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ORIGINAL ARTICLE

Development of a harmonized method for antimicrobial susceptibility testing of *Bordetella avium* using broth microdilution and detection of resistance genes

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Abstract

Aims: In response to a request from the Clinical and Laboratory Standards Institute (CLSI), the objective of this study was to develop a harmonized method for broth microdilution susceptibility testing of *Bordetella* (*B.) avium*, the major causative agent of infectious coryza in poultry.

Methods and Results: To find a suitable test medium, growth curves with four epidemiologically unrelated *B. avium* isolates were created in cation-adjusted Mueller-Hinton broth (CAMHB), CAMHB + 2.5% lysed horse blood and veterinary fastidious medium. All isolates showed good growth in CAMHB, therefore MIC values were determined using this medium and the homogeneity of the values was determined. An essential MIC agreement of 99.7% was calculated. Testing of a larger strain collection ($n = 49$) for their susceptibility to 24 antimicrobials confirmed the suitability of the tested method and revealed some isolates with elevated MICs of florfenicol ($n = 1$), streptomycin ($n = 2$), tetracyclines ($n = 5$), and trimethoprim/sulfamethoxazole ($n = 6$). PCR assays detected the resistance genes *aadA1*, *dfrB1*, *floR*, *sul1*, *sul2* and *tet*(A).

Conclusions: The method used enables easy reading and a good reproducibility of MIC values for *B. avium*.

Significance and Impact of Study: Application of the tested method allows harmonized resistance testing of *B. avium* and identification of isolates with elevated MIC values.

KEYWORDS

aadA1, antibiotics, CAMHB, CLSI, *dfrB1*, *floR*, harmonization, minimal inhibitory concentration values, *sul1*, *sul2*, *tet(A)*

INTRODUCTION

The gram-negative bacterium *Bordetella avium* (*B. avium*) plays a major economic role across the globe being the most important causative agent of the highly contagious bordetellosis in turkeys and other poultry (also termed turkey coryza or *B. avium* rhinotracheitis or BART), along with the closely related pathogen *B. hinzii* (Beach et al., 2012; Register & Jackwood, 2020; Register & Kunkle, 2009). A 2018 report by the U.S. Animal Health Association´s Subcommittee on Turkey Health ranked *B. avium* as the sixth most important issue facing the U.S. turkey production industry (Clark, 2018). Of particular importance, 2- to 6-week-old turkeys show acute respiratory symptoms after infection with *B. avium*, such as sneezing, beak breathing, tracheal collapse, submandibular oedema and conjunctivitis because of protracted inflammation, while older turkeys suffer from a dry cough (Kelly et al., 1986; Knab et al., 2018; Panigrahy et al., 1981; Register & Jackwood, 2020). Although mortality is quite low, coming in at mostly less than 10%, the rapid spread of *B. avium* leads to a high morbidity of about 80%–100% within herds (Knab et al., 2018; Register & Jackwood, 2020; Saif et al., 1980). Thought to be transmitted to humans via contaminated poultry meat or other avian reservoirs (Register & Jackwood, 2020), opportunistic *B. avium* infections have been detected in humans who previously suffered from pneumonia, with symptoms similar to those of *B. pertussis* or *B. bronchiseptica* infections; patients with cystic fibrosis are also vulnerable (Harrington et al., 2009; Lavrenko et al., 2020; Spilker et al., 2008).

To treat *B. avium* infections and the frequently associated secondary pathogens, such as *Escherichia* (*E.*) *coli* in poultry, antimicrobial agents like tetracyclines and penicillins have been used for several decades (Ficken, 1983; Kelly et al., 1986; Register & Jackwood, 2020). Because every use of antimicrobial agents also favours resistance selection, it is particularly important to monitor the susceptibility status of *B. avium*. However, there are only six studies so far that have investigated the susceptibility status of the pathogen. Three of them have used disk diffusion as a method and have examined the susceptibility of 2, 4 and 19 *B. avium* isolates respectively (Erfan et al., 2018; Malik et al., 2005; Szabó et al., 2015). One of these studies and three further studies tested the antimicrobial susceptibility of *B. avium* (also) by broth microdilution (Beach et al., 2012; Blackall et al., 1995; Mortensen et al., 1989; Szabó et al., 2015). However, the broth microdilution methods performed in these studies differ widely, for example, in the choice of the test medium, inoculum density or incubation conditions.

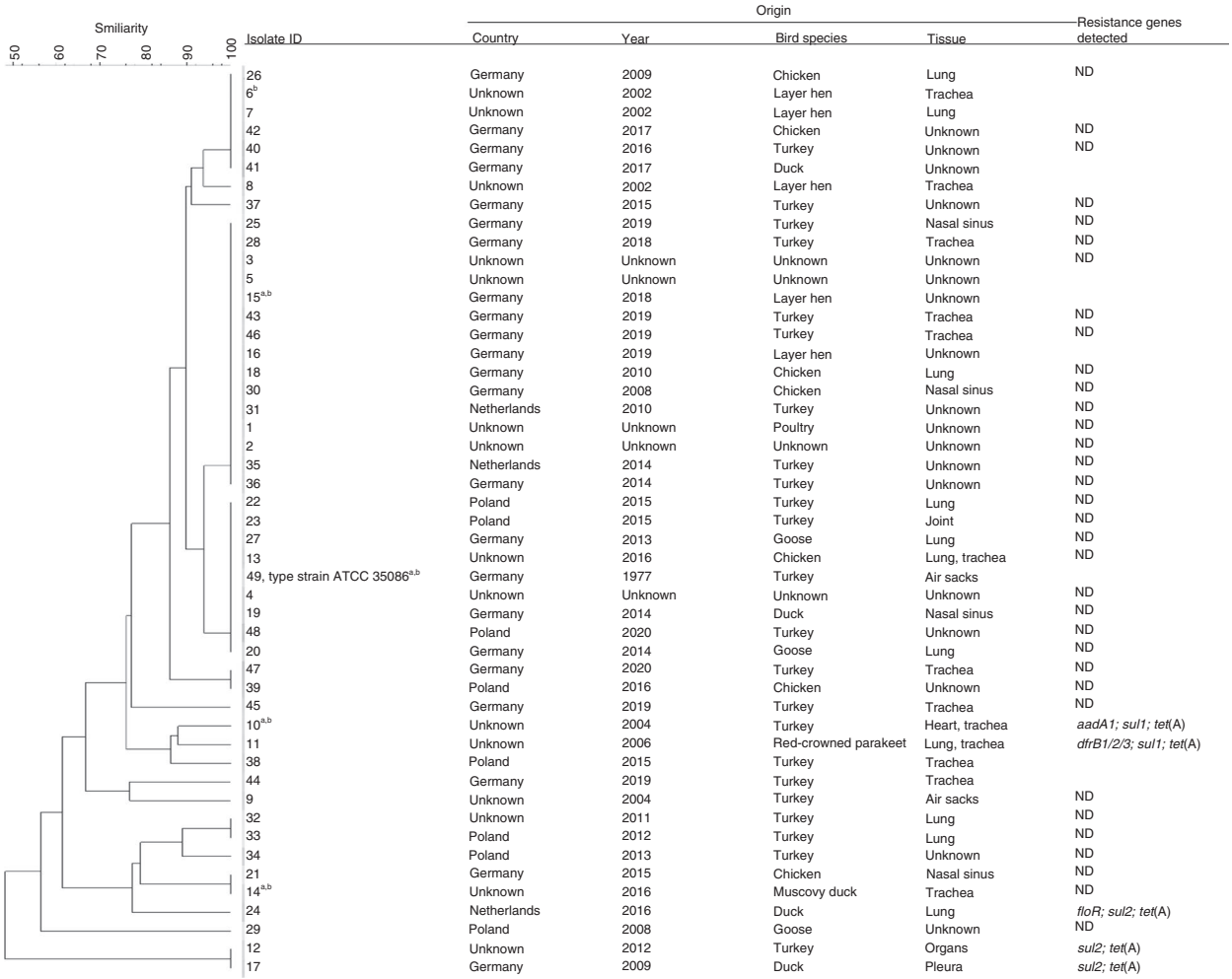
Because there is currently no harmonized method for antimicrobial susceptibility testing of *B. avium* and the methods described differ widely, laboratories may use completely different methods, and the results are not comparable between them (Register & Jackwood, 2020). Therefore, it is highly recommended to harmonize antimicrobial susceptibility testing for this pathogen (CLSI, 2017). Furthermore, it is necessary to know the susceptibility status of a pathogen to achieve a targeted treatment of diseased animals. To achieve this, approved methods for testing the pathogen must be available. To address the problem when it comes to *B. avium*, the Clinical and Laboratory Standards Institute (CLSI) has already requested the development of harmonized antimicrobial susceptibility testing for this species (CLSI, 2017). Hence, the aim of the current study was to evaluate a harmonized method for antimicrobial susceptibility testing of *B. avium* using the broth microdilution method and to determine the antimicrobial susceptibility status of a strain collection of epidemiologically unrelated isolates.

MATERIALS AND METHODS

B. avium isolates included in the study and species confirmation

Between 2019 and 2020, a total of 48 *B. avium* field isolates were collected, originating from different geographical regions in Germany, Poland, the Netherlands and unknown countries (Figure 1). These isolates were provided by various veterinary practices and clinics, diagnostic laboratories and federal institutions. The field isolates originated from different poultry species such as turkeys, chickens and wild waterfowl as well as from exotic birds and unknown species; they were isolated between 2002 and 2020 based on one isolate per flock and year. The *B. avium*-type strain ATCC 35086 (Culture Collection University of Gothenburg, Gothenburg, Sweden) was obtained as a reference for method evaluation. All 49 *B. avium* isolates were incubated for culturing at 37°C for 24 ± 2 h in ambient air on a Columbia blood agar containing 5% defibrinated sheep blood (Oxoid Limited), casein soybean peptone (CASO) agar as an alternative to blood agar plates when they were not available (Merck KGaA) or in a brain-heart infusion broth (BHI) (Merck KGaA).

Genomic DNA of all 49 *B. avium* isolates was isolated from overnight cultures by boiling. For this purpose, overnight cultures were suspended in 300 µl bidistilled water. This suspension was then heated at 99°C for 15 min and centrifuged (13,000*g*) for 2 min. Afterwards, the species of the isolates were confirmed by a previously described species-specific polymerase chain reaction (PCR) assay (Turkyilmaz et al., 2009).



Isolate ID	Origin				Resistance genes detected
	Country	Year	Bird species	Tissue	
26	Germany	2009	Chicken	Lung	ND
6[b]	Unknown	2002	Layer hen	Trachea	
7	Unknown	2002	Layer hen	Lung	
42	Germany	2017	Chicken	Unknown	ND
40	Germany	2016	Turkey	Unknown	ND
41	Germany	2017	Duck	Unknown	
8	Unknown	2002	Layer hen	Trachea	
37	Germany	2015	Turkey	Unknown	ND
25	Germany	2019	Turkey	Nasal sinus	ND
28	Germany	2018	Turkey	Trachea	ND
3	Unknown	Unknown	Unknown	Unknown	ND
5	Unknown	Unknown	Unknown	Unknown	
15[a,b]	Germany	2018	Layer hen	Unknown	
43	Germany	2019	Turkey	Trachea	ND
46	Germany	2019	Turkey	Trachea	ND
16	Germany	2019	Layer hen	Unknown	
18	Germany	2010	Chicken	Lung	ND
30	Germany	2008	Chicken	Nasal sinus	ND
31	Netherlands	2010	Turkey	Unknown	ND
1	Unknown	Unknown	Poultry	Unknown	ND
2	Unknown	Unknown	Unknown	Unknown	ND
35	Netherlands	2014	Turkey	Unknown	ND
36	Germany	2014	Turkey	Unknown	ND
22	Poland	2015	Turkey	Lung	ND
23	Poland	2015	Turkey	Joint	ND
27	Germany	2013	Goose	Lung	ND
13	Unknown	2016	Chicken	Lung, trachea	ND
49, type strain ATCC 35086[a,b]	Germany	1977	Turkey	Air sacks	
4	Unknown	Unknown	Unknown	Unknown	ND
19	Germany	2014	Duck	Nasal sinus	ND
48	Poland	2020	Turkey	Unknown	ND
20	Germany	2014	Goose	Lung	ND
47	Germany	2020	Turkey	Trachea	ND
39	Poland	2016	Chicken	Unknown	ND
45	Germany	2019	Turkey	Trachea	ND
10[a,b]	Unknown	2004	Turkey	Heart, trachea	*aadA1; sul1; tet*(A)
11	Unknown	2006	Red-crowned parakeet	Lung, trachea	*dfrB1/2/3; sul1; tet*(A)
38	Poland	2015	Turkey	Trachea	
44	Germany	2019	Turkey	Trachea	
9	Unknown	2004	Turkey	Air sacks	ND
32	Unknown	2011	Turkey	Lung	ND
33	Poland	2012	Turkey	Lung	ND
34	Poland	2013	Turkey	Unknown	ND
21	Germany	2015	Chicken	Nasal sinus	ND
14[a,b]	Unknown	2016	Muscovy duck	Trachea	ND
24	Netherlands	2016	Duck	Lung	*floR; sul2; tet*(A)
29	Poland	2008	Goose	Unknown	ND
12	Unknown	2012	Turkey	Organs	*sul2; tet*(A)
17	Germany	2009	Duck	Pleura	*sul2; tet*(A)

FIGURE 1 Genetic similarity, origin and antimicrobial genotypes of 49 *Bordetella avium* isolates tested in this study. (a) this isolate was used as a test isolate for the growth experiments. (b) this isolate was used as a test isolate for the method validation of broth-microdilution. *ND, this isolate was included in resistance gene analysis, but no resistance genes were detected (for the listing of resistance genes, see Table 3)

Macrorestriction analysis

To examine the clonality of the 49 *B. avium* isolates, their relationship was investigated by macrorestriction analysis, which was followed by pulsed-field gel-electrophoresis (PFGE), here based on a previously published protocol (Ribot et al., 2006). The restriction enzyme *SpeI* (*BcuI*) (Thermo Fisher Scientific Inc.) was used to enzymatically digest the DNA of *B. avium* isolates into about 34 fragments. XbaI digested DNA from *Salmonella* Typhimurium LT2 was used as a marker. The digested DNA of all the isolates was separated within 20 h in a CHEF DR II system (BioRad) at 6 V, starting at an initial time of 6.8 s and ending at a final time of 35.2 s. Band patterns were evaluated using BioNumerics software (version 7.6; Applied Maths). For the cluster analysis, the Dice coefficient was applied with a setting of 0.5% optimization and 1% position tolerance.

Growth curves

Growth experiments were performed in three different media recommended by the CLSI: cation-adjusted Mueller Hinton broth (CAMHB) (Sigma-Aldrich, Seelze, Germany) for bacteria isolated from animals such as *Enterobacterales* (CLSI, 2020); CAMHB plus 2.5% lysed horse blood (Oxoid Limited, Basingstoke, UK) for fastidious organisms like *Streptococcus* spp.; and veterinary fastidious medium (VFM) prepared according to CLSI specifications for *Histophilus somni* and *Actinobacillus pleuropneumoniae* (CLSI, 2020). To find a suitable medium for antimicrobial susceptibility testing of *B. avium*, the growth of four epidemiologically unrelated isolates (*B. avium*-type strain ATCC 35086 and field isolates no. 10, 14 and 15) was investigated in two independent growth experiments in the three test media. For this, the turbidity of overnight cultures in 0.9% saline solution (Merck KGaA) was adjusted to the 0.5 McFarland standard using the

McFarland densitometer DEN-1B (Biosan SIA). A volume of 50 µl of this bacterial suspension was then added to 5 ml of 0.9% saline solution. To achieve a starting concentration of approximately 1×10^3 cfu ml^{-1}, 50 µl were suspended into 50 ml of the respective test medium. The inoculated media were subsequently incubated at 35°C ± 2°C. Within the first 24 h, the optical density was measured every 4 h and thereafter at 32 and 48 h, each time at 600 nm with a UV-visible spectrometer (Spectrophotometer UV5, Mettler Toledo). In addition, the bacterial counts (cfu ml^{-1}) were determined by culture-based enumeration at eight of the measuring points mentioned above (0, 4, 8, 12, 16, 24, 32 and 48 h). For this purpose, 10-fold serial dilutions were prepared, which were spread in duplicate on CASO agar plates. Agar plates were then incubated at 37 ± 1°C for 24 ± 2 h until the colonies were counted.

To compile the growth curves using Microsoft Excel software, the statistical averages of cfu ml^{-1} and the standard deviations of both repetitions were calculated after counting colonies at two dilution steps. Only those dilutions containing 5–200 cfu ml^{-1} were evaluated.

Method validation of broth microdilution

To verify the suitability of CAMHB as a test medium for *B. avium* and assess the homogeneity of minimal inhibitory concentration (MIC) values, five independent replicates of broth microdilution testing, here following CLSI guidelines, were performed (CLSI, 2020). For these experiments, four *B. avium* isolates (isolates 6, 10, 14 and 15), which were as unrelated as possible according to macrorestriction results, were used in addition to the type strain ATCC 35086. The direct colony suspension method was used for inoculum preparation. In brief, the colonies were selected from an overnight culture of *B. avium* on blood agar plates (incubation at 37 ± 1°C for 20 ± 2 h) and were suspended in 0.9% saline solution to achieve a 0.5 McFarland standard. A volume of 100 µl of this suspension was then diluted into 19.9 ml of CAMHB to yield approximately 5×10^5 cfu ml^{-1}. Using a multichannel pipette (Eppendorf AG), 50 µl of the suspension was then added to each well of the microtiter plates (Sensititre, Trek Diagnostic Systems). A total of 24 antimicrobial agents per isolate were tested. Twenty of these agents are currently licenced for food-producing animals. MICs were read after sealing and incubation for 20 h (±10 min) at the CLSI-specified temperature of 35 ± 2°C in an aerobic atmosphere.

Quality control was performed using *E. coli* control strain ATCC 25922. The inoculum densities were checked by means of culture-based enumerations in which 10-fold serial dilutions were prepared.

Antimicrobial susceptibility testing of a larger strain collection

After method validation, another 44 *B. avium* field isolates were tested for antimicrobial susceptibility using the broth microdilution method evaluated in the current study to assess the suitability of the method for a larger strain population. Following testing, the MIC_{50} and MIC_{90} values were calculated for this strain collection. The MIC_{50} value defines the lowest concentration of an antimicrobial agent at which 50% of the test collective is visibly inhibited, while the MIC_{90} value defines the lowest concentration at which 90% of the test collective is visibly inhibited.

Following broth microdilution susceptibility testing, *B. avium* isolate 21 was tested for the presence of extended-spectrum β-lactamases (ESBLs), here according to CLSI guidelines (CLSI, 2021).

Comparison of the MICs obtained with the medium from different CAMHB manufacturers

To exclude differences between the MICs obtained in CAMHB from different manufactures, susceptibility of the five tested *B. avium* isolates was additionally analysed in CAMHB from another manufacturer (Becton Dickinson). The obtained MIC values were compared with those obtained from the method validation.

Resistance gene analysis

Based on the results of the antimicrobial susceptibility testing, 39 *B. avium* isolates with elevated MIC values against at least one antimicrobial agent compared with the other isolates were included in the resistance gene analysis. For this, previously described primers and associated PCR assays were used to verify the presence of antimicrobial resistance genes. Thus, the presence of the tetracycline resistance genes *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(G), *tet*(H), *tet*(L), *tet*(M) and *tet*(O) and the sulfonamide resistance genes *sul1*, *sul2* and *sul3* was investigated (Prüller et al., 2015b). In addition, PCR-based detection of streptomycin-resistance-mediating genes *strA*, *strB*, *aadA1* and *aadA2* was performed as well as an analysis of the β-lactamase-encoding genes bla_{BOR-1}, bla_{OXA-1}-like and bla_{OXA-2}, bla_{ROB}, bla_{SHV} and bla_{TEM} (Dallenne et al., 2010; Prüller et al., 2015b; Randall et al., 2004). Isolates with higher MICs for quinolones were also tested for the plasmid-encoded resistance genes *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS* and *aac*(6')*-Ib-cr* (Kehrenberg et al., 2006; Vredenburg et al., 2014). In addition, the occurrence of

trimethoprim resistance genes *dfrA1/15/16*, *dfrA5/14*, *dfrA7/17* and *dfrB1/2/3* was analysed (Frech et al., 2003; Prüller et al., 2015b).

RESULTS

Clonal relationship of *B. avium* isolates

Figure 1 illustrates the genetic relationship and origin of all 49 *B. avium* isolates tested in the current study, including their phenotypic and genotypic antimicrobial resistance profiles. *BcuI* macrorestriction analyses revealed a partially high clonality of the isolates. There were three major clusters containing a minimum of 6 and maximum of 15 genetically indistinguishable isolates exhibiting equal numbers and identical band sizes. Although eight isolates formed four additional clusters consisting of two isolates each, the remaining 11 *B. avium* isolates had unique patterns. Thus, four test isolates that were distinguishable according to these results could be selected for the growth experiments and one additional test isolate for the broth microdilution method validation. Selection was based on choosing one representative from each of the larger clusters and additional isolates that differed in fragment patterns so that they were considered unrelated. To define unrelated isolates, the criterion of >6 bands difference was used (Tenover et al., 1995). All isolates also differed in their year of isolation and origin: Isolate 14 was isolated from waterfowl (Muscovy duck), and the other four test isolates originated from turkeys ($n = 2$) and laying hens ($n = 2$) (Figure 1).

Growth curves of *B. avium* in three different test media

Both repetitions of the growth experiments obtained comparable results. At each time point, the standard deviation of both growth trials was calculated and visualized in the growth curves (Figure 2; Supplemental material Figures S1–S3). Looking at the optical density (OD) values, after 20 h at 35 ± 2°C, a mean OD_{600} value of about 0.054 of the four test isolates was measured in CAMHB, while the values in blood-containing media CAMHB + 2.5% LHB and VFM were higher, with mean OD_{600} values of about 0.142 and 0.079 respectively (Figures S4–S7). However, the culture-based enumeration revealed very good growth

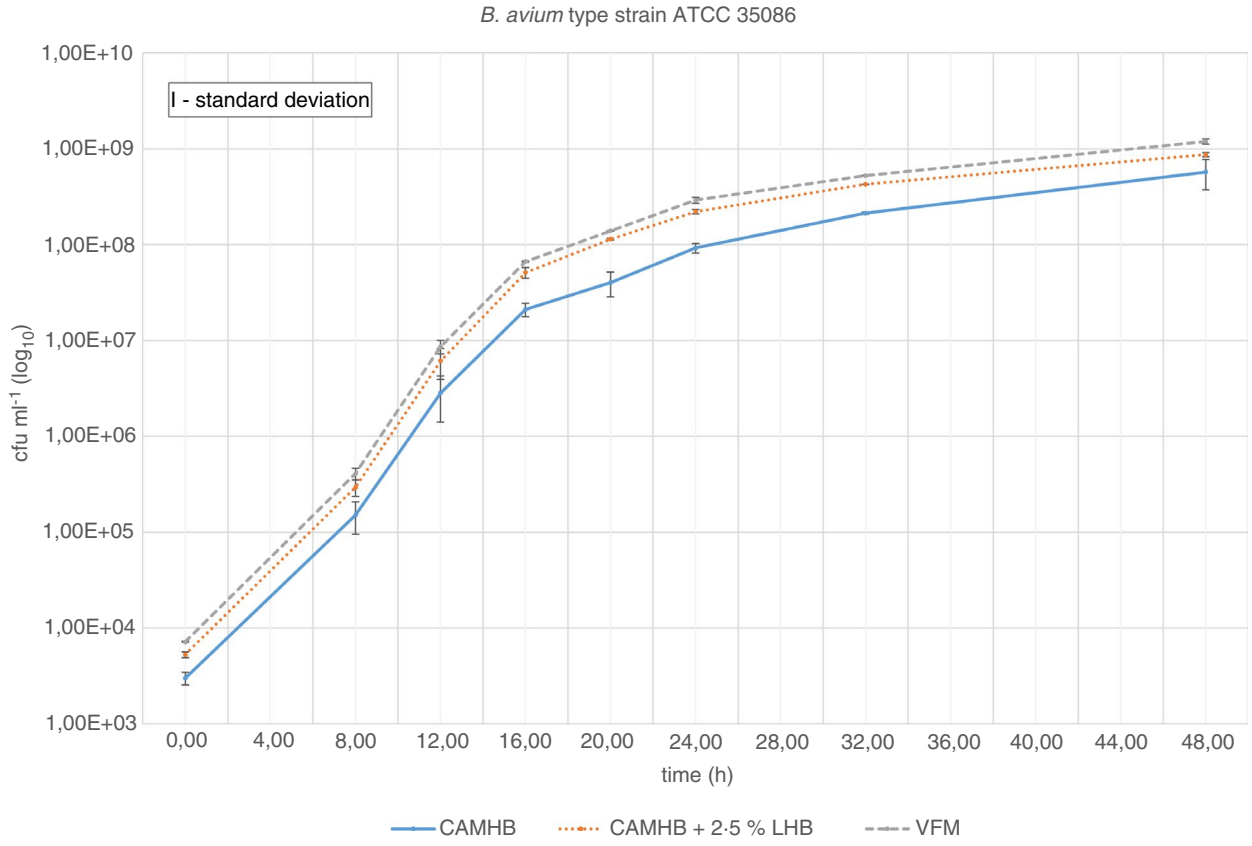


FIGURE 2 Viable counts ($\log_{10}$ cfu ml^{-1}) of *Bordetella avium* type strain ATCC 35086 grown in three different media. CAMHB, cation-adjusted Mueller-Hinton broth; LHB, lysed horse blood; VFM, Veterinary Fastidious Medium

of all test isolates to >10^7 cfu ml^{-1} after 20 h of incubation in all media (Figure 2; Figures S1–S3). Therefore, the commercially available, easy-to-produce and CLSI-approved CAMHB was selected for subsequent antimicrobial susceptibility testing of *B. avium*.

Suitability of the test method and susceptibility status of the field isolates

Table 2 illustrates the calculated exact MIC agreements (MIC values that match the MIC mode of each isolate) and the essential MIC agreements (MIC mode accepting a deviation of ±1 $\log_2$ dilution steps), which were used to evaluate homogeneity (CLSI, 2018a; Prüller et al., 2017). Overall, the MIC values of the five tested *B. avium* isolates demonstrated good reproducibility in CAMHB after an incubation period of 20 h at 35°C ± 2°C (Table 1). For a single antimicrobial agent (imipenem), an exact MIC agreement of 100% (five identical MICs) was calculated, while for 16 of the tested antimicrobial agents an exact MIC agreement of ≥80% was shown. The lowest exact MIC agreements were seen for doxycycline, neomycin and tilmicosin with percentages of 68%. Nevertheless, 23 out of the 24 antimicrobials tested showed an essential MIC agreement of 100%. Only for tiamulin was there a slightly lower essential MIC agreement of 92%. Thus, for the MIC mode accepting a deviation of a single dilution step, 99.7% of MICs were homogeneous.

Because the five *B. avium* isolates used for method validation provided easily readable and reproducible MICs, a larger *B. avium* strain collection was tested to evaluate the suitability of the method for current and older field isolates originating from different poultry species. Table 3 shows all MICs obtained for the isolates and the MIC_{50} and MIC_{90} values calculated. Because there are currently no published MIC breakpoints for *B. avium*, a classification of the isolates as susceptible, intermediate or resistant could not be performed. However, a bimodal distribution of the MICs was observed for some antimicrobial agents, for example florfenicol, streptomycin, tetracyclines and trimethoprim/sulfamethoxazole. Five *B. avium* isolates had elevated MICs (compared with the other isolates) against two to three different classes of antimicrobials. Isolates 12 and 17 were found to have higher MICs of tetracyclines (doxycycline 8–16 μg ml^{-1}; tetracycline 128 μg ml^{-1}) and trimethoprim/sulfamethoxazole (2/38–4/76 μg ml^{-1}) (Table 2). Isolates 10, 11 and 24 showed elevated MICs against three of the following four classes of antimicrobials: tetracyclines (doxycycline 4–16 μg ml^{-1}; tetracycline 64–128 μg ml^{-1}; $n = 3$), streptomycin (128 to ≥1024 μg ml^{-1}, $n = 2$), florfenicol (32 μg ml^{-1}; $n = 1$) or trimethoprim/sulfamethoxazole (1/19-≥64/1216 μg ml^{-1}; $n = 3$). These three isolates were regarded as phenotypically multidrug resistant (Müller et al., 2018).

An almost unimodal distribution of MICs was found for most of the remaining antimicrobial agents tested, such as for fluorquinolones (e.g., ciprofloxacin, enrofloxacin, marbofloxacin), macrolides (e.g., tilmicosin, tulathromycin), aminoglycosides (e.g., neomycin, gentamicin) or cephalotin. However, isolate 21 had higher MICs of ampicillin (2 μg ml^{-1}) and amoxicillin/clavulanic acid (2/1 μg ml^{-1}) compared with the other 48 isolates which exhibited MICs between 0.06 and 1 μg ml^{-1} ampicillin and 0.06/0.03 and 0.5/0.25 μg ml^{-1} amoxicillin/clavulanic acid. Isolate 21 also had slightly higher MICs for third- and fourth-generation cephalosporins such as cefotaxime (8 μg ml^{-1}), cefquinome (8 μg ml^{-1}) and ceftiofur (8 μg ml^{-1}), whereas the other 48 *B. avium* isolates partially showed lower MICs for the antimicrobials cefotaxime (0.12–2 μg ml^{-1}), cefquinome (0.5–8 μg ml^{-1}) and ceftiofur (0.25–4 μg ml^{-1}). Another isolate (3) showed elevated MICs of penicillin (16 μg ml^{-1}) and cefquinome (8 μg ml^{-1}). Moreover, isolate no. 21, with an MIC of 16 μg ml^{-1} nalidixic acid, also had MICs at the right edge of the distribution for fluoroquinolones (enrofloxacin, ciprofloxacin and marbofloxacin; MIC of 1 μg ml^{-1}), and all isolates with tilmicosin MIC of ≥64 μg ml^{-1} also exhibited MIC values at the right edge of the distribution for tulathromycin (32 μg ml^{-1}).

Because isolate 21 presented slightly elevated MICs for some beta-lactam antibiotics, including third- and fourth-generation cephalosporins, this isolate was additionally tested by the CLSI-approved broth microdilution test for detecting extended-spectrum β-lactamase-producing *Klebsiella pneumoniae*, *Klebsiella oxytoca* and *E. coli* (CLSI, 2021). For this, the MIC values of cefotaxime and ceftazidime were tested with and without the addition of 4 μg ml^{-1} clavulanic acid. Because the MIC value for ceftazidime alone was ≤1 μg ml^{-1} and in the presence of clavulanic acid, the MIC values were not reduced by the required three 2-fold dilution steps, so ESBL production could not be confirmed according to the criteria indicative for ESBL production in *Klebsiella* spp. and *E. coli*. The MIC values of the quality control strains *Klebsiella pneumoniae* ATCC 700603 and *E. coli* ATCC 25922 were within the ranges recommended by CLSI (CLSI, 2021).

MIC comparison using CAMHB from different manufacturers

A comparison of results from two manufacturers showed that the MICs of the five *B. avium* isolates used for validation were at maximum one dilution level below the lowest previously determined values, when CAMHB from another manufacturer (Becton Dickinson) was used (Table

TABLE 1 Homogeneity of MIC values of five *Bordetella avium* isolates obtained from five independent test replicates in cation-adjusted Mueller-Hinton broth after incubation in ambient air at 35 ± 2°C for 20 h

Anti-microbial agent	Deviation from MIC mode[a,b]							Exact MIC agreement (%)[c]	Essential MIC agreement (%)[d]
	−3	−2	−1	0	1	2	3		
AMP			2	20	3			80.0	100.0
AMC			4	21				84.0	100.0
XNL				21	4			84.0	100.0
CFP			4*	21				84.0	100.0
CTX			2	20	3			80.0	100.0
CQN			5	19	1			76.0	100.0
CEF			1	22	2			88.0	100.0
CIP				24	1			96.0	100.0
CST			2	21	2			84.0	100.0
DOX			3	17*	5			68.0	100.0
ENRO			1	22	2			88.0	100.0
FFN				23	2			92.0	100.0
GEN				24	1			96.0	100.0
IPM				25				100.0	100.0
MAR			2	18	5			72.0	100.0
NAL			2	22	1			88.0	100.0
NEO			6	17	2			68.0	100.0
PEN			1	22	2			88.0	100.0
STR				20	5			80.0	100.0
TIA		1	2	19	2	1		76.0	92.0
TET				21*	4			84.0	100.0
TIL			4	17	4			68.0	100.0
SXT			2	18	5			72.0	100.0
TUL			1	22	2			88.0	100.0

Abbreviations: AMC, amoxicillin-clavulanic acid; AMP, ampicillin; CEF, cephalotin; CFP, cefoperazone; CIP, ciprofloxacin; CQN, cefquinome; CST, colistin; CTX, cefotaxime; DOX, doxycycline; ENRO, enrofloxacin; FFN, florfenicol; GEN, gentamicin; IPM, imipenem; MAR, marbofloxacin; NAL, nalidixic acid; NEO, neomycin; PEN, penicillin; STR, streptomycin; SXT, trimethoprim-sulfamethoxazole; TET, tetracycline; TIA, tiamulin; TIL, tilmicosin; TUL, tulathromycin; XNL, ceftiofur.

[a]MIC, minimal inhibitory concentration.

[b]Data fulfilling the criteria of the essential MIC agreement are shaded in grey.

[c]Exact MIC agreement, percentage of MIC values that exactly match the MIC mode.

[d]Essential MIC agreement, percentage of MIC values within ±1 dilution step from MIC mode.

*Number of isolates with MIC values equal to or lower or higher than the concentrations of the test range.

S8). Thus, for a few antibiotics (gentamicin, neomycin, tulathromycin and trimethoprim/sulfamethoxazole), the MIC values deviated from the MIC mode by two dilution steps.

PCR amplification of antimicrobial resistance genes

Whole-cell DNA from 39 *B. avium* isolates with (in some cases only moderately) elevated MICs compared with the majority of isolates (Table 3) was analysed by PCR assays for the presence of various resistance genes. The selection of isolates also considered the antimicrobial resistance mechanisms and occurrence of specific resistance genes for the antibiotic classes. Overall, the resistance genes *tet*(A) and *sul2* were detected in both *B. avium* isolates (isolates 12 and 17) with elevated MICs of tetracycline (≥64 μg ml^{-1}) and trimethoprim/sulfamethoxazole (4/76 μg ml^{-1}; 2/38 μg ml^{-1}), while three resistance genes were identified in isolates 10, 11 and 24, which were previously classified as phenotypic multidrug resistant (Figure

TABLE 2 Results from antimicrobial susceptibility testing of 49 *Bordetella avium* isolates in cation-adjusted Mueller-Hinton broth (CAMHB)

Antimicrobial agent	Number of *B. avium* isolates and their MIC[a] (μg ml^{-1}) after 20 h of incubation																		MIC_{50}[b]	MIC_{90}[c]
	0.008	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024		
AMC[d]			[e]	9	22	7	10		1										0.12	0.50
AMP				20	11	9	5	3	1										0.12	0.50
CFP				2*	23	4	4	16											0.12	1.00
CTX					9	19	4	8	8		1								0.25	2.00
CQN							8	22	5	12	2								1.00	4.00
XNL						17	11	7	8	5	1								0.50	4.00
CEF							8	37	4										1.00	1.00
CIP							39	10											0.50	1.00
CST							11	26	10	2									1.00	2.00
DOX				8*	27	8	1			1	2	2							0.12	4.00
ENRO						17	27	5											0.50	1.00
FFN								5	39	4			1						2.00	4.00
GEN								15	32	2									2.00	2.00
IPM						1	16	32											1.00	1.00
MAR						12	34	3											0.50	0.50
NAL									1	19	28	1							8.00	8.00
NEO									25	22	2								2.00	4.00
PEN						1	25	8	6	7	1	1							0.50	4.00
STR											1	27	18	1	1			1*	16.00	32.00
TET					8*	31	4	1						2	3				0.25	64.00
TIA										5	9	13	4	1	17*				16.00	≥128.00
TIL											3	27	5	8	6				16.00	128.00
SXT[f]				1	30	10	2	3	1	1				1*					0.12	1.00
TUL										11	15	8	15						8.00	32.00

Abbreviations: AMC, amoxicillin-clavulanic acid 1:2 ratio; AMP, ampicillin; CEF, cephalotin; CFP, cefoperazone; CIP, ciprofloxacin; CQN, cefquinome; CST, colistin; CTX, cefotaxime; DOX, doxycycline; ENRO, enrofloxacin; FFN, florfenicol; GEN, gentamicin; IPM, imipenem; MAR, marbofloxacin; NAL, nalidixic acid; NEO, neomycin; PEN, penicillin; STR, streptomycin; SXT, trimethoprim-sulfamethoxazole 1:19 ratio; TET, tetracycline; TIA, tiamulin; TIL, tilmicosin; TUL, tulathromycin; XNL, ceftiofur.

[a]MIC, minimal inhibitory concentration.

[b]MIC50 indicates the MIC that is required to inhibit 50% of the isolates.

[c]MIC90 indicates the MIC that is required to inhibit 90% of the isolates.

[d]Data represent the concentration of amoxicillin.

[e]Data represent the concentration of trimethoprim.

[f]The tested range of the antimicrobials is visualized in the white area.

*Number of isolates with MIC values equal to or lower or higher than the concentrations of the test range.

1). These multidrug-resistant isolates harboured *tet*(A) and *sul1* or *sul2* (mediating elevated MICs to tetracyclines and sulfonamides respectively); in addition, they carried *dfrB1/2/3* (trimethoprim resistance), *aadA1* (aminoglycoside resistance) or the florfenicol resistance gene *floR* (Figure 1).

Although isolates 10 and 11 (with MIC values of 128 μg ml^{-1} and ≥1024 μg ml^{-1} for streptomycin) were tested for the presence of four streptomycin resistance-mediating genes, only isolate 10 with a MIC of 128 μg ml^{-1} was found to harbour *aadA1*. No β-lactamase-encoding genes were detected in isolate 21, which showed a MIC of 8 μg ml^{-1} for cefotaxime, ceftiofur and cefquinome, and in isolate 3, with a MIC of 8 μg ml^{-1} cefotaxime. Sulfonamide and trimethoprim resistance genes were detected at MIC values of ≥1/19 μg ml^{-1} trimethoprim/sulfamethoxazole ($n = 6$), though one of three isolates with a MIC of 1/19 μg ml^{-1} did not carry any of the resistance genes tested.

DISCUSSION

So far, only a few studies have analysed the antimicrobial susceptibility of *B. avium* using broth microdilution (Beach et al., 2012; Blackall et al., 1995; Mortensen et al., 1989; Szabó et al., 2015). Comparing these, some differences in the performance of broth microdilution are noticeable regarding inoculum preparation, the use of test media and incubation conditions. These discrepancies in methodology can make it difficult to compare the MIC results between laboratories. Therefore, a suitable standard medium and comparable, harmonized test conditions for antimicrobial susceptibility testing of *B. avium* are required.

In two independent growth experiments, the growth of *B. avium* was tested in three different media, which were already recommended by the CLSI: CAMHB is a commercially available medium that leads to only a few interactions with antimicrobial agents, such as sulfonamides, trimethoprim or tetracyclines, and it shows little batch-dependent variation (CLSI, 2018b). It is recommended for the antimicrobial susceptibility testing of bacteria isolated from animals such as *Enterobacterales*, while CAMHB + 2.5% LHB is recommended for fastidious organisms such as *Streptococcus* spp. (CLSI, 2020). For other fastidious organisms such as *Histophilus somni* and *Actinobacillus pleuropneumoniae*, VFM is recommended (CLSI, 2020). Because *B. avium* was in a CLSI request for data on fastidious organisms, the latter two media were included in the growth experiments of the current study. A Mueller-Hinton broth without the addition of cations can influence the activity of various antimicrobial agents, such as aminoglycosides (CLSI, 2018b), fluoroquinolones (Gürdal et al., 1991; Marshall & Piddock, 1994), polypeptides (Marshall & Piddock, 1994) or various cephalosporins (Chow & Bartlett, 1981). Therefore, this medium, which was used in two previous studies (Beach et al., 2012; Szabó et al., 2015), was excluded from the growth experiments.

After 20 h incubation at 35 ± 2° C, the OD_{600} values of inoculated CAMHB were lower than those of CAMHB plus 2.5% LHB and VFM although in all test media bacterial counts of 10^7 to 10^8 cfu ml^{-1} were obtained. The differences between culture-based enumeration and OD_{600} measurements have already been observed in previous studies performing growth experiments with *B. bronchiseptica* or *Listeria monocytogenes* (Francois et al., 2005; Prüller et al., 2015a; Tyrovouzis et al., 2014). The differences are probably because of components in the media, such as erythrocytes in CAMHB + 2.5% LHB or yeast extract in VFM, which darken during incubation, thus leading to OD shifts. Therefore, the colony counts were used to evaluate the suitability of the media, and CAMHB was selected for further development of the method. The CAMHB provided clearly readable button formations after 20 h incubation of *B. avium* at 35°C ± 2°C in microtiter plates and it is a convenient medium that does not require the addition of supplements, such as lysed horse blood, supplement C or yeast extract as is the case with the other test media. The other susceptibility test conditions were chosen exactly as recommended by CLSI for the broth microdilution of bacteria that grow aerobically (CLSI, 2018b). Under these test conditions, the MIC values were easily readable for all 49 *B. avium* isolates tested; the results were also highly reproducible, as demonstrated in the present study, by analysing five independent replicates of the MIC testing with five epidemiological unrelated isolates. Thus, the current study showed an exact MIC agreement of 68%–100%, here depending on the antimicrobial agent considered and an essential MIC agreement of 100% for 23 of the 24 antimicrobial agents. For tiamulin only, an essential MIC agreement of 92% was calculated. However, because the essential MIC agreement proposed by the CLSI should be ≥90%, the agreement for tiamulin is still in the acceptable range (CLSI, 2018a).

Because there are currently no approved breakpoints for the interpretation of MIC values for *B. avium*, it was not possible to classify the isolates tested as resistant, intermediate or susceptible. Nevertheless, such a classification was made in one study, in which the authors defined high-level resistance as equal to or greater than the maximum dose (Beach et al., 2012). In another study, Blackall et al. (1995) suggested MIC breakpoints for *B. avium*, which were summarized from older studies; the latter authors classified *B. avium* isolates as resistant if they exhibited

TABLE 3 *Bordetella avium* isolates tested and detected resistance genes

Antimicrobial agent	MIC (μg ml^{-1})	Number of isolates tested	Number of isolates with the following detected resistance genes									
Aminoglycosides			*strA*	*strB*	*aadA1*	*aadA2*	*aac(3)-I*	*aac(3)-IV*	*aac(6')-Ib*	*ant(2'')-I*		
Gentamicin	4	2	[a]				—	—	—	—		
Streptomycin	≥128	2	—	—	1	—						
β-lactam antibiotics			*bla*$_{BOR-1}$	*bla*$_{OXA-1}$	*bla*$_{OXA-2}$	*bla*$_{ROB}$	*bla*$_{TEM}$	*bla*$_{SHV}$				
Ampicillin	2	1	—	—	—	—	—	—				
Cefotaxime	≥8	1	—	—	—	—	—	—				
Cefquinome	≥8	2	—	—	—	—	—	—				
Ceftiofur	≥8	1	—	—	—	—	—	—				
Folate pathway inhibitors			*dfrA1/15/16*	*dfrA5/14*	*dfrA7/17*	*dfrB1/2/3*	*sul1*	*sul2*	*sul3*			
Trimethoprim/ Sulfamethoxazole	≥1/19	6	—	—	—	1	2	3	—			
Phenicols			*floR*									
Florfenicol	32	1	1									
Quinolones			*qnrA*	*qnrB*	*qnrC*	*qnrD*	*qnrS*	*aac(6')-lb-cr*				
Enrofloxacin	≥0.5	32	—	—	—	—	—	—				
Marbofloxacin	≥0.5	37	—	—	—	—	—	—				
Tetracyclines			*tet*(A)	*tet*(B)	*tet*(C)	*tet*(D)	*tet*(E)	*tet*(G)	*tet*(H)	*tet*(L)	*tet*(M)	*tet*(O)
Tetracycline[b]	≥64	5	5	—	—	—	—	—	—	—		

[a]Grey shading indicates that the isolates have not been tested for the presence of these genes.

[b]Isolates with tetracycline MIC values of ≥64 μg ml^{-1} also had MIC values for doxycycline of ≥4 μg ml^{-1}.

the following MICs: ampicillin ≥2 μg ml^{-1}, streptomycin ≥16 μg ml^{-1}, tetracycline ≥2 μg ml^{-1} and trimethoprim/sulfamethoxazole ≥64/1216 μg ml^{-1}. Accordingly, one *B. avium* isolate tested in the present study with a MIC for ampicillin of 2 μg ml^{-1} would be considered resistant to ampicillin. This isolate also showed higher MICs to third- and fourth-generation cephalosporins such as cefotaxime (8 μg ml^{-1}), cefquinome (8 μg ml^{-1}), ceftiofur (8 μg ml^{-1}) and cephalotin (4 μg ml^{-1}) when compared with the rest of the strain collection. Because there was no detection of ESBL in the ESBL confirmatory test, it can be assumed that the resistance was because of either an AmpC mechanism or a currently unknown mechanism in *B. avium*. A lower effectiveness of cephalosporins has already been observed in another *Bordetella* species -the pig pathogen *B. bronchiseptica*- which was attributed to a generally low membrane permeability for cephalosporins (Kadlec et al., 2007; Prüller et al., 2015b). Moreover, all except one isolate would be classified as resistant to streptomycin according to the breakpoints proposed by Blackall et al. (1995) because the MICs ranged between 16 and 512 μg ml^{-1}. In addition, according to these breakpoints, five isolates with MICs of 64–128 μg ml^{-1} for tetracycline and a single isolate with a MIC of 32 μg ml^{-1} for trimethoprim/sulfamethoxazole would be considered resistant to the corresponding antimicrobial.

Comparing the MICs obtained in the present study with the MICs previously described, some differences are noticeable. For example, the current study found 21 isolates with higher MICs (32 to ≥1024 μg ml^{-1}) of streptomycin, which was not the case in the study by Blackall et al. (1995). We also found lower MIC values for tetracycline or trimethoprim/sulfamethoxazole than in the studies by Blackall et al. (1995), Mortensen et al. (1989), or Beach et al. (2012). However, some MIC values, such as for doxycycline, were higher than previously found (Szabó et al., 2015). The different MIC values obtained in the few available susceptibility studies including *B. avium* might be because of the variations in the testing methods (e.g., media, incubation times and conditions); changes in the antimicrobial resistance status of the pathogen in recent years (because some studies are more than 15 years old) (Blackall et al., 1995; Mortensen et al., 1989); or differences in the geographic origin of the isolates (Australian, US and European isolates). However, this underlines the need for harmonized methods for antimicrobial susceptibility testing of *B. avium*, which is also a prerequisite for the development of antimicrobial susceptibility testing breakpoints for the pathogen.

Most isolates did not carry any of the resistance genes tested, but this may be because very little is known about the genetic basis of antimicrobial resistance in *B. avium*. PCR assays only detect the target genes, so some genes may have remained undetected. To detect these genes, other methods, such as whole-genome sequencing, must be used. Nevertheless, the presence of at least two resistance genes conferring resistance to different classes of antimicrobial agents was detected in five *B. avium* isolates. Three even harboured three different resistance genes (Figure 1) and were classified as multidrug resistant based on their phenotypic resistance. Although *tet*(A), *sul1* and *aadA1* have been previously described in two *B. avium* isolates (Erfan et al., 2018), the current study was, to the best of our knowledge, the first to demonstrate the occurrence of *floR*, *dfrB1/2/3* and *sul2* in *B. avium*. Therefore, it is of great importance to monitor the antimicrobial resistance of *B. avium* using harmonized antimicrobial susceptibility testing.

It was shown that cation-adjusted Mueller-Hinton broth is a suitable medium for broth microdilution susceptibility testing of *B. avium*. Using the test conditions and incubation times recommended for rapidly growing bacteria from animals in the CLSI documents, valid and reproducible results can be obtained (CLSI, 2018b). Obtaining a harmonized method for testing this pathogen could contribute to monitoring the antimicrobial resistance status of *B. avium* and performing targeted antimicrobial therapy for bordetellosis.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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