b* gene, localized on the mitochondrial genome, has been determined as a powerful marker for identifying species with DNA analytical techniques (Kocher and others 1991; Chikune and others 1994; Forrest and Carnegie 1994; Abdulmawjood and Bülte 2001, 2002) Moreover, mtDNA is present in a much higher copy number compared to nuclear DNA which makes it a useful tool in forensic casework (Wilson and others 1995; Lutz and others 1996). According to Carney and others (1997), Rigaa and others (1997), Yoshizaki and others (1997) and Abdulmawjood and Bülte (2001 2002), mtDNA analysis requires the isolation of the mtDNA molecule and a digestion of the mtDNA with a variety of restriction endonucleases. The resulting fragment patterns are then examined for polymorphisms within and among populations examined. A specific PCR amplification of mtDNA followed by RFLP analysis has already been used in species and stock identification studies (Wilson and others 1995; Ram and others 1996; Yoshizaki and others 1997; Carrera and others 1998, 1999; Cespedes and others 1999; Abdulmawjood and Bülte 2001, 2002).

A second approach for species identification is the construction of species specific primers. A PCR protocol using species-specific mtDNA primer pairs has been developed for the identification of three commercial Russian sturgeon (caviar) species (Desalle and Birstein 1996) and for species differentiation of meat from snails (Abdulmawjood and Bülte 2001)

The present study was designed to investigate PCR methods for the detection of dog and cat meat in meat mixtures and in animal feed. This was based on PCR-RFLP analyses as well as the design of species-specific primers for dog and cat using a conserved mitochondrial DNA region of the *cytochrome b* gene.

## Materials and Methods

### Samples

A total number of 96 muscle samples of different animal species were investigated. These included muscle samples of dogs (*Lupus canis f. familiaris*, n = 30) and cats (*Felis silvestris f. catus*, n = 26). These samples were kindly provided by the Institute of Veterinary Pathology, University of Giessen, Germany. For control purposes, muscle samples from the following animal species were included: cattle (*Bos primigenius f. taurus*, n = 13), pig (*Susscrofa f. domestica*, n = 10), sheep (*Ovis ammon f. aries*, n = 5), chicken (*Gallus gallus gallus*, n = 4) and turkey (*Meleagris gallopavo domestica*, n = 8). These samples were purchased from local butcher shops.

### DNA extraction

The total cellular DNA was isolated using the Dneasy tissue isolation kit (Qiagen, Darmstadt, Germany) according to the manufacturer's instructions. Briefly, 25 mg from the meat sample was lysed and followed by binding of the DNA to the column (Qiagen). After washing steps the DNA was eluted with 100-μL elution buffer (Qiagen).

### Amplification of the mitochondrial subunits using universal oligonucleotide primers

The oligonucleotide primers Cytb-I/ Cytb-II were used to amplify a *cytochrome b*

gene subunit. These primers were designed by Zehner and others 1998. The DNA was amplified by PCR. The reaction mixture (50 µL) contained 1-µL primer 1 (10 pmol/µL), 1-µL primer 2 (10 pmol/µL), 1-µL dNTP (10 mmol, Roche, Mannheim, Germany), 5 µL 10 × thermophilic-buffer (Applied Biosystems, Weiterstadt, Germany), 0.2-µL *Taq* DNA polymerase (5 U/µL, Applied Biosystems) and 39.3-µL aqua bidest. Finally 2.5-µL DNA preparation was added to each reaction tube. The PCR was carried out in a thermal cycler (PE GeneAmp PCR system 9600, Applied Biosystems) with the following program: 1 × 3 min precycle at 93 °C, 35 × [30 sec at 93 °C, 30 sec at 52 °C and 45 sec at 72 °C] followed by a final extension incubation of 72 °C for 5 min. The presence of PCR products was determined by electrophoresis of 10 µL of the reaction product in a 2% agarose gel (Appligene, Heidelberg, Germany), with Tris acetate-electrophoresis buffer TAE (0.04 mol/l Tris, 0.001 mol/l EDTA, pH 7.8) and a DNA ladder (Roche) as molecular marker.

**Table 1—Oligonucleotide primers used in this study**

| Primer | Primer sequence (5′ to 3′) | Primer size | Amplicon size | Reference |
|---|---|---|---|---|
| Cytb-I | CAT CGA CCT TCC AGC CCC ATC AAA CAT | 27-mer | 981 bp | Zehner and others 1998 |
| Cytb-II | TGT TCT ACT GGT TGG CCT CCA ATT CA | 26-mer | | |
| Dog F | **GGA GTA TGC TTG ATT CTA CAG** | 21-mer | 808 bp | this study |
| Dog R | AGA AGT GGA ATG AAT GCC | 18-mer | | |
| Cat F | CTC ATT CAT CGA TCT ACC CA | 20-mer | 672 bp | this study |
| Cat R | GTG AGT GTT AAA ACT AGT ACT AGA AGA | 27-mer | | |

**Table 2—Predicted fragment sizes of the partial *cytochrome b* gene (981 bp) of different animal species after PCR-RFLP analysis**

| Species | Size of the DNA fragments (bp) after digestion with the restriction enzymes | | Accession numbers |
|---|---|---|---|
| | *Alu* I | *Hae* III | |
| Dog | 464, 387, 100, 30 | 652, 248, 81 | X 94920 |
| Cat | 656, 205, 120 | 612, 253, 89, 16, 11 | X 82296 |
| Cattle | 453, 323, 205 | 551, 341, 89 | J 01394 |
| Pig | 581, 243, 130, 27 | 581, 158, 153, 89 | X 56295 |
| Sheep | 518, 450, 13 | 717, 159, 89, 16 | X 56284 |
| Chicken | 981 | 608, 159, 105, 89, 20 | L 08376 |
| Turkey | 981 | 652, 89, 8, 1, 60, 56, 43 | L 08381 |

### Restriction site analysis and enzymatic digestion of the PCR products

The *cytochrome b* gene sequences from dog, cat, cattle, pig, chicken, and turkey obtained form GenBank, were aligned by using the computer program Meg Align, DNASTAR, Inc., Madison, Wis., U.S.A. (Figure 1). In addition, using computer program Clone-Manager (Version 4.1, Scientific Educational Software, Durham, N.C., U.S.A.), the restriction enzymes *Alu* I and *Hae* III (Roche) were selected as suitable candidates of species specific restriction of the *cytochrome b* gene. The PCR products were subjected to restriction digestion with the selected endonucleases without further purification. All reactions were performed in 30-µL volumes using digestion conditions specified by the manufacturer. The DNA fragments were separated by electrophoresis on 2% Metaphor agarose gels (Biozym, Hessisch Oldendorf, Germany).

### Design of species-specific primers

Based on the *cytochrome b* sequence analysis dog and cat specific primers were designed using the OLIGO Primer Analysis Software, ver. 4.0 (National Biosciences Inc., Plymouth, Minn., U.S.A.) The species-specific primers Dog F/R and Cat F/R were selected from a characteristic region of the *cytochrome b* gene of dog and cat, respectively. The reaction mixture and thermal cycler programs were used as described above. All oligonucleotide primers used in present study are summarized in Table 1.

### Determination of the PCR sensitivity

To investigate the detection limit of the PCR system 25 mg of meat mixture of pork and cattle containing 0.01, 0.1, 0.5, 1, 2, 5, and 10% of dog and cat meat were applied for DNA extraction using Dneasy tissue kit. In addition, 21 samples of dog and cat meat, respectively, were heat-treated. The samples were boiled at 100 °C for 2 min (n = 3), 5 min (n = 3), 10 min (n = 3), 30 min (n = 3), 60 min (n = 3) and 90 min (n = 3), respectively. In addition 3 samples of dog and cat meat, respectively were autoclaved at 121 °C at 1.1 bar for 30 min.

### Nucleotide sequences accession numbers

The *cytochrome b* gene sequences used in this study were obtained from EMBL gene bank under the accession numbers: dog (accession number X 94920), cat (accession number X 82296), cattle (accession number J 01394), pig (accession number X 56295), sheep (accession number X 56284), chicken (accession number L 08376) and turkey (accession number L 08381).

## Results and Discussion

THE AMPLIFICATION OF THE GENE ENCODing a part of *cytochrome b* using the universal oligonucleotide primers Cytb-I and Cytb-II yielded an amplicon with a size of 981 bp. The oligonucleotide primers used were designed by Zehner and others (1998). Alignment of this part of the *cytochrome b* gene sequence available in the EMBL GenBank of dog and cat with 5 different common meat-animal species showed differences in their restriction maps and allowed selection of restriction enzymes. On the basis of these results, the enzymes *Alu* I and *Hae* III were chosen for differentiation of the dog and cat *cytochrome b* gene sequences from the sequences of other food animal species. The predicted fragment patterns are shown in Table 2. Using the restriction enzyme *Alu* I, 3 restriction sites were found in dog *cytochrome b* sequence, yielding, as predicted, 4 fragments with a size of 464, 387, 100, and 30 bp, respectively. The *cytochrome b* sequence from cat was digested into 3 fragments with sizes of 656, 205, and 120 bp, respectively. Using the restriction enzyme *Hae* III the *cytochrome b* gene from dog showed 2 restriction sites resulting 3 fragments with sizes of 652, 248, and 81 bp, respectively. The *cytochrome b* gene from cat was digested with *Hae* III into 5 fragments with the sizes of 612, 253, 89, 16, and 11 bp respectively. The results obtained after restriction analysis of the *cytochrome b* PCR amplicon of all dog, cat, cattle, pig, sheep, chicken, and turkey samples investigated did not show any intraspecific polymorphism for the 2 endonucleases tested (Figure 2).

However, 2 or more restriction enzymes

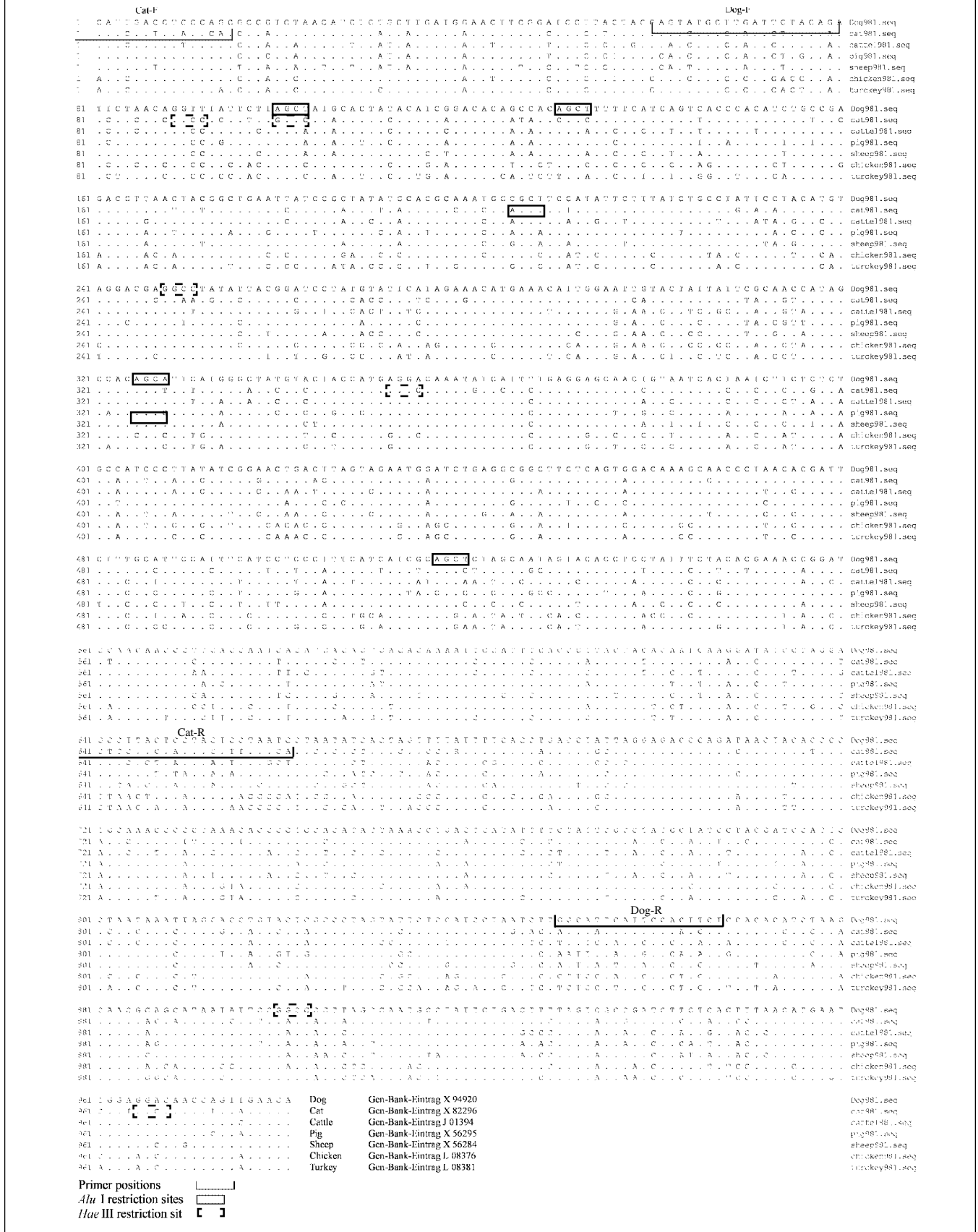


**Figure 1—DNA sequences of the *cytochrome b* gene of dog and cat aligned with the sequences of other meat animal species. Identical nucleotide sequences are indicated by dots. The restriction sites and position of the oligonucleotide primers are marked.**

must be studied to circumvent the problem of non-previously detected intraspecific genetic variability and to obtain an unambiguous identification. Nevertheless, further studies of more individuals from different breeds belonging to dog and cat species are needed to determine the genetic variability of each species in all their distribution range and consequently to increase the confidence level.

The analysis of the *cytochrome b* gene sequences showed several unique nucleotide positions that could be used to identify the species dog and cat. Using the primer pair Dog F and Dog R, a species-specific product with a size of 808 bp could be amplified for all 30 dog samples but not for the other animal species investigated. For the cat samples a species-specific amplicon with a size of 672 bp could be amplified for all 26 samples from cat by using the primer pair Cat F and Cat R (Figure 3). The designed species-specific oligonucleotide primer for dog and cat showed no cross-reaction with any of the meat-animal species used in this study. The comparison of the sequences of the species-specific primer sides of dog and cat *cytochrome b* gene with the other five animal species indicates a low degree of homology especially at the 3' end which is the end recognized by polymerases (Table 3). Desalle and Birstein (1996); Abdulmawjood and Bülte (2001) used intraspecific sequence variations to design species-specific primers for the identification of black caviar and snail meat, respectively.

In the admixture analysis the species-specific PCR system allowed us to detect the presence of dog or cat DNA in meat mixture containing less than 0.01% of dog or cat meat (Figure 3). These species-specific primer can be applied for confirming food authenticity because a specific sequence could be detected very sensitively in a pool of sequences of different origins. This strategy has been followed by Tartaglia and others (1998) who used a specific PCR system for the detection of 0.125% of bovine meat in animal feed.

The PCR reaction was also carried out with DNA extracted from heat-treated dog and cat meat. Comparable to previous studies for beef, chicken, and ostrich meat (Tartaglia and others 1998; Hopwood and others 1999; Abdulmawjood and Bülte 2001) a strong signal appeared even after heat treatment of the meat prior to DNA extraction at 100 °C up to 90 min. In addition the DNA extracted form autoclaved meat yielded a clear Amplicon as well (Figure 4). Tartaglia and others (1998) could also amplify the bovine DNA from autoclaved samples.

According to the present results a simple and efficient method was utilized to detect the presence of dog or cat DNA in meat mixtures containing less than 0.01% meat of one of these two species. The PCR reaction was not considerably affected by a prolonged heat denaturation of the rendering process, and its sensitivity proved to be high also when samples were further subjected to autoclaving at 121 °C for 30 min under 1.1 bar pressure. The use of endonuclease digestion of the PCR product provides a rapid tool to further confirm the origin of the DNA sequences.

**Table 3—Sequence of the species-specific oligonucleotide primers Dog F, Cat F and Dog R, Cat R (coding strand) and the homologous sequences from other meat animal species. Identical nucleotide sequences are indicated by dots.**

| | Primer | |
|---|---|---|
| **Homolog** | **Dog F** | **Dog R (coding strand)** |
| Dog | GGAGTATGCTTGATTCTACAG | GGCATTCATTCCACTTCT |
| Cat | .....C...C.A..CT....A | A...A........A.C.. |
| Cattle | ...A.C...C.A..C.....A | T..TC.A..C..C..A.. |
| Pig | ..CA.C...C.A..CT.G..A | AATT..A..G..CA.A.. |
| Sheep | ..CA.T...T.A...T....G | .T.A..T..A..CC.C.. |
| Chicken | .C...C...C.C..CACC..A | CTTCC.A..C..CT.C.. |
| Turkey | .C........C.C..CACT..A | TCTCC.T..C..CT.C.. |
| | **Cat F** | **Cat R (coding strand)** |
| Cat | CTCATTCATCGATCTACCCA | TCTTCTAGTACTAGTTTTAACACTCAC |
| Dog | .........T..C..C..AG | AGCCT..C.C...C.CC...TC..A.T |
| Cattle | TG..........C..T..AG | GGCC..CT.....A..C..G.T..A.T |
| Pig | .G.......T..C..C..AG | AGCCT..T.TA..A.AC...TC..ACT |
| Sheep | .G.......T.....C..AG | TGC.A.CC.....A.CC.C.TC..CAT |
| Chicken | ...CC.A.....C..C..AG | CT.AACTC.CA..C.CACCC..T..CT |
| Turkey | ......A.....C..C..A. | ...AAC.A.CA..C.AACCC.CT.ACT |

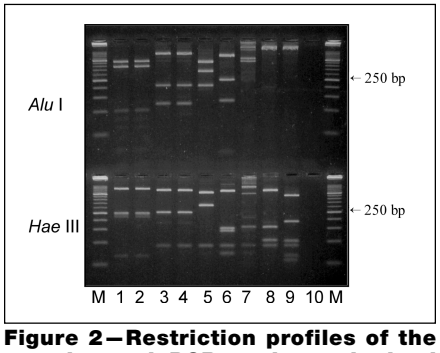


**Figure 2—Restriction profiles of the *cytochrome b* PCR products obtained from dog (1-2); cat (3-4); cattle (5); pig (6); sheep (7) chicken (8); turkey (9) and a negative control (10), after digesting with *Alu* I (top) *Hae* III (bottom). (M) DNA molecular weight marker XIII 50 bp ladder (Roche)**

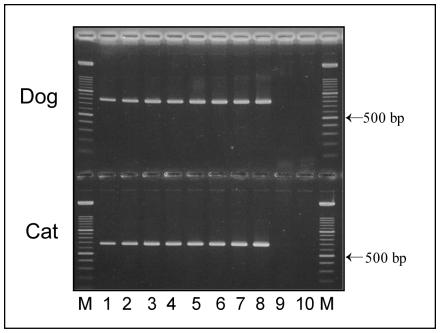


**Figure 3—Specific amplicon of dog (top) and cat (bottom) with a size of 808 bp and 672 bp, respectively. Lane 1 to 9: meat mix containing 0.01, 0.1, 0.5, 1, 2, 5, 10, 100 and 0% of dog and cat meat, respectively; Lane 10 negative control; (M) DNA molecular weight marker XIV 100 bp ladder (Roche)**

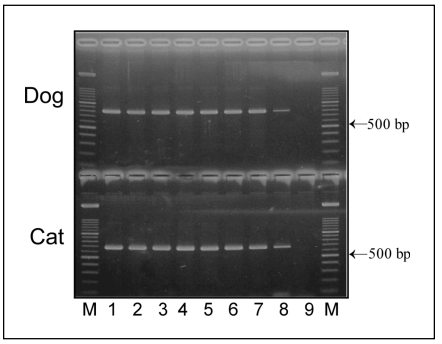


**Figure 4—Specific amplicon of dog (top) and cat (bottom) with a size of 808 bp and 672 bp, respectively. Lane 1 to 7: meat mix after heating at 100 °C for 0 min, 2 min, 5 min, 10 min, 30 min, 60 min and 90 min, respectively. Lane 8: the meat was autoclaved at 121 °C, 1.1 bar for 30 min. Lane 9: negative control; for marker see Figure 3.**

## Conclusion

TO OUR KNOWLEDGE THIS IS THE FIRST report of a molecular approach to test for the presence of dog and cat meat in meat

products and animal feed. In our opinion, this method—being highly sensitive, reproducible, rapid, simple, and not expensive—could be used for food and animal feed analysis.

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# Toward an international standard for PCR-based detection of *Escherichia coli* O157 Part 1. Assay development and multi-center validation

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**Abstract**

As part of a major European research project, a diagnostic PCR assay, including an internal amplification control, was developed and validated in a collaborative trial for the detection of *Escherichia coli* O157. The assay is based on amplification of sequences of the *rfbE* O157 gene. The collaborative trial, including 12 international laboratories, was carried out in two phases: phase (a) was performed with identical PCR reagents, including the internal control, provided by the sending laboratory; phase (b) was performed on the same samples and internal control but using in-house PCR reagents of own choice. Phase (a) showed an inclusivity (detection of target strains) of 96.8% and the exclusivity (negative response from nontarget strains) was 100%. The overall performance resulted of phase (a) in an accordance of 98.8, concordance of 98.6, and a concordance odds ratio of 1.11. Phase (b) results showed an accuracy of 100% with all partners and by using different polymerase types and thermocycler models. This indicates that the assay, under consideration as an international standard, was just as reproducible between laboratories, as repeatable within a laboratory. The assay is taken further for validation on carcass-rinse samples.


*Keywords:* *Escherichia coli* O157; *rfbE* gene; Validation; Standard

## 1. Introduction

Enterohemorrhagic *Escherichia coli* O157:H7 is an important foodborne pathogen and a causative agent of hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS). The most common serotype of this category of diarrheagenic *E. coli* is O157:H7 (Keene et al., 1994; Heuvelink et al., 1996; Pierard et al., 1997; Lindqvist et al., 1998; Little and de Louvois, 1998; Tarr et al., 1999; Schmidt et al., 2000). *E. coli* strains which express the lipopolysaccharide (LPS) O-antigen 157 (O157 strains) are commonly associated with severe clinical manifestations, including bloody diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (Tarr, 1994). The O-antigen is encoded by the *rfb* gene cluster which is comprised of 12 genes. The

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fifth gene, *rfbE*, is specific for *E. coli* O157 (Wang and Reeves, 1998).

Practical and reliable detection methods for screening food, environmental and clinical samples for the presence of *E. coli* O157 are needed for improving food safety and for studying the ecology and epidemiology of this pathogen. The polymerase chain reaction (PCR) offers the possibility of rapid and accurate analysis. However, for a PCR-based method to be completely suitable for implementation as an analytical tool, its diagnostic accuracy must be thoroughly evaluated and demonstrated (Hoorfar and Cook, 2002; Malorny et al., 2003). There are many publications regarding PCR detection of *E. coli* O157 (Desmarchelier et al., 1998; Grant, 2003; Gryko et al., 2002; Maurer et al., 1999; Osek, 2002; Osek and Dacko, 2001; Wang et al., 2002) but none of the methods reported therein have been validated for use by a full scale inter-laboratory collaborative trial. The possibility of successful reproduction of results is an absolute prerequisite for adoption of a PCR-based detection method as a standard diagnostic tool (Hoorfar and Cook, 2002; Malorny et al., 2003). Selectivity and detection limit are the critical parameters which define the accuracy of a PCR assay. The work presented here was part of a major research project, including 34 laboratories from 21 countries (www.pcr.dk), funded by the European Union (Hoorfar, 1999). The work is distinguished from similar approaches by its noncommercial nature, use of open-formula products and complete transparency for the benefit of diagnostic laboratories and general public. We report a PCR assay for *E. coli* O157, which was developed with a view to providing a diagnostic method, which will be suitable for routine adoption and future proposal as a standard. Additionally, the present paper describes the validation of the performance characteristics of the assay by a collaborative trial involving 12 European laboratories.

## 2. Materials and methods

### 2.1. Bacterial strains

A total of 155 strains and isolates (Table 1) were used for evaluation of the selectivity of the PCR assay. These included 32 *E. coli* O157 isolates from humans ($n=17$), bovine ($n=3$), meat and meat products ($n=9$) and milk ($n=3$). The 123 non-O157 isolates and strains included *E. coli* isolates from humans ($n=14$), bovine ($n=29$), meat and meat products ($n=40$) and milk ($n=2$), as well as *Salmonella* spp. ($n=3$), *H. alvei* ($n=2$), *Citrobacter freundii* ($n=1$), *Enterobacter aerogenes* ($n=1$), *Shigella* spp. ($n=2$), *Serratia marcescens* ($n=1$) and *Vibrio* spp. ($n=28$). The cultures, including species or serogroup reference strains, were obtained from the strain collection of the Institute of Veterinary Food Science. The list of strains in Table 1, proposed to the standard organisations, is the result of international scientific consensus on the minimum requirement on any similar validation in the future. The present paper is the first complete presentation of the strains.

### 2.2. DNA extraction

DNA was prepared from whole cell suspensions by the following method. Cultures were gown to exponential phase, then cells were pelleted by centrifugation at 10,000 × *g* for 10 min. The cells were washed in double-distilled water, and boiled for 10 min at 100 °C. After centrifugation (10,000 × *g*, 5 s) to pellet cell debris, the solution was stored at 4 °C. The DNA of the non-*E. coli* strains was isolated by using the DNeasy isolation kit (Qiagen, Darmstadt, Germany) according to the manufacturer's instructions. Briefly, The washed bacteria were lysed and followed by binding of the DNA to the column (Qiagen). After washing steps the DNA was eluted with 100 μl elution buffer (Qiagen).

### 2.3. Oligonucleotide primer design

The oligonucleotide primers Gi-O157-I 5′-CGA GTA CAT TGG CAT CGT G-3′ (position no. 4596) and Gi-O157-II 5′-ATT GCG CTG AAG CCT TTG-3′ (position no. 5079) were selected from a region of the *rfbE* gene sequence of *E. coli* O157 (accession no. AF061251) by using the computer program Oligo, Primer Analysis Software (version 4.0, National Biosciences, Annapolis lane, Plymouth, MN, USA, 1991). The primers were synthesised by MWG-Biotech (Ebersberg, Germany).

Table 1
The reference *E. coli* O157 and non-O157 strains used for the validation of the *E. coli* O157 PCR assay

| No. | Strains | Serovar | Source | Virulence factors | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | *vtx1*[a] | *vtx2* | *vtx2c* | *vtx2d* | *vtx2e* | *eae*[b] |
| 1 | B 2324 | O157.H7 | bovine feces | – | – | + | – | – | + |
| 2 | B 2325 | O157.H7 | bovine feces | – | – | + | – | – | + |
| 3 | B 2482 | O157.H7 | bovine feces | – | – | + | – | – | + |
| 4 | HUS 1249 | O157.H7 | human stool, HUS | + | + | – | – | – | + |
| 5 | HC 2044 | O157.H7 | human stool, HC | + | + | – | – | – | + |
| 6 | 1271-84 | O157.H7 | human stool, HUS | – | + | + | – | – | + |
| 7 | 3526-87 | O157.H7 | human stool, HC | + | + | + | – | – | + |
| 8 | EDL 931 | O157.H7 | human stool, HC | + | – | – | – | – | + |
| 9 | A 9167-1 | O157.H7 | human stool, HC | + | – | + | – | – | + |
| 10 | A 9218-C1 | O157.H7 | human stool, HC | + | + | – | – | – | + |
| 11 | EDL 933 | O157.H7 | human stool, HC | + | + | – | – | – | + |
| 12 | C 7-80 | O157.H7 | human stool, HC | + | – | + | – | – | + |
| 13 | C 1011-87 | O157.H7 | human stool, HC | – | + | + | – | – | + |
| 14 | E 1047 | O157.H7 | human stool, HC | + | + | – | – | – | + |
| 15 | E 1177 | O157.H7 | human stool, HC | + | + | – | – | – | + |
| 16 | E 4884 | O157.H7 | human stool, HC | + | + | – | – | – | + |
| 17 | C 8/ATCC 43895 | O157.H7 | Hamburger | + | + | – | – | – | + |
| 18 | C 12 | O157.H7 | Salami | + | + | – | – | – | + |
| 19 | 3873 5/1 | O157.H7 | raw milk | + | + | – | – | – | + |
| 20 | Sal 4/LXIV/1 | O157.H7 | ground beef | – | + | + | – | – | + |
| 21 | Sal 57/4/1-99 | O157.H7 | ground beef | – | – | + | – | – | + |
| 22 | NCTC 12079 | O157.H7 | human | + | + | – | – | – | + |
| 23 | 26/22 | O157.H$^-$ | human stool | + | – | + | – | – | + |
| 24 | 7579/95 | O157.H$^-$ | human stool, HUS (Bayern) | – | + | – | – | – | + |
| 25 | D 1 | O157.H$^-$ | Nürnberger Rostbratwurst | – | – | + | – | – | + |
| 26 | Sal 2/LI/1 | O157.H$^-$ | ground beef | – | – | – | – | – | – |
| 27 | Schu 2 | O157.H16 | ground beef | – | – | – | – | – | – |
| 28 | H 4 | O157.H16 | ground beef | – | – | – | – | – | – |
| 29 | #51 | O157.H$^-$ | raw milk | – | – | – | – | – | – |
| 30 | ’15/10/2 | O157.H$^-$ | milk | – | + | + | – | – | + |
| 31 | 730 | O157.H$^-$ | intestine, sheep | – | + | + | – | – | + |
| 32 | NCTC 12080 | O157.H$^-$ | human | – | + | + | – | – | + |

Non-O157 strains

| No. | Strains | Serovar | Source | Virulence factors | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | *vtx1* | *vtx2* | *vtx2c* | *vtx2d* | *vtx2e* | *eae* |
| 1 | B 1780 | O75.H8 | bovine feces | – | – | – | + | – | – |
| 2 | B 1834 | O136.H19 | bovine feces | + | – | – | – | – | – |
| 3 | B 1835 | O10.H21 | bovine feces | + | – | – | – | – | – |
| 4 | B 2114 | O39.H40 | bovine feces | – | + | – | – | – | – |
| 5 | B 2405 | O3.H$^-$ | bovine feces | + | – | – | – | – | – |
| 6 | B 2477 | Or.H18 | bovine feces | + | + | – | – | – | – |
| 7 | B 2480 | O126.H20 | bovine feces | – | + | – | – | – | – |
| 8 | T 173 | O22.H8 | bovine feces | – | + | – | – | – | – |
| 9 | T 423 | O82.H8 | bovine feces | + | – | + | – | – | – |
| 10 | T 464 | O116.H21 | bovine feces | + | – | + | – | – | – |
| 11 | T 476 | O105.H18 | bovine feces | + | – | – | – | – | – |
| 12 | T 509 | Ont.H29 | bovine feces | – | + | – | + | – | – |
| 13 | 173a 3422 20/8 | O26.H11 | bovine feces | + | – | – | – | – | + |

*(continued on next page)*

Table 1 (*continued*)

| Non-O157 strains | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| No. | Strains | Serovar | Source | Virulence factors | | | | | |
| | | | | *vtx1* | *vtx2* | *vtx2c* | *vtx2d* | *vtx2e* | *eae* |
| 14 | 173a 3744 37/4 | O103.H2 | bovine feces | + | – | – | – | – | + |
| 15 | 173a 3744 41/1 | O48.H$^-$ | bovine feces | + | – | – | – | – | + |
| 16 | 173a 4339 7/2 | O74.H39 | bovine feces | + | + | – | – | – | – |
| 17 | 173a 4339 17/1 | O118.H$^-$ | bovine feces | + | + | – | – | – | + |
| 18 | 51/9 | O146.H28 | bovine feces | + | – | + | – | – | – |
| 19 | 54/5 | Ont.H$^-$ | bovine feces | + | + | – | – | – | – |
| 20 | T 710 | O91.H10 | bovine feces | – | + | – | – | – | – |
| 21 | 173a 2032 11/1 | Or.H3 | bovine feces | + | – | – | – | – | – |
| 22 | 173a 2032 11/4 | O146.H28 | bovine feces | + | – | + | – | – | – |
| 23 | 173a 2032 12/1 | O8.H21 | bovine feces | + | + | – | – | – | – |
| 24 | 173a 2032 13/1 | O46.H$^-$ | bovine feces | + | + | – | – | – | – |
| 25 | 173a 2032 14/1 | O146.H28 | bovine feces | + | – | + | – | – | – |
| 26 | 173a 3025 17/1 | O32.H8 | bovine feces | – | + | + | – | – | – |
| 27 | 173a 329 1/2 4 | O111.H$^-$ | bovine feces | + | – | – | – | – | + |
| 28 | 173a 1035 31/1 | O118.H$^-$ | bovine feces | + | – | – | – | – | + |
| 29 | 173a 3025 18/2 | O77.H18 | bovine feces | – | + | – | – | – | – |
| 30 | ED 31 | O111.H$^-$ | stool, human | + | + | – | – | – | + |
| 31 | 78/92 | O111.H$^-$ | stool, human | + | – | – | – | – | + |
| 32 | 4166/94 | O26.H$^-$ | stool, human | + | – | – | – | – | + |
| 33 | 7828/95 | O103.H2 | stool, human | + | + | – | – | – | + |
| 34 | O.55 | O55.H$^-$ | stool, human | – | – | – | – | – | + |
| 35 | E 43 | O55 | stool, human | – | – | – | – | – | – |
| 36 | 12/6 | Or.H$^-$ | stool, human | + | + | – | – | – | + |
| 37 | 2704/94 | O8.H$^-$ | stool, human | – | – | – | – | – | + |
| 38 | 3639/96 | O26.H$^-$ | stool, human | – | + | – | – | – | + |
| 39 | 3379/96 | O55.H$^-$ | stool, human | + | – | – | – | – | – |
| 40 | 7465/96 | O145.H$^-$ | stool, human | + | – | – | – | – | + |
| 41 | 3759/96 | O113.H4 | stool, human | – | + | – | – | – | – |
| 42 | 4865/96 | O145.H$^-$ | stool, human | + | – | – | + | – | – |
| 43 | O.26 (5382) | O26.H11 | stool, human | + | – | – | – | – | + |
| 44 | Y2-447 | O55 | beef | – | – | – | – | – | – |
| 45 | Sal 3/LI/3 | O113.H$^-$ | ground beef | + | + | – | – | – | – |
| 46 | Sal 4/LIV/1 | Or.H23 | ground beef | + | + | – | – | – | – |
| 47 | M 13 | Ont.H23 | ground beef | + | + | – | – | – | – |
| 48 | Sal 2/LXVI/3 | O7.H16 | ground beef | + | – | – | – | – | – |
| 49 | Sal 8/LXVI/1 | O91.H$^-$ | ground beef | – | + | + | – | – | – |
| 50 | Sal 2/5/1-98 | O113.H4 | ground beef | – | + | – | – | – | – |
| 51 | Sal 29/5/1-98 | O113.H$^-$ | ground beef | + | + | – | – | – | – |
| 52 | Sal 33/4/1-98 | O22.H8 | ground beef | + | + | – | – | – | – |
| 53 | Sal 34/3/1-98 | Ont.H19 | ground beef | + | + | – | – | – | – |
| 54 | Sal 36/1/1-98 | O82.H8 | ground beef | + | + | + | – | – | – |
| 55 | Sal 39/5/1-98 | O113.H21 | ground beef | – | + | – | – | – | – |
| 56 | Sal 34/1/1-98 | O91.H21 | ground beef | + | + | + | – | – | – |
| 57 | Sal 42/5/1-98 | Ont.H$^-$ | ground beef | + | + | – | – | – | – |
| 58 | L198-17460 SK 1 | O8.H8 | ground beef | + | – | – | – | – | – |
| 59 | W 49/3/1-98 | O113.H4 | ground beef | + | + | – | – | – | – |
| 60 | W 50/2/1-98 | Ont.H18 | ground beef | + | + | + | – | – | – |
| 61 | W 51/1/1-98 | O113.H21 | ground beef | – | – | + | – | – | – |
| 62 | Sal 37/3/1-99 | Ont.H2 | ground beef | + | + | – | – | – | – |
| 63 | Sal 38/3/1-99 | O103.H42 | ground beef | + | + | – | – | – | – |
| 64 | W 51/4/1-98 | O146.H21 | ground beef | + | – | – | + | – | – |

Table 1 (*continued*)

| Non-O157 strains | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| No. | Strains | Serovar | Source | Virulence factors | | | | | |
| | | | | *vtx1* | *vtx2* | *vtx2c* | *vtx2d* | *vtx2e* | *eae* |
| 65 | Sal 54/1/1-98 | O156.H$^-$ | ground beef | + | – | – | – | – | + |
| 66 | Sal 1/2/1-99 | O153.H25 | ground beef | – | + | – | – | – | – |
| 67 | Sal 7/4/1-99 | O113.H21 | ground beef | – | + | – | – | – | – |
| 68 | Sal 16/1/1-99 | Ont.H21 | ground beef | + | – | + | – | – | – |
| 69 | Sal 19/7/1-99 | O113.H4 | ground beef, burger | + | + | – | – | – | – |
| 70 | Si 3303/4 | O9.H10 | mixed ground meat | – | – | – | – | + | – |
| 71 | Y1-552 | O121.H10 | fried sausage | – | – | – | – | + | – |
| 72 | C 6 | O111 | salami | + | + | – | – | – | + |
| 73 | Si 1280 | 065.H$^-$ | mettwurst | + | – | – | – | – | – |
| 74 | L198-18438 SK 1 | O6.H10 | intestine | + | – | – | – | – | – |
| 75 | 8/6 | O138.H8 | milk | – | + | – | – | – | – |
| 76 | DS 1/1-99 | O91.H21 | raw milk | + | + | + | – | – | – |
| 77 | Pt 252 | O156.H25 | sheep, carcas | + | – | – | – | – | + |
| 78 | Pt 256 | O156.H$^-$ | sheep, carcas | + | – | – | – | – | + |
| 79 | Pt 268 | O107.H11 | sheep, carcas | + | – | – | – | – | + |
| 80 | Pt 270 | O156.H25 | sheep, carcas | + | – | – | – | – | + |
| 81 | LK 1 | O4.H$^-$ | lamb meat | + | – | – | + | – | – |
| 82 | D 20 | O8.H$^-$ | lamb kotelett | + | – | – | – | – | – |
| 83 | D 59 | Ont.H$^-$ | lamb meat | + | – | – | + | – | – |
| 84 | 87D8 | Ont.H$^-$ | pork | – | – | – | – | + | – |
| 85 | ED 43 | O101.H$^-$ | pork | – | – | – | – | + | – |
| 86 | *Salmonella typhimurium* SV 4/1 | | | | | | | | |
| 87 | *Salmonella typhimurium* SV 6/3 | | | | | | | | |
| 88 | *Salmonella enteritidis* SV 5/19 | | | | | | | | |
| 89 | *Hafnia alvei* Sal 55/4a-99 | | | | | | | | |
| 90 | *Hafnia alvei* Sal 60/8a-99 | | | | | | | | |
| 91 | *Citrob. freundii* Sal 7/15a-00 | | | | | | | | |
| 92 | *Enterobacter aerogenes* 1779/89 | | | | | | | | |
| 93 | *Shigella sonnii* 7887 | | | | | | | | |
| 94 | *Shigella boydii* Typ 16 2710-54 | | | | | | | | |
| 95 | *Serratia marcescens* 2122/93 | | | | | | | | |
| 96 | *Vibrio cholerae* CH 931 | O11 | | | | | | | |
| 97 | *Vibrio cholerae* CH 933 | O103 | | | | | | | |
| 98 | *Vibrio cholerae* CH 1329 | O27 | | | | | | | |
| 99 | *Vibrio cholerae* CH 1330 | O186 | | | | | | | |
| 100 | *Vibrio cholerae* CH 1332 | O2 | | | | | | | |
| 101 | *Vibrio cholerae* CH 1354 | O51 | | | | | | | |
| 102 | *Vibrio cholerae* CH 1371 | O10 | | | | | | | |
| 103 | *Vibrio cholerae* CH 1373 | O18 | | | | | | | |
| 104 | *Vibrio cholerae* CH 1374 | O42 | | | | | | | |
| 105 | *Vibrio cholerae* CH 1461 | O10 | | | | | | | |
| 106 | *Vibrio cholerae* CH 1464 | O7 | | | | | | | |
| 107 | *Vibrio cholerae* CH 1473 | O40 | | | | | | | |
| 108 | *Vibrio cholerae* CH 1474 | O40 | | | | | | | |
| 109 | *Vibrio cholerae* CH 1475 | O40 | | | | | | | |
| 110 | *Vibrio cholerae* CH 1476 | O40 | | | | | | | |
| 111 | *Vibrio cholerae* CH 1528 | O10 | | | | | | | |
| 112 | *Vibrio parahaemolyticus* CH 1314 | O1:K33 | | | | | | | |
| 113 | *Vibrio parahaemolyticus* CH 1315 | O6:K46 | | | | | | | |
| 114 | *Vibrio parahaemolyticus* CH 1316 | O6:K46 | | | | | | | |

*(continued on next page)*

Table 1 (*continued*)

| Non-O157 strains | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| No. | Strains | Serovar | Source | Virulence factors | | | | | |
| | | | | *vtx1* | *vtx2* | *vtx2c* | *vtx2d* | *vtx2e* | *eae* |
| 115 | *Vibrio parahaemolyticus* CH 1353 | O1:K66 | | | | | | | |
| 116 | *Vibrio parahaemolyticus* CH 1385 | O1:KuK | | | | | | | |
| 117 | *Vibrio vulnificus* CH 988 | O4 | | | | | | | |
| 118 | *Vibrio vulnificus* CH 1345 | O3 | | | | | | | |
| 119 | *Vibrio vulnificus* CH 1593 | O1 | | | | | | | |
| 120 | *Vibrio vulnificus* CH 1595 | O7 | | | | | | | |
| 121 | *Vibrio vulnificus* CH 1596 | rauh | | | | | | | |
| 122 | *Vibrio mimicus* CH 386 | O24 | | | | | | | |
| 123 | *Vibrio mimicus* CH 759 | O111 | | | | | | | |

[a] *vtx1*: Verocytotoxin = *stx*.
[b] *eae*: *E. coli* attaching and effacing gene.

### 2.4. Optimization of the PCR conditions

Several parameters of the PCR were optimised: $MgCl_2$, primer and dNTP concentration, annealing temperature, and cycle number. Optimisation was performed using DNA from 8 target and 10 nontarget strains. The concentration of $MgCl_2$ was tested over in a range of 0.5–2.5 μM. The primer concentration was tested over a range of 0.1–1.25 μM. The dNTP concentration was tested over a range of 40–250 μM. Annealing temperatures were tested over a range of 40–60 °C. Cycle numbers ranged from 20 to 35 cycles. After each reaction was performed, 10 μl was electrophoresed and stained as above. The signal from each test was compared, and the optimal conditions were adopted.

### 2.5. Final PCR conditions

The PCR mixture (25 μl) contained 1 μl of each primer (10 pmol/μl), 1 μl dNTP (10 mmol, Roche, Mannheim, Germany), 2.5 μl 10 × thermophilic buffer with 25 mM $MgCl_2$ (PE Applied Biosystems, Weiterstadt, Germany), 1 μl (5.23 fg) *rfbE* internal amplification control (IAC, Abdulmawjood et al., 2002), 0.1 μl *Taq* DNA polymerase (5 U/μl, PE Applied Biosystems) and 15.9 μl molecular biology-grade water. Finally, 2.5 μl DNA solution or cell suspension was added to each reaction mix. The PCR was carried out in a thermal cycler (PE GeneAmp PCR system 9600, PE Applied Biosystems) with the following program: 1 × 3 min precycle at 93 °C, 30 × 15 s at 93 °C, 15 s at 60 °C and 30 s at 72 °C followed by a final extension incubation of 72 °C for 5 min. The presence of PCR products was determined by electrophoresis of 10 μl of the reaction product in a 2% agarose geld (Appligene, Heidelberg, Germany), with Tris–acetate electrophoresis buffer TAE (0.04 mol/l Tris, 0.001 mol/l EDTA, pH 7.8) and a 50 bp DNA ladder (Roche) as molecular marker.

### 2.6. Construction of an internal amplification control for rfbE gene PCR

An internal amplification control (IAC) was performed according to Abdulmawjood et al. (2002) by using the same *rfbE* specific primer pair and the same gene as template lacking an internal fragment.

### 2.7. Determination of the detection probability

The determination of the detection probability was performed according to the method of Knutsson et al. (2001). *E. coli* O157 (strain no. EDL 931) was grown to exponential phase in BHI broth, then stored at −20 °C until use. To determine the exact cfu $ml^{-1}$, the cell suspension was serially diluted 10-fold in 0.9% (w/v) NaCl within the concentration range of $10^6$–$10^1$ cfu/ml and aliquots plated on PC agar (Casein-peptone Dextrose Yeast Extract Agar, Merck, Darmstadt, Germany). The frozen cell suspension was thawed, then a 10-fold dilution series was prepared in double-distilled water. A PCR was performed on each concentration, using 2.5 μl cell suspension added directly to

the reaction. This was performed in 10 independent experiments, i.e. 10 independently grown cultures were diluted, and the dilutions tested. The total number of positive signals obtained was expressed as a percentage. This was compared with the number of cells contained in the reaction, as calculated from the dilution factor of the original count. The detection probability of the PCR assay was obtained by plotting the relative observed positive PCR reactions against the concentration of the cell suspension (Langton et al., 2002). A sigmoidal line fitting was performed.

### 2.8. Design of the collaborative trial

A total of 12 laboratories participated from Austria, Denmark, England, France, Germany (three laboratories), Ireland, the Netherlands, Slovakia, Spain and Sweden. The ring trial included two phases: phase (a) was performed with PCR reagents provided by institute of Veterinary Food Science. Phase (b) was performed by using in-house PCR reagents of each partner. The primers, internal amplification control (IAC) and coded DNA samples were sent for the originating laboratory in Giessen, Germany. Each laboratory received 17 coded "blind" DNA samples, including seven *E. coli* O157 and 10 non-*E. coli* O157 DNA (Table 2), one negative and one positive control DNA, IAC template and reagents for performing PCR, including Ampli*Taq* polymerase. All DNA samples were prepared from $1 \times 10^8$ cfu/ml cultures. Each participant was sent a detailed trial chronology, a standard operating procedure (SOP; available at http://www.pcr.dk), and a test report on which to record the results and return to the trial leader for analysis.

Table 2
Bacterial strains used in the collaborative trial of the *E. coli* O157 PCR assay

| Strain | Number in validation trial | Serotype |
|---|---|---|
| *E. coli* NCTC 12079 | 1 | O157.H7 |
| *E. coli* ATCC 25922 | 2 | O23:H15 |
| *E. coli* 15/10/2 | 3 | O157.H$^-$ |
| *E. coli* Scheu 2 | 4 | O157.H16 |
| *E. coli* 7465/96 | 5 | O145.H$^-$ |
| *Shigella sonnii* 7887 | 6 | |
| *E. coli* NCTC 12080 | 7 | O157.H$^-$ |
| *E. coli* 173a 3744 37/4 | 8 | O103.H2 |
| *E. coli* Sal 57/4/1-99 | 9 | O157.H7 |
| *E. coli* 7579/95 | 10 | O157.H$^-$ |
| *Vibrio cholerea* CH 933 | 11 | |
| *Shigella boydii* Typ 16 2710-54 | 12 | |
| *E. coli* 173a 2106 43/3 | 13 | O145.H16 |
| *C. freundii* Sal 7/15a-00 | 14 | |
| *Hafnia alvei* Sal 60/8a-99 | 15 | |
| *E. coli* O.55 süd Bayern | 16 | O55.H$^-$ |
| *E. coli* EDL 933 | 17 | O157.H7 |

### 2.9. Statistical analysis of the data

The results of the trial were evaluated according to the methods of Langton et al. (2002). These methods are useful for analyzing collaborative trial data regarding qualitative microbiological methods (Scotter et al., 2001). In this study, the accuracy parameters, sensitivity and specificity of the assay, are termed inclusivity and exclusivity, respectively (Hoorfer and Cook, 2002). Inclusivity is here defined as the percentage of target DNA samples that gives a positive signal. Exclusivity is defined as the percentage of nontarget samples that gives a negative signal (i.e. only the IAC signal appeared). Confidence intervals for the accuracy parameters were calculated by the method of Wilson (1927). Repeatability and reproducibility were determined by calculating the accordance and concordance values (Langton et al., 2002; Scotter et al., 2001). Accordance is defined as the percentage chance of finding the same result (i.e. both positive and negative whether correctly or not) from two identical DNA samples analyzed in the same laboratory under standard repeatability conditions. Concordance is defined as the percentage chance of finding the same result from two identical samples analyzed in different laboratories under standard repeatability conditions. The calculations take account of differing replication in different laboratories by weighting results appropriately. In the present trial, all results were combined for this determination, identical samples being therefore defined as containing either target or nontarget DNA. The concordance odds ratio, or COR (Langton et al., 2002), was calculated in order to assess the degree of between-laboratory variation in results. Confidence intervals for accordance and concordance were calculated by the "bootstrap" method of Davison and Hinckley (1997); laboratories were assumed representative of all laboratories in the "end-user population" of laboratories, not just the participating ones, in this analysis.

## 3. Results

### 3.1. Optimization of rfbE gene PCR

Fig. 1 shows the signals obtained from PCRs containing varying concentrations of $MgCl_2$. No signals were obtained using 0.5 mM $MgCl_2$. Specific and clear bands were obtained using 1.0, 1.5, 2.0 and 2.5 mM. The concentration recommended by the polymerase supplier (1.5 mM) was adopted. The signals obtained from PCRs containing varying concentrations of primers and dNTPs were all similar in their clarity and specificity (not shown). Annealing temperatures of 40, 45, 50 and 55 °C produced bands from nontarget samples. An annealing temperature of 60 °C was chosen. At 20 cycles, faint signals were

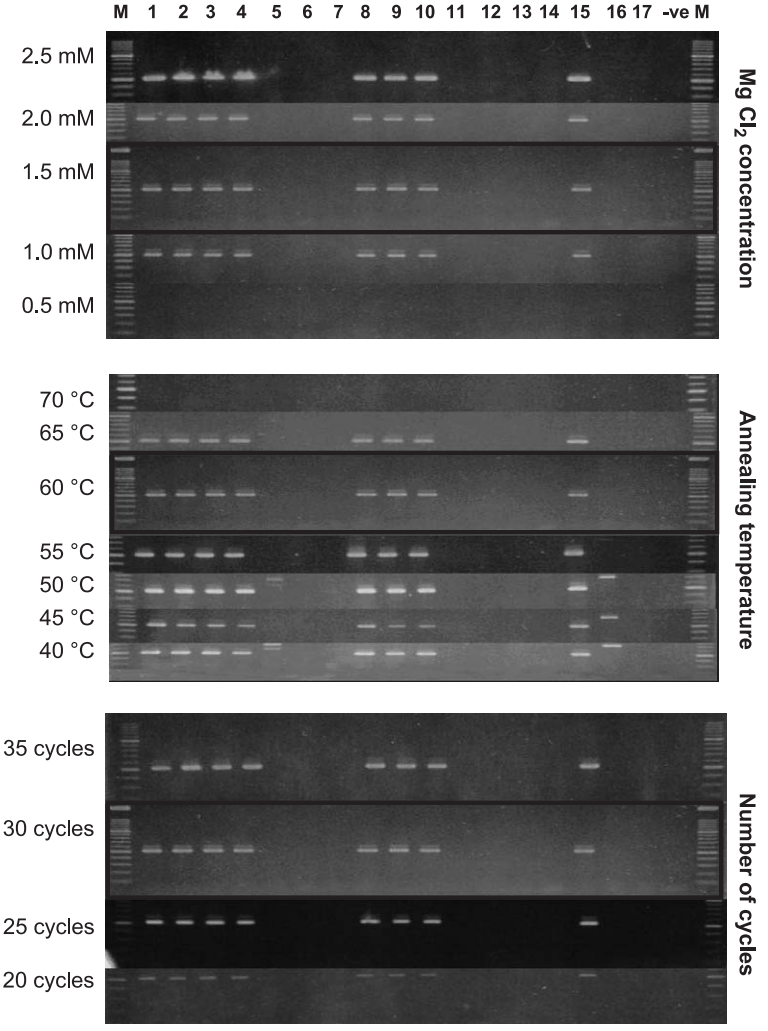


Fig. 1. PCR amplification of DNA from *E. coli* O157 and other bacterial strains using various annealing temperatures, Mg $Cl_2$ concentration and various number of cycles. Lane M, marker 100 bp DNA ladder (Roche); lane 1, *E. coli* O157:H7 (NCTC 12079); lane 2, *E. coli* O157:H7 (Sal 57/4/1-99); lane 3, *E. coli* O157:H16 (Schu 2); lane 4, *E. coli* O157:H? (MG 3/3-00); lane 5, *E. coli* O55:$H^-$ (O55 Südbayern); lane 6, *E. coli* O145:$H^-$ (7465/96); lane 7, *E. coli* O145:H16 (173a 2106 43/3); lane 8, *E. coli* O157:$H^-$ (NCTC 12080); lane 9, *E. coli* O157:$H^-$ (15/10/2); lane 10, *E. coli* O157:$H^-$ (7579/95); lane 11, *H. alvei* (Sal 60/8a-99); lane 12, *C. freundii* (Sal 7/15a-00); lane 13, *Shigella sonnei* (7887); lane 14, *Shigella boydii* Typ 16 (2710-54); lane 15, *E. coli* O157:H7 (EDL 933); lane 16, *E. coli* O23:H15 (ATCC 25922); lane 17, *E. coli* O103 H2 (173a 3744 37/4); lane M, marker 100 bp DNA ladder.

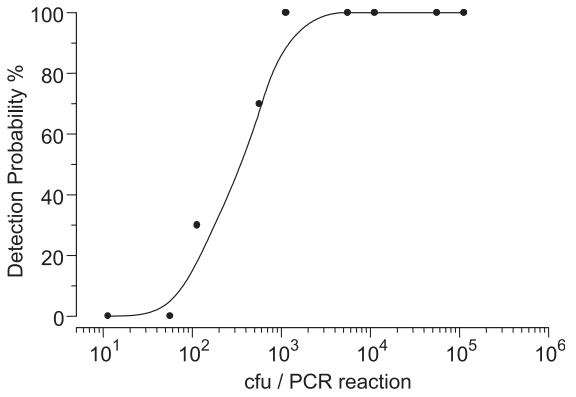


Fig. 2. Detection probability of the *rfbE* PCR assay. The graph shows a sigmoidal fit of data points generated from 10 independent experiments.

produced. Thirty cycles was chosen for the final PCR.

### 3.2. Demonstration of the selectivity of the PCR

The assay was tested on DNA extracted from each strain in Table 1. The assay was 100% inclusive and 100% exclusive. The presence of an IAC signal in all negative results demonstrated that each was a true negative, and not the result of inhibition of the PCR.

Table 3
Participants' results in the collaborative trial (phase 2a) of the *rfbE* PCR assay

| Strain number | Number of positive signals obtained | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Expected[a] | Participant | | | | | | | | | | | |
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9[b] | 10 | 11 | 12 |
| 1 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 2 | 3 | 3 | 3 |
| 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 2 | 2 | 3 | 3 |
| 4 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 2 | 2 | 3 | 3 |
| 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 7 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 2 | 3 | 3 | 3 |
| 8 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 9 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 2 | 3 | 3 | 3 |
| 10 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 2 | 2 | 3 | 3 |
| 11 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 12 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 13 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 14 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 15 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 16 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 17 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 2 | 2 | 3 | 3 |

[a] The number of positive PCR signals expected from triplicate reactions.

[b] Only duplicate reactions performed by this participant.

Table 4
Statistical evaluation of the collaborative trial of the *E. coli* O157 PCR assay

| Inclusivity (%) | Exclusivity (%) | Accordance (%) | Concordance (%) | Concordance odds ratio (COR) |
|---|---|---|---|---|
| 96.8 (92.0, 98.7)[a] | 100 – | 98.8 (96.3, 100) | 98.6 (96.0, 100) | 1.11 (1.00, 1.14) |

[a] Numbers in parentheses are the lower and upper 95% confidence intervals.

### 3.3. Detection probability

Fig. 2 shows the results of the detection limit evaluation. The detection probability at a concentration of $1.125 \times 10^3$ cfu/reaction was 100% with the suitable concentration of IAC. However, the detection probability of a $5.6 \times 10^2$ and $11.2 \times 10$ cfu/reaction was 70% and 30% respectively.

### 3.4. Results of collaborative trial

Table 3 shows each participant results in the collaborative trial phase (a). One participant performed the test in duplicate only. Only one partner reported false negative results. No partner reported false positive results. While in phase (b) (in house reagents) showed an accuracy of 100% with all partners and by using 5 different polymerase and 4 thermocycler models. No false positive or false negative was reported (data not shown).

### 3.5. Statistical analysis of the collaborative trial

Table 4 shows the performance assessments of the assay in relation to its selectivity.

## 4. Discussion

The O157 *rfbE* PCR assay presented here is a highly selective and rapid method for the confirmation of the O157 serotype. The assay detected all strains of

*E. coli* O157, whether or not they are pathogenic. This will be useful as part of a screening method for *E. coli* O157 in many sample types. However, further tests should be performed, such as PCR for detecting the *VT* or *eaeA* genes (Gannon et al., 1992; Karch and Meyer, 1989; Louie et al., 1994), to confirm a pathogenic genotype.

The PCR reported here was extensively optimised to ensure that it detected only its intended target. Selectivity is most effectively evaluated by testing an assay against a large number of target and nontarget strains. The strains against which the assay were tested formed a comprehensive selection of target and nontarget bacteria, and the results of the tests confirmed the selectivity of the *rfbE* primer set. Desmarchelier et al. (1998) and Paton and Paton (1998) published *E. coli* O157 PCRs which were based on *rfbE* sequences, but Chapman et al. (2001) found that they produced false positive signals with various *E. coli* non-O157 strains, even after extensive optimisation. Chapman et al. (2001) stated that the primer set of Maurer et al. (1999) was fully selective against a strain list comprising 12 *E. coli* O157, 46 *E. coli* non-O157 and 12 non-*E. coli*. The *rfbE* gene is found in both *E. coli* O157 and also *Vibrio* spp. (Bilge et al., 1996). However, neither Maurer et al. (1999) nor Chapman et al. (2001) verified that their PCR excluded cross-reaction with *Vibrio* spp. The PCR described in the present paper was evaluated against a more extensive strain list than the ones used by Maurer et al. (1999) and Chapman et al. (2001), and it included 28 *Vibrio* spp, none of which were detected.

This is the first published *E. coli* O157 assay which includes an IAC. In diagnostic PCR, an IAC is necessary in order to identify false negative results. In food samples, it is important to take into account that components of the food sample or the competing microflora may influence the effectiveness of the PCR, especially by reducing the detection limit. This might cause false negative results. Other PCR inhibitors could also be found in body fluids, soil, bacterial cells and nontarget DNA (Al-Sound and Radström, 2000). The presence of a PCR control product in the absence of the target PCR product allows a differentiation of true and false negative results. A simple approach to develop IAC is to produce PCR products, which differ in size and hence can easily be visualized separately from the native product by agarose gel electrophoresis and ethidium bromide staining (Abdulmawjood et al., 2002; Al-Sound and Radström, 2000). The PCR will detect in present of an IAC $1.125 \times 10^3$ cells per reaction with 100% probability. This compares with the detection of $10^4$ cells which appeared to be the limit of the assay of Maurer et al. (1999).

The evaluation of the diagnostic accuracy was performed "in-house" by our laboratory. A standard method should however be proven reliable through evaluation of its performance in several laboratories (Leclerq et al., 2000; Wang and Reeves, 1998).

It is essential, for effective widespread adoption and implementation, that an analytical method is consistent and reliable. The increasing need to have microbiological methods which are standardized (Lahellec, 1998; Leclerq et al., 2000) requires extensive testing of the robustness of such methods. The most effective way of doing this is through collaborative trials. Such trials should thoroughly examine the performance characteristics of a method, i.e. how accurately its results can be repeated by a single laboratory, or reproduced by different laboratories.

The authors propose that, in regard to the outcome of collaborative trials of PCR assays, inclusivity and exclusivity values higher than 90% should signify that the assay is acceptable for implementation in end-user laboratories. In phases (a) and (b) of the trial, the values obtained fulfilled these criteria.

The PCR assay presented here is qualitative only, therefore the measures of "repeatability" and reproducibility" cannot be used to describe its performance characteristics, as they are applicable only to quantitative analyses (Langton et al., 2002). To enable the precision of qualitative methods to be assessed in the same way that repeatability and reproducibility are used with quantitative methods (e.g. direct plating), appropriate statistical methods were employed. In these statistical methods, accordance and concordance parameters are analogous to repeatability and reproducibility values respectively (Langton et al., 2002). In the collaborative trial phase (a) of the *E. coli* O157 PCR assay, these values were high, indicating that the method may be confidently reproduced and applied in other laboratories. The concordance odds ratio reflects the relative magnitude of the accordance and concordance values (Langton et al., 2002). A concordance odds ratio (COR) of 1.00 or less indicates that two

samples sent to different laboratories will probably produce the same result if the same two samples were analyzed by the same laboratory. A COR significantly greater than 1.00 indicates that variability between different laboratories is greater than the variation which occurs in a single laboratory. In this collaborative trial, the CORs for the results of the analysis of both target and nontarget DNA samples were within the 95% confidence intervals, and therefore not significantly greater than 1.00. This shows that the *E. coli* O157 PCR assay was just as reproducible between laboratories, as repeatable within a laboratory, and signifies that it may be used with confidence in any laboratory. In phase (b) of the collaborative trial, in additional tests performed by all participants (not shown), it was found that 100% selectivity could be obtained using the PCR assay containing five different polymerases: *Taq* DNA polymerase (PE Applied Biosystems), Taq DNA polymerase (Roche), Fast Start Taq (Roche), Platinum *Taq* DNA polymerase (Invitrogen, Groningen Netherlands) and QBiotaq (Qbiogene, Heidelberg, Germany) and 4 different thermocycler models: PE GeneAmp PCR system 9600 (PE Applied Biosystems), PE GeneAmp PCR system 9700 (PE Applied Biosystems) Hybaid, Tuch Down (Hybaid, Heidelberg, Germany) and Mastercycler gradient (Eppendorf, Hamburg, Germany).

In conclusion, the results indicate that the end user has some versatility of choice in the provenance of the assay's reagents. The assay was further successfully validated on spiked samples in another collaborative trial (Abdulmawjood et al., in preparation), and is proposed as a new item for international standard.

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FOOD BIOLOGICAL CONTAMINANTS

# Development, Validation, and Standardization of Polymerase Chain Reaction-Based Detection of *E. coli* O157

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**A diagnostic polymerase chain reaction assay was developed for the detection of *E. coli* O157 as the first part of a multicenter validation and standardization project. The assay is based on amplification of sequences of the *rfbE* O157 gene and includes an internal amplification control. The selectivity of the assay was evaluated against 155 strains, including 32 *E. coli* O157, 38 *E. coli* non-O157, and 85 non-*E. coli*. It was shown to be highly inclusive (100%) and exclusive (100%). The assay has a 100% detection probability of approximately $2 \times 10^3$ cells per reaction.**

Enterohemorrhagic *Escherichia coli* O157:H7 is an important foodborne pathogen and a causative agent of hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS). The most common serotype of this category of diarrheagenic *E. coli* is O157:H7 (1–7). *E. coli* strains which express the lipopolysaccharide (LPS) O-antigen 157 (O157 strains) are commonly associated with severe clinical manifestations, including bloody diarrhea, HC, and HUS (8). The O-antigen is encoded by the *rfb* gene cluster which comprises 12 genes. The fifth gene, *rfbE*, is specific for *E. coli* O157 (9).

Practical and reliable detection methods for screening food, environmental, and clinical samples for the presence of *E. coli* O157 are needed for improving food safety and for studying the ecology and epidemiology of these pathogenic bacteria. The polymerase chain reaction (PCR) offers the possibility of rapid and accurate analysis. However, for a PCR-based method to be completely suitable for implementation as an analytical tool, its diagnostic accuracy must be thoroughly evaluated and demonstrated (10, 11). Selectivity and detection limit are the critical parameters which define the accuracy of a PCR assay.

The work presented here was part of the EU-funded FOOD-PCR (www.pcr.dk) research project (12). It is distinguished from similar approaches by its noncommercial nature, no involvement of closed products, and complete transparency for the benefit of the general public and 34 diagnostic laboratories from 21 countries. A PCR assay for *E. coli* O157 was developed to provide a diagnostic method that will be suitable for routine adoption and future proposal as a standard. The assay is fully selective and contains an internal amplification control.

## METHODS

### *Bacterial Strains*

A total of 155 strains and isolates (Table 1) were used for evaluation of the selectivity of the PCR assay. These included 32 *E. coli* O157 isolates from humans ($n = 17$), bovine ($n = 3$), meat and meat products ($n = 9$), and milk ($n = 3$). The 123 non-O157 isolates and strains included *E. coli* isolates from humans ($n = 14$), bovine ($n = 29$), meat and meat products ($n = 40$), and milk ($n = 2$), as well as *Salmonella* ($n = 3$), *Hafnia alvei* ($n = 2$), *Citrobacter freundii* ($n = 1$), *Enterobacter aerogenes* ($n = 1$), *Shigella* spp. ($n = 2$), *Serratia marcescens* ($n = 1$), and *Vibrio* spp. ($n = 28$). The cultures, including species or serogroup reference strains, were obtained from the strain collection of the Institute of Veterinary Food Sciences, Giessen, Germany.

### *Cell Lysis*

DNA was prepared from whole cell suspensions by the following method. Cultures were grown to exponential phase in brain heart infusion (BHI) broth (Merck, Darmstadt, Germany); cells were then centrifuged at $10\,000 \times g$ for 10 min. The cells were washed in ultrapure water, and boiled for 10 min at 100°C. After centrifugation ($10\,000 \times g$, 5 s) to pellet cell debris, the supernatant containing DNA was transferred to a new tube and stored at 4°C.

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**Table 1. Reference *E. coli* O157 and non-O157 strains used in this study**

| No. | Strain | Serogroup | Source | Virulence factors | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | *vtx1*[a] | *vtx2* | *vtx2c* | *vtx2d* | *vtx2e* | *eae*[b] |
| 1 | B 2324 | O157.H7 | Bovine feces | – | – | + | – | – | + |
| 2 | B 2325 | O157.H7 | Bovine feces | – | – | + | – | – | + |
| 3 | B 2482 | O157.H7 | Bovine feces | – | – | + | – | – | + |
| 4 | HUS 1249 | O157.H7 | Human stool, HUS | + | + | – | – | – | + |
| 5 | HC 2044 | O157.H7 | Human stool, HC | + | + | – | – | – | + |
| 6 | 1271-84 | O157.H7 | Human stool, HUS | – | + | + | – | – | + |
| 7 | 3526-87 | O157.H7 | Human stool, HC | + | + | + | – | – | + |
| 8 | EDL 931 | O157.H7 | Human stool, HC | + | – | – | – | – | + |
| 9 | A 9167-1 | O157.H7 | Human stool, HC | + | – | + | – | – | + |
| 10 | A 9218-C1 | O157.H7 | Human stool, HC | + | + | – | – | – | + |
| 11 | EDL 933 | O157.H7 | Human stool, HC | + | + | – | – | – | + |
| 12 | C 7-80 | O157.H7 | Human stool, HC | + | – | + | – | – | + |
| 13 | C 1011-87 | O157.H7 | Human stool, HC | – | + | + | – | – | + |
| 14 | E 1047 | O157.H7 | Human stool, HC | + | + | – | – | – | + |
| 15 | E 1177 | O157.H7 | Human stool, HC | + | + | – | – | – | + |
| 16 | E 4884 | O157.H7 | Human stool, HC | + | + | – | – | – | + |
| 17 | C 8/ATCC 43895 | O157.H7 | Hamburger | + | + | – | – | – | + |
| 18 | C 12 | O157.H7 | Salami | + | + | – | – | – | + |
| 19 | 3873 5/1 | O157.H7 | Raw milk | + | + | – | – | – | + |
| 20 | Sal 4/LXIV/1 | O157.H7 | Ground beef | – | + | + | – | – | + |
| 21 | Sal 57/4/1-99 | O157.H7 | Ground beef | – | – | + | – | – | + |
| 22 | NCTC 12079 | O157.H7 | Human | + | + | – | – | – | + |
| 23 | 26/22 | O157.H$^-$ | Human stool | + | – | + | – | – | + |
| 24 | 7579/95 | O157.H$^-$ | Human stool, HUS (Bayern) | – | + | – | – | – | + |
| 25 | D 1 | O157.H$^-$ | Nürnberger Rostbratwurst | – | – | + | – | – | + |
| 26 | Sal 2/LI/1 | O157.H$^-$ | Ground beef | – | – | – | – | – | – |
| 27 | Schu 2 | O157.H16 | Ground beef | – | – | – | – | – | – |
| 28 | H 4 | O157.H16 | Ground beef | – | – | – | – | – | – |
| 29 | # 51 | O157.H$^-$ | Raw milk | – | – | – | – | – | – |
| 30 | ′15/10/2 | O157.H$^-$ | Milk | – | + | + | – | – | + |
| 31 | 730 | O157.H$^-$ | Intestine, sheep | – | + | + | – | – | + |
| 32 | NCTC 12080 | O157.H$^-$ | Human | – | + | + | – | – | + |
| | | | Non-O157 strains | | | | | | |
| 1 | B 1780 | O75.H8 | Bovine feces | – | – | – | + | – | – |
| 2 | B 1834 | O136.H19 | Bovine feces | + | – | – | – | – | – |
| 3 | B1835 | O10.H21 | Bovine feces | + | – | – | – | – | – |
| 4 | B2114 | O39.H40 | Bovine feces | – | + | – | – | – | – |
| 5 | B2405 | O3.H- | Bovine feces | + | – | – | – | – | – |
| 6 | B2477 | Or.H18 | Bovine feces | + | + | – | – | – | – |
| 7 | B2480 | O126.H20 | Bovine feces | – | + | – | – | – | – |
| 8 | T 173 | O22.H8 | Bovine feces | – | + | – | – | – | – |
| 9 | T423 | O82.H8 | Bovine feces | + | – | + | – | – | – |
| 10 | T464 | O116.H21 | Bovine feces | + | – | + | – | – | – |
| 11 | T476 | O105.H18 | Bovine feces | + | – | – | – | – | – |

**Table 1. (*continued*)**

| No. | Strain | Serogroup | Source | Virulence factors | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | *vtx1*[a] | *vtx2* | *vtx2c* | *vtx2d* | *vtx2e* | *eae*[b] |
| 12 | T509 | Ont.H29 | Bovine feces | – | + | – | + | – | – |
| 13 | 173a 3422 20/8 | O26.H11 | Bovine feces | + | – | – | – | – | + |
| 14 | 173a 3744 37/4 | O103.H2 | Bovine feces | + | – | – | – | – | + |
| 15 | 173a 3744 41/1 | O48.H- | Bovine feces | + | – | – | – | – | + |
| 16 | 173a 4339 7/2 | O74.H39 | Bovine feces | + | + | – | – | – | – |
| 17 | 173a 4339 17/1 | O118.H- | Bovine feces | + | + | – | – | – | + |
| 18 | 51/9 | O146.H28 | Bovine feces | + | – | + | – | – | – |
| 19 | 54/5 | Ont.H- | Bovine feces | + | + | – | – | – | – |
| 20 | T 710 | O91.H10 | Bovine feces | – | + | – | – | – | – |
| 21 | 173a 2032 11/1 | Or.H3 | Bovine feces | + | – | – | – | – | – |
| 22 | 173a 2032 11/4 | O146.H28 | Bovine feces | + | – | + | – | – | – |
| 23 | 173a 2032 12/1 | O8.H21 | Bovine feces | + | + | – | – | – | – |
| 24 | 173a 2032 13/1 | O46.H- | Bovine feces | + | + | – | – | – | – |
| 25 | 173a 2032 14/1 | O146.H28 | Bovine feces | + | – | + | – | – | – |
| 26 | 173a 3025 17/1 | O32.H8 | Bovine feces | – | + | + | – | – | – |
| 27 | 173a 3291/2 4 | O111.H- | Bovine feces | + | – | – | – | – | + |
| 28 | 173a 1035 31/1 | O118.H- | Bovine feces | + | – | – | – | – | + |
| 29 | 173a 3025 18/2 | O77.H18 | Bovine feces | – | + | – | – | – | – |
| 30 | ED 31 | O111.H- | Stool, human | + | + | – | – | – | + |
| 31 | 78/92 | O111.H- | Stool, human | + | – | – | – | – | + |
| 32 | 4166/94 | O26.H- | Stool, human | + | – | – | – | – | + |
| 33 | 7828/95 | O103.H2 | Stool, human | + | + | – | – | – | + |
| 34 | O.55 | O55.H- | Stool, human | – | – | – | – | – | + |
| 35 | E 43 | O55 | Stool, human | – | – | – | – | – | – |
| 36 | 12/6 | Or.H- | Stool, human | + | + | – | – | – | + |
| 37 | 2704/94 | O8.H- | Stool, human | – | – | – | – | – | + |
| 38 | 3639/96 | O26.H- | Stool, human | – | + | – | – | – | + |
| 39 | 3379/96 | O55.H- | Stool, human | + | – | – | – | – | – |
| 40 | 7465/96 | O145.H- | Stool, human | + | – | – | – | – | + |
| 41 | 3759/96 | O113.H4 | Stool, human | – | + | – | – | – | – |
| 42 | 4865/96 | O145.H- | Stool, human | + | – | – | + | – | – |
| 43 | O.26 (5382) | O26.H11 | Stool, human | + | – | – | – | – | + |
| 44 | Y2-447 | O55 | Beef | – | – | – | – | – | – |
| 45 | Sal 3/LI/3 | O113.H- | Ground beef | + | + | – | – | – | – |
| 46 | Sal 4/LIV/1 | Or.H23 | Ground beef | + | + | – | – | – | – |
| 47 | M 13 | Ont.H23 | Ground beef | + | + | – | – | – | – |
| 48 | Sal 2/LXVI/3 | O7.H16 | Ground beef | + | – | – | – | – | – |
| 49 | Sal 8/LXVI/1 | O91.H- | Ground beef | – | + | + | – | – | – |
| 50 | Sal 2/5/1-98 | O113.H4 | Ground beef | – | + | – | – | – | – |
| 51 | Sal 29/5/1-98 | O113.H- | Ground beef | + | + | – | – | – | – |
| 52 | Sal 33/4/1-98 | O22.H8 | Ground beef | + | + | – | – | – | – |
| 53 | Sal 34/3/1-98 | Ont.H19 | Ground beef | + | + | – | – | – | – |
| 54 | Sal 36/1/1-98 | O82.H8 | Ground beef | + | + | + | – | – | – |
| 55 | Sal 39/5/1-98 | O113.H21 | Ground beef | – | + | – | – | – | – |

**Table 1. (*continued*)**

| No. | Strain | Serogroup | Source | Virulence factors | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | *vtx1*[a] | *vtx2* | *vtx2c* | *vtx2d* | *vtx2e* | *eae*[b] |
| 56 | Sal 34/1/1-98 | O91.H21 | Ground beef | + | + | + | – | – | – |
| 57 | Sal 42/5/1-98 | Ont.H- | Ground beef | + | + | – | – | – | – |
| 58 | L198-17460 SK 1 | O8.H8 | Ground beef | + | – | – | – | – | – |
| 59 | W 49/3/1-98 | O113.H4 | Ground beef | + | + | – | – | – | – |
| 60 | W 50/2/1-98 | Ont.H18 | Ground beef | + | + | + | – | – | – |
| 61 | W 51/1/1-98 | O113.H21 | Ground beef | – | – | + | – | – | – |
| 62 | Sal 37/3/1-99 | Ont.H2 | Ground beef | + | + | – | – | – | – |
| 63 | Sal 38/3/1-99 | O103.H42 | Ground beef | + | + | – | – | – | – |
| 64 | W 51/4/1-98 | O146.H21 | Ground beef | + | – | – | + | – | – |
| 65 | Sal 54/1/1-98 | O156.H- | Ground beef | + | – | – | – | – | + |
| 66 | Sal 1/2/1-99 | O153.H25 | Ground beef | – | + | – | – | – | – |
| 67 | Sal 7/4/1-99 | O113.H21 | Ground beef | – | + | – | – | – | – |
| 68 | Sal 16/1/1-99 | Ont.H21 | Ground beef | + | – | + | – | – | – |
| 69 | Sal 19/7/1-99 | O113.H4 | Ground beef, burger | + | + | – | – | – | – |
| 70 | Si 3303/4 | O9.H10 | Mixed ground meat | – | – | – | – | + | – |
| 71 | Y1-552 | O121.H10 | Fried sausage | – | – | – | – | + | – |
| 72 | C 6 | O111 | Salami | + | + | – | – | – | + |
| 73 | Si 1280 | O65.H- | Mettwurst | + | – | – | – | – | – |
| 74 | L198-18438 SK 1 | O6.H10 | Intestine | + | – | – | – | – | – |
| 75 | 8/6 | O138.H8 | Milk | – | + | – | – | – | – |
| 76 | DS 1/1-99 | O91.H21 | Raw milk | + | + | + | – | – | – |
| 77 | Pt 252 | O156.H25 | Sheep, carcass | + | – | – | – | – | + |
| 78 | Pt 256 | O156.H- | Sheep, carcass | + | – | – | – | – | + |
| 79 | Pt 268 | O107.H11 | Sheep, carcass | + | – | – | – | – | + |
| 80 | Pt 270 | O156.H25 | Sheep, carcass | + | – | – | – | – | + |
| 81 | LK 1 | O4.H- | Lamb meat | + | – | – | + | – | – |
| 82 | D 20 | O8.H- | Lamb cutlet | + | – | – | – | – | – |
| 83 | D 59 | Ont.H- | Lamb meat | + | – | – | + | – | – |
| 84 | 87D8 | Ont.H- | Pork | – | – | – | – | + | – |
| 85 | ED 43 | O101.H- | Pork | – | – | – | – | + | – |
| 86 | *Salmonella typhimurium* SV 4/1 | | | | | | | | |
| 87 | *Salmonella typhimurium* SV/ 6/3 | | | | | | | | |
| 88 | *Salmonella enteritidis* SV 5/19 | | | | | | | | |
| 89 | *Hafnia alvei* Sal 55/4a-99 | | | | | | | | |
| 90 | *Hafnia alvei* Sal 60/8a-99 | | | | | | | | |
| 91 | *Citrob. freundii* Sal 7/15a-00 | | | | | | | | |
| 92 | *Enterobacter aerogenes* 1779/89 | | | | | | | | |
| 93 | *Shigella sonnii* 7887 | | | | | | | | |
| 94 | *Shigella boydii Typ* 16 2710-54 | | | | | | | | |
| 95 | *Serratia marcescens* 2122/93 | | | | | | | | |
| 96 | *Vibrio cholerae* CH 931 | O11 | | | | | | | |
| 97 | *Vibrio cholerae* CH 933 | O103 | | | | | | | |
| 98 | *Vibrio cholerae* CH1329 | O27 | | | | | | | |
| 99 | *Vibrio cholerae* CH 1330 | O186 | | | | | | | |

**Table 1. (*continued*)**

| No. | Strain | Serogroup | Source | Virulence factors | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | *vtx1*[a] | *vtx2* | *vtx2c* | *vtx2d* | *vtx2e* | *eae*[b] |
| 100 | *Vibrio cholerae* CH 1332 | O2 | | | | | | | |
| 101 | *Vibrio cholerae* CH 1354 | O51 | | | | | | | |
| 102 | *Vibrio cholerae* CH 1371 | O10 | | | | | | | |
| 103 | *Vibrio cholerae* CH 1373 | O18 | | | | | | | |
| 104 | *Vibrio cholerae* CH 1374 | O42 | | | | | | | |
| 105 | *Vibrio cholerae* CH 1461 | O10 | | | | | | | |
| 106 | *Vibrio cholerae* CH 1464 | O7 | | | | | | | |
| 107 | *Vibrio cholerae* CH 1473 | O40 | | | | | | | |
| 108 | *Vibrio cholerae* CH 1474 | O40 | | | | | | | |
| 109 | *Vibrio cholerae* CH 1475 | O40 | | | | | | | |
| 110 | *Vibrio cholerae* CH 1476 | O40 | | | | | | | |
| 111 | *Vibrio cholerae* CH 1528 | O10 | | | | | | | |
| 112 | *Vibrio parahaemolyticus* CH 1314 | O1:K33 | | | | | | | |
| 113 | *Vibrio parahaemolyticus* CH 1315 | O6:K46 | | | | | | | |
| 114 | *Vibrio parahaemolyticus* CH 1316 | O6:K46 | | | | | | | |
| 115 | *Vibrio parahaemolyticus* CH 1353 | O1:K66 | | | | | | | |
| 116 | *Vibrio parahaemolyticus* CH 1385 | O1:KuK | | | | | | | |
| 117 | *Vibrio vulnificus* CH 988 | O4 | | | | | | | |
| 118 | *Vibrio vulnificus* CH 1345 | O3 | | | | | | | |
| 119 | *Vibrio vulnificus* CH 1593 | O1 | | | | | | | |
| 120 | *Vibrio vulnificus* CH 1595 | O7 | | | | | | | |
| 121 | *Vibrio vulnificus* CH 1596 | Rauh | | | | | | | |
| 122 | *Vibrio mimicus* CH 386 | O24 | | | | | | | |
| 123 | *Vibrio mimicus* CH 759 | O111 | | | | | | | |

[a] *vtx* = Verocytotoxin gene = *stx*.
[b] *eae* = *E. coli* attaching and effacing gene.

### *Oligonucleotide Primer Design*

The oligonucleotide primers GiO157-I 5′-CGA GTA CAT TGG CAT CGT G-3′ and Gi O157-II 5′-ATT GCG CTG AAG CCT TTG-3′ were selected from a region of the *rfbE* gene sequence of *E. coli* O157 (Accession No. AF061251) by using the computer program Oligo, Primer Analysis Software, Version 4.0 (National Biosciences Inc., Plymouth, MN). The primers were synthesised by MWG-Biotech (Ebersberg, Germany).

### *Optimization of PCR Conditions*

Several parameters of the PCR were optimized: $MgCl_2$, primer and deoxynucleotides (dNTP) concentration, annealing temperature, and cycle number. Optimization was performed with DNA from 8 target and 10 nontarget strains. The concentration of $MgCl_2$ was tested over a range of 0.5–2.5 μM. The primer concentration was tested over a range of 0.1–1.25 μM. The dNTP concentration was tested over a range of 40–250 μM Annealing temperatures were tested over a range of 40° to 60°C. Cycle numbers ranged from 20 to 35. After each reaction was performed, 10 mL was electrophoresed and stained with ethidium bromide (0.3 μg/mL for 20 min). The signal from each test was compared, and the optimal conditions were adopted.

### *Optimized PCR Conditions*

The PCR mixture (25 μL) contained 1 μL of each primer (10 pmol/μL), 1 μL dNTP (10mmol; Roche, Mannheim, Germany), 2.5 μL 10 × thermophilic-buffer with 25mM $MgCl_2$ (PE Applied Biosystems, Weiterstadt, Germany), 1 μL (5.23 fg) *rfbE*-internal amplification control (IAC; 13), 0.1 μL *Taq* DNA polymerase (5 U/μL, PE Applied Biosystems), and 15.9 μL $H_2O$. Finally, 2.5 μL DNA solution or cell suspension was added to each reaction mix. The PCR was performed in a thermal cycler (PE GeneAmp PCR system 9600, PE Applied Biosystems) with the following program: 1 × 3 min precycle at 93°C, 30 × 15 s at 93°C, 15 s at 60°C, and 30 s at 72°C

followed by a final extension incubation of 72°C for 5 min. The presence of PCR products was determined by electrophoresis of 10 μL reaction product in a 2% agarose gel (Appligene, Heidelberg, Germany), with Tris acetate-electrophoresis buffer (TAE; 0.04 mol/L Tris, 0.001 mol/L EDTA, pH 7.8), and a 50 base pair (bp) DNA ladder (Roche) as molecular marker.

### *Determination of Detection Probability*

The determination of detection probability was performed according to the method of Knutsson et al. (14). *E. coli* O157 (EDL 933) was grown to exponential phase in BHI broth, and then stored at –20°C until use. To determine the exact colony forming units (CFU)/mL, the cell suspension was serially diluted 10-fold in 0.9% (w/v) NaCl within the concentration range of $10^6$–$10^1$ CFU/mL, and aliquots were plated on PC agar (casein-peptone dextrose yeast extract agar, Merck). The frozen cell suspension was thawed, and a 10-fold dilution series was prepared in ultrapure water.

A PCR was performed on each dilution, using 2.5 μL added directly to the reaction. This was done in 10 independent experiments, i.e., 10 independently grown cultures were diluted, and the dilutions were assayed. The total number of positive signals obtained was expressed as a percentage, e.g., 6 positive signals from 10 reactions = 60%. This was compared with the number of cells contained in the reaction, as calculated from the dilution factor of the original count. The detection probability of the PCR assay was obtained by plotting the relative observed positive PCR reactions against the concentration of the cell suspension (12). A sigmoidal line fitting was performed with the software Eisensmith, S.P. Plot It (Scientific Programing Enterprises, Haslett, MI).

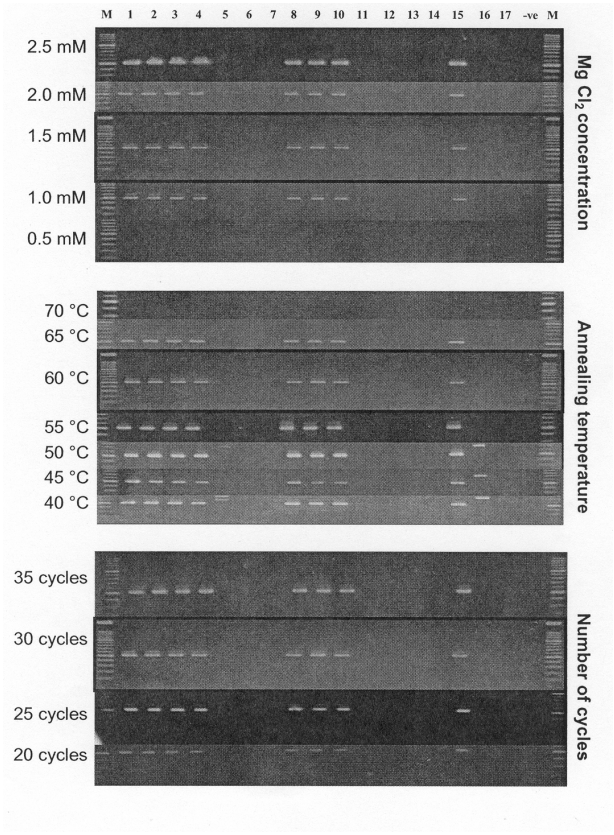


**Figure 1. PCR amplification of DNA from *E. coli* O157 and other bacterial strains using various annealing temperatures, $MgCl_2$ concentration, and various number of cycles. Lane M, marker 100 bp DNA ladder (Roche); Lane 1, *E. coli* O157:H7 (NCTC 12079); Lane 2, *E. coli* O157:H7 (Sal 57/4/1-99); Lane 3, *E. coli* O157:H16 (Schu 2); Lane 4, *E. coli* O157:H? (MG 3/3-00); Lane 5, *E. coli* O55:H$^-$ (O55 Südbayern); Lane 6, *E. coli* O145:H$^-$ (7465/96); Lane 7, *E. coli* O145:H16 (173a 2106 43/3); Lane 8, *E. coli* O157:H$^-$ (NCTC 12080); Lane 9, *E. coli* O157:H$^-$ (15/10/2); Lane 10, *E. coli* O157:H$^-$ (7579/95 Südbayern); Lane 11, *Hafnia alvei* (Sal 60/8a-99); Lane 12, Citrobacter *freundii* (Sal 7/15a-00); Lane 13, *Shigella sonnei* (7887); Lane 14, *Shigella boydii* Typ 16 (2710-54); Lane 15, *E. coli* O157:H7 (EDL 933); Lane 16, *E. coli* O23:H15 (ATCC 25922); Lane 17, *E. coli* O103 H2 (173a 3744 37/ 4); Lane M, marker 100 bp DNA ladder.**

## Results and Discussion

### *Optimization of rfbE Gene PCR*

Figure 1 shows the signals obtained from PCRs containing various concentrations of $MgCl_2$. No signals were obtained with 0.5mM $MgCl_2$. Specific and clear bands were obtained with 1.0, 1.5, 2.0, and 2.5mM. The concentration recommended by the polymerase supplier (1.5mM) was adopted. The signals obtained from PCRs containing various concentrations of primers and dNTPs were all similar in their clarity and specificity (not shown). Annealing temperatures of 40°, 45°, 50°, and 55°C produced bands from nontarget samples. An annealing temperature of 60°C was chosen. At 20 cycles, faint signals were produced. Thirty cycles was chosen for the final PCR. Figure 2 shows the typical amplicon of O157 strains and negative strain with the IAC.

### *Demonstration of the Selectivity of the PCR*

The assay was tested on DNA extracted from each strain in Table 1. Table 2 summarizes the results obtained. The assay was 100% inclusive and 100% exclusive. The presence of an IAC signal in all negative results demonstrated that each was a true negative and not the result of inhibition of the PCR.

### *Detection Probability*

Figure 3 shows the results of the detection limit evaluation. The number of cells per reaction which could be detected with 100% probability was approximately $2 \times 10^3$.

The O157 *rfbE* PCR assay presented here is a highly selective and rapid method for the confirmation of the O157 serogroup. The assay detects all strains of *E. coli* O157, whether or not they are pathogenic. It is most suitable as part of a screening method for *E. coli* O157 in many sample types. Further tests should be performed, such as PCR for detecting the *vtx* or *eaeA* genes (15–17), to confirm a pathogenic genotype.

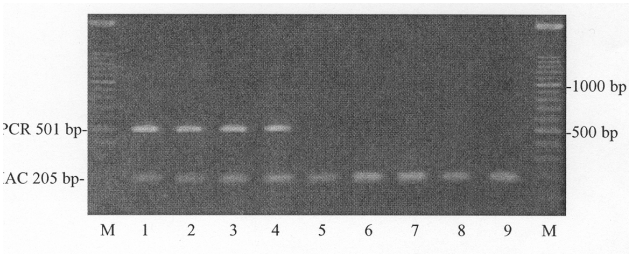


**Figure 2. PCR amplification of DNA from *E. coli* O157 strains with a size of 501 bp and negative strains in presence of IAC (205 bp). Lane M, marker 100 bp DNA ladder (Roche); Lane 1, *E. coli* O157:H7 (EDL 933); Lane 2, *E. coli* O157:H7 (NCTC 12079); Lane 3, *E. coli* O157:H⁻ (NCTC 12080); Lane 4, *E. coli* O157:H⁻ (15/10/2); Lane 5, *Hafnia alvei* (Sal 60/8a-99); Lane 6, *Citrobacter freundii* (Sal 7/15a-00); Lane 7, *Shigella sonnei* (7887); Lane 8, *Vibrio cholerae* CH 933; Lane 9, negative control.**

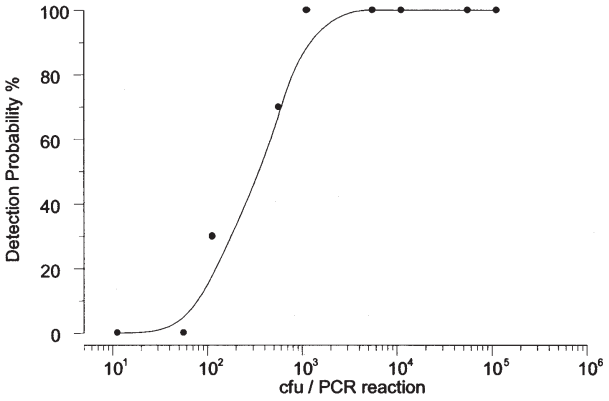


**Figure 3. Detection probability of *rfbE* PCR assay. The graph shows sigmoidal fit of data; each point represents percentage positive of 10 independent experiments.**

The PCR reported here was extensively optimized to ensure that it detected only its intended target. Selectivity is most effectively evaluated by testing an assay against a large number of target and nontarget strains. The strains against which the assay were tested formed a comprehensive selection of target and nontarget bacteria, and the results of the tests confirmed the selectivity of the *rfbE* primer set. Desmarchelier et al. (18) and Paton and Paton (19) published *E. coli* O157 PCRs which were based on *rfbE* sequences, but Chapman et al. (20) found that they produced false-positive signals with various *E. coli* non-O157 strains, even after extensive optimization. Chapman et al. (20) stated that the primer set of Maurer et al. (21) was fully selective against a strain list, comprising 12 *E. coli* O157, 46 *E. coli* non-O157, and 12 non-*E. coli*. The *rfbE* gene is found in both *E. coli* O157 and also *Vibrio* spp. (22). However, neither Maurer et al. (21) nor Chapman et al. (20) verified that their PCR excluded cross-reaction with *Vibrio* spp. The PCR described in the present work was evaluated against a more extensive strain list than the ones used by Maurer et al. (21) and Chapman et al. (20), and it included 28 *Vibrio* spp., none of which were detected.

This is the first published *E. coli* O157 assay which includes an IAC. In diagnostic PCR, an IAC is necessary in order to identify false-negative results (13, 23).

**Table 2. Summary of results of selectivity test of *E. coli* O157 PCR assay**

| Strains | No. tested | No. positive |
|---|---|---|
| *E. coli* O157 | 32 | 32 |
| Other *E. coli* | 85 | 0 |
| Non-*E. coli* spp. | 38 | 0 |

The PCR will detect approximately $2 \times 10^3$ cells per reaction with 100% probability. This compares with the detection of $10^4$ cells, which appeared to be the limit of the assay of Maurer et al. (21). The infectious dose of *E. coli* O157 can be as low as <10 cells (2); therefore, the PCR should not be used directly on environmental, clinical, or food samples without an appropriate pre-enrichment step. The PCR would be also suitable for confirmation of the identity of agar-grown colonies.

The evaluation of the diagnostic accuracy was performed in-house by our laboratory. A standard method should, however, be proven reliable through evaluation of its performance in several laboratories (10, 11). The outcome of the collaborative trials of the assay is reported in the companion paper.

## Acknowledgments

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FOOD BIOLOGICAL CONTAMINANTS

# Toward an International Standard for PCR-Based Detection of Foodborne *Escherichia coli* O157: Validation of the PCR-Based Method in a Multicenter Interlaboratory Trial

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**The performance of a polymerase chain reaction (PCR) method for detection of *Escherichia coli* O157, previously validated on DNA extracted from pure cultures, was evaluated on spiked cattle swabs through an interlaboratory trial, including 12 participating laboratories from 11 European countries. Twelve cattle swab samples, spiked at 4 levels (0, 1–10, 10–100, and 100–1000 colony-forming units, in triplicate) with *E. coli* O157 were prepared centrally in the originating laboratory; the receiving laboratories performed pre-PCR treatment followed by PCR. The results were reported as positive when the correct amplicons were present after gel electrophoresis. The statistical analysis, performed on 10 sets of reported results, determined the diagnostic sensitivity to be 92.2%. The diagnostic specificity was 100%. The accordance (repeatability) was 90.0%, calculated from all positive inoculation levels. The concordance (reproducibility) was 85.0%, calculated from all positive inoculation levels. The concordance odds ratio (degree of interlaboratory variation calculated from all positive inoculation levels) was 1.58, indicating the robustness of the PCR method. Thus, the interlaboratory variation due to personnel, reagents, minor temperature or pH fluctuations and, not least, thermal cyclers, did not affect the performance of the method, which is currently being considered as part of an international PCR standard.**

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Consumption of beef is associated with foodborne infections with *Escherichia coli* O157 (1). Rapid detection of *E. coli* O157 is an important issue in food microbiology (2–6). The challenge is being addressed through development of new diagnostic tests. To encourage acceptance and use of a diagnostic test by the food industry, it is recommended that a test has been thoroughly evaluated through interlaboratory trial of its performance characteristics in several laboratories (7).

Recognizing the need for sensitive, rapid, and cost-effective methods for the detection of foodborne pathogens, a European research project was launched for validation and standardization of open-formula, noncommercial polymerase chain reaction (PCR)-based methods, as alternatives to traditional culture-based methods (8). In the first stages, an *E. coli* O157-specific PCR assay was developed (9). The assay targets the *per* (*rfbE* O157) gene (Accession Nos. NC002655 and AE005175), using the primer set Gi-O157-I, Gi-O157-II (Accession No. AF061251), and includes an internal amplification control (IAC) to indicate false-negative results (10). The assay was evaluated through a European interlaboratory trial and showed an analytical accuracy of 100% (9).

To mediate the detection of *E. coli* O157 in materials used in primary food production, the PCR assay was incorporated in a complete method in which the preceding steps were enrichment overnight followed by a simple and nonproprietary DNA extraction procedure.

Several PCR methods on *E. coli* O157 have been published, using various sample types, enrichment media, and primers (11–17). However, none of the existing PCR methods have included an IAC no been been validated through interlaboratory trials. Inclusion of an IAC and assessment of method performance through interlaboratory trials are essential to accreditation of any

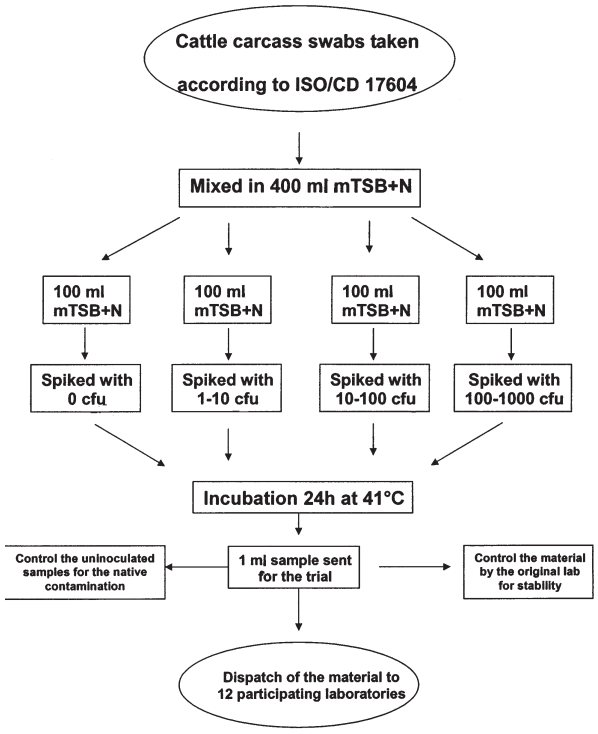


**Figure 1. Flow diagram showing preparation, spiking, testing, and shipping of samples for the PCR interlaboratory trial for detection of *E. coli* O157.**

PCR method to be used as a diagnostic tool in laboratories with quality assurance programs.

A validation of an alternative method according to criteria from the International Standard Organization (ISO; 7) requires interlaboratory trial involving at least 8 participating laboratories that have not been involved in the development of the method. The present study reports the results of a second interlaboratory trial, which involved analysis of artificially contaminated pre-enriched cattle carcass swabs by the PCR-based method for detection of *E. coli* O157.

## Interlaboratory Study

### *Materials and Methods*

The interlaboratory trial was designed according to the recommendations of NordVal (18). Twelve European laboratories from Austria, Denmark, Finland, Germany, Greece, Ireland, Italy, The Netherlands, Portugal, Slovakia, and the United Kingdom participated in the interlaboratory trial. Each participant received 12 coded (blind) 1 mL enriched samples: 12 cattle swab samples spiked with *E. coli* O157 at the following levels of colony forming-units (CFU): 0, 1–10, 10–100, and 100–1000. The shipment also included a positive DNA control, an IAC (10), and ca 6 g of a resin used for pre-PCR treatment (Chelex-100, 142-2832, Bio-Rad Laboratories, Munich, Germany). A detailed standard operating procedure (SOP) explaining how to perform the pre-PCR treatment, as well as the PCR assay (available at www.pcr.dk), was sent to the receiving laboratories. The participants purchased their own batch of primers, *Taq* DNA polymerase, and additional reagents from local suppliers. The SOP also enclosed a reporting sheet, to be returned to an impartial laboratory in the United Kingdom (Participant No. 2) for statistical analysis. In the reporting sheet, participants were required to detail all additional information that could possibly have influenced their results.

### *Preparation of Inoculum*

The cattle swabs, sampled according to the sampling method of ISO/CD 17604 and DIN 10113-1 (19) were obtained from the Giessen slaughterhouse (Giessen, Germany). Briefly, as shown in Figure 1, the carcass surface was delineated by a sterile frame of 100 $cm^2$, which pressed on the carcass. This area was first sampled by a cotton wool swab fixed on plastic stick (Medka, Berlin, Germany) moistened with NaCl–Peptone water (1.0 g/L peptone + 8.5 g/L sodium chloride, pH 7.0) followed by sampling with dry swab. The swabs were stirred in the dilution fluid. The swab samples were immediately transported to the laboratory in a cooled, insulated container at 4°C. The tops were cut off from the holder and placed in a bottle with 400 mL modified trypticase soy broth and novobiocin (mTSB+N). Subsequently, the samples were mixed gently and divided into 4 subsamples of 100 mL. Three of the 4 samples were spiked with 1–10, 10–100, and 100–1000 CFU of *E. coli* O157:H7, respectively, and 1 sample was not inoculated. The samples were incubated overnight at 41°C in mTSB+N. After overnight enrichment, 1 mL samples were drawn and stored at –25°C until sent on ice to the participating laboratories. In order to verify that there was no native contamination of the samples with *E. coli* O157, each noninoculated sample was cultured according to ISO 16654 method (20).

### *Shipment*

The test samples were packaged in containers with solid carbon dioxide labeled in conformity with International Air Transport Association Regulations, and shipped to the participants by a courier company that was experienced in the shipment of infectious materials (The Courier Co., Milton Keynes, UK; www.thecourierco.uk.com).

### *Method Performed by the Receiving Laboratory*

The interlaboratory trial participants performed a resin-based DNA extraction (9) on each sample. Briefly, this involved mixing 200 μL culture to 800 μL $H_2O$, and then sedimentation of the cells by centrifugation at 13 000 × *g*. The pellet was resuspended in 300 μL 6% Chelex-100 resin, and the suspension was incubated at 56°C for 20 min with periodic mixing. The suspension was then incubated at 100°C for 10 min, mixed on a Vortex mixer for 10 s, placed on ice for 2 min, and contrifuged at 13 000 × *g* for 5 min. A 4 μL volume of the supernatant was used directly as template in the PCR reaction. The 25 μL PCR mixture consisted of 2.5 μL 10 × PCR-buffer for *Taq* DNA polymerase with 15mM $MgCl_2$ (Applied Biosystems, Darmstadt, Germany), 0.2 μL *Taq* DNA polymerase (5 U/μL, Applied Biosystems), 0.5 μL 10mM of each dNTP (Roche, Mannheim, Germany), 1 μL of each primer (10 pmol), 1 μL IAC, 14.8 μL PCR grade water, and 4 μL

**Table 1. Reported participants' results from interlaboratory trial of the PCR-based method for detection of *E. coli* O157 in cattle swab samples**

| | Presence of target amplicon | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Participant No. | | | | | | | | | | | |
| Sample inoculation level | Expected[a] | 1 | 2[b] | 3 | 4 | 5 | 6 | 7 | 8 | 9[c] | 10 | 11 | 12 |
| Uninoculated | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Low (1–10 CFU) | 3 | 3 | 1 | 3 | 3 | 3 | 2 | 3 | 3 | 0 | 2 | 3 | 3 |
| Medium (10–100 CFU) | 3 | 3 | 1 | 3 | 3 | 3 | 3 | 3 | 3 | 0 | 1 | 3 | 3 |
| High (100–1000 CFU) | 3 | 3 | 1 | 3 | 2 | 3 | 3 | 3 | 3 | 0 | 1 | 3 | 3 |

[a] From analysis of triplicate samples.
[b] Excluded due to lack of target amplicon in the assay positive control.
[c] Excluded due to omission of the internal amplification control in the PCR mixture.

sample (9). The PCR was performed in a thermal cycler (PE GeneAmp PCR system 9600; Applied Biosystems) with the following program: 1 × 3 min precycle at 93°C, 30 × 15 s at 93°C, 15 s at 60°C, and 30 s at 72°C, followed by a final extension incubation of 72°C for 5 min. The PCR products were detected by gel electrophoresis in a 1.5% agarose gel at 100 V for 30 min, stained with ethidium bromide, and visualized under UV light.

The results were recorded on the reporting sheet by each participant, and were returned together with the original gel pictures for independent observation and statistical analysis to a different laboratory (N. Cook and M. D'Agostino, UK) than the sending laboratory (Giessen, Germany). A result was described as a false negative if no correct signal was obtained from an inoculated sample, or as an inhibited reaction if no IAC signal was obtained.

### *Statistical Analysis*

The interlaboratory trial results were analyzed statistically according to the recommendations of Scotter et al. (21) by the methods of Langton et al. (22). The diagnostic sensitivity was defined as the percentage of positive samples giving a correct positive signal (7). The diagnostic specificity was defined as the percentage of negative samples giving a correct negative signal, and a signal from the IAC (7). Confidence intervals (CI) for diagnostic sensitivity and diagnostic specificity were calculated by the method of Wilson (23). Accordance (repeatability of qualitative data) was defined as the percentage chance of finding the same result, positive or negative, from 2 identical samples analyzed in the same laboratory under predefined repeatability conditions. Concordance (reproducibility of qualitative data) was defined as the percentage chance of finding the same result, positive or negative, from 2 identical samples analyzed in different laboratories under predefined repeatability conditions. These calculations take into account different replication in different laboratories by weighting results appropriately. The concordance odds ratio (COR) was defined as the degree of interlaboratory variation in the results. The COR was expressed as the ratio between accordance and concordance percentages, making it less dependent on the sensitivity. CI for accordance, concordance, and COR were calculated by the method of Davidson and Hinckley (24).

### *Criteria for Inclusion of Results*

The results from each participating laboratory were included unless they fell into the following 2 categories: (*1*) obvious performance deviation from the SOP, and (*2*) lack of target amplicons in assay positive control.

## Results and Discussion

All aliquots of the inoculated test samples were culture- and PCR-positive on the day of shipment, as determined in the

**Table 2. Statistical evaluation of data obtained in the multicenter interlaboratory trial[a]**

| Inoculation level | Sensitivity, % | Specificity, % | Accordance, % | Concordance, % | COR[b] |
|---|---|---|---|---|---|
| Uninoculated | — | 100 (88.7, 100) | 100 | 100 | 1.00 |
| Low | 93.3 (78.7, 98.2) | — | 86.7 (66.7, 100) | 87.2 (71.6, 100) | 0.96 (0.79, 1.00) |
| Medium | 93.3 (78.7, 98.2) | — | 93.3 (80.0, 100) | 86.7 (65.9, 100) | 2.15 (1.00, 2.15) |
| High | 90.0 (74.4, 96.5) | — | 86.7 (66.7, 100) | 81.0 (62.2, 100) | 1.53 (0.91, 2.15) |
| All positive levels | 92.2 (84.8, 96.2) | — | 90.0 (77.8, 97.8) | 85.0 (66.8, 97.8) | 1.58 (0.96, 2.13) |

[a] Numbers in parentheses are the lower and upper 95% confidence intervals.
[b] COR = Concordance odds ratio.

sending laboratory (Giessen). All aliquots of the noninoculated test samples were culture- and PCR-negative.

Table 1 shows the participants' results of the interlaboratory trial of the PCR-based method for the detection of *E. coli* O157 in cattle swab. In agreement with the predefined criteria, the results of Participant Nos. 2 and 9 were excluded, as they did not obtain target amplicons in the assay positive control (data not shown). All remaining results were accepted according to the predefined criteria; thus, the statistical analysis was based on 10 sets of results. Participant No. 10 reported that 1 PCR from a high-inoculated sample was inhibited, as judged by the absence of an IAC signal. The results of Participant No. 10 were included in the statistical analysis, as shown in Table 2.

The interlaboratory trial showed that the method has a diagnostic sensitivity >90% for each inoculation level, and calculating the diagnostic sensitivity for all inoculation levels gave a value of 92.2%. Combination of accordance (repeatability) and concordance (reproducibility) values, expressed as COR value, indicated that there was the same likelihood of obtaining the same result from 2 identical samples, whether they were sent to the same or to 2 different laboratories. In all cases, the COR fell within the 95% CI of 1.0, indicating that the diagnostic sensitivity of the method was as repeatable as it was reproducible. The method has a very high probability of detecting *E. coli* O157 at any contamination level in the sample types tested. Conversely and, more importantly, there is a very low risk of obtaining false-negative responses.

The strength of PCR lies in its potential for rapid identification of negative samples. The PCR-based method here compares favorably with the ISO culture method (20), as the latter takes approximately 3 days to identify negative samples, whereas the method reported here takes only 1 day. The diagnostic specificity, or percentage of correctly identified noninoculated cattle samples, was 100% with complete accordance and concordance. Although Participant No. 6 performed the trial several months after receipt of the samples, the results obtained were acceptable, indicating the robustness of the PCR.

In conclusion, the outcome of this interlaboratory trial indicates that this PCR-based method for detection of *E. coli* O157 is highly sensitive and specific, repeatable as well as reproducible, and robust when applied to enriched cultures from cattle swabs. To the best of our knowledge, no other interlaboratory trial has validated a similar open-formula, noncommercial PCR for *E. coli* O157 (9). This method, unlike real-time fluorescence-based PCR, does not require procurement of costly equipment. These features, in combination with the validation presented here, make it eminently suitable for routine use, and thus appropriate for international standardization.

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FOOD BIOLOGICAL CONTAMINANTS

# Multicenter Validation of PCR-Based Method for Detection of *Salmonella* in Chicken and Pig Samples

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**As part of a standardization project, an interlaboratory trial including 15 laboratories from 13 European countries was conducted to evaluate the performance of a noproprietary polymerase chain reaction (PCR)-based method for the detection of *Salmonella* on artificially contaminated chicken rinse and pig swab samples. The 3 levels were 1–10, 10–100, and 100–1000 colony-forming units (CFU)/100 mL. Sample preparations, including inoculation and pre-enrichment in buffered peptone water (BPW), were performed centrally in a German laboratory; the pre-PCR sample preparation (by a resin-based method) and PCR assay (gel electrophoresis detection) were performed by the receiving laboratories. Aliquots of BPW enrichment cultures were sent to the participants, who analyzed them using a thermal lysis procedure followed by a validated *Salmonella*-specific PCR assay. The results were reported as negative or positive. Outlier results caused, for example, by gross departures from the experimental protocol, were omitted from the analysis. For both the chicken rinse and the pig swab samples, the diagnostic sensitivity was 100%, with 100% accordance (repeatability) and concordance (reproducibility). The diagnostic specificity was 80.1% (with 85.7% accordance and 67.5% concordance) for chicken rinse, and 91.7% (with 100% accordance and 83.3% concordance) for pig swab. Thus, the interlaboratory variation due to personnel, reagents, thermal cyclers, etc., did not affect the performance of the method, which will be proposed as part of a developing international PCR standard.**

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Food products derived from pig and chicken are among the primary sources of foodborne salmonellosis (1). Rapid detection of *Salmonella* in these foodstuffs is an important issue in food microbiology (2). The challenge is being addressed through development of new diagnostic tests. To encourage acceptance and use of a diagnostic test by the food industry, it is recommended that a test be thoroughly evaluated through collaborative trial of its performance characteristics in several laboratories (3).

Recognizing the need for sensitive, rapid, and cost-effective methods for the detection of foodborne pathogens, an international research project was launched for validation and standardization of polymerase chain reaction (PCR)-based methods, with publicized formulations, as alternatives to traditional culture-based methods (4). In the first stages, a *Salmonella*-specific PCR

assay was developed (5). The assay targets the gene, *invA*, using the published primer set 139–141 (6), and includes an internal amplification control (IAC) to indicate false-negative results. The assay was evaluated through a European collaborative trial and showed a test accuracy of 98% (5).

To mediate the detection of *Salmonella* in materials used in primary food production, the PCR assay was incorporated in a complete method in which the steps were enrichment in buffered peptone water (BPW) followed by a simple and nonproprietary DNA extraction procedure. A precollaborative study of this method, involving 4 laboratories, investigated 4 different sets of presumably naturally contaminated samples and compared the results with these from the standard microbiological culture method (7). The interlaboratory diagnostic accuracy was shown to be 99.3% for pig carcass swabs and 100% for chicken carcass rinse. Applying the method to artificially contaminated minced beef and chicken carcass rinse resulted in detection of <5/25 g meat or 100 mL chicken rinse (7).

A validation of an alternative method according to criteria from the International Standard Organization (ISO; 3) requires an interlaboratory trial involving at least 8 participating laboratories that have not been involved in the development of the method. The present study reports the results of a full-scale interlaboratory trial, which involved analysis of artificially contaminated pre-enriched carcass rinse from whole chicken and pig swabs by the PCR-based method for detection of *Salmonella*.

## Interlaboratory Study

The interlaboratory study was designed according to the recommendations of NordVal, the Noridc countries' validation protocol (8). Fifteen European laboratories from Denmark, the United Kingdom, Austria, Germany, Greece, France, Czech Republic, Slovakia, Spain, Italy, Finland, The Netherlands, and Poland participated. Each participant received 24 coded (blind) 1 mL test samples. Of these, 12 were obtained from whole chicken carcass rinses inoculated with strain *Salmonella enterica* Enteritidis phage type PT 4 [Bundesinstitut für gesundheitlichen Verbraucherschutz und Veterinärmedizin (BgVV 98-425)], and 12 from pig swab samples inoculated with *S. enterica* Typhimurium phage type DT104 (BgVV 96-51K61). In addition, a positive *S. enterica* DNA control (strain BgVV 96-51K61), an IAC (498 bp PCR product; 5), and 0.36 g of the resin Chelex-100 (142-2832, BioRad Laboratories, CA) for DNA extraction was included in each package. A detailed standard operating procedure (SOP) describing how to perform the pre-PCR treatment and the PCR assay (available at www.pcr.dk) was sent to each participating laboratory. The DNA extraction method (7) and the PCR assay (5) have been previously described. The participants purchased primers 139-141 (LC purified; 6), platinum *Taq* polymerase and supplied buffer (Invitrogen, Karlsruhe, Germany), bovine serum albumin (BSA) Fraction V, and deoxyribonucleoside triphosphates (dNTPs). The model of thermal cycler usage was left to the laboratory.

### *Preparation of Inoculum*

The cultures to be used as inocula of strains *S. Enteritidis* phage type PT4 (BgVV 98-425, Germany) and *S. enterica* Typhimurium phage type DT104 (BgVV 96-51K61) were aerobically grown in BPW (Merck, Darmstadt, Germany) at 37°C to the exponential phase, and appropriately diluted. Both strains are well-characterized (molecularly and by antibiotic resistance profiling) and are used as reference strains in the National Reference Laboratory for Salmonella in Berlin, Germany. BgVV 98-425 was isolated in 1998 from chicken meat. BgVV 96-51K61 was isolated in 1996 from pig feces. Viable cell count was obtained by plating each of 3 appropriate cell concentrations ($10^{-5}$, $10^{-6}$, and $10^{-7}$ dilutions) made in 0.9% (w/v) sodium chloride onto Luria-Bertani agar (9). The plates were incubated at 37°C for 24 h. The cell concentration of each dilution was estimated by calculating the average number of the colony forming units (CFU) from 5 agar plates. The same dilutions were taken as inocula for the samples (*see* below). The estimated inoculated values of CFUs/100 mL sample is given in Tables 1 and 2.

### *Preparation of Test Samples and Verification*

Three frozen chickens were purchased at local retailers. Suspensions of carcass rinses were prepared as recommended in ISO/CD 6887 (10; Figure 1). Each chicken was thawed overnight in a refrigerator in a sterile plastic bag. BPW (500 mL) at 4°C was added. The chicken was rinsed manually by gently shaking for 1 min. From each of the 3 chicken rinses, a 100 mL aliquot was taken in order to verify the absence of natural *Salmonella* contaminants (*see* below). The remaining BPW suspension was dispensed into 100 mL aliquots. From each chicken sample, one aliquot was left uninoculated, one was inoculated with a 1 mL aliquot of the $10^{-7}$ cell dilution (1–10 CFU/100 mL, low spike), one with a 1 mL aliquot of the $10^{-6}$ cell dilution (10–100 CFU/100 mL, medium spike), and one with a 1 mL aliquot of the $10^{-5}$ cell dilution (100–1000 CFU/100 mL, high spike) of BgVV 98-425. Twelve pig carcass swabs, sampled in accordance with the ISO/FDIS 17604 (11), were obtained from a local German slaughterhouse. Swabs were rinsed in 110 mL BPW. A 10 mL aliquot of each sample was taken in order to verify the absence of *Salmonella* (*see* below). The remaining 100 mL aliquots were used for artificial contamination. Three broth samples were left uninoculated, 3 were inoculated with a 1 mL aliquot of the $10^{-7}$ cell dilution (1–10 CFU/100 mL), 3 with a 1 mL aliquot of the $10^{-6}$ cell dilution (10–100 CFU/100 mL), and 3 with a 1 mL aliquot of the $10^{-5}$ cell dilution (100–1000 CFU/100 mL) of BgVV 96-51K61. After inoculation, a pre-enrichment step was performed at 37°C for 20 h. The pre-enriched test samples were aliquoted in 1 mL portions and stored at –30°C (for <4 weeks) until shipment to the participants.

**Table 1. Reported participants' results from interlaboratory trial of PCR-based method for detection of *Salmonella* in chicken carcass rinse samples**

| Chicken samples | Presence of target amplicon | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Participant No. | | | | | | | | | | | | | | |
| Sample inoculation level | Expected[a] | 1 | 2[b] | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
| Uninoculated | 0 | 0 | 3 | 0 | 0 | 3 | 0 | 0 | 2 | 2 | 0 | 0 | 1 | 0 | 0 | 0 |
| Low (5 CFU/100 mL) | 3 | 3 | 2 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| Medium (26 CFU/100 mL) | 3 | 3 | 2 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| High (474 CFU/100 mL) | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |

[a] From analysis of triplicate samples.
[b] Excluded due to presence of target amplicons in the PCR-negative control.

The cultural method was considered as the gold standard in order to verify the absence and presence of *Salmonella*. Uninoculated and inoculated pre-enriched carcass samples were tested according to the standard culture method (12) with slight modification. A modifed semisolid Rappaport Vassiliadis (MSRV) agar (Merck; Figure 1) was used as the first enrichment broth. MSRV is widely used, and it was shown that MSRV performed better than RV for isolation of *Salmonella* (13). Tetrathionate brilliant green bile (TBG; Merck) broth was used as the second selective enrichment. MSRV agar plates were spotted with 100 µL pre-enriched culture and incubated 22–24 h at 42°C. A 10 mL aliquot of TBG broth was inoculated with 100 µL pre-enriched culture and incubated 22–24 h at 37°C. For the plating out step, xylose lysine desoxycholate agar (XLD; Merck) and Rambach agar (Merck) was used. If the presumptive *Salmonella* strain migrated around the inoculation spot, a loopful from MSRV agar was streaked onto a selective agar plate. A 10 µL aliquot of the TBG enrichment culture was streaked onto the selective agar plates. The plates were incubated 24 h at 37°C. Presumptive colonies were serologically and biochemically confirmed as described in ISO 6579:2002 (12). Cultural tests and PCR of one aliquot, respectively, were performed 1 day after storage at –30°C and on the day of shipment to verify the stability of the material and continued detection of *Salmonella*.

Enumeration of viable cells derived from the natural background flora of the carcass rinses was determined by plating appropriate 10-fold dilutions of carcass rinses (in 0.9%, w/v, sodium chloride) on double-strength nutrient agar (9) in triplicate. After aerobic incubation for 24 h at 37°C, the total number of CFU per mL carcass rinse was calculated.

### *Shipment*

The test samples were packaged in containers with solid carbon dioxide, labeled in conformity with International Air Transport Association Regulations, and shipped to the participants by a courier company that was experienced in the shipment of infectious materials.

**Table 2. Reported particpants' results from interlaboratory trial of PCR-based method for detection of *Salmonella* in pig carcass swab samples**

| Pig samples | Presence of target amplicon | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Participant No. | | | | | | | | | | | | | | |
| Sample inoculation level | Expected[a] | 1 | 2[b] | 3 | 4 | 5 | 6 | 7 | 8[b] | 9 | 10 | 11 | 12 | 13 | 14 | 15[c] |
| Uninoculated | 0 | 0 | 3 | 0 | 0 | 3 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Low (10 CFU/100 mL) | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| Medium (66 CFU/100 mL) | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| High (560 CFU/100 mL) | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |

[a] From analysis of triplicate samples.
[b] Excluded due to presence of target amplicon in the PCR-negative control.
[c] Excluded due to omission of the IAC in the PCR mixture.

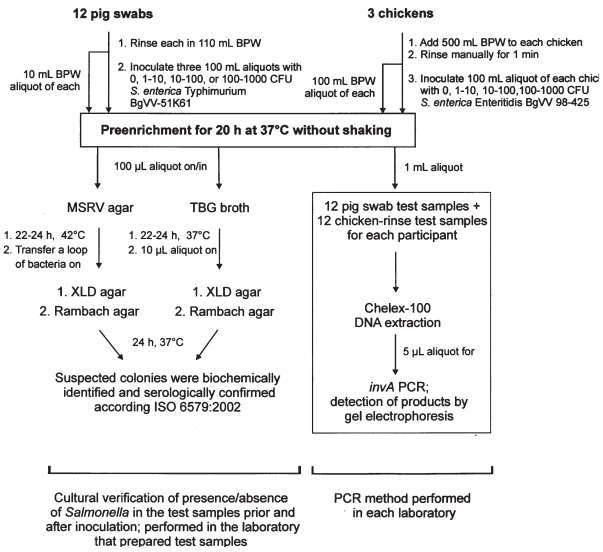


**Figure 1. Flow diagram showing preparation, inoculation, pre-enrichment, cultural confirmation, and *Salmonella* PCR detection method performed in each of the 15 participating laboratories. Sample preparation including pre-enrichment and verification of absence/presence of *Salmonella* before and after inoculation were performed centrally in one laboratory. Aliquots of pre-enriched uninoculated and inoculated samples were sent to 15 laboratories performing DNA extraction and PCR.**

### *Method Performed by the Receiving Laboratory*

Each participating laboratory was sent an SOP to perform the detection of *Salmonella* by PCR method.

In order to avoid cross-contamination, the chicken and pig samples were analyzed separately in each participating laboratory. Frozen pre-enriched samples were thawed, and a resin-based DNA extraction using Chelex-100 was performed (7). Briefly, this involved mixing 200 µL culture to 800 µL $H_2O$, and then pelleting the cells by centrifugation at 13 000 × *g*. The pellet was suspended in 300 µL 6%, w/v, Chelex-100 resin, and the suspension incubated at 56°C for 20 min with periodic mixing. The suspension was then incubated at 100°C for 8 min, roughly mixed for 10 s, and placed on ice for 2 min. The suspension was centrifuged at 13 000 × *g* for 5 min. A 5 µL aliquot of the supernatant was used directly as template in the PCR reaction.

For PCR assay, a 25 µL PCR mixture consisted of 0.4 µM of each primer 139 and 141 (LC purified; 6), 200 µM of each dNTP, 1 X PCR reaction buffer [20mM Tris-HCl (pH 8.4), 50mM KCl], 1.5mM $MgCl_2$, 1 µg/µL BSA Fraction V, 1 Unit Platinum *Taq* polymerase (Invitrogen), 300 copies of IAC (498 bp purified PCR product; 5), and 5 µL Chelex-100 treated sample. The incubation conditions were 95°C for 1 min, followed by 38 cycles of 95°C for 30 s, 64°C for 30 s, and 72°C for 30 s. A final extension of 72°C for 4 min was applied. The PCR products were detected by gel electrophoresis in a 1.8% agarose gel at 100 V for 60 min, stained with ethidium bromide, and visualized under UV light (5). The results were returned for statistical analysis.

### *Statistical Analysis*

The raw data sent by each laboratory were statistically analyzed according to the recommendations of Scotter et al. (14) by the methods of Langton et al. (15). The diagnostic sensitivity was defined as the percentage of positive samples giving a correct positive signal (4). The diagnostic specificity was defined as the percentage of negative samples giving a correct negative signal and a signal from the IAC (3). Confidence intervals for diagnostic sensitivity and diagnostic specificity were calculated by the method of Wilson (16). Accordance (repeatability of qualitative data) was defined as the percentage chance of finding the same result, positive or negative, from 2 identical samples analyzed in the same laboratory under predefined repeatability conditions. Concordance (reproducibility of qualitative data) was defined as the percentage chance of finding the same result, positive or negative, from 2 identical samples analyzed in different laboratories under predefined repeatability conditions. These calculations take into account different replication in different laboratories by weighting results appropriately. The concordance odds ratio (COR) was defined as the degree of interlaboratory variation in the results, and expressed as the ratio between and accordance × (100 – concordance) and concordance × (100 – accordance), where accordance and concordance are expressed as percentages (15). Confidence intervals for accordance, concordance, and COR were calculated by the method of Davidson and Hinckley (17); laboratories were considered representative of all laboratories in the population of laboratories, not just those participating in this analysis.

Results from each participating laboratory were excluded if an obvious performance deviation from the SOP was recognized, and if target amplicons were present in negative PCR controls, indicating contamination during PCR master mix setup.

## Results and Discussion

All aliquots of the inoculated test samples were investigated in the laboratory that prepared the samples and were culture- and PCR-positive on the day of shipment. All aliquots of the uninoculated test samples were culture- and PCR-negative.

### *Chicken Samples*

Table 1 shows the participants' results of the interlaboratory trial of the PCR-based method for the detection of *Salmonella* in chicken rinse. In agreement with the predefined criteria, the results of Participant No. 2 were excluded, as they reported target amplicons in the PCR-negative control (data not shown). All remaining results were accepted according to the predefined criteria; thus, the statistical analysis was based on 14 sets of results. Table 3

**Table 3. Statistical evaluation of data obtained from interlaboratory trial with PCR-based method for detection of *Salmonella* in chicken rinse samples[a]**

| Inoculation level | Sensitivity, % | Specificity, % | Accordance, % | Concordance, % | COR[b] |
|---|---|---|---|---|---|
| Uninoculated | — | 80.1 (66.7, 90.0) | 85.7 (71.4, 100) | 67.5 (50.7, 95.2) | 2.89 (1.00, ∞) |
| Low | 100 (91.0, 100) | — | 100 | 100 | 1.00 |
| Medium | 100 (91.0, 100) | — | 100 | 100 | 1.00 |
| High | 100 (91.0, 100) | — | 100 | 100 | 1.00 |
| All positive levels | 100 (91.0, 100) | — | 100 | 100 | 1.00 |

[a] Numbers in parentheses are the lower and upper 95% confidence intervals.
[b] COR = Concordance odds ratio.

shows the statistical evaluation of the results. The background flora ranged between 4.9 and $8.0 \times 10^6$ CFU/mL for chicken carcass rinses (data not shown).

### *Pig Samples*

Table 2 shows the participants' results of the interlaboratory trial of the PCR-based method for the detection of *Salmonella* in pig carcass swabs. In agreement with the predefined criteria, the results of Participant Nos. 2 and 8 were excluded, as they reported target amplicons in the assay negative control (data not shown). The results of Participant No. 15 were also excluded, as they did not use IAC in the PCR mixtures. All remaining results were accepted according to the predefined criteria; thus, the statistical analysis was based on 12 sets of results. Table 4 shows the statistical evaluation of the results. The background flora ranged between 2.7 and $3.7 \times 10^2$ CFU/mL for pig swab rinses (data not shown).

In the laboratory trial using the method on chicken rinse and pig swab, the diagnostic sensitivity was 100% for all inoculation levels with complete accordance and concordance. Thus, the method has a very high probability of detecting *Salmonella* at any contamination level in the sample types tested. Conversely, and more importantly, there is a very low risk of obtaining false-negative responses. The strength of PCR lies in its potential for rapid identification of negative samples. The PCR-based method here compares favorably with the ISO culture method (12); the latter takes approximately 3 days to identify negative samples, whereas the method reported here takes only 1 day.

The diagnostic specificity, or percentage of correctly identified uninoculated chicken samples, was 80.1%. There was a higher degree of variation between laboratories than within laboratories, reflected in the concordance value of 67.5%. The COR value can be interpreted as the likelihood of getting the same result from 2 identical samples, whether they are sent to the same or to 2 different laboratories. The closer the value is to 1.0, the higher the likelihood is of getting the same result. With the uninoculated samples, the COR fell within the 95% confidence interval (CI). The upper 95% CI of infinity was due to one laboratory (Participant No. 5) obtaining false-positive results from all uninoculated samples, whereas all other laboratories reported at least one correct negative identification.

In the interlaboratory trial using the method on pig samples, the diagnostic specificity was 91.7%. An infinite value for the COR was calculated because the accordance value was 100%, but this was solely due to a single laboratory (Participant No. 5) reporting 3 false-positive results. The infinite upper CI produced thereby is a nuance of the statistical procedure.

Where uninoculated samples were reported as containing *Salmonella*, this was most likely due to contamination during sample treatment by the trial participant(s). During routine application of this method, any positive result obtained would be

**Table 4. Statistical evaluation of data obtained from interlaboratory trial with PCR-based method for detection of *Salmonella* in pig swab samples[a]**

| Inoculation level | Sensitivity, % | Specificity, % | Accordance, % | Concordance, % | COR[b] |
|---|---|---|---|---|---|
| Uninoculated | — | 91.7 (78.2, 97.1) | 100 | 83.3 (59.1, 100) | ∞ (1.00, ∞) |
| Low | 100 (89.6, 100) | — | 100 | 100 | 1.00 |
| Medium | 100 (89.6, 100) | — | 100 | 100 | 1.00 |
| High | 100 (89.6, 100) | — | 100 | 100 | 1.00 |
| All positive levels | 100 (96.3, 100) | — | 100 | 100 | 1.00 |

[a] Numbers in parentheses are the lower and upper 95% confidence intervals.
[b] COR = Concordance odds ratio.

confirmed by re-analyzing the retained BPW-enriched broth using the complete ISO 6579 method. The lower COR value for uninoculated chicken rinse than for uninoculated pig swab samples seems not to be statistically significant ($p < 0.05$), and therefore is not necessarily dependent on the sample matrix. In general, laboratories having false-negative results from pig swabs also had false-negative results from chicken rinses. Only 2 laboratories (Participant Nos. 9 and 12) found *Salmonella* DNA in uninoculated chicken samples but not in uninoculated pig swabs.

In order to focus only on the combination of the sample extraction method and PCR assay steps, the pre-enrichment step was performed centrally. Individual deviations in sampling set-up and pre-enrichment were avoided by this approach. The next phase of validation is planned to include the pre-enrichment step.

To the best of our knowledge, no other interlaboratory trial has validated a similar nonproprietary formulation PCR-based method for *Salmonella*. The method, unlike real-time fluorescence-based PCR, does not require procurement of costly equipment. These features, in combination with its repeatability and reproducibility, and the high diagnostic accuracy (97.5%) obtained on various naturally contaminated samples (7), make it eminently suitable for routine use, and thus appropriate for international standardization.

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# Letter to the Editor

## DIAGNOSTIC PCR: MAKING INTERNAL AMPLIFICATION CONTROL MANDATORY

The explosive increase since the beginning of the 1990s in the number of publications reporting PCR-based methods for detection or molecular typing of foodborne pathogens has attracted the attention of end-user laboratories.

However, the well-recognized difficulties in reproducing published tests because of variation in performance of PCR thermal cyclers (Schoder *et al.* 2003), in efficiency of different DNA polymerases, and in the presence of PCR inhibitors in the sample matrix, have hampered implementation in end-user laboratories. This particularly applies to laboratories with quality-assurance programmes.

It is necessary to have PCR-based methods available as internationally recognized standards (Hoorfar and Cook 2002). Currently, lack of international standards often forces end-user laboratories to spend substantial resources on adaptation of the published tests. Although many commercial PCR kits are available, it is important that end-users and reference laboratories have access to open-formula, noncommercial and nonproprietary PCRs in which the information on target gene and reagents are fully available.

The prerequisite for a PCR, published in the scientific literature, to be adopted as a standard is that it has to be nonproprietary, and to have been validated through multicentre collaborative trial according to the international criteria (Anon. 2001, 2002a; Hoorfar and Cook 2002). Multicentre trial validation of noncommercial PCRs for detection of zoonotic pathogens has been performed by a European validation and standardization project (FOOD-PCR: http://www.pcr.dk) involving 35 laboratories from 21 countries (Hoorfar 1999; Malorny *et al.* 2003).

A major drawback of most published PCRs, surprisingly even to date, is that they do not contain an internal amplification control (IAC). An IAC is a nontarget DNA sequence present in the same sample reaction tube, which is co-amplified simultaneously with the target sequence. In a PCR without an IAC, a negative response (no band or signal) can mean that there was no target sequence present in the reaction. But, it could also mean that the reaction was inhibited, as a result of malfunction of thermal cycler, incorrect PCR mixture, poor polymerase activity and, not least, the presence of inhibitory substances in the sample matrix. Conversely, in a PCR with an IAC, a control signal will always be produced when there is no target sequence present. When neither IAC signal nor target signal is produced, the PCR reaction fails. Thus, when using a PCR-based method in routine analysis, an IAC, if the concentration adjusted correctly, will indicate false-negative results. It is the false-negative results that turn a risk into a threat for the population, whereas a false-positive result merely leads to a clarification of the presumptive results by re-testing the sample.

The European Standardization Committee (CEN), in collaboration with International Standard Organization (ISO) has proposed a general guideline for PCR testing that requires the presence of IAC in the reaction mixture (Anon. 2002b). Therefore, only IAC-containing PCRs may undergo multicentre collaborative trials, which is a prerequisite for standardization.

Scientific journals must provide the source of new PCR-based methods suitable for standardization. Therefore, we propose that henceforward the editorial boards of applied microbiology journals require inclusion of an IAC in diagnostic PCR reported in submitted manuscripts. This could be performed by providing a specific section devoted to PCR in their Instruction to Authors.

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# Novel Molecular Method for Detection of Bovine-Specific Central Nervous System Tissues as Bovine Spongiform Encephalopathy Risk Material in Meat and Meat Products

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**The emergence of a new variant of Creutzfeldt-Jacob disease during the bovine spongiform encephalopathy epidemic has focused attention on the use of tissues from the central nervous system (CNS) in food. For efficient consumer protection, European legislation prohibits several bovine tissues, encompassing mainly the central nervous system, from the food chain. A quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) was designed to identify bovine spongiform encephalopathy risk material in meat and meat products. This was based on an mRNA assay that used bovine, ovine, and caprine glial fibrillary acidic protein (GFAP) encoding gene sequences as a marker. The real-time RT-PCR assay allowed the detection of bovine, ovine, or caprine CNS tissues in meat and meat products. Bovine brain at a concentration of 0.01% yielded a positive PCR reaction. The real-time RT-PCR assay included a housekeeping gene as an endogenous control. The detection was not affected by heat treatment of the meat products. The quantitative real-time RT-PCR detection of GFAP mRNA appeared to be useful as a routine diagnostic test for the detection of illegal use of CNS tissues in meat and meat products. The stability of the specific region of GFAP mRNA also allows the detection of CNS tissues after meat processing steps. The use of organ- and species-specific subunits of mRNA might be a promising approach for the detection of other banned tissues. *(J Mol Diagn 2005, 7:368–374)***

Since 1996, evidence has been increasing for a causal relationship between ongoing outbreaks in Europe of a bovine spongiform encephalopathy (BSE) in cattle and the variant Creutzfeldt-Jakob disease in humans. However, some member states are not able to give an exact overview about the occurrence and the development of BSE disease. This is based in part on the late start with area-wide BSE tests and on the delayed implementation of prohibition of meat and bone meal for animal feeding. In the U.S., the first BSE case was announced in December 2003.[1] However, there is strong evidence that BSE can cause variant Creutzfeldt-Jakob disease, most likely via the oral route of infection.[2–4] Public health concerns require the efficient exclusion of ruminant tissues containing high accumulations of the causative agent ($PrP^{SC}$),[5] particularly brain and spinal cord, from the food chain. This is demanded by European laws. It should be mentioned that porcine central nervous system (CNS) is not affected by this legislation.[6]

For further risk assessment, the member states are classified according to their individual situation of reported and expected BSE cases. Hence, for the maintenance of the "BSE-free status" of the affected countries and the strong prohibition of specified bovine offal from the food chain, the detection of BSE risk material is one of the highest priority tasks for food analysts. Several phenotypic methods for detection of BSE risk material have been developed, including enzyme-linked immunosorbent assay, HPLC, Western blot, and immunohistochemical methods.[7–11] However, these methods allowed neither differentiation of CNS tissues of banned species from CNS tissues of other animal species nor an exact quantification of the detected CNS. In addition, a test system should also allow an effective control of heat-treated samples.

In the present study, a real-time reverse transcriptase-polymerase chain reaction (RT-PCR) method based on a species-specific glial fibrillary acidic protein (GFAP) mRNA region was developed for the detection of bovine, ovine, and caprine CNS tissues in raw and heat-treated meat products. The relative quantitative technique was evaluated in CNS and other tissues of various animal origins.

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## *Materials and Methods*

### *Samples and the Internal Reference Material (IRM)*

The raw meat samples used in the present investigation were purchased from a slaughterhouse in Giessen, Germany. The materials used were muscle ($n = 3$), liver ($n = 3$), heart ($n = 3$), kidney ($n = 3$), lung ($n = 3$), spleen ($n = 3$), lymph nodes ($n = 3$), peripheral nerves (in particular sciatic and axillaries nerves) ($n = 6$), and spinal cord ($n = 3$) from bovine subjects as well as muscle of ovine, caprine, and porcine origin. In addition, brains from bovine ($n = 10$), ovine ($n = 3$), caprine ($n = 2$), and porcine ($n = 5$) subjects were included. These were removed directly from skulls; porcine brains were taken from carcasses of pig halves. The samples were transported immediately on ice to the laboratory. Additionally, brain tissues from chicken ($n = 2$), turkeys ($n = 2$), and ducks ($n = 1$) were kindly provided by the Klinik für Vögel, Reptilien, Amphibien und Fische, Justus-Liebig-Universität Giessen. Likewise, cooked and raw sausages with varying additives of bovine brain ($n = 4$ of each) were produced as IRM according to the basic recipes provided by the guiding principles of the German Food Code.[12] The preparation of the sausages is described by Lücker et al.[9] The sausage filling contained 50% porcine meat (max 10% fat), 5% bovine meat, 25% porcine fat, 20% ice, 20 g/kg curing salt (4 to 5 g/kg $NaNO_2$ in NaCl; Enders, Reiskirchen, Germany), 3 g/kg phosphate-based stabilizer (di-Na-phosphate; Kirchheimbolanden, Germany), 5 g/kg ready mixed spices for cooked sausages (Delikatess Aufschnitt, Gewürzmüller, Stuttgart, Germany), and bovine brain in varying concentrations (20, 8, 1, 0.1, and 0.01%). For preparing the cooked sausages, the contents were heated for 90 minutes at 80°C in a steam heater (Fessmann, Winnenden, Germany).

### *DNA Extraction*

Total cellular DNA was isolated from bovine, ovine, caprine, and porcine muscle samples using the DNeasy tissue isolation kit (Qiagen, Darmstadt, Germany) according to the manufacturer's instructions. Briefly, 25 mg of the sample was lysed, followed by binding of the DNA to the spin column (Qiagen). After washing steps, the DNA was eluted with 100 µl of elution buffer; 2.5 µl was used as a DNA template.

### *Amplification of GFAP-DNA Subunits*

A segment of bovine, ovine, caprine, and porcine GFAP gene was amplified and sequenced. For this purpose, sequencing primers were designed using human (accession number AF028784), rat (accession number Z48978), and bovine (accession number Y08255) GFAP gene sequences already published in National Center for Biotechnology Information GenBank.

The PCR reaction mixture (50 µl) contained 1 µl of primer 1 (10 pmol/µl), 1 µl of primer 2 (10 pmol/µl), 1 µl of dNTP (10 mmol; Roche Diagnostic, Mannheim, Germany), 5 µl of 10× thermophilic-buffer (Applied Biosystems, Darmstadt, Germany), 0.2 µl of *Taq*DNA polymerase (5 U/µl; Applied Biosystems), and 39.3 µl of double-distilled water. Finally, a 2.5-µl of DNA preparation was added to each reaction tube. The PCR was carried out in a thermal cycler (PE GeneAmp PCR system 9600; Applied Biosystems) with the following program: one 3-minute precycle at 93°C; and 35 times for 30 seconds at 93°C, 30 seconds at 52°C, and 45 seconds at 72°C, followed by a final extension incubation of 72°C for 5 minutes. The presence of PCR products was determined by electrophoresis of 10 µl of the reaction product in a 2% agarose gel (Appligene, Heidelberg, Germany) with Tris acetate-electrophoresis buffer (0.04 mol/L Tris and 0.001 mol/L EDTA, pH 7.8) and a 100-bp DNA ladder (Roche) as molecular marker.

### *Sequencing of GFAP Subunit*

Sequencing of the amplified subunits of bovine, ovine, caprine, and porcine GFAP gene was performed using the facilities of the university (Institut für Medizinische Mikrobiologie und Virologie, Justus-Liebig-Universität Giessen) with the MegaBACE 1000 DNA Sequencing System (Amersham Pharmacia Biotech, Freiburg, Germany) with protocols described by the manufacturer. The sequence data were further studied and analyzed with the computer program SeqMan (Lasergene; DNASTAR, Inc., Madison, WI).

### *Design of Specific Oligonucleotide Primers and the Fluorogenic Probe*

Based on the generated bovine, ovine, caprine, and porcine GFAP gene sequences, a GFAP gene region was determined to be characteristic for the three ruminant species but not for porcine GFAP gene. The specific oligonucleotide primers were designed using the Primer Express Software (version 2.0; Applied Biosystems). The forward primer RTGcowM56F2a was selected from a region located on exon 5, whereas the reverse primer RTGcowM56R2a was selected from a region of exon 6. To avoid the amplification of DNA, the TaqMan fluorogenic minor groove binder (MGB)-probe was selected in the exon 5/exon 6 junction region and conjugated with 6-carboxy-fluorescein (FAM). The sequences of the oligonucleotide used in the real-time PCR reaction were as follows: the forward primer RTGcowM56F2a, 5′-ACC TGC GAC CTG GAG TCC T-3′; the reverse primer RTGcowM56R2a, 5′-CTC GCG CAT CTG CCG-3′; and the fluorogenic MGB probe OptiR, 6-FAM-ACT CGT TCG TGC CGC GC-MGB. The oligonucleotide primers and the fluorogenic probe were synthesized by Applied Biosystems.

### *RNA Extraction*

Total cellular RNA was isolated from the samples using the RNeasy Lipid Tissue mini kit (Qiagen) according to

the manufacturer's instructions. Briefly, 1000 μl of Qiazol was added to a 50-mg sample, and the mixture was transferred to a glass matrix tube (FastRNA Green; Q BIOgene, Heidelberg, Germany) for cell lysis. The mixture was processed in a spin/rotation instrument for cell lysis (FastPrep-120; Q BIOgene), with a speed setting of 6 and a time setting of 45 seconds. After processing, 200 μl of chloroform was added to the mixture. The aqueous and organic layers were separated by microcentrifugation for 15 minutes at room temperature at 10,000 × *g*. The aqueous phase, containing the RNA, was removed and 200 μl of ethanol (70%) was added, followed by binding of the RNA to the spin-column (Qiagen). After DNase digestion with 80 μl of RNase-free DNase (Qiagen), the total RNA was washed and eluted with 50 μl of elution buffer.

### *Real-Time RT-PCR Analysis*

Total RNA from each sample was subjected to reverse transcription using TaqMan Reverse Transcriptase Reagents kit with uracil-*N*-glycosylase (Applied Biosystems) according to the manufacturer's protocol. The reactions were incubated at 25°C for 10 minutes and 48°C for 30 minutes followed by a final reverse transcriptase inactivation at 95°C for 5 minutes. Real-time PCR reactions were subsequently carried out in a 50-μl reaction mixture with final concentrations of 300 nmol/L of each oligonucleotide primer, 200 nmol/L of the fluorogenic probe, and 1× TaqMan Universal PCR Master mix (Applied Biosystems) in an ABI Prism 7000 Sequence Detection System (Applied Biosystems). Thermal cycling conditions comprised an initial UNG incubation at 50°C for 2 minutes, an AmpliTaq Gold DNA Polymerase activation at 95°C for 10 minutes, 40 cycles of denaturation at 95°C for 15 seconds, and an annealing and extension at 60°C for 1 minute. Each measurement was performed at least in duplicate, and the threshold cycle ($C_t$) (the fractional cycle number at which the amount of amplified target reached a fixed threshold) was determined.

### *Construction of Standard Curve*

The GFAP cDNA concentration was estimated by using an UV spectrophotometer (DU 640; Beckman). Real-time PCR amplifications of the serially diluted GFAP cDNA were performed using the same PCR conditions mentioned above. The $C_t$ value was defined as the number of PCR cycles required for the fluorescence signal to exceed the detection threshold value (background noise). All of the reactions were run in triplicate, and the normalized reporter signal ΔRn and $C_t$ were averaged from the values obtained in each reaction. A standard curve was then constructed by plotting the $C_t$ of known concentration of each standard sample. The quality of the standard curve can be judged from the slope and the correlation coefficient (*r*). The slope of the line can be used to determine the efficiency of the target amplification (Ex) using the equation $Ex = (10^{-1/slope}) - 1$.[13]

### *Endogenous Control and Relative Quantitative Analysis*

The comparative $C_t$ method was used for the relative quantitative detection of the expression of GFAP gene in different organs with the 18S rRNA as an endogenous control for each reaction. The 18S rRNA probe was labeled with the fluorescent dye FAM (Assay on Demand; Applied Biosystems). Real-time data were analyzed using the comparative $C_t$ method. This method is similar to the standard curve method, except that it uses the arithmetic formula $2^{\Delta\Delta Ct}$ to achieve relative quantification (Applied Biosystems User Bulletin 2). The relative expression of GFAP gene in the different organs was determined with reference to the muscle after normalization against 18S rRNA as implemented in the ABI PRISM 7000 Sequence Detection System software.[14] A prior validation experiment was performed to demonstrate that amplification efficiencies of GFAP cDNA and 18S rRNA primer/probe sets.

## *Results*

### *Sequences of GFAP Gene Subunit*

According to the published human, rat, and bovine GFAP gene sequences, oligonucleotide primers were designed that allowed an amplification of subunits of bovine, ovine, caprine, and porcine GFAP genes. These partial sequences were used for the design of specific oligonucleotide primers and a fluorogenic probe and were published in GenBank under the accession numbers AY617158, AY617159, AY617160, and AY617161, respectively.

### *Specificity of the Real-Time GFAP RT-PCR*

The designed oligonucleotide primers RTGcowM56F2a and RTGcowM56R2a allowed after reverse transcription an amplification of specific regions of bovine, ovine, and caprine GFAP mRNA isolated from brain with a size of 86 bp. No amplification could be observed with GFAP mRNA of porcine origin. Amplification of a bovine DNA preparation as positive PCR control yielded an amplicon with a size of 219 bp (Figure 1). The oligonucleotide primers were subsequently used together with a fluorogenic MGB probe for TaqMan RT-PCR detection of GFAP mRNA. The real-time RT-PCR amplified bovine, ovine, and caprine GFAP mRNA, but not bovine DNA or porcine mRNA and DNA. In addition, mRNA or DNA from turkey, chicken, and duck brain tissues showed no positive signal.

### *Evaluation of Housekeeping Gene as an Endogenous Control and Determination of the Detection Limit of the Real-Time GFAP RT-PCR*

Using a serial dilution of a known quantity of a bovine brain GFAP cDNA standard, ranging from 9.66E − 04 to

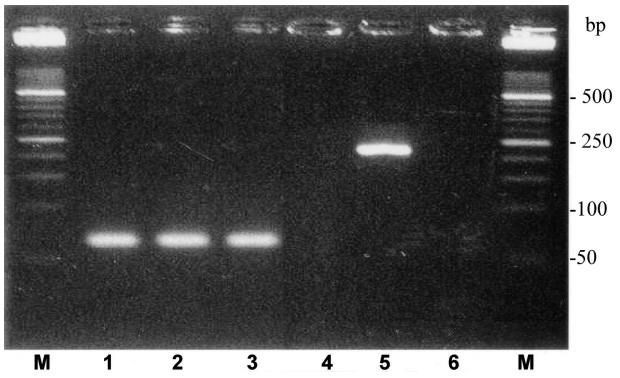


**Figure 1.** Typical amplification products of bovine (**lane 1**), ovine (**lane 2**), and caprine (**lane 3**) GFAP cDNA with a size of 86 bp; negative RT-PCR of porcine GFAP cDNA (**lane 4**); positive PCR control by using genomic DNA with a size of 219 bp (**lane 5**); and negative control (**lane 6**); M, DNA molecular weight marker XIII 50-bp ladder (Roche).

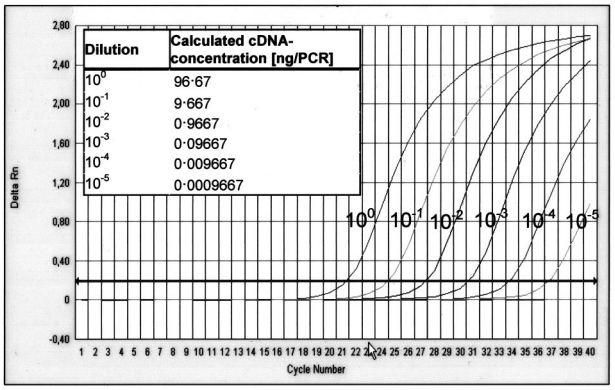


**Figure 3.** Real-time GFAP RT-PCR amplification curves of undiluted and 10-, 100-, 1000-, 10,000-, and 100,000-fold dilutions of bovine cDNA. On the *y* axis, the absolute emission intensity is indicated; the *x* axis shows the number of PCR cycles. The calculated cDNA concentrations are shown in the table.

96.67 ng/PCR, it was possible to obtain a series of amplification plots and to determine their relative $C_t$ values. Standard curves were then constructed by plotting the $C_t$ of the known concentration of each standard sample with the GFAP and 18S rRNA RT-PCR. Each sample was performed in triplicate, and the standard curves showed slopes of −3.561 and −3.630, respectively. The correlation coefficient (*r*) was >0.999 of both GFAP RT-PCR and 18S rRNA RT-PCR. This indicated that relative differences in target genes could be calculated by the comparative $C_t$ method (Figure 2). Because the $C_t$ value decreased linearly with the increasing amount of GFAP cDNA concentration in the standard sample, the absolute quantity of the GFAP transcript in an unknown sample could be accomplished by measuring the $C_t$ value and using the standard curve to calculate the absolute GFAP cDNA concentration. The detection limit of this real-time GFAP RT-PCR system was approximately $1.0 \times 10^{-12}$ g/PCR reaction of absolute GFAP cDNA. This concentration could be detected by a $C_t$ value of around 36 cycles (Figure 3).

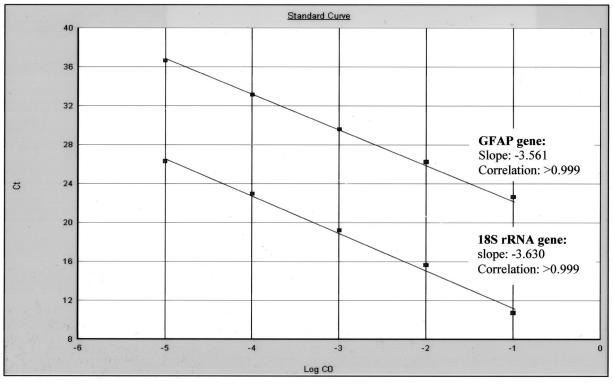


**Figure 2.** Relative standard curves show comparable amplification efficiencies of GFAP gene (target) PCR and 18S rRNA gene (housekeeping gene) real-time RT-PCR. The Δ slope was <0.1.

## *Organ Specificity and the Relative Quantification of Real-Time GFAP RT-PCR*

The mRNA of GFAP gene of three different samples of brain, spinal cord, peripheral nerves, liver, lung, spleen, heart, lymph node, and muscle from bovine subjects was determined in triplicate transcripts by relative quantitative real-time PCR. For all samples, the expression level of the GFAP gene was calculated relative to the organ muscle in the presence of the endogenous control (18S rRNA gene). The endogenous control shows a positive signal with all cDNA samples investigated. The $C_t$ values of all non-neural organs investigated were greater than 35 cycles. The values of the relative amount of GFAP cDNA obtained from non-neural organs varied between 2.00E − 03 with kidney samples to 3.90E + 00 in investigating lymph node samples. The CNS tissues including brain and spinal cord contained 1.46E + 05 and 1.88E + 06, respectively. The relative amount of peripheral nerves was 2.41E + 02 (Table 1). No fluorescent signal was detected in any of DNA samples of the various organs or in the negative RT-PCR control. Figure 4 demonstrates the $Log_{10}$ values of the relative amount of the GFAP expression in different organs investigated.

## *Detection of Bovine GFAP in IRM*

To test the stability and detectability of bovine GFAP mRNA after meat processing and heat treatment, cooked and raw sausages with varying additives of bovine brain (20, 8, 1, 0.1, and 0.01%) were investigated by quantitative real-time PCR (Figure 5). The bovine brain was detectable in raw as well as in heat-treated sausages. The $C_t$ values of the raw sausages with various brain concentrations ranged from 20.17 cycles for sausages containing 20% bovine brain to 32.23 cycles for sausages that comprised 0.01% of bovine brain. The $C_t$ values of the cooked sausages ranged from 20.18 cycles for sausages containing 20% bovine brain to 33.42 cycles for sausages with 0.01% of bovine brain. The bovine DNA and the negative control as well as the sausages without

**Table 1.** Relative Quantitative Detection of GFAP mRNA in Different Bovine Organs Using Comparative $C_t$ Method ($\Delta\Delta C_t$)

| Organs | GFAP | 18S rRNA | GFAP | $\Delta\Delta C_t$† | GFAP | |
|---|---|---|---|---|---|---|
| | Average $C_t$ | Average $C_t$ | Average $\Delta C_t$* | | RQ to Muscle‡ | RQ $Log_{10}$§ |
| Brain | 18.445 | 14.349 | 4.096 | −17.154 | 1.46E + 05 | 5.16 |
| Spinal cord | 14.737 | 14.327 | 0.410 | −20.840 | 1.88E + 06 | 6.27 |
| Peripheral nerves | 29.206 | 15.867 | 13.339 | −7.911 | 2.41E + 02 | 2.38 |
| Heart | 35.813 | 14.582 | 21.231 | −0.019 | 1.01E + 00 | 0.01 |
| Kidney | 46.851 | 16.697 | 30.154 | 8.904 | 2.00E − 03 | −2.68 |
| Liver | 46.042 | 15.077 | 30.965 | 9.715 | 1.00E − 03 | −2.92 |
| Lung | 36.514 | 14.703 | 21.811 | 0.561 | 6.78E − 01 | −0.17 |
| Lymph node | 36.105 | 16.819 | 19.286 | −1.964 | 3.90E + 00 | 0.59 |
| Muscle | 36.234 | 14.984 | 21.250 | 0.000 | 1.00E + 00 | 0.00 |
| Spleen | 37.669 | 14.663 | 23.006 | 1.756 | 2.96E − 01 | −0.53 |

*GFAP $\Delta C_t$ is the $C_t$ for the target gene normalized to an endogenous control (18S rRNA) ($C_{t\ GFAP} - C_{t\ 18S\ rRNA}$).
‡The calculation of $\Delta\Delta C_t$ involves subtraction by the $\Delta C_t$ calibrator value (muscle) ($\Delta C_{t\ organ} - \Delta C_{t\ muscle}$).
†Relative quantitative of GFAP determined by this expression:($2^{-\Delta\Delta Ct}$).
§Amount of the target GFAP relative to the calibrator as $log_{10}$ of the $\Delta\Delta C_t$ value.

added bovine brain yielded a fluorogenic signal under the threshold level (Table 2). The endogenous control shows a positive signal with all cDNA of IRM investigated even after heat treatment.

## *Discussion*

In the present study, a sensitive detection assay for bovine, ovine, and caprine CNS tissues was developed using an mRNA-based quantitative real-time RT-PCR with the GFAP gene as marker. This protein is expressed at high levels in the astrocyte cells of the CNS tissues but not or only at very low levels in the cellular elements of other organs and peripheral nerves.[7] GFAP protein had been previously used as a marker for the detection of CNS tissues. A detection was performed by Western blotting,[9,10] by enzyme-linked immunosorbent assay,[7,8] and by immunohistochemistry.[11] The disadvantage of these GFAP detection methods is that none of these methods appeared to be species specific. A species specificity could be determined by gas chromatography spectrometric analysis of the fatty acid composition of meat components[15] or by PCR-RFLP analysis of GFAP mRNA.[16] However, the spectrometric analysis requires expensive laboratory equipment, and the latter shows cross reactions with other organs, eg, muscle or heart. In addition, the PCR-RFLP method used a conventional detection by nonquantitative gel electrophoresis.

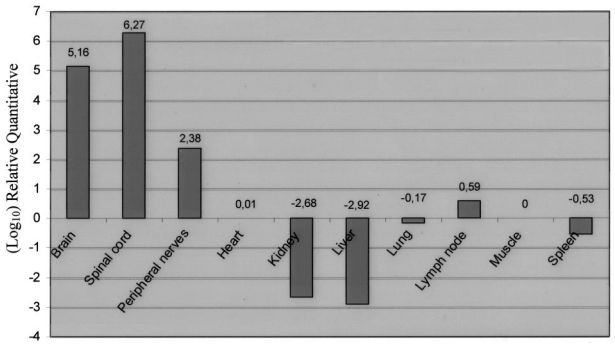


**Figure 4.** Relative quantitative value ($Log_{10}$) of different organs calculated relative to the organ muscle, normalized to the housekeeping gene.

In the present study, partial gene sequences of bovine, ovine, caprine, and porcine GFAP genes were analyzed to find conserved and variable regions. These regions were used for the selection of species-specific oligonucleotide primers and a fluorogenic probe to amplify RT-PCR products that allow an amplification of species-specific regions of mRNA of bovine, ovine, and caprine origin but not of mRNA of porcine origin. The assay allowed the detection of CNS tissues and in parallel the identification of the species. An mRNA-based analytical test to determine the presence of CNS tissues in meat should be sensitive and should reliably avoid false-positive results of DNA contamination. Based on this consideration, we designed a MGB TaqMan probe, which was selected in the junction of exon-exon region. The presented real-time RT-PCR amplified and detected only mRNA but not bovine or porcine DNA.

The sample preparation is one of the most critical aspects of mRNA assays because this might cause false-negative results. The RNA isolation method, using a combination of a mechanical step (Fast Prep) and the RNeasy Lipid Tissue kit, as performed in this study provided a good quality of total RNA. The high lysis efficiency of this method enables extraction of a sufficient amount of total RNA in about 1 hour. In comparison with classical RNA extraction protocols, it has been shown that the selected method increased the sensitivity of the detection of GFAP mRNA in meat products (data not shown).

The typical standard curves for the quantitative real-time must be generated with an optimal correlation coefficient of >0.99. The efficiency of cDNA synthesis from an mRNA template is important for the downstream processing of cDNA quantitation. The relative quantitation standard was reverse transcribed in a batch process that included the RNA samples to be quantitated. This procedure allowed for accurate quantitation between different assays, regardless of differences in reverse transcription efficiency, as aliquots of the same standard RNA were used. The endogenous control for quantitative RT-PCR experiments functions as a control for reverse transcription and PCR reaction, as well as mRNA quantity, quality, and integrity. rRNAs are frequently used as internal controls for quantification experiments, particularly

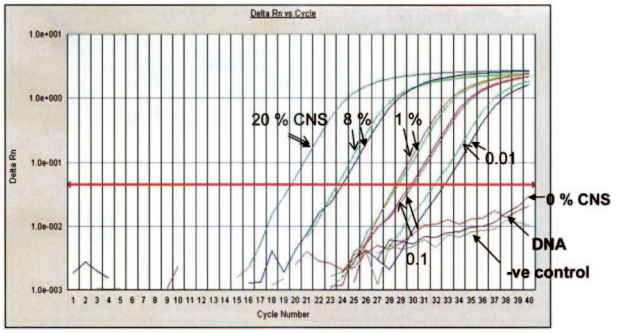


**Figure 5.** Detection of bovine GFAP mRNA in sausages with varying quantities of homogenized brain tissue added with (➞) and without (→) heat treatment of the samples.

because stable expression levels of 18S rRNA relative to other housekeeping genes have been described for rat, mouse, and human tissues.[17,18] However, 18S and 28S rRNA are distinct from messenger RNAs, constituting up to 80% of total cellular RNA.[19] The correlation coefficients for standard curves and efficiency values for detection of GFAP and 18S rRNA gene were excellent. The mean correlation coefficient for these response curves obtained was >0.999. The efficiency of amplification was very similar (efficiencies were within 0.1). According to the results of this study the 18S rRNA gene can be recommended to serve as an endogenous control to avoid the false-negative results as well as for relative quantitative detection of GFAP gene in different tissues. The detection limit of this system was <0.01% of CNS tissues in meat products, which is 100 time less than the detection limit of two previously described commercial kits.[20] In addition, both of these detection kits are based on immunological reactions and are not species specific.

In general, mRNA can have a short half-life within viable cells and is rapidly degraded by specific enzymes (RNases), which are themselves very stable even in environments outside the cell itself.[21] It has been previously reported, that mRNA sequences from *Escherichia coli* could be amplified for up to 30 hours after cell death.[22] In mammalian cells, the abundance of a particular mRNA can be many folds more than in bacteria. It has been demonstrated that mRNA degradation can be dependent on the sequence content[23] or regions thereof.[21] In the present study, the functional activity or the structural integrity of the GFAP mRNA was not investigated; only a small species-specific region of GFAP mRNA has been used as a marker of CNS tissues. This can explain the stability of this region during the food processing steps even after heat treatment.

For determination of expression level of GFAP mRNA in different organs, $2^{\Delta\Delta Ct}$ method was used, which is included an endogenous control and a calibrator organ. The purpose of the endogenous control is to normalize the PCRs for the amount of the mRNA added to the reverse transcription reactions. The choice of calibrator for $2^{\Delta\Delta Ct}$ method depends on the type of target gene. The data are presented as the fold change in gene expression normalized to the 18S rRNA gene and relative to the calibrator organ (in our case muscle as a primary component of the meat product). The present assay detected very low levels of GFAP in non-neural organs. The relative amount of GFAP expression obtained from all of these organs was less than 3.90E + 00. The peripheral nerve samples yielded a relative value of 2.41E + 02. This was in contrast to brain and spinal cord values of 1.46E + 05 and 1.88E + 06, respectively; corresponding to about 1000- and 10,000-fold more of GFAP mRNA content. The results of the GFAP levels in the different neural tissues obtained in the present study were comparable with the results demonstrated previously by other authors.[7,8]

The $C_t$ value obtained from all of these organs was above 35 cycles, whereas a concentration of 0.01% of brain exhibited a signal at $C_t$ value of 32.23 (± 0.12) cycles. The peripheral nerve samples yielded a $C_t$ value of 29.21 (± 0.10). The $C_t$ values of brain and spinal cord were 18.45 (± 0.01) and 14.74 (± 0.16), respectively.

The peripheral nerve signal does not present a realistic problem in the analysis of CNS detection in meat and meat products. Peripheral nerves would be detected if present as one of the primary components of the meat product. Thus, if the peripheral nerves comprised more than 10% of the meat product, a fluorescent signal would be detected at the $C_t$ value of 34.00 (± 1.20) cycles; this is equivalent to 0.047 ng cDNA/PCR reaction (data not shown). This signal is about one-seventeenth of the concentration value of 3.28 ng cDNA/PCR resulting from 0.1% brain. Therefore, the use of a standard curve and a cut-off value of significant CNS contamination of about 0.1% will circumvent this problem.

Analysis of IRM containing bovine brain homogenate and of bovine brain homogenate alone revealed GFAP mRNA RT-PCR signal stability. Enzymes released from minced muscle and brain tissue apparently did not influence the detectability of the bovine GFAP mRNA signal. Analogous results were found for the study of a heated meat product with varying amounts of bovine brain homogenate added. Ingredients and additives commonly used in sausages (see Materials and Method) and other meat products also did not influence the detection of

**Table 2.** Detection of Bovine GFAP mRNA in Sausages with Varying Quantities of Homogenized Brain Tissue with and without Heat Treatment of the Samples

| IRM | $C_t$*values of investigated sausages with varying brain homogenate concentration | | | | | |
|---|---|---|---|---|---|---|
| | 20% | 8% | 1.0% | 0.1% | 0.01% | 0% |
| Raw | 20.17 | 23.33 | 28.58 | 29.99 | 32.23 | Undetected |
| Heat treated† | 20.18 | 23.45 | 28.74 | 30.09 | 33.42 | Undetected |

*$C_t$ value is the cycle number at which the measured fluorescent signal exceeds a calculated background threshold indicating the amplification of the target sequence.
†Heat treatment at 80°C for 90 minutes.

bovine CNS tissue. In addition, it was demonstrated that the applied pasteurization conditions (core temperature, 80°C) had no significant negative effect on the bovine GFAP mRNA signal. Because 0.01% bovine brain homogenate was successfully detected in all of the experiments conducted and no false-negative results were obtained, we conclude that the detection limit is below 0.01% and must be determined in a future study along with the influence of extended heating (eg, sterilization, 121°C) and a long storage period.

According to the present results, an efficient method was used to detect the presence of CNS tissues in meat mixtures containing a minimum amount of bovine CNS tissues. The small mRNA region of the bovine GFAP was not considerably affected by a prolonged storage and heat denaturation of the rendering process, and its sensitivity proved to be high also when samples were further subjected to 80°C for 90 minutes. On the basis of these results and previously published studies,[7–11,16] it can be stated that bovine GFAP mRNA exhibits storage and heat stability.

In conclusion, quantitative real-time RT-PCR detection of GFAP appears to be useful as a routine diagnostic test for detection of the illegal use of bovine CNS tissue in meat and meat products. Bovine GFAP mRNA exhibits certain stability, facilitating the detection of CNS tissue in raw and heat-treated sausages. The technique should be evaluated through a ring trial to confirm the ability of the test in different laboratories. According to our knowledge, this is the first report of a real-time RT-PCR method for the species- and tissue-specific detection of bovine CNS tissue in meat and meat products. Furthermore, this approach seems to be promising for the species-specific detection of other banned tissue.

## *Acknowledgments*

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# From farm to fork – *Mycobacterium avium* ssp. *paratuberculosis* (MAP) als Zoonoseerreger?

## *From farm to fork – Mycobacterium avium ssp. paratuberculosis (MAP) as zoonotic agent?*

Michael Bülte, Holger Schönenbrücher, Amir Abdulmawjood

*(Herrn Prof. Dr. Dr. h.c. mult. Dieter Großklaus zum 75. Geburtstag gewidmet)*

**Zusammenfassung**

*Mycobacterium avium* ssp. *paratuberculosis* (MAP) ist der Erreger der Paratuberkulose (Para Tb) bei Wiederkäuern. Aufgrund der vergleichbaren pathomorphologischen Veränderungen bei der als Morbus Crohn (MC) bezeichneten chronisch entzündlichen Darmveränderung des Menschen wird seit langer Zeit eine Beteiligung von MAP an dieser Krankheit diskutiert. Es ist einerseits mehrfach gelungen, MAP-DNA in verändertem Gewebe beim Menschen nachzuweisen sowie, in jüngster Zeit, den Erreger aus dem Blut Erkrankter anzuzüchten; andererseits gibt es eine Vielzahl von Veröffentlichungen, die einen Zusammenhang widerlegen.
In kritischer Bewertung bisher vorliegender Daten kann daher allenfalls die Hypothese formuliert werden, daß MAP möglicherweise am MC des Menschen beteiligt sein könnte. Die Verifizierung oder Falsifizierung dieser Hypothese wird sicherlich erst möglich sein, wenn zum einen die diagnostischen Verfahren, die z. T. ein erhebliches Defizit bezüglich der Sensitivität und/oder Spezifität aufweisen, verfeinert werden und statistisch einwandfreie Fall-Kontroll-Studien erhoben werden, an denen es derzeit noch mangelt.
Abgesehen von einer vorstellbaren Erregerübertragung auf den Menschen durch den direkten Tierkontakt werden aufgrund entsprechender MAP-Nachweise, selbst in pasteurisierter Milch, auch Lebensmittel tierischen Ursprungs als mögliche Vektoren diskutiert. Die vornehmlich aus blutserologischen Untersuchungen stammenden Prävalenzdaten liegen für Milchrinderbestände in vielen Bundesländern über 80 %, bei einer Einzeltierprävalenz, die sich regelmäßig zwischen 1 % und 17 % bewegt. Vergleichbare Daten liegen auch aus anderen Staaten sowie für kleine Wiederkäuer vor. Es handelt sich somit um ein globales Problem. Die ebenfalls hohe Verbreitung von MAP in Wildtierpopulationen sowie die beachtliche Überlebensfähigkeit des Erregers in der Umwelt unterhalten insgesamt einen Infektions- und Kontaminationszyklus über belebte und unbelebte Vektoren, der kaum zu durchbrechen sein dürfte. Vor diesem Hintergrund bedarf es intensiver Forschungsbemühungen zur Fortentwicklung methodischer Verfahrenstechniken als Basis für valide epidemiologische Erhebungen bei Tieren, beim Menschen und in Lebensmitteln.

**Schlüsselwörter:** *Mycobacterium avium* ssp. *paratuberculosis* (MAP), Wiederkäuer, Lebensmittel, Morbus Crohn (MC), MAP-DNA, molekulargenetische Nachweisverfahren.

**Summary**

*Mycobacterium avium* ssp. *paratuberculosis* (MAP) is the causative agent of the paratuberculosis (Para Tb) in ruminants. In addition, this pathogen has been suspected to be implicated in the pathogenesis of Morbus Crohn disease (MC), causing chronic inflammatory intestine changes of humans. The participation of MAP in this illness is discussed intensively and has very contradictory opinions. On the one hand several times succeeded in proving MAP DNA in changed human tissues as well as, in recent time, the bacteria has been isolated from patient's blood. On the other hand there are many publications which support the opposite opinion.

In critical evaluation of already available data, therefore the hypothesis can be formulated that MAP could possibly take part in the MC of humans. The reliable verification of this hypothesis will only be possible, if the diagnostic procedures can be refined upon the substantial deficit concerning the sensitivity and/or specificity of the diagnostic methods. In addition, till now there is lack of optimized statistically case control studies.
The conceivable transmission of the bacteria to humans by the direct animal contact has been considered as possible vector, furthermore, MAP has been detected in pasteurised milk and other food of animal origin. The prevalence data, usually estimated by ELISA for milk cattle stock show over 80 % prevalence in many counties of the Federal Republic of Germany with an individual case prevalence ranging between 1 % and 17 % in different stocks. Comparable data are present also from other countries as well as for small ruminants.
MAP has been concerned as a global problem, moreover the high spreading rate of MAP in wild animal populations as well as the considerable ability of the bacteria to survive in different stages of the infectious- and contamination-cycle, which might hardly be broken through. Thus it requires intensive research efforts for the development of the methodical diagnostic process as basis for valid epidemiological investigations of animals, humans and food.

**Keywords:** *Mycobacterium avium* ssp. *paratuberculosis* (MAP), Ruminants, Food, Morbus Crohn (MC), MAP-DNA, detection methods.

## Einleitung

*Mycobacterium avium* ssp. *paratuberculosis* (MAP) wurde erstmalig 1895 als ätiologisches Agens bei einer chronisch-entzündlichen Darmerkrankung einer Kuh in Deutschland beschrieben (Johne und Frothingham, 1895). Der Dedikationsname Johne's Erkrankung wurde für diese bei Wiederkäuern, aber auch weiteren Säugetieren auftretende Paratuberkulose (ParaTb) eingeführt. Es handelt sich um eine Erkrankung, die, mit Ausnahme von Finnland und Schweden, die als ParaTb-frei gelten, weltweit verbreitet ist und zu erheblichen wirtschaftlichen Schäden führt.

MAP besitzen eine hohe Tenazität und sind in der Umwelt weit verbreitet. Die Überlebensdauer im Boden wird mit bis zu 11 Monaten angegeben (Gay und Sherman, 1992). Die Überlebenszeit in Fließgewässern wird mit 163, in stehenden mit 270 Tagen sowie in Rinderfäzes mit 330 Tagen angegeben (Chiodini et al., 1984b). Damit ist die Gefahr einer Infektion auch in der nachfolgenden Weidesaison gegeben. Das Ausbringen von Gülle oder Dung auf Grünflächen, die der Futtermittelgewinnung dienen, erhöht das Infektionsrisiko erheblich.

Aufgrund der vergleichbaren pathomorphologischen Veränderungen der beim Menschen als Morbus Crohn (MC) bezeichneten chronisch-entzündlichen Darmerkrankung wurde bereits zu Beginn des vorigen Jahrhunderts der Verdacht geäußert, daß solche Mykobakterien ätiologisch auch hierbei eine Rolle im Infektionsgeschehen spielen könnten. Namensgebend war die Beschreibung von acht Fällen einer Ileitis durch Crohn, Ginzburg und Oppenheimer (1932) am Mount Sinai Hospital in New York. Die erste exakte Charakterisierung des klinischen Verlaufs erfolgte bereits 1913 durch Dalziel in Glasgow. Der Verlauf und die Ausdehnung der Erkrankung sind variabel. Das Entzündungsgeschehen sowie rezidivierende Schübe können grundsätzlich an jeder Stelle des Verdauungstraktes auftreten. Für die Betroffenen bedeutet dieses zumeist eine erhebliche Einschränkung der Lebensqualität. Die Letalität liegt bei ca. 6 %. Die großen Ähnlichkeiten zwischen den pathomorphologischen Veränderungen bei der bovinen ParaTb und der humanen intestinalen Tb und MC sind nicht zu leugnen. Einigen Forschergruppen gelang der MAP-Nachweis aus Gewebeproben Erkrankter (Burnham et al., 1978; Chiodini et al., 1984a). Dieses wurde als Hinweis gedeutet, daß MAP entweder als mitverursachendes oder die Erkrankung unterhaltendes Agens beteiligt sein könnte. Mit der Verbesserung der Nachweismethoden und der Einführung der PCR-Methodik gelang der Nachweis von MAP bzw. MAP-DNA beim Menschen immer häufiger (Lisby et al., 1994; Brown et al., 1996; Mishina et al., 1996; Sechi et al., 2001). Mittels Pulsfeld-Gel-Elektrophorese (PFGE) konnte überdies ein hoher genetischer Verwandtschaftsgrad der jeweiligen Isolate gefunden werden. Weiterhin belegen einige Untersuchungen einen geographischen und zeitlichen Zusammenhang zwischen der Prävalenz von ParaTb und MC (Mayberry und Hitchens, 1978; Tamboli, 1996). Die MAP-Hypothese wurde weiterhin dadurch gestützt, daß eine antimikrobielle Therapie, z. B. mit Makroliden, zu einer Verbesserung des Krankheitsbildes führte, und die Annahme zumindest einer Beteiligung von MAP am Infektionsgeschehen damit erhärtet wurde (Gui et al., 1997). Insgesamt also wird die Beteiligung von MAP an MC nach wie vor sehr widersprüchlich diskutiert. Während einige Autoren den Zusammenhang als nicht gesichert charakterisieren (Horowitz und Lien, 1997), sehen andere Wissenschaftler einen Zusammenhang als erwiesen an (Hermon-Taylor und Bull, 2002).

Basierend auf einer entsprechenden Literaturstudie mit über 300 Veröffentlichungen war Chiodini (1998) davon überzeugt, einen Zusammenhang zwischen MC und MAP liefern zu können. Nach einer neueren Literaturrecherche zur Frage, ob die Exposition mit MAP zu einem erhöhten Risiko führt, an MC zu erkranken, finden sich „keine validen wissenschaftlichen Erkennt-

nisse" (Schrauder et al., 2004). Die für diese Recherche einbezogenen Arbeiten wurden als anfällig für systematische Fehler bewertet. Gleichwohl erlauben sie es, „bestenfalls die Hypothese aufzustellen bzw. zu unterstützen, daß MAP am Krankheitsgeschehen bei MC beteiligt sein könnte". Allerdings verdichten sich aufgrund jüngster Publikationen über den signifikanten Nachweis von MAP in Biopsieproben MC-Erkrankter (Bull et al., 2003) sowie die Anzüchtung des Erregers aus dem Blut von MC-Patienten (Naser et al., 2004) die Hinweise für eine Verifizierung der Hypothese. Für die nahe Zukunft wird die Publikation einer australischen Studie erwartet, die einen sehr bedeutsamen Beitrag für die Ätiopathogenese des MC liefern könnte. Dabei handelt es sich um eine mehrjährige doppelblinde, randomisierte, Plazebo-kontrollierte Studie, bei der auch die Behandlung mit Makroliden berücksichtigt wurde.

Aus der Bundesrepublik ist ein Pathogenesekonzept vorgestellt worden, das die ursächliche Beteiligung sowie die Aufrechterhaltung der Erkrankung durch MAP eher unwahrscheinlich erscheinen läßt (Schmid et al., 2004). Es basiert auf der Theorie, daß über eine Mutation im NOD2-Gen, die bei MC-Patienten nachweisbar ist, eine verminderte Defensinexpression stattfindet. Defensine sind endogene antibakterielle Peptide mit antibiotischer Wirkung und spielen eine entscheidende Rolle bei der Aufrechterhaltung der intestinalen Mucosabarriere. Eine reduzierte Defensinbildung kann daher zu einer vermehrten bakteriellen Invasion, u.a. auch von MAP, führen, da die antibakterielle Aktivität der Darmschleimhaut fehlt.

Eine Multi-Center-Studie für Europa besagt, daß die Inzidenz, d. h. die Neuerkrankungen für MC 5,6 Fälle pro 100 000 Einwohner pro Jahr beträgt; damit sind ca. 200 000 Menschen betroffen (Shivananda et al., 1996). Dabei konnte ein Gefälle von nördlichen (z. B. Island: 24,5) nach südlichen Ländern (Südportugal: 1,6) festgestellt werden. Die Prävalenz des MC für Deutschland wurde mit 1/500 bis 1/800 angegeben (Timmer et al., 1999), was in etwa 200.000 Erkrankten entspricht. Die Inzidenz lag bei diesen Untersuchungen in Deutschland zwischen 4,9 bis 5,2 pro 100 000 Einwohner, in Abhängigkeit vom Untersuchungszeitraum (1980–1984 und 1991–1995). Dabei konnte die höchste Inzidenzrate bei den 15- bis 24Jährigen (10,5 bzw. 10,2) ermittelt werden.

In der Diskussion über mögliche Vektoren stehen Milch und Milcherzeugnisse im Vordergrund (Millar et al., 1996). Aufgrund der hohen Tenazität von MAP in der belebten und unbelebten Umwelt müssen aber auch weitere Eintragungs-/ Infektionswege bedacht werden. Dazu zählen Gemüse (Pavlik et al., 2002), Trinkwasser (Mayberry und Hitchens, 1978) sowie Fleisch und Fleischerzeugnisse (Gwozdz et al., 1997; Chamberlin, 2001; Rubery, 2002). Die nachfolgenden Ausführungen stellen den Versuch dar, den aktuellen epidemiologischen Kenntnisstand widerzuspiegeln.

**MAP bei Nutz- und Wildtieren**

Die Paratuberkulose kommt insbesondere bei der Tierart Rind, seltener bei den kleinen Wiederkäuerarten vor, ist aber auch bei einer Reihe von Wildwiederkäuern, z. B. Hirscharten sowie auch Mufflons beschrieben worden (Williams et al., 1985; Godfroid et al., 2000; Machackova et al., 2004). Daneben gelang der Nachweis des Erregers bei einer Vielzahl recht unterschiedlicher Tierarten (Schadnager, Füchse, Dachse, Kaninchen, aber auch Vögel [Beard et al., 2001; Deutz et al., 2005]). In diesem Zusammenhang ist anzumerken, daß MAP regelmäßig auch in Insekten und Würmern nachgewiesen werden konnten, die somit als biologische Vektoren im Gesamtgeschehen von Bedeutung sein können (Lloyd et al., 2001; Whittington et al., 2001; Fischer et al., 2004). Aufgrund der hohen Tenazität des Erregers in der Umwelt spielen möglicherweise solche biotischen ebenso wie abiotische (z. B. Fäzes, Wasser, Futtermittel [Chiodini et al., 1984b]) Faktoren eine entscheidende Rolle bei der wechselseitigen Übertragung von MAP zwischen Nutz- und Wildtieren. Eine solch enge Vernetzung derartiger Infektions- und Kontaminationsketten ist beispielsweise für die hochpathogenen *E. coli*-Stämme des Serovars O157 belegt (Shere et al., 1998; Bülte, 2004). Eine Auflistung aktueller Befunde über die Nachweisquoten von MAP bei Wildtieren in Österreich ist in der Tabelle 1 enthalten (Deutz et al., 2005).

Nicht zuletzt aufgrund der ökonomischen Aspekte stehen Milchrinder im Vordergrund des Geschehens. Die Infektion der Wiederkäuer erfolgt überwiegend fäkal-oral in den ersten Lebenstagen der Tiere, wobei entsprechend infiziertes oder kontaminiertes Kolostrum, Milch oder Wasser die entscheidenden Vektoren darstellen (Chiodini et al., 1984b; Collins, 1997). Die Inkubationszeit beträgt in der Regel 2–3 Jahre, mit einer Streuungsbreite von 6 Monaten bis zu 15 Jahren (Chiodini und van Kruiningen, 1986). Auch die intrauterine ebenso wie eine Sperma-bedingte Übertragung sind beschrieben worden (Sweeney, 1996; Clarke, 1997; Ayele et al., 2004). Nach der oralen Aufnahme invadieren MAP-Zellen die Darmschleimhaut und werden von Makrophagen aufgenommen (Momotani et al., 1988). Der weitere Ablauf der Infektionskaskade, insbesondere auch die damit einhergehende immunologische Reaktion des betroffenen Wirtes konnte aufgrund von Infektionsversuchen mit Kälbern zu einem wesentlichen Teil aufgeklärt werden (Koo et al., 2004). Diese Untersuchungen sind vor dem Hintergrund einer frühzeitigen und zuverlässigen Diagnostik äußerst bedeutsam. Eine klinische ParaTb wird nur von etwa 10–15 % der infizierten Tiere entwickelt, die dann immer letal endet. Aufgrund der vorausgehenden Persistenzphase mit entsprechender Erregerausscheidung ist zu befürchten, daß nach Einstallung von infizierten Tieren eine Vielzahl weiterer Tiere angesteckt werden.

Es liegen bisher kaum Daten über genetische Faktoren in Zusammenhang mit dem Infektionsgeschehen vor. So wurden vereinzelt tierartliche und rassespezifische Unterschiede vermutet (Clarke, 1997). Als signifikanter Faktor wurde bereits sehr frühzeitig die Familienzugehörigkeit der Tiere beschrieben (Hole und MacClay, 1959). Eine Beteiligung erblicher Komponenten am Infektionsgeschehen ist auch aufgrund der geschätzten Heritabilitätswerte für die Empfänglichkeit von Rindern für MAP-Infektionen anzunehmen (Koets et al., 2000). In einer neueren Studie an Dänisch-Holstein Kühen konnte eine signifikante Heritabilität der Antikörper-Produktion bei Paratuberkulose ermittelt werden, hingegen keine genetische Korrelation zwischen täglicher Milchproduktion und der Höhe der Antikörperproduktion (Mortensen et al., 2004).

Basierend auf bis seinerzeit nur vereinzelt vorliegenden Ergebnissen zur Einschätzung der Prävalenz von MAP in der Bundesrepublik vermutete ein nationales Sachverständigengremium (Anonymus, 2001) bis zu

30 % der Rinderbestände als positiv. Mittlerweile sind aktuelle Daten bekannt geworden, die eine durchgehend höhere Prävalenz vermuten lassen, legt man die Nachweisquoten blut- bzw. milchserologischer oder bakteriologischer Reagenten zu Grunde. So erwiesen sich beispielsweise in Hessen 11 von 12 Herden als blutserologisch positiv (Doll, persönliche Mitteilung). Die auf einem kürzlich in Stendal abgehaltenen Symposium vorgelegten Ergebnisse lassen für einige Bundesländer Herdenprävalenzen von über 80 % vermuten, bei Einzeltierprävalenzen zwischen ca. 1 % bis 17 %, selten höher (Hacker et al., 2004; Böttcher et al., 2005; Donat, 2005; Elschner, 2005). Über die Situation bezüglich der Paratuberkulose in Schaf- und Ziegenbeständen liegen nur vereinzelte Meldungen vor. Die Untersuchungen aus Ziegenbeständen in vier Bundesländern ergaben in 17–62 % der Bestände serologisch positive Reaktionen (Anonymus 2001). All diese Daten bedürfen einer vorsichtigen Interpretation, da sie teilweise mit unterschiedlichen Methoden und an differierenden Altersgruppen ermittelt wurden. Ein verläßliches flächendeckendes Überwachungssystem existiert jedenfalls für die Bundesrepublik Deutschland nicht (Köhler et al., 2003). Allerdings liegen Daten über klinische ParaTb-Fälle bei Rindern vor, da es sich um eine meldepflichtige Tierkrankheit handelt. So wurden für das Jahr 2004 238 und für das Jahr 2003 281 Fälle gemeldet.

Auch aus anderen EU-Mitgliedstaaten sind Daten zur Prävalenz von MAP in Wiederkäuerbeständen vorgelegt worden. Untersuchungen in den Niederlanden an fast 16 000 Milchkühen aus 378 Herden ergaben mit 2,5 % zwar eine recht geringe mittlere Anzahl serologisch positiver Tiere, aber in fast 55 % aller Betriebe wurde mindestens ein MAP-positives Tier ermittelt. Im Hinblick auf die unzureichende Sensitivität des serologischen Antikörpernachweises für MAP wurde von den Autoren die tatsächliche Zahl MAP-positiver Bestände auf bis zu 71 % geschätzt (Muskens et al., 2000). Aus Belgien liegen Untersuchungsergebnisse von 13 317 Rindern aus insgesamt 556 Beständen vor. Bei einer Herdenprävalenz von 18 % wurde die Einzeltierprävalenz zu 2,9 % ermittelt (Boelaert et al., 2000). Bezüglich der Tierart Schaf liegen entsprechende Informationen aus Griechenland vor, wo die Prävalenz zu fast 10 % bestimmt wurde (Dimarelli-Malli et al., 1992). Noch höher lagen die Werte bei dieser Tierart in Spanien (Juste et al., 1992). Bei einer Herdenprävalenz von 44,7 % ergab sich innerhalb der Herden eine Einzeltierprävalenz von 33 bis 46 %.

Vor diesem Hintergrund stellt der EU-weite Länderinterne und -übergreifende Transport von Wiederkäuern sowie der Zukauf von Tieren aus nicht kontrollierten Beständen eine enorme Herausforderung dar. Am Beispiel der neuen Bundesländer kann dieses verdeutlicht werden: Während die Paratuberkulose in den 1980er Jahren kaum in Erscheinung trat, gab es Anfang der 1990er Jahre durch unkontrollierten Zukauf infizierter Tiere eine deutliche Zunahme (Köhler et al., 2003).

## MAP in Nahrungsmitteln

In der Diskussion, ob MAP als Zoonoseerreger beim Menschen ätiologisch eine Bedeutung beizumessen sei, werden als Vektoren regelmäßig Milch und Milcherzeugnisse angeführt. Neben dieser als wesentliche Expositionsquelle charakterisierten Infektionsmöglichkeit sind MAP auch in Trinkwasser, in Lebensmitteln pflanzlichen Ursprungs sowie in Gewebeproben von Schafen und Schlachtrindern nachgewiesen worden. Die Übertragungsmöglichkeiten durch pflanzliche Lebensmittel oder auch Fleisch und Fleischerzeugnisse sind wissenschaftlich bisher jedoch nicht weitergehend verfolgt worden.

### *Milch und Milcherzeugnisse*

DieAusscheidung von MAP über die Milch erwies sich mit zwei bis acht Kolonie-bildenden Einheiten pro 50 ml als recht gering (Sweeney et al., 1992). Die fäkale Sekundärkontamination ist wohl der entscheidende Eintragsweg, da einmal infizierte Tiere den Erreger streuen können, wobei eine intermittierende Ausscheidung charakteristisch zu sein scheint (Whitlock et al., 1986). An Paratuberkulose erkrankte Tiere können mit dem Kot bis zu $10^{12}$ MAP/g ausscheiden, persistent infizierte Tiere wohl immer noch mehr als $10^{8}$ MAP/g (Chiodini et al., 1984b). Bereits sehr geringe fäkale Verunreinigungen können daher zu einer erheblichen Kontamination der Milch führen. Eine Abschätzung der Keimzahl ist aufgrund des von der Melkhygiene abhängigen Eintrags durch fäkale Kontamination, die wiederum von der Melkhygiene der Betriebe abhängig ist, nicht möglich. Da Paratuberkulose auch bei Schaf und Ziege häufig vorkommt, ist auch hier mit einem Eintrag in die Milch zu rechnen. Für Deutschland gibt es hierzu allerdings keinerlei Untersuchungen, Angaben für andere europäische Länder sind ebenfalls spärlich und widersprüchlich (Grant et al., 2000; Djonne et al., 2003).

Abgesehen von dem – quantitativ unerheblichen – Verzehr von Rohmilch in der Bundesrepublik Deutschland werden ca. 45 % als pasteurisierte Milch bzw. daraus hergestellte Milcherzeugnisse konsumiert. Den restlichen Anteil stellt nahezu ausschließlich die UHT-Milch (Hammer und Knappstein, 1998). Da es sich bei MAP um hitzetolerante Mikroorganismen handelt, ist es nicht verwunderlich, dass auch in pasteurisierter Milch dieser Erreger anzutreffen ist (Grant et al., 1998; Übersicht: Hammer und Knappstein, 1998; Hammer et al., 2002; ILSI, 2004). Zwar kommt es zu einer Keimreduzierung, da aber das Absterbeverhalten einer logarithmischen Abhängigkeit folgt, ist – je nach Ausgangsbelastung der Rohmilch – mit dem Überleben eines bestimmten Anteils der MAP-Population zu rechnen. Dieser Anteil ist allerdings in aller Regel quantitativ sehr niedrig, da bei der üblichen Pasteurisation eine Reduzierung um 99,999 % (D5-Konzept) erfolgt. Gegen Ende des Pasteurisierungsprozesses kann es zu einem zunehmend asymptotischen Verlauf der Abtötungsrate kommen ("tailing"). Dies kann durch die langsameren Stoffwechselvorgänge bei MAP erklärt werden. Nur bis zu ca. 10 % einer frischen Kultur erscheint bei der sogenannten Vitalfärbung als stoffwechselaktiv, der restliche Anteil befindet sich offensichtlich in einer Art Ruhezustand (Hammer et al., 2002). Zum Vorkommen von MAP in Milcherzeugnissen, außer Konsummilch, liegen bisher kaum Untersuchungen vor. Für im Laborversuch hergestellten Käse (Hartkäse, halbfester Schnittkäse) wurde allerdings in zwei Arbeiten eine relativ lange Überlebensdauer (28–45 Tage) beschrieben (Sung und Collins, 2000; Spahr und Schafroth, 2001). Daher muß davon ausgegangen werden, daß insbesondere in mit MAP-kontaminiertem Rohmilchkäse eine entsprechende Überlebensdauer des Erregers möglich ist.

Eine ausführliche und aktuelle Zusammenstellung bisheriger experimenteller Untersuchungen zur Hitzeinaktivierung bzw. Überlebensfähigkeit von MAP ist bei kritischer Würdigung der jeweiligen Vorgehensweise erst kürzlich erschienen (ILSI, 2004). Von besonderem Interesse sind die von Grant et al. (2002) durchgeführten Untersuchungen, die an mit MAP natürlich kontaminierten Milchproben vorgenommen wurden.

*Fleisch und Fleischerzeugnisse*

Erstaunlicherweise liegen Daten zum Vorkommen von MAP in Fleisch und Fleischerzeugnissen nach ausführlicher Sichtung der Literatur kaum vor. Den diesbezüglichen Ausführungen von Collins (1997), Chamberlin et al. (2001) sowie Rubery (2002) ist allerdings zu entnehmen, daß über solche Lebensmittel eine bisher nicht berücksichtigte Eintragsquelle für MAP gegeben sein könnte. Die schlachttechnologische Herrichtung von Rindern und Schafen führt häufig zu einer fäkalen Kontamination der Tierkörperoberflächen. Da MAP bereits in der Inkubationsphase nach persistierender Infektion mit den Fäzes ausgeschieden wird, ist dieser Eintragsweg in die Nahrungsmittelkette als durchaus realistisch anzusehen. Ein solch fäkaler, sekundärer Kontaminationsweg ist für die Milch als bedeutsamste Eintragsquelle für MAP bewiesen (Rubery, 2002). Auch eine primäre, also *intra vitam* erfolgende Kontamination mit einer hämatogenen, überwiegend aber wohl lymphogenen Streuung von MAP mit anschließender Besiedlung von Muskulatur und Organen ist beschrieben worden (Collins, 1997; Gwozdz et al., 1997). So konnten Rossiter und Henning (2001) bei 34 % von 189 „dünnen" Milch- und bei 3 % von 350 Fleischkühen MAP in Darmlymphknoten nachweisen. Weiterhin erwiesen sich bei den Milchkühen 11,1%, bei den Fleischkühen 0,7 % MAP-positiv in der Leber. Vor dem Hintergrund, daß die Vermarktung eines Rinderschlachttierkörpers ohne weiteres 300-400 Verbraucher erreichen kann, sind diese Zahlen besonders zu gewichten. Der auch als Translokation charakterisierte Übertritt von Erregern ist auch für andere Mikroorganismen, wie z. B. Salmonellen, eindrucksvoll belegt worden (Fehlhaber und Alter, 1999). Eine Untersuchung der Kotproben und Ileocäcallymphknoten von 536 geschlachteten Rindern im Regierungsbezirk Arnsberg in 1993 ergab beim kulturellen Nachweis eine Prävalenz von 1,7 %, bei Anwendung verschiedener serologischer Verfahren eine solche von 16,8 % (Böttcher, 1997). In Kenntnis der schwierigen Kultivierung des Erregers sowie der geringen Sensitivität der in dieser Arbeit eingesetzten serologischen Tests (33,3–44,4 %) muß davon ausgegangen werden, dass die Prävalenz höher anzusetzen ist. Sollte der Eintragsweg von MAP in die menschliche Nahrungsmittelkette über Tierkörper und Nebenprodukte der Schlachtung von Bedeutung sein, so stellt sich die Frage nach der Überlebensfähigkeit von MAP in prozessierten Fleischerzeugnissen. Hierzu gibt es bisher keine Untersuchungen.

**MAP in Pflanzen**

Auf die Möglichkeit einer Infektion bzw. Reinfektion bei Wiederkäuern durch mit MAP kontaminierte Weideflächen ist bereits hingewiesen worden. Ein weiterer bedeutsamer Aspekt ist aber auch der vorstellbare Eintrag von MAP in die menschliche Nahrungsmittelkette über Gemüse und Obst. MAP konnte in Stängeln, Blättern und Früchten von Tomaten, Radieschen und Salat nachgewiesen werden, die mit Rinderdung gedüngt worden waren (Pavlik et al., 2002). Die Überlebensfähigkeit betrug bei <6 °C mindestens 113 Tage. Im Rahmen der besonders in der Bundesrepublik zunehmenden ökologischen Produktionsausrichtung spielt die natürliche Düngung eine große Rolle. Über die damit einhergehenden Gefahrenpotentiale liegen bisher bezüglich MAP keine weitergehenden Untersuchungen vor.

**MAP im Trinkwasser**

Auf die Bedeutung des Trinkwassers als Vektor für Infektionen ist in der wissenschaftlichen Literatur mehrfach hingewiesen worden (Mayberry und Hitchens, 1978; Chiodini et al., 1984b). Als primäre Eintragsquelle ist das mit Fäzes MAP-infizierter Tiere kontaminierte Oberflächenwasser anzusehen. Aufgrund der Resistenz des Erregers gegenüber einer Chlorierung ist die Überlebensfähigkeit auch bei der üblichen Trinkwasseraufbereitung für den menschlichen Verzehr gegeben (Whan et al., 2001).

**Diagnostik von MAP-Infektionen und -Kontaminationen**

Für die Detektion von *Mycobacterium avium* ssp. *paratuberculosis* stehen kulturelle, mikroskopische, immunchemische und molekulargenetische Methoden zur Verfügung, die je nach vorliegender Probenmatrix unterschiedlich geeignet sind. Dabei ist festzustellen, daß insbesondere die bisher etablierten molekulargenetischen Methoden nicht ausreichend validiert und ihre Routinetauglichkeit reproduzierbar unter Beweis gestellt worden sind.

Bei den als „golden standard" (Bartos et al., 2003) angesehenen kulturellen Methoden mit den verschiedenen Festmedien auf Eibasis (Herrolds Egg Yolk Medium [HEYM]), Nährboden nach Löwenstein-Jensen) und auf Agarbasis (Middlebrook 7H10-Agar als transparentes Medium mit stereomikroskopisch gestützter Auswertung, die eine Früherkennung ermöglicht) ist die Erregeranzucht von mindestens 8–16 Wochen als großer Nachteil anzusehen. Radiometrisch gestützte Analyseverfahren (BACTEC) setzen im Vorfeld hohe Anschaffungskosten für das Analysegerät und teilweise weitere Modifikationen der Flüssigmedien in Bezug auf die zugesetzten Antibiotika voraus (Kühn, 2003). Dieses System ist überdies nicht für alle Probenmatrizes standardisiert. Grundsätzlich ist immer auch die Eignung für die Detektion von subletal geschädigten MAP bzw. von in Sphäroplastenform vorliegenden Zellen zu berücksichtigen. Dabei muß sowohl bei Antibiotikazusatz als auch bei inadäquater Dekontamination mit falsch-negativen Ergebnissen gerechnet werden (Grant et al., 2000). Die Mikroskopie dient nur bei hohen Keimzahlen als geeignetes Verfahren zum Erregernachweis. Weiterentwicklungen in Form von einer an In-Situ-Hybridisierung gekoppelten konfokalen Laser Scanning Mikroskopie sind für die Aufarbeitung klinischen Sektionsmaterials von Naser et al. (2002) vorgestellt worden. Die Autoren selbst werten ihr Verfahren als methodische Ergänzung zur weiteren Erforschung der Morbus-Crohn-Pathogenese.

Indirekte Verfahren zum Nachweis der Antikörperbildung (KBR, AGID, ELISA) sind ebenfalls etabliert. Die bislang verfügbaren ELISA-Systeme sind durch unterschiedliche Spezifität und Sensitivität gekennzeichnet, was bei der Verwendung zur Detektion subklinisch infizierter Tiere zu berücksichtigen ist. Die zelluläre Immu-

nantwort wurde mittels Haut-Hypersensitivitäts-Test (Johnin-Test) ermittelt, ist jedoch nicht spezifisch für die Diagnose (Olsen et al., 2002). Eine Kombination von kulturellen und serologischen Untersuchungsmethoden stellt daher und bisher in der Routinediagnostik das erfolgreichste und am weitesten verbreitete Untersuchungsprozedere dar (Rossiter und Burhans, 1996).

Der Standardnachweis von MAP mittels Polymerase-Kettenreaktion erfolgt seit Jahren über die *IS*900-Sequenz, die zum einen relativ spezifisch für MAP ist und zum anderen in hoher Kopienzahl vorkommt. Mit diesem Verfahren wurden in Großbritannien bisher die umfangreichsten Untersuchungen zum Vorkommen von MAP in Konsummilch (n = 312) durchgeführt, wobei 22 MAP-positive Befunde (7 %) ermittelt wurden (Millar et al., 1996). Allerdings konnten nur 50 % der positiven Befunde kulturell bestätigt werden, und immerhin 16 % der PCR-negativen Kontrollproben (n = 36) ergaben beim kulturellen Nachweis ein MAP-positives Ergebnis. Ein weiterer Nachteil beim Nachweis von MAP über DNA-Sequenzen ist darin zu sehen, daß nicht zwischen lebensfähigen und abgetöteten Erregern differenziert werden kann.

Der MAP-Erregernachweis mittels PCR gestaltet sich aufgrund der heterogen-komplexen Matrizes (Fäzes, Lebensmittel, Biopsiematerial), mit einer Vielzahl von PCR-Inhibitoren sehr schwierig (Bickley et al., 1996; Stevenson und Sharp, 1997; Al-Soud und Radstroem, 2000; Riffon und Sayasith, 2001). Der Einsatz sog. Interner Kontrollen/Internal Controls (IC) wurde bislang nur in wenigen Arbeiten für den PCR-basierten MAP-Nachweis aus gespikten Biopsieproben umgesetzt (Englund et al. 2001). Die Sensitivität des PCR-Verfahrens wird überdies durch die zur Dekontamination verwendeten Reagentien Hexadecyl-pyridinium-chlorid (HPC) (Whipple et al., 1991, Reddacliff et al., 2003) und N-Acetyl-L-Cystein (NALC) herabgesetzt. Dabei ist die Verwendung neuer, als MAP-spezifisch angesehener Marker zu berücksichtigen (Strommenger at al., 2001; Stratmann et al., 2002).

Die Nachweisgrenze bei Anwendung magnetischer Separationstechniken liegen bis zu $10^3$ KbE/g Fäzes. Dabei ist zu berücksichtigen, daß die Mehrzahl der Verfahren eine deutlich schlechtere Sensitivität aufwies; auch fiel die Reproduzierbarkeit geringer aus. Exemplarisch verdeutlichten Grant et al. (1998 u. 2000) den Anteil an arbeitsintensiven und schwer standardisierbaren Vorgehensweisen bei der Untersuchung von Milchproben. Mit dem von Stratmann et al. (2002) vorgestellten phagengestützten „Biopanning" ist die Nachweisgrenze zwar auf bis zu $10^2$ Phage Forming Unit (PFU)/ml Milch zu reduzieren. Eine interne Kontrolle bleibt jedoch auch hier unberücksichtigt. Vergleichenden Untersuchungen zur Einsatzfähigkeit und Eignung unterschiedlicher Markersequenzen sind Gegenstand aktueller Forschung (Möbius und Köhler, 2005).

### Wirtschaftlich-ökonomische Aspekte

Die Paratuberkulose in den Tierbeständen stellt ein zunehmendes Problem dar, das zu erheblichen wirtschaftlichen Verlusten führen kann. Dabei ist das Ausmaß der Verbreitung (Prävalenz) sowie der Neuinfektionen (Inzidenz) derzeit nur zu schätzen. Nach Angaben des Sachverständigengremiums im BfR (Anonymus, 2001) muß von bis zu 30 % MAP-positiven Rinder – sowie bis ca. 60 % MAP-positiven Ziegenbeständen ausgegangen werden. Die wirtschaftlichen Folgen durch Leistungsrückgang (v.a. Milchproduktion), Tierverluste, verminderte Fertilität, eine erhöhte Bereitschaft für andere Infektionen sowie die Behandlungskosten werden auf ca. 250,- € pro Rind und Jahr sowie auf ca. 100,- € pro Schaf und Jahr in infizierten Beständen geschätzt (Benedictus et al., 1987; Ott et al., 1999).

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FOOD COMPOSITION AND ADDITIVES

# Collaborative Trial for Validation of a Real-Time Reverse Transcriptase-Polymerase Chain Reaction Assay for Detection of Central Nervous System Tissues as Bovine Spongiform Encephalopathy Risk Material: Part 1

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**A collaborative trial was conducted to evaluate a real-time reverse transcriptase-polymerase chain reaction (RT-PCR) assay for detection of central nervous system (CNS) tissues in meat products (e.g., sausages). The method is based on the detection of ruminant glial fibrillary acidic protein (GFAP) mRNA by applying real-time RT-PCR. The assay was evaluated through a multicenter trial involving 12 participating laboratories that received coded cDNA obtained from 3 different types of sausages. The participants used 5 different real-time detection systems. The results obtained in this validation revealed that this real-time RT-PCR assay performed well in the different laboratories with a detection limit of at least 0.1% CNS in those test materials that contained strongly heat-treated samples (sausages cooked at 120°C) and the medium heat-treated samples (sausages cooked at 80°C). The detection limit of liver sausages was determined to be 0.2% of CNS. Neither the samples with no CNS additive nor the bovine DNA and the negative control containing 100% swine brain gave any positive signals. The presented results indicate that the real-time RT-PCR assay was just as reproducible between laboratories, as repeatable within a laboratory, could reliably be used for detection of bovine spongiform encephalopathy risk material in meat and meat products, and signify that it may be used with confidence in any laboratory.**

There is strong evidence that bovine spongiform encephalopathy (BSE) can cause variant Creutzfeldt-Jakob Disease (vCJD), most likely via the oral route of infection (1–3). Public health concerns require the efficient exclusion of ruminant tissues containing high accumulations of the causative agent prion protein gene ($PrP^{SC}$; 4), particularly brain and spinal cord, from the food chain. BSE specified risk material (SRM; e.g., brain and spinal cord) has been specified by the European Commission (EC) in Annex V Commission Regulation EC No. 999/2001 and EC No. 1326/2001 (5, 6). The use of SRM in food destined for human consumption was banned by EU Directives; the removal and destruction of SRM is mandatory to protect human and animal health from the risk of BSE. In meat products such as sausages, the content and type of meat must be labeled in addition to other animal materials used (e.g., liver, heart, and intestine).

According to Comer and Huntly (7), 3 possible routes of exposure through food containing brain or spinal cord must be considered: Direct consumption or incorporation of brain into meat before it was banned, or the contamination of food with head meat or tongue. Bovine central nervous system (CNS) tissues (e.g., brain and spinal cord) have been used as emulsifying agents for the production of hamburgers as well as for the fabrication of liver sausages (8). Mechanically recovered meat (MRM) as another important source of BSE infectivity was included from 5 to 10%, in some cases up to 30% in beef burgers or 10 to 20% in frozen minced meat (9). Therefore, in order to ensure consumer protection and to enforce food-labeling legislation, the monitoring of tissues of the CNS in retail meat products is mandatory for the benefit of both consumers and producers.

The challenge is being addressed through development of a new diagnostic test. To encourage acceptance of this test for use in routine control by the respective authority and by the food industry, it is recommended that the system be thoroughly evaluated through collaborative trial of its performance in several laboratories with variant real-time detection systems.

The method used in this study is a recently developed real-time reverse transcriptase-polymerase chain reaction (RT-PCR) based on the detection of glial fibrillary acidic protein (GFAP) mRNA, which can distinguish animal species of CNS tissues in heat-treated meat products such as cooked sausages (10). In order to determine the performance characteristics of this assay, a collaborative trial was conducted to evaluate this method for the specific detection of CNS tissues in different meat products (sausages). The assay

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was evaluated through a collaborative trial involving 12 participating German laboratories and using 5 different real-time systems.

## Materials and Methods

### *Collaborative Study*

The collaborative trial was designed according to the recommendations in CEN standard (CEN/TC 275/WG 6/TAG 3N 0119), which included the general requirements of real-time PCR (11). Twelve laboratories participated in the multicenter trial. These included government laboratories ($n$ = 6), research centers ($n$ = 3), university laboratories ($n$ = 2), and a private bioanalytic company ($n$ = 1). Each participant received in addition to 4 standard cDNA dilutions, control positive (100% bovine brain) and control negative (100% swine brain) and 9 coded (blind) cDNA samples, which included strong heat-treated sausages with 0.1% brain ($n$ = 1), strong heat-treated sausages with 1% brain ($n$ = 1), medium heat-treated sausages with 0.1% brain ($n$ = 1), medium heat-treated sausages with 1.0% brain ($n$ = 1), liver sausages ($n$ = 2) with 0.2% brain, liver sausages ($n$ = 2) with 0.5% brain, and sausages without CNS tissue additive ($n$ = 1). The shipment also included 1.5 mL 2x TaqMan® Universal PCR Master Mix Reagents Kit (Applied Biosystems, Darmstadt, Germany) with real-time PCR buffer and AmpliTaq® Gold-DNA-Polymerase; forward primer (RTGcowM56F2a); reverse primer (RTGcowM56R2a); and TaqMan fluorogenic minor groove binder (MGB)-probe conjugated with FAM (6-carboxy-fluorescein). The sequences of the oligonucleotide primers and the fluorogenic MGB probe (OptiR) were described by Abdulmawjood et al. (10). A detailed standard operating procedure (SOP), including a time schedule that explained how to perform the real-time RT-PCR, was sent to the receiving laboratories. The SOP also enclosed a reporting sheet, to be returned to the Institute of Veterinary Food Science for statistical analysis. Participants were required to detail in the reporting sheet all additional information that could possibly have influenced their results.

### *Preparation of Samples*

Cooked sausages with varying additives of bovine brain were produced according to the basic recipes provided by the guiding principles of the German Food Code (12). The preparation of the sausages had been described by Abdulmawjood et al. (10) and Lücker et al. (13). Briefly, the sausage filling contained 50% porcine meat (maximum 10% fat), 5% bovine meat, 25% porcine fat, 20% ice, 20 g/kg curing salt (4–5 g/kg $NaNO_2$ in NaCl; Enders, Reiskirchen, Germany), 3 g/kg phosphate-based stabilizer (di-Na-phosphate; Kirchheimbolanden, Germany), 5 g/kg of ready-mixed spices for cooked sausages (Delikatess Aufschnitt, Gewürzmüller, Stuttgart, Germany), and bovine brain in varying concentrations (0.1 and 1.00%). For preparing the cooked sausages, the contents were strong heated at 120°C for 15 min and medium heated at 80°C for 90 min. For the preparation of liver sausages, 80% porcine meat and 18% porcine liver were homogenized, and 2.5 g/kg curing salt, 0.5 g/kg emulsifier, and 5 g/kg ready-mixed spices for liver sausages ("Kalbsleberwurst Morenoperle", Gewürzmüller) were added. Cans were filled with sausage meat and heated for 60 min at 80°C.

RNeasy® Lipid Tissue Mini Kit (Qiagen, Hilden, Germany) was used to isolate the total cellular RNA according to the manufacturer's instructions. Briefly, 1000 µL Qiazol was added to 50 mg of each sample and the mixture was transferred to a glass matrix tube (FastRNA Green; Q BIOgene, Heidelberg, Germany) for cell lysis. The mixture was processed in a spin/rotation instrument for cell lysis (FastPrep®-120; Q BIOgene), with a speed setting of 6 and a time setting of 45 s. After processing, 200 µL chloroform was added to the mixture. The aqueous and organic layers were separated by microcentrifugation for 15 min at room temperature at 10 000 × $g$. The aqueous phase, containing the RNA, was removed and 200 µL ethanol (70%) was added, followed by binding of the RNA to the spin-column (Qiagen). After DNase digestion with 80 µL RNase free DNase (Qiagen), the total RNA was washed and eluted with 50 µL elution buffer. The total RNA from each sample was subjected to reverse transcription using TaqMan® Reverse Transcriptase Reagents kit with uracil-N-glycosylase (UNG; Applied Biosystems), according to the manufacturer's protocol. The reactions were incubated at 25°C for 10 min and 48°C for 30 min, followed by a final RT inactivation at 95°C for 5 min. The cDNAs were sent cooled to the collaborative trial participants.

### *Method Performed by the Receiving Laboratory*

The collaborative trial participants performed the real-time PCR reactions in 5 different real-time detection systems, which included ABI 5700 Real-Time PCR system ($n$ = 1), ABI PRISM 7000 SDS ($n$ = 3), ABI PRISM 7500 fast Real-Time PCR ($n$ = 4), ABI PRISM 7700 SDS ($n$ = 2), and ABI PRISM 7900 SDS ($n$ = 2). All systems were obtained from Applied Biosystems. The real-time PCR reactions were performed in a 50 µL reaction mixture with final concentrations of 300 nM of each oligonucleotide primer, 200 nM of the fluorogenic probe, and 1x TaqMan® Universal PCR Master Mix (Applied Biosystems). The oligonucleotide primers and the fluorogenic probe were synthesized by Applied Biosystems. The thermal cycling conditions comprised an initial UNG incubation at 50°C for 2 min, an AmpliTaq Gold® DNA polymerase activation at 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 s, and an annealing and extension at 60°C for 1 min. The assay was described by Abdulmawjood et al. (10). Each measurement was performed in triplicate and the threshold cycle ($C_t$), which is the fractional cycle number at which the amount of amplified target reached a fixed threshold, was determined. The threshold and base line value were adjusted automatically by the software. The results were recorded on the reporting sheet by each participant and returned for statistical analysis.

**Table 1. Reported participants' $C_t$ values from the collaborative trial of the real-time RT-PCR assay detecting cDNAs obtained from 3 different sausages and sausages without CNS tissues as negative control**

| | | $C_t$ values, mean ($s^2$) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Participant No. | | | | | | | | | | | |
| Coded sample | *n* | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| Sausage (cooked at 120°C) | | | | | | | | | | | | | |
| 0.1%[a] | 3 | 37.81 (±0.76) | 36.53 (±0.17) | 36.02 (±0.08) | 36.88 (±0.35) | 42.76 (±1.39) | 36.88 (±0.35) | 36.51 (±0.25) | 37.19 (±0.11) | 36.66 (±0.32) | 33.33 (±2.55) | 37.16 (±0.89) | 35.31 (±0.39) |
| 1.0% | 3 | 34.83 (±0.23) | 34.06 (±0.51) | 33.89 (±0.02) | 34.39 (±0.02) | 36.59 (±0.00)[b] | 34.39 (±0.02) | 34.67 (±0.29) | 32.89 (±0.61) | 34.07 (±0.31) | 30.30 (±0.18) | 33.29 (±0.21) | 32.57 (±0.32) |
| Sausage (cooked at 80°C) | | | | | | | | | | | | | |
| 0.1% | 3 | 33.62 (±0.20) | 32.35 (±0.02) | 32.65 (±0.32) | 32.77 (±0.15) | 35.20 (±0.00)[b] | 32.77 (±0.15) | 32.46 (±0.07) | 33.07 (±0.29) | 32.73 (±0.19) | 29.69 (±0.59) | 32.81 (±0.82) | 31.65 (±0.20) |
| 1.0% | 3 | 28.83 (±0.11) | 27.72 (±0.05) | 27.79 (±0.05) | 28.23 (±0.05) | 32.29 (±1.76) | 28.23 (±0.05) | 28.17 (±0.06) | 27.69 (±0.24) | 28.04 (±0.08) | 23.94 (±0.33) | 27.49 (±0.23) | 26.18 (±0.04) |
| Liver sausage | | | | | | | | | | | | | |
| 0.2% (I) | 3 | 35.07 (±0.29) | 34.06 (±0.39) | 33.93 (±0.17) | 34.26 (±0.21) | 38.36 (±0.47) | 34.26 (±0.21) | 34.39 (±0.44) | 33.80 (±0.54) | 34.00 (±0.26) | 31.16 (±0.09) | 34.38 (±0.36) | 32.78 (±0.23) |
| 0.2% (II) | 3 | 35.35 (±0.50) | 30.94 (±0.26) | 33.85 (±0.20) | 34.43 (±0.60) | 36.19 (±0.00)[b] | 34.43 (±0.60) | 34.74 (±0.38) | 34.65 (±0.30) | 34.29 (±0.24) | 30.68 (±0.47) | 33.39 (±0.41) | 32.96 (±0.09) |
| 0.5% (I) | 3 | 33.62 (±0.09) | 30.94 (±0.07) | 30.79 (±0.19) | 31.16 (±0.11) | 33.68 (±0.00)[b] | 31.16 (±0.11) | 31.16 (±0.17) | 31.05 (±0.02) | 31.13 (±0.04) | 27.26 (±0.25) | 30.28 (±0.07) | 29.82 (±0.38) |
| 0.5% (II) | 3 | 33.76 (±0.21) | 32.50 (±0.12) | 32.53 (±0.10) | 33.33 (±0.15) | 36.08 (±1.60) | 33.33 (±0.15) | 33.09 (±0.24) | 33.19 (±0.23) | 32.82 (±0.18) | 29.05 (±0.27) | 32.53 (±0.36) | 31.41 (±0.08) |
| Sausage | | | | | | | | | | | | | |
| 0.0% | 3 | —[c] | — | — | — | — | — | — | — | — | — | — | — |

[a] Content of CNS tissues.
[b] Only 1 of 3 was positive.
[c] — = Undetected.

### *Statistical Analysis*

The collaborative trial results were analyzed statistically, according to the recommendations of Scotter et al. (14) and by the methods of Langton et al. (15).

### *Accuracy (Sensitivity and Specificity)*

In a collaborative trial with quantitative data, whenever possible, results are compared with the true contents of the sample(s) in order to demonstrate the accuracy of the method. The equivalent statistics for qualitative data are straightforward. For positive samples, this is known as sensitivity and is the percentage of samples correctly identified as positives. For the purposes of this calculation, it must be assumed that all supposedly positive samples do in fact contain the target tissue. As the sensitivity can depend on circumstances such as the food matrix, a reported sensitivity applies only to the set of circumstances under which it was measured. For the negative samples, the percentage of samples correctly identified as being negative is recorded; this is known as the specificity.

### *Accordance (Repeatability)*

The qualitative equivalent of repeatability has been defined as accordance; this is the (percentage) chance that 2 identical test materials analyzed by the same laboratory under standard repeatability conditions will both be given the same result (i.e., both found positive or both found negative). To calculate the accordance, we take each laboratory in turn and calculate the probability that 2 samples will give the same result, and then average this probability over all laboratories. In general, when a laboratory has $n$ results and $k$ of these are positive, then the accordance for that food matrix is estimated using the formula:

$$\text{Accordance for food sample} = \{k(k-1) + (n-k)\,(n-k-19)\} \,/\, n\,(n-1)$$

### *Concordance (Reproducibility)*

The equivalent of reproducibility is concordance, which is the (percentage) chance that 2 identical test materials sent to different laboratories will both be given the same result (i.e., both found positive or both found negative). The most intuitive way to calculate concordance is simply to enumerate all possible between-laboratory pairings in the data.

Concordance can be calculated from accordance using the formula:

$$\text{(Estimated) concordance} = \{2r(r - nL) + nL\,(nL-1) - AnL(n-1)\}/\{(n^{**}2)\,L(L-1)\}$$

**Table 2. Reported participants' results from the collaborative trial of the real-time RT-PCR assay for the detection of bovine-specific CNS tissues in sausages**

| | | Presence of fluorescent signal of the target amplicon | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Participant No. | | | | | | | | | | | |
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| Sample | Expected[a] | Positive ($n$ = 9) | | | | | | | | | | | |
| Sausage (cooked at 120°C) | | | | | | | | | | | | | |
| 0.1%[b] | 3/3 | 3 | 3 | 3 | 3 | 2 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| 1.0% | 3/3 | 3 | 3 | 3 | 3 | 1 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| Sausage (cooked at 80°C) | | | | | | | | | | | | | |
| 0.1% | 3/3 | 3 | 3 | 3 | 3 | 1 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| 1.0% | 3/3 | 3 | 3 | 3 | 3 | 2 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| Liver sausage | | | | | | | | | | | | | |
| 0.2% | 6/6 | 6 | 6 | 6 | 6 | 3 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| 0.5% | 6/6 | 6 | 6 | 6 | 6 | 3 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| Bovine brain 100% | 1/1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | | Negative ($n$ = 3) | | | | | | | | | | | |
| Sausage 0.0% | 0/3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Bovine-DNA 100% | 0/1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Swine brain 100% | 0/1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

[a] From analysis of triplicate samples: No. of positive signals/No. of samples investigated.

[b] Content of CNS tissue.

where $r$ = total number of positives, $L$ = number of laboratories, $n$ = replications per laboratory, and $A$ = accordance, expressed as a proportion. The concordance odds ratio (COR) was defined as the degree of interlaboratory variation in the results. The COR was expressed as the ratio between accordance and concordance percentages, making it less dependent on the sensitivity.

### *Criteria for Inclusion of Results*

The results from each participating laboratory were included unless they fell into one of the following categories: (*1*) obvious performance deviation from the SOP; (*2*) lack of target amplicons in assay positive control; and (*3*) cross reaction (at least one false-positive signal) of one negative control.

## Results and Discussion

The results of the collaborative trial of the real-time PCR-based assay for the detection of CNS tissues in cDNA obtained from different types of sausages were received by the Institute of Veterinary Food Science at the scheduled date given in the SOP. All results obtained from 12 partners were accepted according to the predefined criteria; thus, the statistical analysis was based on 12 sets of results (Table 1). Partner 5 reported 1 and 2 false-negative signals of the investigated cDNA from strong heat-treated sausage samples containing 0.1 and 1.0% CNS tissue, respectively, and 2 and 1 false-negative signals by medium heat-treated samples containing 0.1 and 1.0% CNS tissue, respectively. The same partner showed 3 false-negative results of each of 6 cDNA of the investigated liver sausages containing 0.2 and 0.5% CNS tissues. All 11 other partners had results with accuracy of 100% (Table 2).

The means of the $C_t$ values of the 4 cDNA standards reported from the 12 participant were 25.28 (±1.58), 28.38 (±1.96), 31.54 (±1.42), and 34.91 (±1.17), respectively. The means of $C_t$ values of the 9 coded cDNA are summarized in Table 1. Partner No. 5 reported variations of $C_t$ values of ±0.047 to ±1.60 in the samples that were correctly detected. The overall results of all the other participants showed a variation of $C_t$ values of less than 1 cycle with one exception by partner No. 10, which displayed a standard deviation of ±2.55 by cDNA from strongly heat-treated sausages containing 0.1% CNS tissues.

Table 3 shows the statistical evaluation of the results. The qualitative results obtained from the collaborative trial showed that the PCR assay has a diagnostic specificity of 100% for all cDNA negative samples, including swine brain and bovine DNA, and from sausages without addition of CNS tissues. The calculating diagnostic sensitivity for all positive samples gave a value of 96.43%. The accordance (repeatability) was 95.37%, calculated as the percentage of

**Table 3. Statistical evaluation of the data obtained in the multicenter collaborative trial[a]**

| | Accuracy | | | | |
|---|---|---|---|---|---|
| | Sensitivity, % | Specificity, % | Accordance, % | Concordance, % | COR[b] |
| | | | Positive ($n$ = 9) | | |
| Sausage (cooked at 120°C) | | | | | |
| 0.1%[c] | 97.22 | — | 94.40 (83.33, 100) | 94.40 (84.34, 100) | 1.00 (0.92, 1) |
| 1.0% | 94.44 | — | 94.40 (83.00, 100) | 88.90 (70.71, 100) | 2.13 (1, 2.13) |
| Sausage (cooked at 80°C) | | | | | |
| 0.1% | 94.44 | — | 94.40 (83.00, 100) | 88.90 (70.71, 100) | 2.13 (1, 2.13) |
| 1.0% | 97.22 | — | 94.40 (83.33, 100) | 94.40 (84.34, 100) | 1.00 (0.92, 1) |
| Liver sausage | | | | | |
| 0.2% | 95.83 | — | 95.00 (85.00, 100) | 91.70 (77.27, 100) | 1.73 (1, 1.7) |
| 0.5% | 95.83 | — | 95.00 (85.00, 100) | 91.70 (77.27, 100) | 1.73 (1, 1.7) |
| Bovine brain, 100% | 100 | — | 100 | 100 | 1.00 |
| All positive samples | 96.43 | — | 95.70 (87.00, 100) | 92.00 (78.09, 100) | 1.92 (1, 1.9) |
| | | | Negative ($n$ = 3) | | |
| Sausages, 0.0% | — | 100 | 100 | 100 | 1.00 |
| Bovine-DNA, 100% | — | 100 | 100 | 100 | 1.00 |
| Swine brain, 100% | — | 100 | 100 | 100 | 1.00 |

[a] Numbers in parentheses are the lower and upper 95% confidence intervals.
[b] COR = Concordance odds ratio.
[c] Content of CNS tissue.

correctly identified samples from all positive samples. The concordance (reproducibility) was 92.85%, calculated from all positive inoculation levels. Combination of accordance (repeatability) and concordance (reproducibility) values, expressed as COR value, was 1.53, indicating that there was the same likelihood of obtaining the same result from 2 identical samples, whether they are sent to the same or to 2 different laboratories. The method has a very high probability of detecting cDNA from CNS tissues at a very low level in the sample types tested. Conversely, and more importantly, there is a very low risk of obtaining false-negative responses.

The strength of this real-time RT-PCR-based assay lies in its potential for species-specific identification of the CNS tissues and the possibility for quantitative detection of the illegal use of CNS tissues as BSE risk material in meat products.

From the overall results of the outcome of this collaborative trial, it has been concluded that the detection limit of both methods is at least as low as 0.1% for analysis of strong- or medium-heated material. Regarding the liver sausages, the detection limit was determined to be 0.2% of CNS tissues. This real-time RT-PCR-based method for detecting CNS tissues in meat products is highly sensitive and specific, repeatable as well as reproducible, and robust even when applied with heat-treated and variant types of samples. To our knowledge, at present no other collaborative trial has validated a similar real-time RT-PCR-based method for detection of CNS tissues in meat and meat products. It has been highly recommended to standardize all steps of this method by the 2nd part of the validation, which included the RNA isolation method and the transcription steps. These features, in combination with the validation presented here, make this method eminently suitable for routine use.

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# Detection of central nervous system tissues in meat products: Validation and standardization of a real-time PCR-based detection system

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**Abstract**

Several phenotypic as well as genotypic methods have been published describing the detection of central nervous system (CNS) tissues that are part of the bovine spongiform encephalopathy (BSE) risk material in food products. However, none of these methods is able to differentiate between CNS tissue of the banned ruminant species and tissues of other animal species.

A quantitative and species-specific real-time RT-PCR method has been developed that enables the reliable identification of CNS tissues in meat and meat products. This method is based on a messenger (m)RNA assay that uses bovine, ovine and caprine glial fibrillary acidic protein (GFAP) encoding gene sequences as markers. The in-house validation studies evaluated the tissue specificity of up to 15 bovine tissues and the standardization of absolute as well as relative quantitative measurement. The specific amplification of spinal cord and brain tissue GFAP cDNA has been shown previously. In addition, two commercially available ELISA kits were used for the comparative analysis of artificially contaminated minced meat. Small quantities of bovine brain that had been stored over the recommended period of 14 days were examined. The real-time PCR method proved to be suitable for the detection of 0.1% CNS tissue. No false negative results were observed.

The quantitative detection of GFAP mRNA using real-time RT-PCR seems a suitable tool in routine diagnostic testing that assesses the illegal use of CNS tissue in meat and meat products. The stability of the selected target region of the GFAP mRNA also allows the detection of CNS tissues after the meat has been processed.


*Keywords:* Real-time PCR; Central nervous system tissues; BSE risk material

## 1. Introduction

Belonging to the group of diseases known as transmissible spongiform encephalopathies (TSEs), bovine spongiform encephalopathy (BSE) has been

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causally associated with a new variant of Creutzfeldt–Jakob disease (vCJD) in humans. To effectively protect consumers from contracting vCJD, European legislators have set up numerous guidelines to reduce risk, in which national eradication programmes including the area-wide BSE testing of cattle and the prohibition of the use of specified risk material (SRM, e.g. brain and spinal cord) of cattle, sheep and goat in the food chain have become of particular importance. SRMs are age-dependent and their use is defined in the regulation (EC) No. 999/2001 (Annex V) of the European Commission (European Commission, 2001). It should be mentioned that porcine CNS tissues are not affected by this legislation. Ruminant brain and spinal cord have been shown to contain the highest infectivity titre of the causative agent $PrP^{Sc}$ (EFSA, 2005). There is also strong evidence that BSE is most likely transferred to humans by way of the oral route of infection (Bruce et al., 1997; Cousens et al., 2001; Comer and Huntly, 2003).

Methods enabling the detection of BSE risk material in food products include enzyme-linked immunosorbent assays (Schmidt et al., 1999, 2001), GC–MS (Biedermann et al., 2004), Western blot and immunohistochemical methods (Lücker et al., 1999, 2000; Wenisch et al., 1999). However, none of these methods allows the differentiation of CNS of banned ruminant species from tissues of other animal species.

This study focussed on the validation and the standardisation of a real-time reverse transcriptase polymerase chain reaction (RT-PCR) assay for the species-specific detection of bovine, ovine and caprine CNS tissues in raw and heat-treated meat products. The evaluation of the tissue specificity was based on up to 15 bovine tissues. In addition, real-time RT PCR and two commercially available ELISA kits were compared for their efficiency in detecting BSE pathogens in artificially contaminated minced meat.

## 2. Materials and methods

### 2.1. Parameters of the real-time PCR assay

The real-time PCR-based method used was standardised and validated according to the requirements of the ISO documents TAG 3 N 145 rev, ISO TC 34/SC 9 N containing general requirements for real-time PCR (Anonymous, 2006a) and ISO/CD TAG 3 N 0144 rev, ISO TC 34/SC 9 N ISO/CD containing general performance characteristics of molecular detection methods (Anonymous, 2006b). The parameters of the real-time PCR assay are summarised in Table 1. Two-step reverse transcriptive PCR was carried out as described previously (Schönenbrücher et al., 2004a; Abdulmawjood et al., 2005). For diagnostic quality assurance, an internal amplification control (IAC) was included to avoid false negative results. Briefly, the RNeasy® Lipid Tissue miniKit (Qiagen, Hilden, Germany) was used to isolate total cellular RNA. To achieve an efficient cell lysis, the mixture of the sample or the sample obtained with the swab with the Qiazol lysis reagent (Qiagen, Hilden, Germany) was transferred to a glass matrix tube (FastRNA Green, Q BIOgene, Heidelberg, Germany) processed in a spin/rotation instrument (FastPrep® -120; Q BIOgene, Heidelberg). The total cellular RNA was subsequently reverse transcribed into cDNA using the TaqMan® Reverse Transcriptase Reagents kit with UNG (Applied Biosystems, Darmstadt, Germany) according to the manufacturer's protocol. The transcribed cDNA was stored at −20 °C until further use.

Table 1
Parameters of the real-time PCR assay

| Target gene | Glial fibrillary acidic protein (GFAP) messenger (m)RNA |
|---|---|
| Detected animal species | Cattle, sheep and goat |
| Fluorescent probes | TaqMan®$_{mgb}$-probes |
| Quantitative measurement | Absolute or relative |
| Internal amplification control[a] | puc19-plasmid |
| Samples | Raw meat, swab samples[b] and heat-treated meat products (*F*-values 5.4) |
| Initial weight | 100 mg |
| Time requirement | 5 h |

[a] If using absolute quantification.
[b] Swab samples taken from carcasses, heads of cattle used for meat cutting, pieces of meat.

### 2.2. Collection of tissue samples and quantification of GFAP content

To estimate tissue specificity, 15 bovine tissue samples from three different animals were collected at an abattoir in Giessen, Germany, and immediately processed according to the aforementioned protocol. The material included brain, spinal cord, peripheral nerves (in particular sciatic and axillary nerves), fat, heart, kidney, liver, lung, lymph nodes, muscle, spleen, adrenal gland, pancreas, parotid gland and thymus. Total cellular RNA of each tissue sample was prepared three times; all real-time PCR runs were performed in triplicate, resulting in a set of 27 quantitative data per sample. The absolute quantification was standardised by using a standard curve out of a serial dilution of a known bovine brain GFAP cDNA standard in triplicate (Fig. 1). The relative quantification was based on an 18S rRNA RT-PCR (Applied Biosystems, Darmstadt, Germany) as a housekeeping gene (Abdulmawjood et al., 2005). For this purpose a selection of 10 tissue samples encompassing brain, spinal cord, peripheral nerves (in particular sciatic and axillary nerves), heart, kidney, liver, lung, lymph nodes, muscle and spleen was evaluated.

### 2.3. Production of internal reference material (IRM)

Minced meat and cooked sausages were prepared according to recipes provided in the guidelines of the German Food Code (Bundesanzeiger, 1994) and artificially contaminated with low concentrations of bovine brain homogenate. Minced meat (50% pork, 50% beef) with 0.1% (duplicate), 0.2%, 0.5% and 1% of CNS tissue was prepared. Two batches of raw IRM were prepared and stored at $\leq$+2 °C for 14 days. Samples without CNS tissue and bovine brain homogenate were processed using the same protocols for quality–control reasons.

The sausages were prepared as described by Lücker et al. (2000). The sausages were boiled at 120 °C for 15 min, representing strong heat treatment, or medium heated at 80 °C for 90 min. The liver sausages were prepared by homogenising 80% porcine meat and 18% porcine liver. Curing salt (2.5 g/kg), emulsifier (0.5 g/kg) and ready mixed herbs (5 g/kg, ''Kalbsleberwurst Morenoperle'', Gewürzmüller, Korntal-Münchingen, Germany) were added. The sausage meat was put in cans and heated for 60 min at 80 °C. The liver sausages were then stored at $\leq$+10 °C. All RNA preparations were done in duplicate.

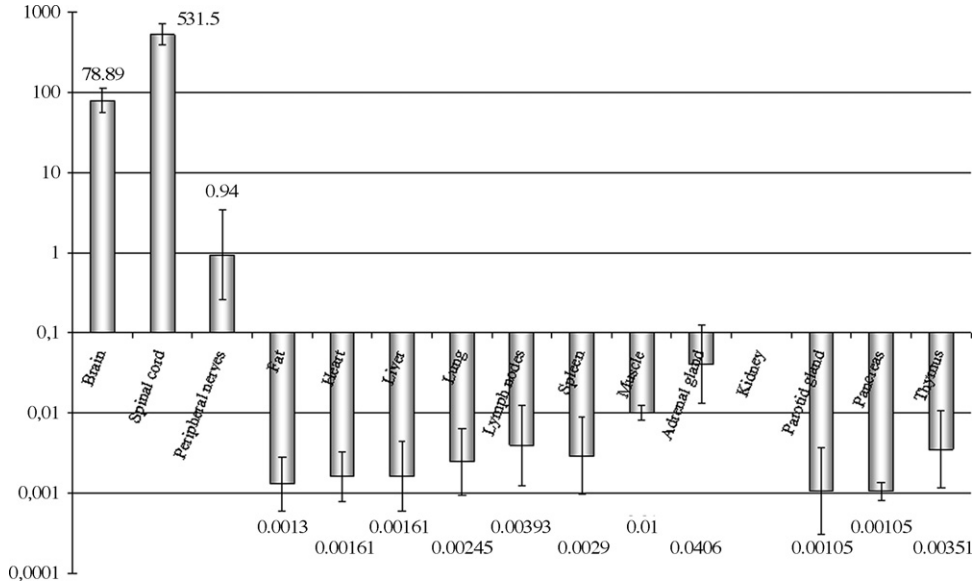


Fig. 1. Absolute quantification: GFAP cDNA-contents ($\bar{x}_g$) per PCR-reaction (ng) presented with a logarithmic scale.

### 2.4. Comparison of real-time PCR with commercially available ELISA kits

The minced meat was analysed using the ScheBo® Brainostic GFAP ELISA kit (ScheBo Biotech AG, Giessen, Germany) and the RIDASCREEN® Risk Material 10/5 ELISA (R-Biopharm AG, Darmstadt, Germany) according to the manufacturers' instructions. For the RIDASCREEN® Risk Material 10/5 the protocol was modified by using an initial weight of 50 mg instead of swab samples. The optical density (OD) values of the samples were examined by applying the Tecan Sunrise™ plate reader (Tecan Austria GmbH, Grödig, Austria).

## 3. Results

The oligonucleotide primer sequences were combined with a TaqMan®$_{mgb}$ fluorogenic probe (Abdulmawjood et al., 2005) and the sequences were amplified according to the parameters given in Table 1. The 100% limit of detection, which was calculated with a serial dilution of a known quantity of a bovine brain GFAP cDNA standard, gave a value of 1 pg bovine GFAP cDNA per PCR reaction (Abdulmawjood et al., 2005). The standard curve showed a slope of −3.124 and a correlation coefficient ($R$) > 0.99 (data not shown).

### 3.1. Examination of the tissue specificity including absolute and relative quantitative measurements

For the examination of the tissue specificity, absolute as well as relative quantitative measurements were standardised for the real-time PCR technique. Both methods showed that the highest amounts of GFAP were expressed in bovine neuronal organs, spinal cord and brain. The same was found for sheep and goat neuronal tissues (data not shown). Considerably lower amounts of GFAP were detected in sciatic and axillary nerves. With regard to the absolute quantification and according to the logarithmic scale presented in Fig. 1, the arithmetic mean of the GFAP cDNA content ranged from 531.5 in the spinal cord, 78.89 in the brain and 0.94 in the peripheral nerves. The GFAP cDNA values ($\bar{x}_g$) obtained for the non-neuronal soft tissues varied between 0.00105 in parotid gland, pancreas and 0.0406 in adrenal glands. The corresponding threshold ($C_t$)-values, including the standard deviation for each tested tissue sample quantified against the known GFAP standard, are given in Fig. 2. The $C_t$-values of all non-neuronal tissues investigated were greater than 34, except that of the adrenal gland (29.5). The peripheral nerves gave a value of 27.9. The relative quantitative consideration of ten selected tissues ranged from 1.88E+06 in the spinal cord, 1.46E+05 in the brain, 2.41E+02 in the peripheral nerves down to 2.00E−03 in

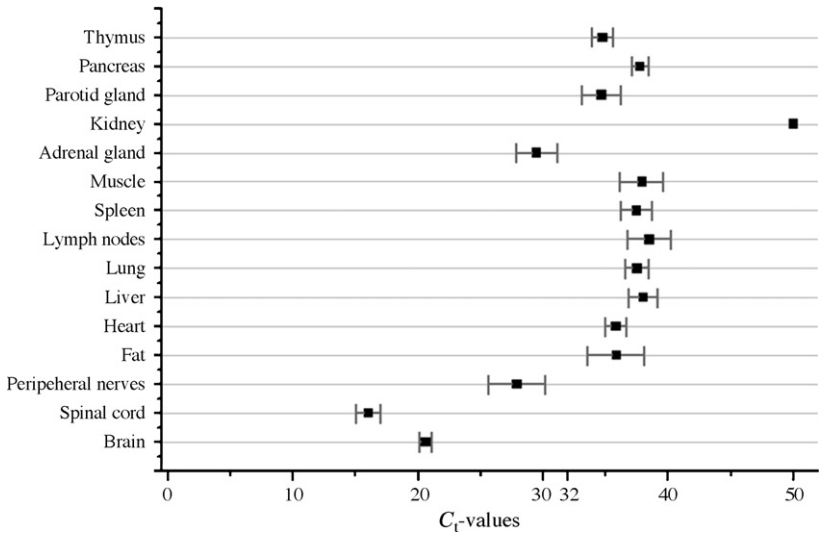


Fig. 2. Absolute quantification: corresponding $C_t$-values.

the kidney (Fig. 3). Independent from the quantification technique used, no fluorescent signal was detected in any of the DNA samples prepared from the different tissues used as quality controls, negative controls or blank values.

### 3.2. Comparison of commercially available ELISA kits and the real-time PCR

The limit of detection (LOD) of the real-time PCR assay was assessed by comparing the results with those obtained with the ScheBo® Brainostic GFAP ELISA kit and the RIDASCREEN® Risk Material 10/5 ELISA. These two enzyme immunoassays are based on the detection of the GFAP antigen and are the only ELISA kits that were commercially available in Germany at the time of the investigation. The LOD of the real-time PCR assay in artificially contaminated minced meat was 0.1% bovine brain as described earlier (Schönenbrücher et al., 2004a; Abdulmawjood et al., 2005). The 0.1% IRM was prepared in duplicate to minimise the effect of a potentially inhomogeneous distribution of the 0.1% content of bovine brain which would have resulted in false negative results. All three test systems allowed the detection of 0.5% of bovine brain over the 14-day testing period (Table 2). The real-time PCR protocol still enabled the correct detection of as little as 0.1% CNS tissue after 14 days. With regard to the criteria described in Table 1 for the classification of positive or negative results, the Brainostic GFAP ELISA kit enabled the identification of 0.2% and 0.1% CNS tissue. In contrast, the RIDASCREEN® Risk Material 10/5 ELISA detected 0.2%, but failed to detect 0.1% CNS tissue on 4 and 3 days out of the 14-day trial period, respectively.

The real-time PCR did not show any false positive nor false negative results for the samples without CNS tissue (0%), the blank values, bovine DNA and porcine (negative controls). The ScheBo® Brainostic GFAP ELISA kit gave two false positive results out of 10 samples that did not contain CNS tissue. The diagnostic sensitivity of the real-time PCR assay was 100%. The sensitivity of the ScheBo® Brainostic GFAP ELISA kit was 92.5%. With the swab sampling technique and a cut-off value of 0.1% (as recommended by the supplier), the RIDASCREEN® Risk Material 10/5 ELISA gave a sensitivity of 52.5%. A cut-off value of 0.2% raised the sensitivity to 95%. Subsequently, the sample preparation of the RIDASCREEN® Risk Material 10/5 ELISA was modified by using 50 mg instead of a swab sample. This resulted in a significant increase in sensitivity up to 90% (cut-off 0.1%, data not shown).

## 4. Discussion

A real-time PCR-based method was standardised and evaluated in terms of the species-specific

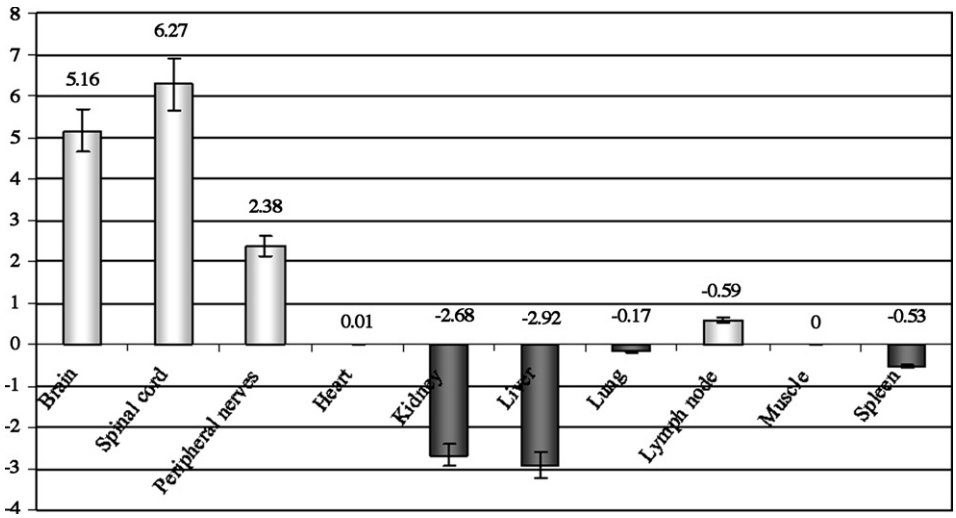


Fig. 3. Relative quantification: logarithmic values of the GFAP cDNA-contents ($\bar{x}_g$) per PCR reaction (ng).

Table 2
Comparative study of real-time PCR and two ELISA kit results of artificially contaminated minced meat

| Brain concentrations | Storage time | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Day 0 | | Day 1 | | Day 3 | | Day 7 | | Day 14 | |
| | Sample A | Sample B | Sample A | Sample B | Sample A | Sample B | Sample A | Sample B | Sample A | Sample B |
| 0% | | | | | | | | | | |
| Risk material 10/5[a] | – | – | – | – | – | – | – | – | – | – |
| Brainostic[b] | – | – | – | – | (+) | (+) | – | – | – | – |
| Real-time PCR[c] | – | – | – | – | – | – | – | – | – | – |
| 0.1% | | | | | | | | | | |
| Risk material 10/5 | – | – | – | – | – | – | – | – | – | – |
| Brainostic | – | (+) | – | + | (+) | (+) | + | + | + | + |
| Real-time PCR | + | + | + | + | + | + | + | + | + | + |
| Risk material 10/5 | – | (+) | – | – | – | (+) | – | – | – | – |
| Brainostic | (+) | (+) | (+) | + | (+) | (+) | + | + | + | + |
| Real-time PCR | + | + | + | + | + | + | + | + | + | + |
| 0.2% | | | | | | | | | | |
| Risk material 10/5 | (+) | (+) | + | (+) | (+) | (+) | (+) | (+) | (+) | – |
| Brainostic | – | + | + | (+) | + | (+) | + | + | + | + |
| Real-time PCR | + | + | + | + | + | + | + | + | + | + |
| 0.5% | | | | | | | | | | |
| Risk material 10/5 | + | + | + | + | + | + | (+) | (+) | (+) | (+) |
| Brainostic | + | + | + | + | + | + | + | + | + | + |
| Real-time PCR | + | + | + | + | + | + | + | + | + | + |

[a] RISASCREEN® Risk Material 10/5-ELISA; +: optical density $(OD)_{sample} \geq$ OD of standard (S)2 (0.1%); (+): $OD_{sample} \geq 2 \times OD_{S1(0\%)}$; –: $OD_{sample} < 2 \times OD_{S1(0\%)}$.

[b] Brainostic GFAP-ELISA; +: optical density $(OD)_{sample} \geq$ OD of standard (S)1 (0.1%); (+): $OD_{sample} > 70\%$ of $OD_{S1}$; –: $OD_{Sample} \leq 70\%$ of S1.

[c] +: threshold cycle ($C_t$) $\leq 32$; –: $C_t > 32$.

detection of CNS tissues in raw meat and heat-treated samples. The experimental studies were conducted according to the ISO documents ISO/CD TAG 3 N 145 rev, ISO TC 34/SC 9 N containing general requirements for real-time PCR (Anonymous, 2006a) and ISO/CD TAG 3 N 0144 rev ISO TC 34/SC 9 N, outlining general performance characteristics of molecular detection methods (Anonymous, 2006b). Hence, two commercially available ELISA kits were included in the evaluation as well as an external validation that was conducted in a multicentre trial (Abdulmawjood et al., 2006).

Since food matrices vary considerably, sample preparation is a crucial step in mRNA assays. Different sampling techniques were evaluated in which swab sampling was seen as an easy-to-use technique for the examination of surfaces. An initial weight of 100 mg is recommended for processed meat and heat-treated meat products (Table 1).

### *4.1. Examination of the tissue specificity including absolute and relative quantitative measurements*

The absolute and relative expression levels of GFAP mRNA were determined. The applicability of the standard curve used for absolute quantification (Fig. 1) was proven by a slope of −3.124 and a correlation coefficient ($R$) of >0.99. The relative expression of GFAP mRNA was achieved by using the $2^{\Delta\Delta C_t}$ method with muscle as a calibrator tissue as described earlier (Abdulmawjood et al., 2005). The endogenous control served as normalisation for the mRNA added to the reverse transcription PCR and as control reaction to avoid false negative results. Both methods revealed that the highest amounts of GFAP were expressed in bovine brain and spinal cord (Figs. 2 and 3). Because of their cross-sectional dimension and large anatomic expansion, sciatic and axillary nerves

represent the most important peripheral nerves in meat processing. The ratio of the absolute values generated for the peripheral nerves corresponded to about an 80 times higher GFAP mRNA content for the brain and a 530 times higher GFAP mRNA content for the spinal cord. Relative quantification gave a ratio of 1000-fold (brain) and 10,000-fold (spinal cord, Abdulmawjood et al., 2005). Low levels of GFAP in non-neuronal tissues could be detected (Figs. 2 and 3). A GFAP signal obtained from 100% adrenal gland tissue corresponded to 0.1% bovine brain, examined by absolute quantification. A low signal was also measured in fat, which could only be explained by a minor contamination of this tissue by peripheral nerves during sampling. As can be seen in the $C_t$-values generated (Fig. 3), the diagnostic sensitivity was not affected by the low GFAP mRNA content described above.

### 4.2. Comparison of commercially available ELISA kits and real-time PCR

Since 2004, enzyme-linked immunosorbent assay tests based on the GFAP antigen have been the official German reference method for the detection of CNS tissues according to Sec. 64 of the Foodstuff, Commodities and Animal Feed Act (Anonymous, 2005). The GFAP antigen is almost exclusively found in the CNS (Schmidt et al., 1999, 2001; Herde et al., 2005; Rencova, 2005; Reddy et al., 2006; Hossner et al., 2006). The multicentre trial presented by Agazzi et al. (2002) proved the suitability of ELISA methods for the analysis of heat-treated samples. The ScheBo® Brainostic GFAP ELISA kit offered the highest sensitivity of 92.5%. Application of the recommended swab sampling technique gave a sensitivity of 52.5% for the RIDASCREEN® Risk Material 10/5 ELISA. Hughson et al. (2003) evaluated the ELISA kit by using minced meat provided by the UK Food Standards Agency but could not detect CNS concentrations as low as 0.1%. Hossner et al. (2006) could not detect 0.2% of CNS tissue and reported highly variable results for the RIDASCREEN® Risk Material 10/5 ELISA. The results gained in the present study suggest that the sensitivity of the assay can be increased by modifying the preparation method of the samples. As an alternative, a cut-off value of 0.2% could be used to improve the sensitivity up to 90%. No differences were observed between the two 0.1% batches. This showed the comparability and the homogenous distribution of the two batches. The real-time PCR assay enabled the reproducible detection of 0.1% bovine brain after a storage time of 14 days.

Furthermore, the performance of the real-time PCR assay was evaluated by a multicentre trial including government laboratories as well as private bioanalytical companies. The investigation showed that the detection limit of the methods is at least as low as 0.1 when analysing strongly heated or medium-heated material. A detection limit of 0.2% was obtained for liver sausages. A detailed statistical analysis of the data is given by Abdulmawjood et al. (2006). The second part of the ring trial will investigate the effect of sample preparation as well as the results obtained with different thermocycler models.

Previously Seyboldt et al. (2003) and Lange et al. (2003) showed the possible applicability of conventional PCR methods for the detection of CNS tissues. Seyboldt et al. (2003) used a restriction fragment length polymorphism (RFLP) system based on the GFAP mRNA to differentiate between several animal species. This approach showed cross-reactions with raw heart as well as muscle tissues. Lange et al. (2003) used GFAP mRNA sequences as well as myelin basic protein (MBP) mRNA sequences. The oligonucleotide primers selected for the marker GFAP enabled a non-species-specific detection of brain tissue. The MBP oligonucleotide primers differentiated bovine, ovine and caprine from porcine brain tissues. The negative PCR results for brain tissues of goose, ostrich and chicken were considered to be preliminary, indicating the need for further investigation of the species-specific detection of CNS tissues. However, both groups did not present reliable data on the critical influence of peripheral nervous system (PNS) tissues. Seyboldt et al. (2003) lacked the investigation of peripheral nerves. Lange et al. (2003) did not explain the consequences of their detection of GFAP in sciatic nerve (e.g. on the detection limit of the method). The occurrence of the mRNA target region of the MBP in sciatic nerve was not conducted.

With regard to the immunohistochemical methods, Lücker et al. (1999, 2001) combined the detection of cholesterol and neuron-specific enolase (NSE). Furthermore, NSE and the CNS-specific GFAP were

combined for Western blot analysis (Lücker et al., 2000), which proved unsuitable for strongly heat-treated meat products (>80 °C). Immunohistological staining of NSE was also unsuitable for testing heat-treated meat products (Aupperle et al., 2002; Tersteeg et al., 2002). Herde et al. (2005) showed that MBP was still detectable by Western blot analysis after meat processing, which also included the addition of spices as well as heat treatment and took storage stability into account.

By using gas chromatography–mass spectrometry (GC–MS), Lücker et al. (2004) identified cerebronic acid as a reliable target for the tissue-specific detection of CNS tissues in meat products. The technique offers the potential for the species- and also the age-dependent quantification of the CNS content. Therefore, several analytical steps are required. At first, the identification of a CNS positive sample can be achieved by using cerebronic acid. The relationship of isomers of the tetracosenic acid is used to investigate species and age of the CNS (Biedermann et al., 2004). As a prerequisite, standards of CNS containing CNS of the adequate species and age must be used. Further and more extensive studies are needed to elucidate the limits of the age- and species-specific detection. Poerschmann et al. (2006) proved the sequential pressurized liquid extraction to be superior to the commonly used exhaustive lipid extraction method. As a consequence, the GC–MS technique achieves further methodological improvement.

However, all of the methods published so far have some drawbacks and do not allow the reliable detection of specified risk material taking into account the age of the animals or the differentiation of CNS tissues obtained from countries with or without a geographical BSE risk (e.g. Argentina). Knowledge about the spread of the causative BSE agent is steadily increasing (Thomzig et al., 2004; Angers et al., 2006) which underlines the importance of setting up reference methods.

As an important part of public health concerns, the EU recommends the development of reliable methods for the detection of CNS in food. The specific detection of CNS tissues of banned animal species using the real-time PCR-based method presented here can be conducted in a single run. Porcine CNS tissues can be specifically detected in a second real-time PCR assay (Schönenbrücher et al., 2004b). As far as the authors know this is the first report that evaluates a real-time PCR-based method in conjunction with a multicentre trial according to ISO requirements and it is also the first report that compares it with two commercially available ELISA kits. This study showed that, in minced meat, the GFAP mRNA target region remains detectable with RT-PCR after several days of storage. With regard to potential economic consequences for the meat-producing industry, should a CNS positive sample be detected, there is a very low risk of obtaining false positive and false negative responses.

The validation data presented here offer a highly suitable method for routine use as well as a large sample throughput test. However, a further multi-centre trial considering the sample preparation will have to be conducted before the method can be put into routine practice.

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# New Triplex Real-Time PCR Assay for Detection of *Mycobacterium avium* subsp. *paratuberculosis* in Bovine Feces[▽]

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**In the present study, a robust TaqMan real-time PCR amplifying the F57 and the IS*Mav2* sequences of *Mycobacterium avium* subsp. *paratuberculosis* from bovine fecal samples was developed and validated. The validation was based on the recommendations of International Organization for Standardization protocols for PCR and real-time PCR methods. For specificity testing, 205 bacterial strains were selected, including 105 *M. avium* subsp. *paratuberculosis* strains of bovine, ovine, and human origin and 100 non-*M. avium* subsp. *paratuberculosis* strains. Diagnostic quality assurance was obtained by use of an internal amplification control. By investigating six TaqMan reagents from different suppliers, the 100% detection probability was assessed to be 0.1 picogram *M. avium* subsp. *paratuberculosis* DNA per PCR. The amplification efficiency was 98.2% for the single-copy gene F57 and 97.8% for the three-copy insertion sequence IS*Mav2*. The analytical method was not limited due to instrument specificity. The triplex real-time PCR allowed the reliable detection of *M. avium* subsp. *paratuberculosis* DNA using the ABI Prism 7000 sequence detection system, and the LightCycler 1.0. TaqMan$_{mgb}$ and locked nucleic acid fluorogenic probes were suitable for fluorescent signal detection. To improve the detection of *M. avium* subsp. *paratuberculosis* from bovine fecal samples, a more efficient DNA extraction method was developed, which offers the potential for automated sample processing. The 70% limit of detection was assessed to be $10^2$ CFU per gram of spiked bovine feces. Comparative analysis of 108 naturally contaminated samples of unknown *M. avium* subsp. *paratuberculosis* status resulted in a relative accuracy of 98.9% and a sensitivity of 94.4% for fecal samples containing <10 CFU/g feces compared to the traditional culture method.**

*Mycobacterium avium* subsp. *paratuberculosis* is the causative agent of ruminant paratuberculosis (Johne's disease), which has become a worldwide problem. There is controversy regarding its zoonotic capacity and potential role in the human Crohn's disease (14). Because of these reasons, a rapid, cost-effective, and automated diagnosis of this pathogen is a high priority task not only for animal breeders but also for the food production industry and for public health institutions. Culture-based detection of *M. avium* subsp. *paratuberculosis* is time-consuming, labor-intensive, and therefore not suitable. The PCR has been shown to be a powerful tool in microbiological diagnostics (12, 43). Guidelines for diagnostic quality assurance have been set by the International Organization for Standardization (7, 8). Standardized PCR and real-time PCR methods should fulfill numerous criteria, including a high detection probability with regard to the investigated matrix, the sample preparation, and DNA extraction as well as high specificity, robustness, and user-friendly protocols. In this context the real-time PCR technology offers the possibility for a one-step and closed-tube reaction (13).

As a molecular reference marker for the confirmation of *M. avium* subsp. *paratuberculosis*, the insertion sequence IS*900* is commonly used (15, 24). Because of a considerably high sequence similarity with IS*900*-like elements or other genetic elements, cross-reactions might give false-positive results (16, 22; for a review of diagnostic tests, see reference 26). According to numerous authors, PCR analysis was unable to match the sensitivity of fecal culture for identifying minute quantities of *M. avium* subsp. *paratuberculosis* (49, 56). An increased sensitivity of the PCR analysis can be achieved by improved DNA extraction protocols guaranteeing the efficient removal of PCR inhibitors such as phytic acid, polyphenolics, polysaccharides, and hemin (4, 5, 18, 37, 52).

The aim of the present study was the development and careful validation of a new real-time PCR assay, considering the existing guidelines for PCR- and real-time PCR-based detection methods. The assay should offer the potential to be used as a stand-alone application for the detection of *M. avium* subsp. *paratuberculosis* from bovine fecal samples without additional PCR confirmation tests. For this purpose, the *M. avium* subsp. *paratuberculosis* marker sequences F57 and IS*Mav*2 were combined with an internal amplification control (IAC) into a triplex real-time PCR assay. Broad-range applicability was assessed by considering PCR reagents from different suppliers. Robustness testing included two different fluorogenic probe formats and two different real-time thermocycler models, both representing widely used technologies. In addition, the fecal sample preparation and DNA extraction protocol was optimized. The applicability of our method was compared to those of the cultural gold standard and IS*900* nested PCR (12) by testing 108 bovine fecal samples of unknown *M. avium* subsp. *paratuberculosis* status.

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## MATERIALS AND METHODS

**Bacterial reference strains.** For estimation of the specificity of the developed real-time PCR assay, a total of 205 strains were used (Table 1). The 105 *M. avium* subsp. *paratuberculosis* strains collected for sensitivity testing contained two official type collection strains (DSM 44133 and DSM 44135) and 103 *M. avium* subsp. *paratuberculosis* field strains of bovine ($n = 95$), ovine ($n = 5$), and human ($n = 3$) origin. These strains had not been characterized for the marker genes F57 and IS*Mav2* before. The 34 non-*M. avium* subsp. *paratuberculosis* strains and the 65 nonmycobacterial strains used for specificity testing were selected because of their close genetic relationship to *M. avium* subsp. *paratuberculosis* or because they are found in the same environment and grow under similar conditions. The *Mycobacterium* strains were cultured on Herrolds egg yolk medium (HEYM) (Becton Dickinson, Heidelberg, Germany) with Mycobactin J (Synbiotics Corporation, France). The other control strains were grown on required solid media.

**Preparation of DNA samples.** The preparation of the reference DNA from the mycobacterial strains was performed by using a protocol for gram-positive bacteria in the Qiagen DNeasy blood and tissue kit (Qiagen, Hilden, Germany). The procedure was slightly modified by including a mechanical cell homogenization and disruption with the Fastprep Ribolyzer (Q-biogene, Heidelberg, Germany) to achieve efficient cell lysis. In case of gram-negative control strains, the protocol for gram-negative microorganisms in the Qiagen DNeasy blood and tissue kit (Qiagen, Germany) was used. Approximately $10^7$ CFU was used as a template in the PCR assay.

For the determination of the detection probability, assay precision, and robustness, *M. avium* subsp. *paratuberculosis* DNA standards from different *M. avium* subsp. *paratuberculosis* strains were prepared. Single colonies of the bovine strains 423 and 428, the ovine strain JD131 and the human strain SN5 were grown separately in mycobacterial growth indicator tubes (Becton Dickinson) containing oleic acid-albumin-dextrose-catalase enrichment and PANTA (both from Becton Dickinson) and Mycobactin J as recommended by the supplier. The DNA for each *M. avium* subsp. *paratuberculosis* standard was extracted by using 1-ml aliquots of the suspension according to the modified protocol described above. UV spectroscopic measurement of the total DNA quantity and quality was performed on a BioMate3 (Thermo Scientific, WI). The bacterial cells in 1 ml of each *M. avium* subsp. *paratuberculosis* suspension were mechanically sheared by repeated drawing and spilling through a syringe needle (gauge $26_G3/8$). Cell numbers were calculated after counting the cells in an aliquot of the *M. avium* subsp. *paratuberculosis* stock solution in a Tuerk counter chamber and comparative cultivation on Middlebrook 7H10 (Becton Dickinson) agar plates. Serial dilutions of *M. avium* subsp. *paratuberculosis* cells from $10^0$ to $10^{-7}$ were prepared. DNA was extracted from 1-ml aliquots of each dilution as described above.

**Primer, TaqMan$_{mgb}$ probe, and LNA probe design.** The design of sequence-specific oligonucleotide primers was based on the *M. avium* subsp. *paratuberculosis* reference sequences for F57 (accession numbers X70277 and AE016958) and IS*Mav2* (accession numbers AF286339 and AE016958) published in the National Center for Biotechnology (NCBI) GenBank (Table 2). Similar sequences were identified according to the scientific literature, by comparative searches of GenBank and the Comprehensive Microbial Resource (CMR) database of The Institute for Genomic Research (TIGR) and were added to the alignments. The TaqMan$_{mgb}$ probes for both primer sets were adopted by using Primer Express version 2.0 (Applied Biosystems, Darmstadt, Germany) with respect to the guidelines from Applied Biosystems and labeled at the 5′ end with VIC (for F57) (Applied Biosystems) or 6-carboxyfluorescein (FAM) (for IS*Mav2*) (Applied Biosystems). The 3′ end contained a minor groove binder (mgb) and the nonfluorescent Eclipse DarkQuencher (Applied Biosystems). Both probe sequences were also synthesized as locked nucleic acid (LNA) probes (Eurogentec, Cologne, Germany) labeled with Yakima Yellow or FAM and the Black Hole Quencher (BHQ1) (all from Eurogentec). All oligonucleotide and probe sequences were submitted to the NCBI and TIGR databases for specificity testing, including broad-range and comparative genome basic local alignment search tool (BLAST) analysis.

**IAC.** An IAC was selected as described previously (1). Briefly, the IAC was synthesized in one PCR, using the plasmid pUC19 vector as a template (M11662; Promega). The oligonucleotide sequences were identical to the F57 diagnostic primers possessing 5′ overhanging ends, whereas their 3′ ends were complementary to the pUC19 plasmid sequences. Separate TaqMan$_{mgb}$ probes were designed according to the pUC19 vector sequence and labeled at the 5′ end with NED (Applied Biosystems). The PCR product was purified, and adjustment of the number of copies for use in the conventional PCR assays and the real-time PCR assays was done as described previously (34). The optimal copy number was assessed to be 175.

TABLE 1. *Mycobacterium avium* subsp. *paratuberculosis*, non-*M. avium* subsp. *paratuberculosis*, and nonmycobacterial strains used for sensitivity and specificity testing

| Species, subspecies, and/or strain(s) | No. of strains | Source | PCR result | |
|---|---|---|---|---|
| | | | IS*Mav2* | F57 |
| *M. avium* subsp. *paratuberculosis* | | | | |
| DSM 44133 | 1 | Bovine feces | + | + |
| ATCC 19698 | 1 | Bovine feces | + | + |
| SN5, SN7, Pat. 7 | 3 | Human (United Kingdom, Germany) | + | + |
| 51/91, JD 131, JD 8, | 5 | Ovine (United Kingdom) | + | + |
| JD 146, F 162 | 95 | Bovine (feces, milk) | + | + |
| Total | 105 | | | |
| Non-*M. avium* subsp. *paratuberculosis* | | Reference stock and field strains[a] | | |
| *M. abscessus* | 1 | | − | − |
| *M. avium* subsp. *avium* | 3 | | − | − |
| *M. avium* subsp. *hominissuis* | 3 | | − | − |
| *M. avium* subsp. *silvaticum* | 1 | | − | − |
| *M. avium* (subspecies not determined) | 3 | | − | − |
| *M. bovis* | 2 | | − | − |
| *M. celatum* | 2 | | − | − |
| *M. chelonae* | 2 | | − | − |
| *M. fortuitum* | 2 | | − | − |
| *M. gordonae* | 3 | | − | − |
| *M. intracellulare* | 2 | | − | − |
| *M. kansasii* | 2 | | − | − |
| *M. scrofulaceum* | 2 | | − | − |
| *M. smegmatis* | 2 | | − | − |
| *M. phlei* | 2 | | − | − |
| *M. tuberculosis* | 2 | | − | − |
| Total | 34 | | | |
| Nonmycobacteria | | Reference stock[b] | | |
| *Bifidobacterium* sp. | 2 | | − | − |
| *Citrobacter freundii* | 4 | | − | − |
| *Clostridium perfringens* | 1 | | − | − |
| *Clostridium bifermentans* | 2 | | − | − |
| *Escherichia coli* | 12 | | − | − |
| *Enterococcus faecium* | 3 | | − | − |
| *Lactobacillus fermentans* | 1 | | − | − |
| *Lactobacillus plantarum* | 1 | | − | − |
| *Lactobacillus reuteri* | 1 | | − | − |
| *Lactobacillus amylovorus* | 1 | | − | − |
| *Listeria monocytogenes* | 2 | | − | − |
| *Listeria innocua* | 1 | | − | − |
| *Listeria ivanovii* | 1 | | − | − |
| *Listeria welshimeri* | 1 | | − | − |
| *Listeria seeligeri* | 1 | | − | − |
| *Salmonella* sp. | 12 | | − | − |
| *Shigella flexneri* | 1 | | − | − |
| *Shigella somnei* | 1 | | − | − |
| *Shigella* (serogroup not tested) | 1 | | − | − |
| *Shigella boydii* | 1 | | − | − |
| *Streptococcus agalactiae* | 1 | | − | − |
| *Streptococcus equi* | 2 | | − | − |
| *Streptococcus epidermicus* | 3 | | − | − |
| *Staphylococcus aureus* | 2 | | − | − |
| *Staphylococcus epidermidis* | 1 | | − | − |
| *Staphylococcus hyicus* | 1 | | − | − |
| *Yersinia enterocolitica* | 6 | | − | − |
| Total | 66 | | | |
| Total | 205 | | | |

[a] Includes reference strains from official type strain culture collections (e.g., DSMZ) and field and clinical strains obtained from bovine, human, porcine, and avian sources.

[b] Pure culture strains as stored in the house reference stock were selected based on the full availability of results of serotyping, biochemical and virulence properties, and genotyping.

**Triplex real-time PCR assay.** The optimized 50-μl PCR mixture for the triplex real-time PCR assay using TaqMan probes contained 300 nM of the primers F57-F/F57-R, 200 nM of the primers IS*Mav2*-F/IS*Mav2*-R, 250 nM of the target probes (F57 and IS*Mav2*), 175 copies of the IAC, 25 μl of 2× qPCR MasterMix Plus without uracil-*N*-glycosylase (UNG) (Eurogentec), and a 5-μl aliquot of the DNA sample. The PCRs were performed in a 96-well plate format on the ABI

TABLE 2. Oligonucleotide primers, fluorogenic probes, and internal amplification control sequences used in this study[a]

| Designation | Sequence[b] | Positions[c] | Melting temp (°C) |
|---|---|---|---|
| F57-F | 5′-TAC GAG CAC GCA GGC ATTC-3′ | 244–263 | 58.8 |
| F57-R (reverse) | 5′-CGG TCC AGT TCG CTG TCA T-3′ | 288–307 | 58.8 |
| F57 TaqMan$_{mgb}$ probe | VIC-CCT GAC CAC CCT TC-MGB | 268–282 | 68.6 |
| F57 LNA probe | Yakima Yellow-cCt gAc Cac CctT-BHQ1 | 268–282 | 69 |
| pUC19-IAC-F (forward) | 5′-CCG GTT CCC AAC GAT CAA G-3′ | 1991–2009 | 60 |
| pUC19-IAC-R (reverse) | 5′-ACT GCG GCC AAC TTA CTT CTG-3′ | 2076–2096 | 58 |
| F57 TaqMan$_{mgb}$ IAC probe | NED-CGA GTT ACA TGA TCC C-MGB | 2011–2026 | 68 |
| IS*Mav2*-F (forward) | 5′-CGG CAA AAT CGA GCA GTT TC-3′ | 1645–1665 | 57.3 |
| IS*Mav2*-R (reverse) | 5′-TGA GCC GGT GTG ATC ATC TTT-3′ | 1786–1807 | 57.9 |
| IS*Mav2* TaqMan$_{mgb}$ probe | FAM-CGC TGA GTT CCT TAG-MGB | 1678–1693 | 65.9 |
| IS*Mav2* LNA probe | FAM-cGc tGa GtT cCt TaG-BHQ1 | 1678–1693 | 69 |

[a] All oligonucleotide primers were synthesized by MWG Biotech (Ebersberg, Germany), TaqMan$_{mgb}$ fluorogenic probes were manufactured by Applied Biosystems, and LNA probes were synthesized by Eurogentec.
[b] Uppercase letter represent LNA bases.
[c] Positions refer to GenBank accession numbers X70277 (F57), AF286339 (IS*Mav2*), and M11662 (pUC19).

Prism 7000 sequence detection system (Applied Biosystems). Thermal cycling conditions comprised a hot-start DNA polymerase activation at 95°C for 10 min, 50 cycles of denaturation at 95°C for 15 seconds, and annealing and extension at 60°C for 1 min. Each measurement was performed in duplicate, and the threshold cycle ($C_T$), defined as the fractional cycle number at which the amount of amplified target reached a fixed threshold, was determined. Additional melting curve analysis was performed with the QuantiTect Sybr green PCR kit (Qiagen), the Absolute QPCR Sybr green mix (ABGene, Hamburg, Germany), and the Sybr green PCR master mix (Applied Biosystems) according to the manufacturers' instructions. DNAs from bovine, ovine, and human *M. avium* subsp. *paratuberculosis* strains were investigated.

**Determination of detection probability.** The detection probability of the real-time PCR assay was first determined by analyzing serial dilutions of known *M. avium* subsp. *paratuberculosis* DNA standards prepared from the bovine strains 423 and 428, the ovine strain JD131, and the human strain SN5. Serial dilutions prepared from the *M. avium* subsp. *paratuberculosis* stock solution ranged from $10^7$ to $10^1$ CFU per ml. Second, 5-μl aliquots of DNA extracted from serial dilutions of homogenized *M. avium* subsp. *paratuberculosis* cells were used as a template for real-time PCR amplification. All experiments were done three times in triplicate format. The copy numbers for F57 and IS*Mav2* corresponding to the lowest limit of detection (LOD) were calculated. Briefly, the mass of the *M. avium* subsp. *paratuberculosis* genome in picograms was calculated by dividing the *M. avium* subsp. *paratuberculosis* genome size of 4,829,781 bp (33) through the number of copies per gene of interest.

**Assessment of amplification efficiency, precision, and robustness.** Assessments of the amplification efficiency and the precision of the assay under optimized conditions were performed by triplicate analysis of serial dilutions of a known *M. avium* subsp. *paratuberculosis* DNA standard. The same operator repeated the experiment three times on different days with the same dilutions. The arithmetic mean of the $C_T$ values and the corresponding standard deviation (SD) were calculated for each sample. Standard curve construction was performed for both PCR marker genes. The slopes were used for the calculation of amplification efficiency ($E$) by using the equation $E = 10^{(-1/\text{slope})} - 10$ (9).

The robustness of the assay was investigated as follows. Duplicates of the serial dilutions of *M. avium* subsp. *paratuberculosis* DNA of a known standard were run with optimized and suboptimal concentrations of the PCR reagents. This included 10% more or less of the qPCR MasterMix Plus without UNG (Eurogentec) and variations of the annealing temperature of 62°C and 65°C. In addition, the influence of different TaqMan PCR reagents was investigated by considering six ready-to-use products from five different suppliers according to the manufacturer's instructions. The PCR reagents were TaqMan Universal PCR Mastermix (Applied Biosystems), the QuantiTect Multiplex PCR kit (Qiagen), Absolute QPCR mix (ABgene), qPCR MasterMix Plus plus UNG and qPCR MasterMix Plus without UNG (both from Eurogentec), and the LightCycler TaqMan Master (Roche, Mannheim, Germany). Analysis was conducted in triplicate format and done three times.

**Assessment of the LOD on the ABI Prism 7000 sequence detection system and the LightCycler 1.0 real-time PCR thermocycler.** In general, the experiments were performed on the ABI Prism 7000 sequence detection system (Applied Biosystems) as described above. Comparative assessment of the LOD on the LightCycler 1.0 (Roche) was conducted with the serial dilution of an *M. avium* subsp. *paratuberculosis* DNA standard. The optimized 20-μl PCR mixture for the duplex real-time PCR assay using TaqMan probes contained 400 nM of the primers F57-F/F57-R or 300 nM of the primers IS*Mav2*-F/IS*Mav2*-R, 250 nM of the target probes (F57 and IS*Mav2*), 175 copies of the IAC, 7.5 μl of the LightCycler TaqMan Master (Roche), and a 5-μl aliquot of the DNA sample. The PCRs were performed in a 32-capillary rotor. Thermal cycling conditions comprised a hot-start DNA polymerase activation at 95°C for 10 min, 50 cycles of denaturation at 95°C for 10 seconds, annealing and extension at 60°C for 1 min, and extension at 72°C for 10 seconds. A cooling step was added at 40°C for 30 s. Fluorescence data were collected with the acquisition mode "single" during the extension with channel 1. Each measurement was performed in duplicate, and the $C_T$ was determined.

**Preparation of DNA samples from bovine fecal samples.** For the real-time PCR assay for the detection of *M. avium* subsp. *paratuberculosis* from bovine fecal samples, two DNA sample preparation protocols were evaluated by analyzing artificially contaminated bovine fecal samples. Serial dilutions were prepared from the *M. avium* subsp. *paratuberculosis* stock solution of bovine strain 423, ranging from $10^6$ to $10^1$ CFU per ml. Beforehand the detection probability was assessed by determining the influence of an additional 5-μl aliquot of DNA isolated from three bovine fecal samples. The samples had been confirmed as *M. avium* subsp. *paratuberculosis* negative by culture. The DNA was prepared according to the modified protocol described below. Comparative analysis of the serial dilutions of the known *M. avium* subsp. *paratuberculosis* DNA standards with and without the background DNA was done in duplicate and repeated twice.

DNA extraction was done by using a modified protocol of the QIAamp DNA stool minikit (Qiagen), as follows. After preheating of 140 ml ASL buffer at 70°C, 350 μl DX buffer was added and the solution was mixed. One gram of bovine feces was mixed with 5 ml of the DX-ASL buffer to obtain a homogenous suspension. The mixture was subsequently incubated at 95°C for 10 min, and 1.3 ml of the supernatant was added to a 2-ml lysing matrix D tube (Q-biogene). Afterwards, mechanical cell disruption was done with a FastPrep-120 (Q-biogene) by four repetitions of 20 seconds at a speed setting of 6 followed by mixture of the tube contents. To separate the solid phase from the liquid phase, tubes were centrifuged at 5,000 × *g* for 5 min, and 1.2 ml of the resulting supernatant was transferred to a new 2-ml Eppendorf tube. After addition of one Inhibitex tablet (Qiagen) per sample to remove PCR inhibitors, the sample was mixed for 1 min and subsequently centrifuged at 15,800 × *g* for 6 min; 300 μl of each supernatant was then transferred to a new 1.5-ml Eppendorf tube which already contained 20 μl proteinase K (20 mg/ml; Qiagen) and mixed with 300 μl AL buffer. Proteinase K was incubated at 70°C for 5 min and subsequently at 95°C for 10 min. Further processing was done according to the kit manual. Eluted DNA was stored at 4°C for direct use or stored at −20°C.

**Analysis of naturally contaminated samples of unknown *M. avium* subsp. *paratuberculosis* status.** A total of 108 bovine fecal samples of unknown *M. avium* subsp. *paratuberculosis* status were analyzed in duplicate by triplex real-time PCR as described above, by IS*900* nested PCR (12), and by conventional microbiological culture (6). A 0.75% solution of hexadecylpyridiniumchloride was used for overnight decontamination before inoculation of tubes of HEYM with Mycobactin J. Fecal samples were collected from cattle herds suspected of having paratuberculosis, as identified by Pourquier paratuberculosis enzyme-linked im-

TABLE 3. $C_T$ values generated with the TaqMan$_{mgb}$ and LNA probes for bovine, ovine, and human *M. avium* subsp. *paratuberculosis* DNA extractions

| Origin of *M. avium* subsp. *paratuberculosis* strains | $C_T$ value with the indicated probe (fluorescent dye) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | TaqMan$_{mgb}$ | | | | LNA | | | |
| | F57 (VIC) | | IS*Mav2* (FAM) | | F57 (Yakima Yellow) | | IS*Mav2* (FAM) | |
| | Mean | SD | Mean | SD | Mean | SD | Mean | SD |
| Bovine | 20.41 | 1.45 | 19.75 | 1.57 | 20.66 | 1.54 | 18.71 | 1.57 |
| Ovine | 16.99 | 3.00 | 16.47 | 2.48 | 17.20 | 2.73 | 15.46 | 2.82 |
| Human | 16.67 | 0.68 | 15.99 | 0.91 | 17.00 | 0.91 | 15.85 | 0.83 |

munosorbent assay (Institute Pourquier, Montpellier, France) on individual milk samples collected per animal, or from clinically infected animals admitted to the clinic for ruminants and pigs, Justus Liebig University, Giessen, Germany.

**Statistical analyses.** Statistical analyses used the program packages BMDP for XP, release 8.1 (17), and BiAS for Windows, release 8.2 (3). The comparison of the PCR master mix results was done by a three-way analysis of variance for nested designs and mixed-effect models using the program BMDP8V. This test was followed by a pairwise comparison of the master mixes according to the Tukey test (BMDP7D). The statistical terms relative sensitivity, specificity, and diagnostic accuracy were calculated as described elsewhere (2) and were completed by calculation of the 95% confidence intervals with the program BiAS.

## RESULTS

**Selectivity.** Table 1 summarizes the results of the sensitivity and specificity testing. All 105 *M. avium* subsp. *paratuberculosis* strains were identified correctly with the primer and oligonucleotide probe sets designed for F57 and IS*Mav2*. The sequences of the primers, the TaqMan$_{mgb}$, the LNA fluorogenic probes, and the IAC are given in Table 2. The sizes of the amplified PCR products were 62 bp (F57), 164 bp (IS*Mav2*), and 105 bp (IAC). No amplification was observed using the 34 non-*M. avium* subsp. *paratuberculosis* strains and the 65 nonmycobacterial strains. $C_T$ values for various DNA extractions from the bovine, ovine, and human *M. avium* subsp. *paratuberculosis* strains tested did not differ significantly between the different sources and ranged between 14.87 and 21.43 for F57 and between 14.71 and 20.86 for IS*Mav2* generated with the TaqMan$_{mgb}$ probes (Table 3). The end point fluorescence values (delta Rn) normalized against the passive reference dye ROX were 0.68 (SD, 0.05) for F57 and 1.19 (SD, 0.04) for IS*Mav2*. The LNA probes gave $C_T$ values of 15.27 and 21.75 (F57) and 13.46 and 19.82 (Table 3) and end point fluorescence values (delta Rn) of 1.01 (SD, 0.1) for F57 and 2.33 (SD, 0.04) for IS*Mav2*. For both probe chemistries, a baseline was set manually at 3 to 12 and a threshold line of 0.06 was used. Melting curve analysis conducted after Sybr green-based amplification gave melting temperatures of 80.4°C for the F57 amplicon, 85.4°C for the IS*Mav2* amplicon, and 84°C for the IAC.

**Determination of detection probability.** The broad linear range of detection was $10^7$ CFU/ml to 10 CFU/ml of *M. avium* subsp. *paratuberculosis*. The 100% lower LOD was assessed with six different PCR master mixes from five different suppliers. It corresponded to 0.1 picogram bovine, ovine, or human *M. avium* subsp. *paratuberculosis* DNA per PCR, indicated as a serial dilution of $10^{-7}$ g of *M. avium* subsp. *paratuberculosis* DNA per PCR (Tables 4 and 5). This was equivalent to detection of 19 copies of F57 and 57 copies of IS*Mav2*. These results were obtained with the TaqMan Universal PCR Mastermix (Applied Biosystems), the Absolute QPCR mix (ABgene), the qPCR MasterMix Plus plus UNG and qPCR MasterMix Plus without UNG (both from Eurogentec), and the LightCycler TaqMan Master (Roche). Intra-assay SDs ranged between 0.21 and 0.35 for F57 and IS*Mav2*, while the interassay SD was 0.37 to 2.5 $C_T$ values. No significant differences for each of the marker genes were observed by analysis of variance for $10^{-7}$ CFU per ml, the lowest dilution investigated. For $10^0$ and $10^{-6}$ CFU per ml, overall significant differences were found ($P < 0.0001$). Pairwise comparison according to the Tukey test gave significant differences ($P < 0.01$) between all reagents investigated except between Absolute QPCR mix (ABgene) and the QuantiTect multiplex PCR kit (Qiagen).

The addition of background DNA obtained from *M. avium* subsp. *paratuberculosis*-negative bovine fecal samples did not affect the LOD or the amplification efficiency. In artificially contaminated fecal samples, an LOD of 100% for $10^3$ CFU and $10^2$ CFU of *M. avium* subsp. *paratuberculosis* per g bovine feces was achieved with the modified protocol of the QIAmp stool minikit (Qiagen, Hilden).

**Assessment of amplification efficiency, precision, and robustness.** After optimization of the PCR master mix and the PCR setup, the amplification efficiency for both PCR marker genes was evaluated with regard to serial dilution of an *M. avium* subsp. *paratuberculosis* DNA standard. The amplification effi-

TABLE 4. $C_T$ values generated with the TaqMan Universal PCR Mastermix (Applied Biosystems) and the qPCR MasterMix Plus without UNG (Eurogentec) in the presence of 175 IAC copy numbers

| Serial dilution of *M. avium* subsp. *paratuberculosis* DNA standard | $C_T$ value with the indicated TaqMan$_{mgb}$ probe (fluorescent dye) | | | | | |
|---|---|---|---|---|---|---|
| | F57 (VIC) | | IS*Mav2* (FAM) | | IAC (NED) | |
| | Mean | SD[a] | Mean | SD | Mean | SD |
| $10^0$ | 18.02 | 0.23 | 17.67 | 0.31 | 0[b] | 0 |
| $10^{-1}$ | 20.67 | 0.32 | 19.69 | 0.28 | 0 | 0 |
| $10^{-2}$ | 24.00 | 0.12 | 22.96 | 0.18 | 0 | 0 |
| $10^{-3}$ | 27.33 | 0.13 | 26.57 | 0.25 | 0 | 0 |
| $10^{-4}$ | 30.65 | 0.22 | 30.06 | 0.31 | 39.20 | 1.39 |
| $10^{-5}$ | 33.60 | 0.15 | 33.49 | 0.37 | 35.95 | 0.49 |
| $10^{-6}$ | 36.59 | 0.22 | 36.28 | 0.39 | 35.91 | 0.31 |
| $10^{-7}$ | 42.91 | 1.9 | 40.45 | 0.32 | 35.62 | 0.19 |
| Blank | 0 | 0 | 0 | 0 | 35.62 | 0.25 |

[a] The SD was calculated for three runs of each PCR master mix with three replicates each.
[b] No fluorescent signal detected or no exponential amplification curve.

TABLE 5. $C_T$ values generated with four different ready-to-use PCR master mixes

| Real-time PCR reagent | Serial dilution of *M. avium* subsp. *paratuberculosis* DNA standard | $C_T$ value with the indicated TaqMan$_{mgb}$ probe (fluorescent dye) | | | |
|---|---|---|---|---|---|
| | | F57 (VIC) | | IS*Mav2* (FAM) | |
| | | Mean | SD | Mean | SD |
| QuantiTect Multiplex PCR Kit | $10^0$ | 15.66 | 0.21 | 13.84 | 0.39 |
| | $10^{-6}$ | 35.83 | 0.28 | 35.08 | 0.21 |
| | $10^{-7}$ | ND[a] | | ND | |
| Absolute QPCR mix | $10^0$ | 15.02 | 0.12 | 14.03 | 0.23 |
| | $10^{-6}$ | 35.20 | 0.41 | 35.57 | 0.43 |
| | $10^{-7}$ | 40.51 | 2.49 | 41.87 | 4.3 |
| qPCR MasterMix Plus plus UNG | $10^0$ | 18.89 | 0.32 | 19.02 | 0.29 |
| | $10^{-7}$ | 41.98 | 1.46 | 40.70 | 0.52 |
| LightCycler TaqMan Master | $10^0$ | 20.49 | 0.8 | 20.43 | 2.17 |
| | $10^{-7}$ | 41.60 | 2.09 | 40.91 | 1.8 |

[a] ND, not detected.

ciency for the F57 primer and fluorogenic probe set was 98.2%, and that for the IS*Mav2* primer and fluorogenic probe set was 97.8%. The correlation coefficient ($r$) for both values was >0.99. Results were obtained with the 2× qPCR MasterMix Plus without UNG (Eurogentec). Precise amplification over the upper and lower LODs was shown by three consecutive runs, including triplicate analysis within each run, by using an *M. avium* subsp. *paratuberculosis* DNA serial dilution. The $C_T$ values for the F57 primer and fluorogenic probe set (fluorescent dye, VIC), the IS*Mav2* primer and fluorogenic probe set (fluorescent dye, FAM), and the IAC (fluorescent dye, NED) corresponded to the initial concentration of target DNA. $C_T$ values for F57 ranged between 15.02 and 20.49, and those for IS*Mav2* ranged between 13.84 and 20.43 (Tables 4 and 5).

An increase of 10% of the optimized PCR reagents lowered the PCR efficiency (data not shown), while a decrease of 5% had no significant effect on the assay. Increasing the annealing temperature to 62°C showed an increase of the $C_T$ values of about 0.75 and a slight reduction of the lower LOD. $C_T$ values increased by about three cycles with an annealing temperature of 65°C and a 70% lower LOD.

**Application of the assay with different thermocycler models.** The performance of the TaqMan assay with different real-time PCR thermocycler models was assessed by comparative analysis of a serial dilution of *M. avium* subsp. *paratuberculosis* DNA on the Applied Biosystems 7000 Prism sequence detection system and the LightCycler 1.0. The LightCycler 1.0 does not support the simultaneous detection of the fluorescent dyes VIC (F57) and FAM (IS*Mav2*) due to technical limitations. Therefore, both primer and oligonucleotide sets were applied in separate runs. The LightCycler 1.0 allowed the detection of the lower LOD of 0.1 picogram *M. avium* subsp. *paratuberculosis* DNA per sample.

**Analysis of naturally contaminated fecal samples.** Growth of *M. avium* subsp. *paratuberculosis* was observed and confirmed by real-time PCR and IS*900* nested PCR with 53 of the 108 bovine fecal samples investigated (Table 6). According to the CFU/g feces detected, samples were divided into groups: 0 CFU/g (0), <10 CFU/g (+1), 10 to 50 CFU/g (+2), and >50 CFU/g (+3). Nineteen and 11 samples were positive by culture and direct triplex real-time PCR of the +2 and +3 groups, respectively. One of the +2 samples was not detected by IS*900* nested PCR, resulting in a sensitivity of 90.9% (95% confidence interval, 58.7 to 99.8%). In the +1 group, the triplex real-time PCR gave 3 false-negative results out of the 13 false-negative results shown by IS*900* nested PCR. Diagnostic specificity was 100% for both PCR methods. The sensitivity was 43.5% (95% confidence interval, 23.2 to 65.5%) with IS*900* nested PCR and 87.00% (95% confidence interval, 66.4 to 97.2%) with the real-time PCR assay. This corresponded to a diagnostic accuracy of 87.03% with nested PCR and 97.22% with real-time PCR analysis.

## DISCUSSION

In this study, a new triplex real-time PCR assay was developed for the detection of *M. avium* subsp. *paratuberculosis* in bovine fecal samples. The overall analysis time was approximately 24 h, in contrast to up to 12 weeks of incubation time for the traditional culture method used. The high selectivity of the oligonucleotide primers and probes was confirmed by percent sequence identity comparisons using two different databases (NCBI GenBank and TIGR CMR database) and sequence alignments obtained from genetic elements with high similarity. Analysis of 105 *M. avium* subsp. *paratuberculosis* strains, 34 representative non-*M. avium* subsp. *paratuberculosis*

TABLE 6. Comparative statistical analysis of 108 possibly naturally contaminated bovine fecal samples

| Culture status[a] | No. of samples positive/no. tested by: | | |
|---|---|---|---|
| | Traditional culture | IS*900* nested PCR | Triplex real-time PCR |
| +3 | 19/19 | 19/19 | 19/19 |
| +2 | 11/11 | 10/11 | 11/11 |
| +1 | 23/23 | 10/23 | 20/23 |
| 0 | 55 | 0 | 0 |

[a] Based on the CFU of *M. avium* subsp. *paratuberculosis* per g feces obtained by traditional culture, the samples were divided into four groups: +3, >50 CFU/g; +2, 10 to 50 CFU/g; +1, <10 CFU/g; 0, culture negative.

mycobacteria, and 66 nonmycobacterial strains revealed high selectivity of the triplex assay. No false-positive or -negative results were reported.

F57 and IS*Mav2* have been described as *M. avium* subsp. *paratuberculosis*-specific markers (41, 48). Nested PCR was performed with oligonucleotide primers for the single-copy gene F57 (53). Conventional PCR primers for the three-copy element IS*Mav2* were used for analysis of bulk milk samples (44, 46, 47). Therefore, F57 and IS*Mav2* were selected as two well-characterized candidate genes offering the potential for the specific detection of *M. avium* subsp. *paratuberculosis*. In addition, other genetic elements have been recently identified and described as possibly being *M. avium* subsp. *paratuberculosis* specific (32, 40). The important characterization of diagnostic specificity based on developed oligonucleotide primers or probes was presented for the single-copy elements IS*Map02* (45) and Hsp X (20). References to the following IS*900* primers were made in more than 30 scientific publications: IS*900* and 150C, IS*900* and 921 (55), p36 and p1 (38), MK5 and MK6 (19), P90 and P91 (36), and P21 and P8 (36). Their applicability for routine diagnosis is limited, because of possible cross-reactions with other mycobacterial strains (16, 22, 50). Optimized nested PCR primer pairs for IS*900* that do not show cross-reactions with IS*900*-like elements have been published and were therefore selected as a PCR control system (12). For diagnostic quality assurance, the use of an IAC is thought to be mandatory in diagnostic PCR (28). Several of the recently published real-time PCR methods are still hampered by not considering an IAC (23, 27, 31, 39). The IAC included in the PCR presented here could reliably exclude false-negative PCRs, especially in the presence of small amounts of *M. avium* subsp. *paratuberculosis* DNA ($10^5$ to $10^7$ picogram DNA per PCR). For a high excess of *M. avium* subsp. *paratuberculosis* DNA, the amplification of the IAC template was suboptimal due to the competitive PCR.

Precision of the amplification was demonstrated in consecutive runs. The SDs of the $C_T$ values ranged between 0.5% and 3% (F57 PCR product), 0.7% and 0.9% (IS*Mav2* PCR product), and 0.53% and 0.55% (IAC PCR product). A decrease of the amplification efficiency can occur due to inadequate primer and probe design, nonoptimized PCR reagents, and amplification conditions. The addition of an IAC might cause PCR inhibition (2, 34). The standardized PCR setup was shown to be well optimized, because the addition of an IAC did not result in a disadvantageous effect on the LOD or amplification efficiency during multiplexing.

All ready-to-use TaqMan reagents enabled the reliable detection of the investigated *M. avium* subsp. *paratuberculosis* strains. The TaqMan Universal PCR Mastermix (Applied Biosystems) and the qPCR MasterMix Plus without UNG (Eurogentec) gave very similar results and enabled the lowest LOD of 0.1 picogram DNA per PCR, corresponding to 19 copies of F57 and 57 copies of IS*Mav2*. None of the six PCR master mixes investigated offered a significant improvement in sensitivity (Tables 4 and 5). DNA polymerase enzymes and buffers can vary substantially, with some being more prone to inhibition by harsh inhibitors of feces than others. No disadvantageous effect was reported for the selected ready-to-use master mix used in the standardized protocol. Besides the cost-effective handling of diagnostic reagents, the possible effect of different fluorescent compounds and PCR reagents on the performance of the PCR method has to be considered for each diagnostic test. TaqMan$_{mgb}$ probes and LNA probe chemistries remained stable over 50 PCR cycles, and no instability resulting in a slowly increasing fluorescence signal in nontemplate controls was observed. No differences were observed in the sensitive detection of DNA derived from bovine, ovine, and human *M. avium* subsp. *paratuberculosis* strains. The fluorescent dye Sybr green offers the possibility for a simple and reasonably priced evaluation of newly developed primer pairs. Specificity of the PCR product is confirmed by melting curve analysis (58). The three Sybr green ready-to-use master mixes investigated in this study did not show differences in the detectability of *M. avium* subsp. *paratuberculosis* strains of bovine, ovine, or human origin, but the fluorescent signal of Sybr green was slightly inhibited in the analysis of DNA extracted from fecal samples (data not shown). Sybr green should therefore be used only for the confirmation of possible *M. avium* subsp. *paratuberculosis* colonies obtained by classical cultivation.

International standard providers encourage method developers to validate methods on a variety of instruments to prove that the analytical method is not limited due to different fluorescent compounds or instrument specificity. Several companies offer thermocycler models for the detection of TaqMan fluorogenic probes. The reactions can be performed in 96-well plates. The LightCycler 1.0 is typically used with hybridization probes. The fluorescent signal is measured through capillaries, and the temperature is adjusted with a heater fan instead of heat blocks. Therefore, the ABI 7000 Prism sequence detection system and the LightCycler 1.0 represent two technically different thermocycler platforms which are widely used. The presented real-time PCR assay gave identical LODs of 0.1 picogram *M. avium* subsp. *paratuberculosis* DNA on both instruments, with a probability of 100%. It should be mentioned that in contrast to the LightCycler 2.0, the LightCycler 1.0 cannot detect the two separate emission spectra of FAM and VIC in a duplex or even triplex PCR. For this purpose, the oligonucleotides and fluorescent probes developed for the marker genes F57 or IS*Mav2* have to be combined separately with the IAC primer and probe set. Promising further experiments are under way using the improved six-channel detection system of the LightCycler 2.0, enabling the application of the complete triplex assay in the configuration described above.

Investigation of bovine fecal samples by culture is the gold standard for the estimation of the prevalence of *M. avium* subsp. *paratuberculosis* on the herd level (21, 42). For PCR applications, the extraction of DNA from small amounts of different bacteria from fecal samples is hampered due to different PCR inhibitors (57). In addition, *M. avium* subsp. *paratuberculosis* is known to form clumps and to be highly resistant to chemical and enzymatic lysis (12). The robust real-time PCR assay was combined with a modified DNA extraction procedure to achieve maximum sensitivity for the detection of *M. avium* subsp. *paratuberculosis* from bovine fecal samples. Addition of nonspecific background DNA isolated with the optimized protocol from bovine feces did not decrease the LOD of the PCR. Numerous methods have been used for the extraction of *M. avium* subsp. *paratuberculosis* DNA from fecal samples, including immunomagnetic separation (31), buoyant density centrifugation (25), addition of resins (35), DNA sequence

capture extraction (54), and silica membrane-based kits (11). The mechanical homogenization of the sample and disruption of the *M. avium* subsp. *paratuberculosis* cells included in the modified QIAamp DNA stool minikit protocol in this study showed an advantageous effect on the LOD (data not shown). The lower LOD in artificially spiked samples was 100% for $10^3$ CFU/per gram feces and 100% for $10^2$ CFU/g feces, compared to an LOD of 70% for $10^3$ CFU/per gram feces with the standard protocol. Mechanical shearing of the fecal samples suspected to contain *M. avium* subsp. *paratuberculosis* by using a bead beater resulted in the formation of foam from the ASL buffer. This could not be reliably removed by centrifugation and hampered further processing. The addition of DX buffer to the ASL buffer reduced the foam formation significantly.

In addition, real-time PCR requires the investigation of the interaction of a probe dye with feces in order to remove any possible quenching effect of the matrix on the fluorescence activity of the probes. Silica membrane columns have been previously shown to provide a convenient method for DNA purification and especially removal of PCR inhibitors (29, 30). The analytical sensitivity obtained from spiking experiments is comparable to the results of other studies. An LOD of 100% for an IS*900* real-time PCR was achieved with a silica membrane-based kit at 500 CFU/g feces (10). An LOD of 100% was described for 100 CFU/g feces (11) in combination with an F57 real-time PCR (51). The analysis of 108 naturally contaminated fecal samples of unknown status revealed a statistically better sensitivity and accuracy than IS*900* nested PCR (Table 6). In accordance with the results of classical cultural investigation, 50 of 53 samples were confirmed to be *M. avium* subsp. *paratuberculosis* positive. However, for three of the samples it was not possible with this real-time PCR to determine definite concentrations of *M. avium* subsp. *paratuberculosis* in fecal samples. Similarly, those samples and 10 additional samples were not identified by using IS*900* nested PCR. These three samples were poorly contaminated and produced growth of 2, 5, and 8 CFU, respectively, on HEYM agar. Although HEYM slants do not allow exact quantification, the detection limit obtained in this study was comparable to that in the spiking experiments and should be sufficient for detection of *M. avium* subsp. *paratuberculosis* in fecal samples contaminated at high or low levels. One sample contained *M. avium* subsp. *avium* as confirmed by sequence analysis. No false-positive reactions with this or any other sample were obtained with the real-time PCR.

Different strategies have been proposed to be suitable for the reliable detection of *M. avium* subsp. *paratuberculosis* by using real-time PCR, including use of conventional IS*900* PCR primers (31), the combination of two separate real-time PCR runs for IS*900* and F57 (27), and the use of F57-derived oligonucleotide primers and hybridization probes restricted to the LightCycler (51). The main advantages of our triplex real-time PCR assay can be summarized as the combination of the reliability of two different marker genes (F57 and IS*Mav2*) and an IAC and its detailed validation according to the requirements made by international standard providers (7, 8). The latter includes applicability on different thermocycler models. Although not described yet and carefully excluded by the strains considered during this study, scientific evidence of possible cross-reacting strains might arise, as described for the former reference marker IS*900*. The application of this assay makes it very unlikely that cross-reactions with both marker genes will be obtained. Being not restricted to a distinct supplier or provider of PCR reagents makes the method easier to adopt for individual needs, and the IAC guarantees diagnostic quality assurance. Considering these factors in combination with the optimized DNA extraction protocol for fecal samples offering potential for automized sample preparation, we think that the assay presented here contributes to the improvement of routine diagnostic procedures for *M. avium* subsp. *paratuberculosis*.

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# Detection of *Mycobacterium avium* Subspecies *Paratuberculosis*-Specific DNA by PCR in Intestinal Biopsies of Dogs

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**Background:** *Mycobacterium avium* subspecies *paratuberculosis* (MAP) is the cause of paratuberculosis. MAP infections have not been reliably detected in dogs, but a reemerging debate about the link between MAP and Crohn's disease has renewed interest about the occurrence of MAP in pets.

**Hypothesis:** This study was undertaken to examine canine intestinal biopsies for the presence of MAP-specific DNA.

**Animals:** Forty-two dogs with chronic vomiting, diarrhea, or both; and 14 dogs with no gastrointestinal disease.

**Methods:** All dogs with signs of gastrointestinal disease had a standard work-up for chronic gastrointestinal disease. Endoscopically obtained intestinal biopsies were submitted for histopathologic and molecular investigations. Biopsies were screened for MAP-specific DNA by 3 polymerase chain reaction (PCR) methods (nested, seminested, and triplex real-time PCR). Samples from control dogs were obtained during necropsy.

**Results:** Histopathology of the biopsies was indicative of inflammatory bowel disease (IBD) in 17 and neoplasia in 6 dogs. Six dogs showing nonspecific changes responded to diet and were classified as having food-responsive enteropathy. In 13 dogs a final diagnosis was not established. MAP-specific DNA was detected and confirmed by sequencing in 8 dogs (19%). These dogs were diagnosed with food-responsive enteropathy (n = 3), IBD (n = 2), and open diagnosis (n = 3). MAP-specific DNA was not detected in dogs with no gastrointestinal disease.

**Conclusions and clinical importance:** MAP-specific DNA was detected in approximately one fifth of dogs with chronic gastrointestinal disease and might play a role as a pathogenic agent. Apart from animal welfare, the zoonotic aspect warrants further studies addressing the viability of MAP organism in canine intestinal biopsies by culture.

**Key words:** Canine; Gastroenterology; Inflammatory bowel disease; Molecular biology.

Mycobacteriosis in dogs can generally be divided into 2 groups, classic tuberculosis (*Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium avium*, *Mycobacterium microti*, and *Mycobacterium simiae*) and opportunistic mycobacteriosis (*Mycobacterium fortuitum*, *Mycobacterium chelonae*, *Mycobacterium smegmatis*, *Mycobacterium phlei*, *Mycobacterium thermoresistibile*, *Mycobacterium terrae*, *Mycobacterium genavense*, and *Mycobacterium xenopi*).[1,2] Despite these well-described mycobacterioses, infections with *M. avium* subspecies *paratuberculosis* (MAP) have not been reliably detected in dogs, except the description of a possible clinical case of paratuberculosis in 1 dog.[3] MAP is the causative agent of paratuberculosis, also known as Johne's disease, a severe chronic incurable granulomatous bowel disease affecting domestic and wild ruminants.[4,5] The disease is responsible for a substantial morbidity and significant economic loss in the dairy and beef cattle industry in various countries.[6,7] A reemerging debate about the link between MAP and Crohn's disease, a human chronic granulomatous ileocolitis, has increased awareness of public health concerns about the occurrence of MAP infections in pets.[8–11]

The etiology of inflammatory bowel disease (IBD) has not been well defined in humans (Crohn's disease and ulcerative colitis) or dogs. In humans, the current consensus hypothesis is that in genetically predisposed individuals (as suggested by epidemiologic and genetic data), exogenous factors (infectious agents and normal flora) and host factors (intestinal epithelium, vascular supply, hormones and neuronal activity) act together to cause and maintain a chronic state of dysregulated mucosal immune function that might be affected by specific environmental factors.[8,12] The infectious etiology hypothesis includes a possible linkage of MAP to patients with Crohn's disease.[8–11] Transmission of the bacterium to humans by direct animal contact has been considered possible. The presence of MAP in milk has been reported for milk supplies, including bulk milk at the farm level[13,14] and milk at the processing level for various countries.[15,16] The occurrence of MAP in bovine lymph nodes and meat has been described,[17,18] but was recently considered to be less important.[19]

Culture-based detection of MAP is time-consuming, labor-intensive, and often not sensitive enough. In contrast, the polymerase chain reaction (PCR) has been shown to be a powerful diagnostic tool in microbiology. The insertion sequence IS*900* has been widely used as a MAP reference marker to detect MAP in clinical samples from both animals and humans.[20,21] However, because of a considerable high sequence similarity with IS*900*-like elements or other genetic elements, cross reactions might give false-positive results.[22–24]

The gene locus F57 and the insertion sequence IS*Mav2* have also been described as MAP-specific markers.[25,26] Therefore, F57 and IS*Mav2* were selected as 2 other well-characterized candidate genes offering the potential for detection of MAP.

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This study was conducted to examine intestinal biopsies from dogs with and without a history of chronic diarrhea or vomiting or both for the presence of MAP-specific deoxyribonucleic acid (DNA) using (a) nested PCR (IS*900*), (b) a seminested PCR (F57), and (c) a recently developed triplex real-time PCR (IS*Mav2*, F57, internal amplification control [IAC]) and to determine whether the detection of MAP-DNA is associated with a specific histopathologic diagnosis in dogs with chronic gastrointestinal disease.

## Material and Methods

### *Clinical Cases and Gastrointestinally Healthy Dogs*

One group of animals consisted of dogs with persistent signs of gastrointestinal disease of at least 3 weeks duration and the other group consisted of gastrointestinally healthy dogs. All animals with signs of gastrointestinal disease (n = 42) included in the study had a standard work-up for chronic diarrhea, vomiting, or both (CBC, biochemical profile, urinalysis, serum trypsin-like immunoreactivity, fecal parasitology and bacteriology, abdominal ultrasound, and gastrointestinal endoscopy with biopsies for histopathologic examination). Thirty-nine of the 42 dogs showed predominantly small bowel disease; in 14 dogs additional signs of large bowel disease were noted and 3 dogs solely had signs consistent with large bowel disease.

Gastrointestinally healthy dogs (n = 14) were presented to the Pathology Department of the University of Giessen for necropsy.

### *Collection of Samples*

Food was withheld 24–48 hours before endoscopy. Under general anesthesia, multiple mucosal biopsy specimens were obtained from the stomach, duodenum, and, in most cases, the colon with a flexible video endoscope. For histopathologic examination, biopsy samples were formaldehyde-fixed. The endoscope and biopsy forceps were routinely cleaned in an automatic washing device with glucoprotamine[a] after every procedure. The histopathologic examination based on previously published grading schemes was performed by board-certified pathologists on sections stained with hematoxylin and eosin (H&E).[27]

Samples for molecular investigation were placed in 1 mL saline solution and stored at −20 °C until further analysis.

Samples for molecular investigation were also obtained from gastrointestinally healthy dogs (n = 14) during necropsy with endoscopic biopsy forceps.

### *DNA Extraction*

To avoid cross-contamination of the DNA preparations, the maximum capacity of biopsies of 5 dogs was processed per day. The DNA of the biopsy samples was extracted by using a modified protocol of the DNeasy Blood and Tissue Kit[b] for the extraction of DNA from Gram-positive bacteria. In most cases, 2 biopsies of identical locations per animal were pooled. Briefly, 180 µL of lysis buffer were added to the samples and incubated at 37 °C for 1.5 hours. Subsequently, 50 µL proteinase K and 200 mL lysis buffer (AL buffer) were added, and the mixture was incubated overnight at 70 °C until complete digestion of the tissues was achieved, resulting in a clear solution. In case of undigested tissue, the mixture was transferred to a glass matrix tube for cell lysis.[c] The mixture was processed in a spin/rotation instrument for cell lysis (FastPrep-120),[c] with a speed and time setting of 6 and 45 seconds, respectively. The DNA was bound to spin columns and washed twice. Subsequently, the DNA was eluted with 100 µL elution buffer. The quality of the DNA preparation was examined using the previously published 16S ribosomal RNA oligonucleotide primers.[28]

### *PCR Analysis*

For the detection of MAP, 3 different PCR systems were set up in parallel, including a nested PCR[20] with IS*900* oligonucleotide primers TJ1//TJ2 and TJ3//TJ4, a seminested PCR[29] with F57 oligonucleotide primers F57//R57 and F57//Rn57, and a triplex real-time PCR amplifying F57 and IS*Mav2* fragments—for primers see Table 1.[30] The PCR reaction mixture for the nested PCR (50 µL) contained 1 µL primer 1 (10 pmol/µL), 1 µL primer 2 (10 pmol/µL), 1 µL dNTP (10 mmol),[d] 5 µL 10 × thermophilic-buffer,[e] 0.5 µL $MgCl_2$ (25 mM),[e] 0.5 µL Ampli *Taq* Gold DNA polymerase (5 U/µL),[e] and 36 µL double-distilled water. Finally, 5 µL of the DNA preparation was added to each reaction tube.

The PCR reaction mixture (25 µL) for the seminested PCR was prepared as follows: 1 µL primer 1 (10 pmol/µL), 1 µL primer 2 (10 pmol/µL), 1 µL dNTP (10 mmol),[d] 2.5 µL 10 × thermophilic-buffer,[e] 0.5 µL $MgCl_2$ (25 mM),[e] 0.5 µL Ampli *Taq* Gold DNA polymerase (5 U/µL),[e] 1.25 µL dimethyl sulfoxide (DMSO), and 12.25 µL double-distilled water. Finally, 5 µL DNA preparation was added to each reaction tube. For the nested and seminested PCR reactions, a 1 : 10 dilution of each first-round PCR product was prepared and 5 µL of this dilution was added to a 50 µL reaction mixture containing the same components as indicated previously.

The PCR reactions were performed with a GeneAmp 9600 Sequence Detection System[e] with the following program: 1 × 10 minutes precycle at 95 °C, 35 × 30 seconds at 95 °C, 30 seconds at 60 °C (nested PCR), and 30 seconds at 72 °C, followed by a final extension step of 72 °C for 5 minutes. For the seminested PCR, an annealing temperature of 61 °C was used.

PCR products were visualized by electrophoresis with 10 µL of the reaction product in a 1.5% agarose gel,[f] with Tris acetate-electrophoresis buffer (TAE) (0.04 mol/L Tris, 0.001 mol/L EDTA, pH 7.8) and a 100 bp DNA ladder[d] as molecular marker. For nucleotide sequence analysis, amplification products were purified with the Qiaquick PCR Purification Kit.[b] Sequencing was performed by Sequence Laboratories GmbH (Göttingen, Germany). Sequencing data were compared with the sequence entries of the National Center for Biotechnology Information (NCBI, Bethesda, MD) by the Basic Local Alignment Search Tool (BLAST 1).

The triplex real-time PCR assay was performed as previously described.[30] The fluorescent data were generated by $TaqMan_{mgb}$

**Table 1.** Primer sequences used for triplex real-time PCR analysis according to Schönenbrücher et al.[30]

| Designation | Sequence |
|---|---|
| F57-F | 5′-TAC GAG CAC GCA GGC ATTC-3′ |
| F57-R (reverse) | 5′-CGG TCC AGT TCG CTG TCA T-3′ |
| F57 $TaqMan_{mgb}$ probe | VIC-CCT GAC CAC CCT TC-MGB |
| F57 $TaqMan_{mgb}$ IAC probe | NED-CGA GTT ACA TGA TCC C –MGB |
| IS*Mav2*-F (forward) | 5′-CGG CAA AAT CGA GCA GTT TC-3′ |
| IS*Mav2*-R (reverse) | 5′-TGA GCC GGT GTG ATC ATC TTT-3′ |
| IS*Mav2* $TaqMan_{mgb}$ probe | FAM-CGC TGA GTT CCT TAG-MGB |

IAC, internal amplification control; PCR, polymerase chain reaction.

probes applied on the ABI Prism 7000 Sequence Detection System according to the instructions of the supplier. Briefly, the 50 μL PCR mixture for the triplex real-time PCR assay contained 25 μL of the 2× qPCR MasterMix Plus w/o UNG,[g] 0.6 μL of each primer (F57-F/F57-R, IS*Mav2*-F/IS*Mav2*-R), 2 μL of each of the fluorogenic probes (F57, IS*Mav2*, IAC), 5 μL of the IAC, 4.6 μL of double-distilled water, and a 5-μL aliquot of the DNA sample. The PCRs were performed in a 96-well plate on the ABI Prism 7000 Sequence Detection System.[e] Thermal cycling conditions comprised a Hot Start DNA Polymerase activation at 95 °C for 10 minutes, 50 cycles of denaturation at 95 °C for 15 seconds, and annealing and extension at 60 °C for 1 minute. Samples were analyzed in duplicate, and the threshold cycle ($C_t$) values were determined.

## Results

The 42 dogs with signs of gastrointestinal disease included in this study were 5.9 ± 3.2 years old and weighed 21.0 ± 10.1 kg. The following breeds were included: mixed breeds (n = 9), German Shepherd Dog (n = 7), Labrador Retriever (n = 3), Golden Retriever (n = 3), Yorkshire Terrier (n = 2), English Springer Spaniel (n = 2), Beagle (n = 2), and Australian Cattle Dog, Bernese Mountain Dog, Border Collie, Boxer, Bull Terrier, Cocker Spaniel, Dachshound, Hungarian Vizsla, Jack Russell Terrier, Loewchen, Rottweiler, Weimaraner, Welsh Terrier, and West Highland White Terrier (1 of each). The main clinical signs were vomiting (n = 24), small bowel diarrhea (n = 16), large bowel diarrhea (n = 11), hematochezia (n = 6), melena (n = 1), and hematemesis (n = 1). Histopathologic evaluation of the biopsy samples was indicative for IBD in 17 cases and neoplasia in 6 cases (adenocarcinoma). Based on previously published histologic grading schemes,[27] 6 dogs showed no or only a mild degree of inflammation and cellular infiltrate. However, these dogs responded to dietary changes (consisting of either a novel protein source or hydrolyzed diet) and were classified as having food-responsive enteropathy.

In 13 dogs, the histopathologic examination revealed only mild nonspecific changes secondary to a possible extragastrointestinal disorder (eg, congestion), and a final diagnosis for the clinical signs was not established.

MAP-specific DNA was detected and confirmed by sequencing of the PCR amplicons in 8 (19%) of 42 dogs with gastrointestinal signs. Nested PCR (IS*900*) was positive in all 8 dogs, real-time PCR (F57 and IS*Mav2*) was positive in 7 dogs, whereas samples from only 3 dogs were positive in the seminested PCR (F57). The 8 dogs with positive MAP results were diagnosed with food-responsive enteropathy (n = 3), IBD (n = 2), and nonspecific gastrointestinal changes (n = 3). The dogs with positive PCR results were 6.5 ± 1.0 years old. Breeds included German Shepherd Dog (n = 2, both diagnosed with food-responsive enteropathy), mixed breed (n = 1, diagnosed with food-responsive enteropathy), Golden Retriever (n = 1, nonspecific gastrointestinal changes), Loewchen (n = 1, nonspecific gastrointestinal changes), Hungarian Vizsla (n = 1, nonspecific gastrointestinal changes), West Highland White Terrier (n = 1, diagnosed with IBD), and Jack Russell Terrier (n = 1, diagnosed with IBD).

One sequence of the IS*900* nested PCR and the F57 seminested PCR was exemplarily submitted to Genbank (Genbank accession numbers: EU092638, EU092639). All other sequences obtained, including those of the real-time PCR products, were identical and showed a 100% identity with the reference sequence entries IS*900*, F57, and IS*Mav2*.

The 14 gastrointestinally healthy dogs were 6.1 ± 1.3 years old and belonged to the following breeds: mixed breeds (n = 6), Dachshund (n = 2), and American Pitbull, Australian Cattle Dog, Beagle, Newfoundland, West Highland White Terrier, and Yorkshire Terrier (1 of each). Necropsies were performed in consequence of cardiorespiratory disease (n = 5), neoplasia (n = 2), trauma (n = 2), sudden death, immune-mediated hemolytic anemia, renal failure, gastrointestinal foreign body, and hepatic failure. Gastrointestinal disease was not present based on history and postmortem examination in any of the cases. MAP-specific DNA was not detected in any of the samples.

## Discussion

The pathogenesis of Crohn's disease in human medicine is a topic of intense debate. A particular focus has been whether or not infection with MAP plays an important role.[8–11] As a consequence, there is an increasing public interest in the occurrence of MAP infections in domestic animals. This is the 1st study to address the occurrence of MAP in the canine population. Using 3 different PCR methods, MAP-specific DNA was reliably detected in intestinal biopsies of 8 dogs with a history of chronic diarrhea or vomiting or both by nested PCR (8 of 8), real-time PCR (7 out of 8), seminested PCR (3 of 8), and subsequent sequencing of the PCR amplicons. The primer pairs included the widely used MAP reference marker IS*900*[21] as well as the MAP unique sequences F57[25] and IS*Mav*.[26]

To date, the molecular detection of MAP-DNA has often been hampered by insufficient specificity of the selected primers and probes, inadequate DNA extraction methods resulting in insufficient sensitivity, and possible contamination as a consequence of the PCR techniques used.[22] Therefore, this study selected 2 nested PCR systems and a real-time PCR method instead of conventional PCR sets, which ensured a highly sensitive approach. The nested PCR used was developed by Bull et al[20] and is based on the widely used MAP reference marker IS*900*. The oligonucleotide primer sequences were specifically adapted to show no cross reactions with the previously described IS*900*-like elements.[23,24] All positive IS*900* PCR products detected in the 8 dogs were sequenced and corresponded to MAP IS*900* but not IS*900*-like sequences.

Dogs have been described as a reservoir host of different *Mycobacterium* subspecies, including those closely related to MAP.[31,32] Therefore, as additional in-house validation, the specificity of the oligonucleotide primers was examined on 18 different *Mycobacteria* species during this study, none of which gave false-positive results.[30] These investigations included the closely related

*M. avium* ssp. *avium* as well as *M. chelonae* and *M. fortuitum* for which false-positive results by using IS*900*-based oligonucleotide primers have been described.[24,33] The single copy gene F57 was validated to be MAP specific by Coetsier et al[34] and more recently by Tasara and Stephan.[35] Therefore, the seminested PCR[29] was added as a 3rd method to ensure high MAP specificity and diagnostic sensitivity.

The closed tube detection of the real-time PCR technique offers the major advantage of avoiding possible cross-contamination, which is one of the main drawbacks of nested PCR systems.[36] This technique has also been proven to be a highly sensitive refinement of conventional PCR.[37] Recently, a triplex real-time PCR assay combining the MAP-specific gene sequences F57, IS*Mav2*, and an IAC for diagnostic quality assurance was developed. The PCR method was carefully validated by using more than 200 references and field strains with different kinds of sample preparation techniques on artificially contaminated and naturally infected bovine fecal samples. The assay uses TaqMan fluorogenic probe chemistry, and the detection limit is $<0.1$ pg of MAP-DNA per PCR reaction.[30] In accordance with our findings, IS*Mav2* has been shown to be highly suitable for exclusive detection of MAP.[26,38,39]

Based on 3 different PCR methods, false-positive results because of unspecific binding of oligonucleotide primer pairs were highly improbable. The risk of cross-contamination between the samples was also reduced by limiting the DNA preparations to a certain amount of samples per day, simultaneous setup of the PCR reactions, and the processing of the nested or seminested PCR reactions, without opening the PCR tubes before performing gel electrophoresis. The use of an IAC has been shown to be mandatory for diagnostic PCR applications.[40] The included IAC of the real-time PCR assay revealed that none of the real-time PCR reactions was inhibited. In contrast to the seminested PCR, the nested PCR and the real-time PCR gave similarly high numbers of PCR-positive results. This proved similar sensitivity and robustness of both methods because very low amounts of MAP-DNA were expected in the biopsies.[20]

As a general prerequisite, routinely performed disinfection after each endoscopy minimized possible cross-contamination during sampling. Bacteriologic surveillance of the endoscopic equipment after disinfection was not performed because culturing MAP is time-consuming, labor-intensive, and also not considered sensitive enough. However, based on the order in which the positive PCR results were obtained, contamination by endoscopic procedures seemed unlikely.

Histologic examination of the biopsies did not reveal specific gastrointestinal lesions being associated with the presence of MAP-specific DNA in dogs. The 8 dogs with positive MAP results were diagnosed with food-responsive disease, IBD, and nonspecific gastrointestinal changes. A similar distribution of histologic results was seen in dogs negative for MAP-DNA.

This is the 1st time that MAP-DNA has reliably been detected in dogs. Because cats and dogs are the most important companion animals, these findings might be very important in light of the zoonotic potential. The detection of MAP-specific DNA in canine intestinal biopsies can represent a true infection or a transient colonization by viable MAP or MAP-DNA only. Detection of viable MAP by cultural investigation in the canine intestinal tract will be helpful to investigate the relationship of a possible linkage between MAP and Crohn's disease in humans and dogs being a possible reservoir for MAP. A recent study showed MAP infections in feral cats.[41] Although typical histopathologic lesions for paratuberculosis were not seen in these cases, the isolation of MAP from mesenteric lymph nodes suggests that cats were truly infected and not just transiently colonized by MAP passing through the gastrointestinal tract. The means by which these cats were infected remained unclear. Direct transmission through unintended consumption of non-pasteurized waste milk of subclinically infected cows was suspected as well as fecal-oral transmission and ingestion of MAP-infected prey.[41,42] The 8 MAP-DNA–positive dogs in this study had access to rural environments, but information about the prevalence of paratuberculosis in these areas was not available. The potential of MAP to survive in the environment has been well characterized.[43] Further studies might benefit from an evaluation of the living environments of patients and medical histories of their owners in light of chronic intestinal symptoms.

The fact that no specific histopathologic lesion in MAP-DNA–positive dogs was seen cannot rule out any clinical significance of a possible infection. The fact that MAP-specific DNA was not found in any samples of the control group—consisting of gastrointestinally healthy dogs—emphasizes a potential role of MAP in canine chronic gastrointestinal disease. Further assessment of the clinical relevance of these findings is warranted and should include cultural investigation of viable MAP from intestinal biopsies of dogs (including clinically healthy animals as a negative control group) as well as repeated assessment of the occurrence of MAP in these animals. Also, positive cultures of biopsies from regional lymph nodes (eg, obtained during exploratory celiotomy) would give evidence of a true infection.

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## Footnotes

[a] Sekusept Plus, Ecolab, Düsseldorf, Germany
[b] Qiagen, Hilden, Germany
[c] FastRNA Green, Q BIOgene, Heidelberg, Germany
[d] Roche Diagnostic, Mannheim, Germany
[e] Applied Biosystems, Darmstadt, Germany
[f] Appligene, Heidelberg, Germany
[g] Eurogentec, Seraing, Belgium

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