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Molecular characterization of *Escherichia coli* isolates recovered from broilers with cellulitis

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ABSTRACT Avian cellulitis in broilers, caused by avian pathogenic *Escherichia coli*, is a major cause for carcass rejections during meat inspection, resulting in significant economic losses. In this study, we analysed *E. coli* isolates obtained from broiler chickens affected by cellulitis for their genetic relatedness and antimicrobial resistance phenotype and genotype. The objective was to determine whether there is a clonal spread or whether these clinical isolates differ. For this purpose, *E. coli* was isolated from swab samples collected from diseased broilers across 77 poultry farms in Germany, resulting in 107 isolates. These isolates were subjected to serotyping, PCR-based phylotyping and macrorestriction analysis with subsequent pulsed-field gel-electrophoresis for typing purposes. In addition, the presence of virulence genes associated with avian pathogenic *E. coli* (**APEC**) was investigated by PCR. Antimicrobial susceptibility of the isolates was examined by the disk diffusion method according to CLSI guidelines and subsequently, the presence of corresponding resistance genes was investigated by PCR. Typing results revealed that a significant proportion of the isolates belonged to serotype O78:K80, which is one of the major APEC serotypes. Phylogenetic grouping showed that phylogenetic group D was most commonly represented (n = 49). Macrorestriction analysis showed overall heterogenous results, however, some clustering of closely related isolates was observed. The level of antimicrobial resistance was high, with 83.8% of isolates non-susceptible to at least one class of antimicrobial agents and 40% of isolates showing resistance to at least three classes. The most frequently observed resistance was to ampicillin, mediated by bla_{TEM} (n = 56). However, few isolates were non-susceptible to ciprofloxacin (n = 8) and none of the isolates was resistant to 3rd generation cephalosporins or carbapenems. Overall, the results show that genetically diverse APEC associated with avian cellulitis can be found among and within German poultry farms. While most isolates were antimicrobial resistant, resistance levels to high(est) priority critically important antimicrobials were low.

Key words: *Escherichia coli*, avian cellulitis, APEC, antimicrobial susceptibility, broiler

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INTRODUCTION

Avian cellulitis is an economically important disease in broilers and among the main reasons for carcass rejections during meat inspection. In the year 2021, it accounted for almost a third (29.4%) of all discarded carcasses in German abattoirs, followed at some distance by ascites as the second most common reason (16.3%) (DESTATIS, 2022). Notably, the proportion of rejections due to cellulitis has seen a marked increase. Ten years ago, in 2013, it was responsible for only approximately 18% of all broiler carcass rejections in German slaughterhouses (DESTATIS, 2014). Cellulitis is mainly caused by *Escherichia coli* and is characterized by subcutaneous inflammation accompanied by fibrinous plaques, mostly affecting the inner thighs and abdominal wall (Löhren, 2012; Schulze Bernd et al. 2022). Live broilers suffering from cellulitis often show no obvious symptoms, and the condition can be hard to detect prior to meat inspection (Löhren, 2012). If it is detected in flocks prior to slaughter, or if pre-catching of part of the flock reveals high incidences of cellulitis during meat inspection, antimicrobial agents can be used in an effort to reduce impending condemnation rates in case of subsequent systemic infection (Schulze Bernd et

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al. 2020). However, high rates of antimicrobial resistance are observed in poultry-associated *E. coli* isolates, especially to ampicillin, sulfamethoxazole, trimethoprim and tetracycline, for which they can function as a reservoir of resistance genes (European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC), 2023; Martínez-Álvarez et al. 2022; Xu et al. 2022). Besides threatening the treatment success in broiler flocks, this is additionally concerning as avian pathogenic *E. coli* (**APEC**) and extraintestinal pathogenic *E. coli* (**ExPEC**) in humans can show remarkable genetic similarity, indicating a zoonotic potential if APEC isolates reach the consumer via the food chain (Jørgensen et al. 2019; Kathayat et al. 2021). Consequently, resistance to antimicrobials deemed high or highest priority critically important by the World Health Organziation (categorie „Watched" according to the AWaRe classification) such as 3rd generation cephalosporins, carbapenems or quinolones, is of particular relevance (World Health Organization 2019; 2021).

The aim of this study was to examine *E. coli* recovered from broilers suffering from cellulitis in order to evaluate the genetic relatedness, the antimicrobial resistance phenotype and genotype, and the presence of virulence genes associated with avian pathogenicity in isolates occurring in German broiler flocks, in order to investigate whether a single pathogenic clone is responsible for condemnations by cellulitis or whether genetically different isolates are involved.

MATERIALS AND METHODS

Isolation of E. coli*, Species Identification and Serotyping*

Between July 2018 and August 2019, samples were collected from broiler chickens that suffered from cellulitis across a total of 77 farms. Swab samples were taken primarily from affected areas of the subcutis (n = 73). If the birds suffered from arthritis or polyserositis, swabs were also taken from joints (n = 23), pericardium (n = 7) or bone marrow of the femur (n = 1). At most 2 samples were taken per farm per year, mostly from different barns amounting to a total of 104 swabs (Supplementary Table 1). In the laboratory, swabs were streaked onto McConkey agar as well as Columbia agar with 7% sheep blood (Thermo Scientific, Germany) and incubated for 24 h at 37°C. Plates were checked for growth indicative of *E. coli* (red colonies on McConkey) and a single colony of typical morphology was subcultured and included in further analyses. Only if 2 visually distinct presumptive *E. coli* colony types were observed, a single colony of each morphology was investigated. The species of presumptive *E. coli* isolates was then confirmed by a PCR assay targeting the species-specific glutamate decarboxylase-alpha gene (*gadA*) using previously published primers (Doumith et al. 2012). Serotyping was performed by slide agglutination (sifin diagnostics gmbh, Berlin, Germany).

Molecular Typing and Detection of Virulence-Associated Genes

Based on the PCR-analyzed presence or absence of *chuA, yjaA* and the DNA fragment TSPEC4.C2, the isolates were assigned to one of the four major phylogenetic groups of *E. coli*, as previously described (Doumith et al. 2012). All isolates were further tested for genes indicative of avian pathogenic *E. coli* by PCR. This included *hylF, iroN, iss, iutA,* and *ompT* (Johnson et al. 2008).

They were further subjected to *Xba*I macrorestriction analysis and pulsed-field gel-electrophoresis (**PFGE**) to provide a conclusion about their clonal relationship (Ribot et al. 2006). Similarity values of band patterns were determined by UPGMA analysis (dice coefficient, 0.5% optimization, and 1% position tolerance) using the Bionumerics software v. 7.6. (Applied Maths, Sint-Martens-Latem, Belgium).

Antimicrobial Susceptibility Testing

The susceptibility of all isolates towards a panel of 15 antimicrobials was tested by disk diffusion according to the specifications provided by the Clinical and Laboratory Standards Institute (**CLSI**) (Clinical and Laboratory Standards Institute (CLSI), 2018). All antimicrobial disks were purchased from Oxoid (Wesel, Germany) and comprised the following substances (disk content in brackets): amoxicillin/clavulanic acid (20/10 μg), ampicillin (10 μg), cefazolin (30 μg), cefoxitin (30 μg), cefpodoxime (10 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), gentamicin (10 μg), imipenem (10 μg), nalidixic acid (30 μg), streptomycin (10 μg), sulfisoxazole (300 μg), trimethoprim (5 μg), tetracycline (30 μg), and florfenicol (30 μg). *E. coli* ATCC25922 served as quality control strain. Zone diameters were interpreted according to CLSI breakpoints for Enterobacterales (Clinical and Laboratory Standards Institute (CLSI) 2020; 2023).

Detection of Antimicrobial Resistance Genes

The presence of antimicrobial resistance genes was determined by PCR assays using a panel of previously published primers and protocols (Müller et al. 2018). This included a multiplex-PCR detecting the β-lactamase genes bla_{TEM}, bla_{SHV} and $bla_{\text{OXA-1-like}}$ (Dallenne et al. 2010), as well as a PCR assay targeting $bla_{\text{OXA-2}}$ (Prüller et al. 2015). Isolates resistant to tetracyclines were tested for the presence of *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(G), *tet*(H), *tet*(L), *tet*(M) and *tet*(O) (Prüller et al. 2015). The panel further included primers targeting *sul1, sul2* and *sul3,* mediating resistance to sulfonamides; *dfrA1/15/16, dfrA5/14, dfrA7/17*, mediating resistance to trimethoprim; *strA, strB, aadA1, aadA2, aac(3)-II, aac(3)-IV, ant(2")-I* (Sandvang and Aarestrup 2000), mediating resistance to aminoglycosides; phenicol resistance genes *catA1, catA2, catA3, catB2, catB3, cmlA* and *floR* (Arcangioli et al. 1999; Prüller et al. 2015); colistin resistance genes *mcr*-1 (Liu

et al. 2016), *mcr*-2, *mcr*-3, *mcr*-4, *mcr*-5 (Rebelo et al. 2018), *mcr*-6, *mcr*-7, *mcr*-8, and *mcr*-9 (Borowiak et al. 2020); and *aac(6')-Ib-cr, qnrA, qnrB, qnrC, qnrD* and *qnrS* (Kehrenberg et al. 2006; Vredenburg et al. 2014), associated with plasmid-mediated quinolone-resistance. If amplicons were obtained with any of the primes targeting *dfr*-genes, they were sequenced (Eurofins Genomics Ebersberg, Germany) in order to determine the specific *dfr*-gene present.

RESULTS

Isolation and Typing of E. coli *Isolates*

A total of 107 confirmed *E. coli* isolates were obtained from the samples (Supplementary Table 1). In 3 cases, 2 morphologically distinct colony types were observed, of which both were included in further analyses. Subsequent results confirmed that in each of these 3 cases, the 2 isolates were distinct from one another (Figure 1, Supplementary Table 1).

Macrorestriction analysis showed an overall heterogenous result. However, some clustering of isolates could be observed. Three pairs of isolates with 100% identical band patterns were identified (Figure 1). Two pairs of identical isolates were recovered from swab samples of the subcutis and joint of the same 2 birds that showed a purulent arthritis in addition to cellulitis (E40 and E41; E85 and E86), whereas the isolates from the third pair differed in origin (E4 and E5). In all cases, both isolates of each pair shared the same phylogenetic group (B1 or D), APEC genes and resistance phenotype and genotype. In addition, a somewhat notable cluster of eight closely related APEC of phylogenetic group B1 showed a similarity value of over 90%, with several isolates showing over 94% similarity (Figure 1; isolates E15, E19, E22, E26, E70, E72 B, E93, E100). These isolates were collected from different farms and in different years, with the exception of isolates E93 and E100, which originated from the same farm and were isolated six days apart. However, these isolates did not show identical band patterns and differed in their antimicrobial resistance phenotype and genotype. In general, apart from the closely related isolates mentioned above, isolates originating from the same farm were often unrelated, showing very different band patterns (e.g., isolates E8 and E9, E35 and E59, E80 and E90). Thirteen isolates produced no bands after *Xba*I macrorestriction and they were regarded as nontypeable by this method.

For evaluation purposes, the indistinguishable isolates obtained from the same animal (isolates E40 and E41; E85 and E86) were considered duplicates and counted as a single isolate, respectively. Thus, the typing results, antimicrobial resistance phenotypes and genotypes of 105 isolates were considered in the following data analysis.

Determination of phylogenetic groups by PCR showed that the majority of the isolates (data excluding duplicate isolates) belonged to group D (n = 49), followed by group B1 (n = 27), group B2 (n = 16) and group A (n = 13). With the exception of three isolates, all carried virulence-associated genes predictive of APEC (Johnson et al. 2008). In the vast majority of these isolates, all five tested genes were detected with only few isolates missing either *iutA* (n = 5) or *iss* (n = 1) (Supplementary Table 1).

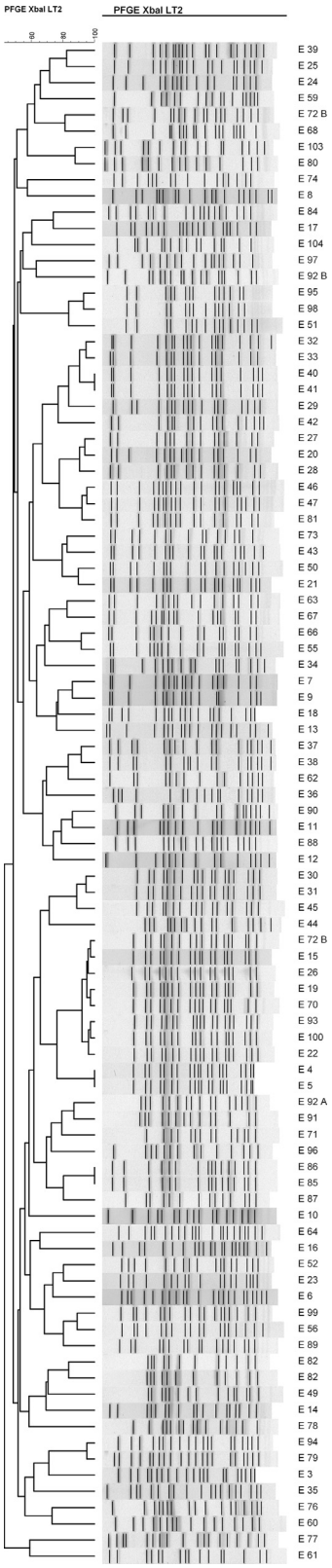

Figure 1. Genetic relatedness of *E. coli* isolates as determined by *Xba*I macrorestriction analysis.

The most common serotype among the isolates was O78:K80 (n = 23). Another 6 isolates could be assigned to serogroups O2 and 3 to O18.

Antimicrobial Resistance Phenotypes and Genotypes

The vast majority of the isolates showed resistance to at least one antimicrobial agent. Only 17 of the isolates (16.2%) showed no resistance to any of the tested antimicrobials whereas 44 isolates (41.9%) showed resistances towards three or more classes of antimicrobials and were therefore considered multi-resistant. Of these, a significant proportion (11 isolates) showed resistance to at least five classes of antimicrobials.

The most common resistance detected among the isolates was to ampicillin (n = 56), which was mediated by bla_{TEM} in all cases (Table 1). Despite this common occurrence of β-lactam resistance, none of the isolates showed resistance to imipenem, cefpodoxim or cefoxitin, which would have been indicative of carbapenemases, extended-spectrum- (**ESBL**) or acquired AmpC-β-lactamases, respectively. Resistance to streptomycin was also very common among the isolates. Full resistance was detected in 50 isolates, with another 21 isolates showing intermediate resistance. Detected resistance genes comprised *aadA1, aadA2, strA* and *strB*, either alone or in combination (Table 1). None of the tested genes were present in any of the isolates with an intermediate resistant phenotype. Resistance to nalidixic acid was the third most commonly detected resistance. However, only three isolates carried plasmid-mediated resistance genes *qnrB* (n = 2) or *qnrS* (n = 1), the latter of which also mediated intermediate resistance to ciprofloxacin. Resistance to folate pathway inhibitors, sulfisoxazole and trimethoprim, was also commonly observed, with 18 isolates carrying both *sul* and *dfrA* resistance determinants. Resistance to phenicols or gentamicin was observed in far fewer isolates, with 6 and 3 resistant isolates, respectively (Table 1). Three isolates were resistant to colistin and all 3 harbored *mcr-1*. A full overview of the resistance phenotype and genotype of each individual isolate is given in Supplementary Table 1.

Table 1. Number of resistant isolates and corresponding genotypes.

Resistance	Resistant isolates	Intermediate resistant isolates	Detected resistance genotype
Ampicillin	56	0	bla_{TEM} (n = 56)
Chloramphenicol	6	0	*catA1* (n = 3)
			cmlA (n = 2)
			catA1, floR (n = 1)
Ciprofloxacin	6	2	*qnrS* (n = 1)
			None detected (n = 7[1])
Colistin	3	0	*mcr-1* (n = 3)
Florfenicol	1	0	*floR* (n = 1)
Gentamicin	3	0	*aac(3)-I* (n = 1)
			None detected (n = 2)
Nalidixic acid	36	2	*qnrB* (n = 2[2])
			qnrS (n = 1)
			None detected (n = 35[1])
Streptomycin	50	21	*aadA1* (n = 15)
			strA, strB (n = 10)
			aadA1, strA, strB (n = 4)
			aadA1, aadA2 (n = 2)
			aadA1, aadA2, strA, strB (n = 1)
			None detected (n = 39[2])
Sulfisoxazole	29	0	*sul2* (n = 22)
			sul1, sul2 (n = 3)
			sul1 (n = 2)
			sul3 (n = 2)
Tetracycline	24	0	*tet*(A) (n = 19)
			tet(B) (n = 4)
			tet(A), *tet*(B) (n = 1)
Trimethoprim	21	0	*dfrA1* (n = 18)
			dfrA5 (n = 4)

[1]PCR-analysis comprised plasmid-mediated resistance genes.
[2]Including all intermediate resistant isolates.

DISCUSSION

In this study, we demonstrated that APEC isolates from broilers suffering from avian cellulitis from German farms show high rates of antimicrobial resistance and are frequently multiresistant. Although some isolates shared a close genetic relationship, overall genetic diversity was observed among the isolates.

While all isolates in this study were recovered from broilers suffering from cellulitis, 3 isolates did not harbor virulence-associated genes considered indicative of APEC. However, it is not always possible to accurately predict a specific pathotype from a genotype in *E. coli* (Rodriguez-Siek et al. 2005). So, these isolates, while perhaps not specifically adapted to poultry as a host, may still be pathogenic for broilers. Another possibility might be the co-occurrence of different isolates within the same diseased chicken. Indeed, we identified 2 distinct APEC isolates in some of our swab samples. If 2 isolates showed no morphologically distinct growth, a commensal *E. coli* might have been isolated while the co-occurring APEC was not investigated further. However, this does not seem likely as one would expect the causative pathogen to be most abundant in an infection site, rather than a purely commensal *E. coli*. Serotyping of the isolates showed that a significant proportion of slightly over 21% belonged to serotype O78:K80. This serotype appears to be strongly associated with poultry rather than to human ExPEC isolates (Rodriguez-Siek et al. 2005). In fact, it is one of the predominant APEC serotypes, fueling efforts to develop a vaccine specifically targeting this serotype in an attempt to combat the associated economic burden (Ebrahimi-Nik et al. 2018). In contrast, serogroups O2 and O18 have been associated with clinical isolates from both poultry and humans (Rodriguez-Siek et al. 2005). Consequently, a higher likelihood of zoonotic potential in these isolates may be expected.

Interestingly, different distinct APEC isolates could be isolated from the same farm in several instances in our study. For example, Farm 2 was sampled once in July of 2018 and again twice, albeit from different barns, in December 2018 (Supplementary Table 1). Yet, all 3

isolates (E2, E50, E51) showed distinct PFGE results, with one isolate presenting as untypeable and the other two showing less than 60% similarity, as well as distinct antimicrobial resistance phenotypes and genotypes. In other cases, 2 morphologically distinct APEC isolates could even be recovered from the same swab sample (e.g., E92A and E92B), demonstrating that not a single circulating clone was responsible for cellulitis cases on these farms. Yet, closely related isolates − and even indistinguishable ones − were recovered from different farms, indicating a spread of these lineages among poultry farms. Previous studies have indicated that while APEC comprise genetically diverse isolates, local spread and regional variations occur, which could explain our findings (Papouskova et al. 2020; Newman et al. 2021).

Antimicrobial susceptibility testing of our isolates revealed overall high levels of antimicrobial resistance. However, these comprised mostly resistance to antimicrobials frequently used in poultry farms such as ampicillin, folate pathway inhibitors and tetracylines, for which high levels of resistance have previously been reported in *E. coli* from poultry (European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC), 2023; Martínez-Álvarez et al. 2022; Xu et al. 2022). In contrast, not a single isolate was resistant to 3rd generation cephalosporins or carbapenems and we only rarely observed resistances to colistin, ciprofloxacin, gentamicin or phenicols. Regarding poultry in particular, 3rd and 4th generation cephalosporins should not be used according to a referral by the European Medicines Agencys Committee for Medicinal Products for Veterinary Use CVMP (2012). In general, increased awareness, stringent monitoring and restrictions on the use of 3rd and 4th generation cephalosporins appear to have resulted in a decrease in ESBL-producing isolates in poultry and other livestock in recent years. According to a French study, resistance levels in France peaked around 2011 in clinical *E. coli* isolates, with highest rates found in isolates from diseased broilers (over 25%) (Bourély et al. 2018). Since then, resistance rates have dropped to below 3% by the end of the studied period in 2016 (Bourély et al. 2018). A recently published report by the European Food Safety authority similarly shows a reduction of the prevalence of presumptive ESBL/AmpC-producing *E. coli* in broilers in many member states. For Germany in particular, a decrease of 31% was reported between 2016 and 2020 (European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC), 2023). While the EFSA report covers routine monitoring of *E. coli* rather than specifically targeting clinical isolates, and the reported trend related to selective monitoring of presumptive ESBL/AmpC-producing *E. coli*, a similar downward trend may be expected for APEC. According to the German national resistance monitoring program (GE*RM*-Vet), resistance to cephalosporins in clinical isolates from broilers has been at a stable level for several years now, with MIC_{90} values between 0.12 μg/ml and 0.5 μg/ml (Bundesamt für Verbraucherschutz und Lebensmittelsicherheit BVL, 2023). Overall, it is interesting − and promising − that not a single ESBL/AmpC isolate was detected among the APEC in the present study. Similarly, it is reassuring that no carbapenemase producers were detected. However, resistances to carbapenems are rarely observed so this is not unexpected. In 2020, a single carbapenemase-producing isolate from a cecal sample of a slaughtered broiler was reported in the course of EU monitoring (European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC), 2023). Similarly, other studies also generally report very low or no occurrence of carbapenemase-producing *E. coli* among APEC (El-shaer, Awad, and Younis 2023; Kathayat et al. 2021). In contrast, resistance to fluoroquinolones, another class of clinically important antimicrobials, is reported more frequently in other studies compared to ours (European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC), 2023; Temmerman et al. 2020). While we did observe resistance to nalidixic acid in over a third of our isolates, only eight of these were non-susceptible to ciprofloxacin. We also observed a low prevalence of plasmid-mediated quinolone-resistance. High-level resistance to quinolones is usually associated with mutations in the chromosomal gyrase (*gyrA, gyrB*) and topoisomerase (*parC, parE*) genes (Temmerman et al. 2020), which is most likely the case for the majority of our isolates as well. However, plasmid-mediated resistance is of particular concern as it is a form of transferrable resistance so it can be seen as favorable that only few of our isolates harbored such genes. Similar to quinolone resistance, isolates with high resistance levels to colistin usually carry chromosomal mutations but transferable, plasmid-mediated *mcr*-genes have become a concern more recently (Liu et al. 2016). Resistance to colistin is of particular relevance considering it is regarded as critically important and of highest priority for human medicine by the WHO. Indeed, all three colistin-resistant isolates in the present study carried *mcr-1*. In general, colistin resistance is still reported relatively rarely in *E. coli* from broilers in the EU, considering both commensal and APEC, but prevalences range of up to 10.1% in broilers were reported by the EFSA (European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC), 2023; Mead et al. 2022). A study investigating the colistin resistance of a large (commensal) *E. coli* strain collection from the German national monitoring on zoonotic agents reported a prevalence of 6.7% colistin-resistant isolates from broilers, with the vast majority of these isolates carrying *mcr*-1 (Irrgang et al. 2016).

Overall, our study demonstrated that a diverse set of APEC can be found among and within German poultry farms. Despite this, the detection of closely related isolates on different farms indicates the spread of certain successful lineages. Antimicrobial resistance, including multidrug-resistance, was common among the isolates. However, resistance levels to substances of particular importance, such as 3rd and 4th generation

cephalosporins were rather favorable. This may reflect the results of ongoing efforts to restrict the use of these substances, particularly in poultry, and highlights the importance of antimicrobial stewardship and monitoring programs.

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Ethical Approval: All of the animals were housed in accordance with EU (European Directive 2007/43/EC) and national law (Tierschutzgesetz, Tierschutz-Nutztierhaltungsverordnung). In compliance with European Directive 2010/63/EC Article 1 5. (f), the present study did not imply any invasive procedure or treatment to the animals. The authors declare that the study was in accordance with current German law. This study was reviewed and received approval from the Animal Welfare Officer of the University of Veterinary Medicine Hannover, Foundation, Germany.

DISCLOSURES

The authors declare no conflicts of interest.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2024.103704.

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