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Towards a Standardized Method for Broth Microdilution Susceptibility Testing of *Haemophilus parasuis*

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ABSTRACT Currently, there is no agreed method available for broth microdilution susceptibility testing of *Haemophilus parasuis*, one of the most important bacterial pathogens in pig production. Therefore, the aim of this study was to develop a method that could be easily performed by diagnostic laboratories and that appears suitable for a harmonized susceptibility testing. Growth determinations using one type strain and three field isolates revealed no visible growth of *H. parasuis* in media which have proven to be suitable for susceptibility testing of fastidious organisms. Therefore, a new medium, cation-adjusted Mueller-Hinton broth (CAMHB) plus NADH and sterile filtered heat-inactivated chicken serum, was developed. The reproducibility of MICs obtained in this medium was evaluated and statistically analyzed, considering a model with two different variables (precondition of five identical MICs and MIC mode accepting a deviation of ± 1 dilution step, respectively). No significant differences for both variables were seen between two time points investigated and between results obtained with the recently proposed test medium broth (TMB). Nearly all MICs of quality control strains were in the acceptable range. Subsequently, 47 *H. parasuis* isolates representing 13 serovars were tested with the newly developed medium and TMB. Statistical analysis of all isolates and 15 antimicrobial agents and antimicrobial combinations showed no significant difference between MICs obtained in supplemented CAMHB and TMB. Because of a simplified implementation in routine diagnostic and a lower chance of interference between medium components and antimicrobial agents, supplemented CAMHB is recommended with an incubation time of 24 h.

KEYWORDS broth microdilution, susceptibility testing, MICs, *Haemophilus parasuis*, fastidious organism, TMB, supplemented CAMHB

Haemophilus parasuis is an abundant colonizer of the upper respiratory tract in swine (1). Although in *H. parasuis* endemic herds, young piglets are usually protected by maternal immunity, several environmental stress factors may lead to disease as well as infections of naive populations. The acute form of disease, Glässer's disease, is characterized by clinical signs of polyarthritis, polyserositis, meningitis, and pneumonia (among others) and can lead to sudden death of animals, especially in weaner pigs (2, 3). At least 15 different *H. parasuis* serovars have been identified so far, with the most common serovars being 4 and 5 (4, 5).

As antimicrobial agents are used for the treatment of disease, a reliable method for susceptibility testing of the pathogen is essential. *H. parasuis* is a fastidious organism which requires specific ingredients or supplements to achieve visible growth in broth, and hence, media which are used to test rapidly growing bacteria are not adequate (6, 7). As a consequence, no validated and standardized method is available (7, 8), and many diagnostic laboratories are currently unable to offer susceptibility testing of this very important pathogen in pig production. However, a few studies investigated the susceptibility status of *H. parasuis* isolates quantitatively by using media such as veterinary fastidious medium (VFM) or *Haemophilus* test medium (HTM) for broth microdilution susceptibility testing (7, 9–16). VFM is recommended in CLSI standard VET01-A4 for susceptibility testing of *Actinobacillus pleuropneumoniae* and *Histophilus somni* (17), while HTM and cation-adjusted Mueller-Hinton broth (CAMHB) supplemented with 2.5% to 5% lysed horse blood (LHB) are CLSI-approved broth media for the testing of fastidious organisms, with HTM being recommended for *Haemophilus influenzae* and *Haemophilus parainfluenzae* (18). As it has been seen that VFM is not suitable for all *H. parasuis* isolates, a broth microdilution susceptibility testing method with test medium broth (TMB) has recently been proposed (7). TMB is a complex medium consisting of 1% (wt/vol) Biosate peptone, 1% (wt/vol) sodium chloride, 0.1% (wt/vol) starch, 0.1% (wt/vol) glucose, and 0.05% (wt/vol) yeast extract, which is supplemented with 0.0025% (vol/vol) NADH, 0.0005% (vol/vol) thiamine HCl, 1% (vol/vol) sterile filtered heat-inactivated chicken serum, and 5% (vol/vol) oleic acid bovine albumin complex. The oleic acid bovine albumin complex is composed of saline with 0.06% (vol/vol) oleic acid and 5% (vol/vol) 0.05 N NaOH, which is finally enriched with 4.75% (vol/vol) bovine serum albumin (7).

Therefore, this study was aimed at (i) comparatively analyzing the suitability of different broth media for susceptibility testing of preferably a wide range of different *H. parasuis* serovars and (ii) identifying a broth medium for a harmonized routine diagnostic approach. For this, a new medium, CAMHB with a reduced content of supplements, was developed and used for testing of 46 *H. parasuis* field isolates, including representatives of 13 serovars, in comparison to TMB. Subsequently, statistical analysis was performed to examine the homogeneity of MIC data and to investigate the difference between supplemented CAMHB and TMB. Hence, this study might contribute to establish a standardized broth microdilution susceptibility testing method for *H. parasuis*.

RESULTS

Growth of *H. parasuis* in broth media. To determine the adequacy of the broth media for susceptibility testing, type strain DSM 21448 and three *H. parasuis* field isolates of serovars 5 (two isolates) and 14 (one isolate) were chosen. The isolates of serovar 5 were included because this serovar is regarded as the most pathogenic *H. parasuis* serovar in Germany. As well, two of the isolates (no. 24 of serovar 14 and 52 of serovar 5) were selected because they are extremely slow-growing isolates and difficult to culture. The growth curves from two independent experiments revealed that all isolates showed almost consistent growth in VFM and TMB. However, in CAMHB plus 2.5% LHB and HTM, a decrease of the isolates in CFU values over time was detected after only a few hours, except for type strain DSM 21448 (see Fig. S1 to S5 in the supplemental material).

In addition, visible growth of 28 *H. parasuis* field isolates of various serovars was assessed in microtiter plates with and without antibiotic coating, and in agreement with results from growth determination, no growth was detected in any of the wells when HTM and CAMHB plus 2.5% LHB were used. With VFM, visible growth could be detected only for seven isolates, although the *H. parasuis* type strain and all three field isolates showed a sufficient increase in CFU/ml during growth curve determination. However, MIC endpoints could be determined in VFM only for the type strain. In contrast to these findings, visible growth of all tested *H. parasuis* isolates was detected in microtiter plate wells while testing TMB.

TABLE 1 Supplementation of CAMHB and determination of visible growth of 15 *H. parasuis* isolates

CAMHB supplement[a]				
NADH (0.0025%)	Heat-inactivated chicken serum (1%)	Thiamine (0.0005%)	O-A complex (5%)	Visible growth in microtiter plates[b]
+	+	+	+	+++
+	+	+	−	+++
+	+	−	+	+++
+	−	+	+	+++
−	+	+	+	−
+	+	−	−	+++
+	−	−	+	+++
+	−	+	−	−
−	+	+	−	−
−	+	−	+	−
−	−	+	+	−
+	−	−	−	−
−	−	−	−	−

[a]+, supplement was added to CAMHB; −, supplement was not added to CAMHB.
[b]The following scheme was used to grade the visible growth of *H. parasuis*: +++, excellent growth (size of buttons is comparable to the size of buttons with TMB); ++, fair growth; +, poor growth; −, no growth.

Supplemented CAMHB for susceptibility testing of *H. parasuis*. CAMHB enriched with the four supplements used in TMB led to visible growth of *H. parasuis* isolates tested in 96-well plates. The amount of growth in the wells was comparable to the amount of growth in microtiter panels with TMB medium (Table 1). Using only three supplements with CAMHB, tests revealed that NADH was essential for the growth of all *H. parasuis* isolates and omitting one of the supplements thiamine, sterile filtered inactivated chicken serum, and O-A complex showed no difference in visible growth. To further reduce the number of supplements, it was evaluated if growth of the *H. parasuis* isolates varied when only two supplements were added to CAMHB. Results of these experiments showed that supplementation of sterile filtered inactivated chicken serum or O-A complex is necessary to obtain visible growth of *H. parasuis* isolates in CAMHB (Table 1). As O-A complex consists of two different components (oleic acid and bovine serum albumin) and therefore is a complex supplement to prepare, sterile filtered inactivated chicken serum together with NADH was chosen to supplement CAMHB for broth microdilution susceptibility testing of *H. parasuis* isolates. Finally, the recipe for the supplemented CAMHB was CAMHB plus 0.0025% (vol/vol) NADH and 1% (vol/vol) sterile filtered heat-inactivated chicken serum.

Subsequently, colony count determinations during culturing of type strain DSM 21448 and three *H. parasuis* field isolates in supplemented CAMHB were performed, and adequate growth of all isolates could be detected (see Fig. S6 in the supplemental material).

Control of different batches and producers. MICs of type strain DSM 21448 and three field isolates using two different batches and another two producers of NADH and chicken serum showed comparable results after 24 h of incubation, with a maximum deviation of one dilution step.

Test ranges of QC strains. To investigate the influence of test media on MICs of quality control (QC) strains, broth microdilution susceptibility testing of four QC strains was performed using TMB and supplemented CAMHB, although QC ranges have been established in unsupplemented CAMHB or in VFM (*Actinobacillus pleuropneumoniae*). Threefold-repeated susceptibility testing revealed that MICs from 261 out of 270 QC strain-antimicrobial agent combinations were within acceptable ranges in both media (17). The only exceptions for TMB were the combinations *A. pleuropneumoniae* with gentamicin and *A. pleuropneumoniae* with the antibiotics gentamicin and penicillin in supplemented CAMHB in all three repetitions.

TABLE 2 Homogeneity of MICs obtained with supplemented CAMHB by using two different incubation times

Antimicrobial agent[b]	Incubation time, 16–20 h									Incubation time, 24 h								
	Deviation from MIC mode[a]							Exact MIC agreement of isolates (%)	Essential MIC agreement of isolates (%)	Deviation from MIC mode[a]							Exact MIC agreement of isolates (%)	Essential MIC agreement of isolates (%)
	−3	−2	−1	0	1	2	3			−3	−2	−1	0	1	2	3		
AMP			2	11	2			73.3	100.0			2	11	2			73.3	100.0
AUG			3	12				80.0	100.0			3	12				80.0	100.0
CEQ				15				100.0	100.0			2	13				86.7	100.0
CIP				15				100.0	100.0				15				100.0	100.0
COL			2	9	4			60.0	100.0			3	10	2			66.7	100.0
DOX			5	10				66.7	100.0			3	12				80.0	100.0
ENRO				15				100.0	100.0				14	1			93.3	100.0
FFN				13	2			86.7	100.0			1	14				93.3	100.0
FOP				15				100.0	100.0			2	13				86.7	100.0
FOT			1	14				93.3	100.0			1	14				93.3	100.0
GEN			1	14				93.3	100.0				13	2			86.7	100.0
IMI				12	3			80.0	100.0			2	11	2			73.3	100.0
NAL			1	13	1			86.7	100.0			1	13	1			86.7	100.0
NEO			2	12	1			80.0	100.0			2	11	2			73.3	100.0
PEN			2	9	3	1		60.0	93.3	1	2	2	8	2			53.3	80.0
STR		3	4	8				53.3	80.0			3	12				80.0	100.0
SXT			1	10	4			66.7	100.0			3	10	2			66.7	100.0
TET		1	4	10				66.7	93.3			2	11	2			73.3	100.0
TIA		2	4	6	3			40.0	86.7			1	9	3	2		60.0	86.7
TIL			1	14				93.3	100.0			1	11	3			73.3	100.0
XNL				15				100.0	100.0				15				100.0	100.0

[a]Data fulfilling the criteria of the essential MIC agreement are in the gray area.
[b]AMP, ampicillin; AMC, amoxicillin-clavulanic acid; CIP, ciprofloxacin; COL, colistin; CQN, cefquinome; DOX, doxycycline; ENRO, enrofloxacin; FFN, florfenicol; FOP, cefoperazone; FOT, cefotaxime; GEN, gentamicin; IMI, imipenem; MAR, marbofloxacin; NAL, nalidixic acid; NEO, neomycin; PEN, penicillin; STR, streptomycin; SXT, trimethoprim-sulfamethoxazole; TET, tetracycline; TIA, tiamulin; TIL, tilmicosin; XNL, ceftiofur.

Homogeneity of *H. parasuis* MICs. To assess the homogeneity of MICs obtained with supplemented CAMHB and TMB with two different incubation times, the *H. parasuis* type strain and the two field isolates included in the growth curve determinations were used. As the third *H. parasuis* field isolate (no. 52) showed visible growth only after 48 h in supplemented CAMHB, this isolate was omitted from this analysis. The MIC determinations were repeated in five independent experiments. A panel of 21 antimicrobials and antimicrobial combinations was included in the repeated tests, and two different incubation times, 16 to 20 h and 24 h, were analyzed. Results from the repeated tests are shown in Tables 2 and 3. The multifactorial logistic regression analysis with the 11 antibiotics fulfilling statistical requirements revealed that no significant influence of the different incubation times on MICs obtained in both media was observed, either for the evaluation of data by exact MIC agreement ($P = 0.5165$) or by essential MIC agreement accepting a deviation of ± 1 dilution step ($P = 0.1913$). For the exact MIC agreement, the percentage of homogeneous MICs was 25.8% after 16 to 20 and 30.3% after 24 h of incubation. The percentages were 92.4% after 16 to 20 h and 97.0% after 24 h of incubation for the essential MIC agreement. When comparing the homogeneity of MICs (i.e., all five MICs were identical) obtained in the two media, supplemented CAMHB showed 27.3% homogeneous MICs, while TMB gave a figure of 28.8%, with the difference not being significant ($P = 0.8282$). For the MIC mode accepting a deviation of ± 1 dilution step, 90.9% of MICs showed homogeneous MICs for supplemented CAMHB, while 98.5% of the MICs were homogeneous for TMB ($P = 0.0567$). No significant influence on the homogeneity of MICs was seen for the exact ($P = 0.5584$) and the essential MIC agreement ($P = 0.9954$) for the antimicrobial agents. In contrast, the bacterial strains had a significant influence on the homogeneity of MICs for the exact MIC agreement ($P = 0.0005$), whereas no significant influence was seen for the essential MIC agreement ($P = 0.4757$).

Results from MIC determinations of *H. parasuis* field isolates. Using both media, supplemented CAMHB and TMB, there was no difficulty in determining visible growth of all 47 *H. parasuis* isolates in the wells of microtiter plates and reading of MIC

TABLE 3 Homogeneity of MICs obtained with TMB by using two different incubation times[a]

Antimicrobial agent[b]	Incubation time, 16–20 h									Incubation time, 24 h								
	Deviation from MIC mode							Exact MIC agreement of isolates (%)	Essential MIC agreement of isolates (%)	Deviation from MIC mode							Exact MIC agreement of isolates (%)	Essential MIC agreement of isolates (%)
	3	−2	−1	0	1	2	3			−3	−2	−1	0	1	2	3		
AMP			3	12				80.0	100.0			1	13	1			86.7	100.0
AUG				14	1			93.3	100.0				12	3			80.0	100.0
CEQ			1	12	2			80.0	100.0			1	12	2			80.0	100.0
CIP				15				100.0	100.0				15				100.0	100.0
COL				13	2			86.7	100.0			1	13	1			86.7	100.0
DOX			1	14				93.3	100.0				14	1			93.3	100.0
ENRO				15				100.0	100.0				15				100.0	100.0
FFN			1	13	1			86.7	100.0			1	14				93.3	100.0
FOP				15				100.0	100.0				14	1			93.3	100.0
FOT				13	2			86.7	100.0				13	2			86.7	100.0
GEN		1	2	10	2			66.7	93.3			1	12	2			80.0	100.0
IMI				13	2			86.7	100.0				14	1			93.3	100.0
NAL			2	10	3			66.7	100.0				12	3			80.0	100.0
NEO			2	10	3			66.7	100.0		2		11	2			73.3	100.0
PEN		2	2	10	1			66.7	86.7				11	4			73.3	100.0
STR			4	10	1			66.7	100.0			1	12	2			80.0	100.0
SXT			1	12	2			80.0	100.0				13	2			86.7	100.0
TET				14		1		93.3	93.3				13	1	1		86.7	93.3
TIA			3	9	3			60.0	100.0			1	12	2			80.0	100.0
TIL			3	10	1	1		66.7	93.3			1	11	3			73.3	100.0
XNL				15				100.0	100.0				15				100.0	100.0

[a]Data fulfilling the criteria of the essential MIC agreement are in the gray area.
[b]AMP, ampicillin; AMC, amoxicillin-clavulanic acid; CIP, ciprofloxacin; COL, colistin; CQN, cefquinome; DOX, doxycycline; ENRO, enrofloxacin; FFN, florfenicol; FOP, cefoperazone; FOT, cefotaxime; GEN, gentamicin; IMI, imipenem; NAL, nalidixic acid; NEO, neomycin; PEN, penicillin; STR, streptomycin; SXT, trimethoprim-sulfamethoxazole; TET, tetracycline; TIA, tiamulin; TIL, tilmicosin; XNL, ceftiofur.

endpoints was feasible. Control of inoculum density showed the required inoculum size of 5×10^5 CFU/ml. The MICs of the isolates are presented in Table 4 and in Table S1 in the supplemental material.

Considering the results from supplemented CAMHB, a bimodal distribution of MICs was detected for the antimicrobial agents ampicillin, ciprofloxacin, colistin, doxycycline, enrofloxacin, nalidixic acid, neomycin, penicillin, streptomycin, tetracycline, and tilmicosin, whereas a unimodal distribution was determined for the remaining antibiotics tested. However, the broadest distribution of MICs was found for the antibiotic combination trimethoprim-sulfamethoxazole. As there are no CLSI-recommended breakpoints for *H. parasuis* isolates available, a classification of isolates as susceptible or resistant was not possible. However, the majority of isolates proved to have very low $MIC_{50}s$ and $MIC_{90}s$ (equal to or below 0.5 μg/ml) for all β-lactam antimicrobials, and only a couple of isolates showed elevated MICs in comparison to the majority of isolates (Table 4).

Comparative analysis of MICs. For simplified interpretability of MICs, an incubation time of 24 h was chosen for comparative analysis. Comparing $MIC_{50}s$ and $MIC_{90}s$ of supplemented CAMHB and TMB, slight differences were detected. Five out of 21 $MIC_{50}s$ were up to two dilution steps higher when using TMB, while it was seen that one MIC_{50} was one dilution step lower. The $MIC_{90}s$ of cefquinome, florfenicol, streptomycin, and tiamulin differed by one dilution step, with lower values detected while using TMB.

The entire comparison of MICs of 47 isolates and 15 antimicrobial agents fulfilling the inclusion criteria for statistical analysis, independent of $MIC_{50}s$ and $MIC_{90}s$, showed no significant difference ($P = 0.2076$) between the two media. However, statistical analysis of individual antimicrobial agents showed significant differences of MICs between the two media for the following antimicrobial agents, which were significantly higher in TMB for the antimicrobials colistin ($P = 0.0038$), gentamicin ($P = 0.0006$), and neomycin ($P < 0.0001$) and significantly lower in TMB for doxycycline ($P = 0.0010$), florfenicol ($P = 0.0003$), streptomycin ($P = 0.0127$), and tiamulin ($P = 0.0110$).

TABLE 4 MIC distribution of 47 *H. parasuis* isolates after 24 h of incubation in supplemented CAMHB

Antimicrobial agent	No. of isolates with indicated MIC (μg/ml) after 24 h of incubation[a]																		MIC_{50}	MIC_{90}
	0.008	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1,024		
Ampicillin			17*	9	9	8	3						1						0.06	0.25
Amoxicillin-clavulanic acid, 2:1 ratio[b]			6*	20	13	4	4												0.06	0.25
Ceftiofur			46*	1															≤0.03	≤0.03
Cefquinome		37*	5	3	2														≤0.015	0.06
Cefoperazone				47*															≤0.06	≤0.06
Cefotaxime		43*	3	1															≤0.015	≤0.015
Ciprofloxacin	39*	2	5					1											≤0.008	0.03
Colistin			1	25	11	3		2	2	3									0.06	2
Doxycycline					10	25	6	3	1		2								0.25	1
Enrofloxacin	28*	12	3	3							1								≤0.008	0.03
Florfenicol					6*	29	12												0.25	0.5
Gentamicin						1	6	26	14										1	2
Imipenem		4*	11	20	12														0.06	0.12
Nalidixic acid							7	29	3		2	4	1	1					1	16
Neomycin			1*	2	4		1	2	14	19	4								2	4
Penicillin		10*	7	8	4	13	3	1						1*					0.06	0.5
Streptomycin									1	3	13	18	6	1	1			4	16	128
Tiamulin						1	1	5	18	17	5								2	8
Tetracycline					1*	7	29	2		1	2	4	1						0.5	16
Tilmicosin						6	14	16	10						1				1	2
Trimethoprim-sulfamethoxazole (1:19)[c]		6*	4	1	6	6	12	5	2	4		1							0.5	4

[a]Asterisked numbers are the number of isolates with MICs equal to or higher or lower than concentrations of the tested range. The tested range of the antimicrobials agents is represented in the white area.
[b]Data represent the concentration of amoxicillin.
[c]Data represent the concentration of trimethoprim.

DISCUSSION

In a recent study, VFM was reported to be unsuitable for broth microdilution susceptibility testing of Australian *H. parasuis* isolates, and TMB was proposed (7). However, currently there is no standardized method available in the CLSI guidelines for *H. parasuis* and the usage of different methods can lead to variable MIC results and poor comparability of results (17, 19). Furthermore, due to high losses on pig farms worldwide caused by *H. parasuis* (20), finding an appropriate and agreed-upon method of susceptibility testing for this important veterinary pathogen is urgently required.

In this study, colony count determinations during culturing in four different media detected growth of *H. parasuis* field isolates in VFM and TMB, whereas only growth of type strain DSM 21448 could be detected in HTM and CAMHB plus 2.5% LHB. Surprisingly, visible growth in microtiter plate wells was detected in only 25% of the tested *H. parasuis* field isolates cultured in VFM. The reason why no visible growth in microtiter plates was seen in VFM, although growth could be obtained in growth curves using culture enumeration, is not clear. The absence of visible growth in CAMHB plus 2.5% LHB, HTM, and VFM indicate that all three media are not suitable for broth microdilution susceptibility testing of *H. parasuis* isolates. In contrast, TMB promotes the growth of all *H. parasuis* isolates from Germany and even slow-growing isolates, which were difficult to culture on agar plates, showed visible growth in microtiter plate wells. TMB is not commercially available, and preparing the medium in routine diagnostic laboratories is a markedly complex task, as the medium consists of several components and supplements. Additionally, there are issues about batch-to-batch reproducibility and a low content of inhibitors that affect antimicrobial agents that still have to be addressed. If a nonapproved medium is used for broth microdilution susceptibility testing, validation of the medium has to undergo an extensive procedure. One important factor is the applicability of QC strains and their MICs within a specified range. In this study, nearly all MICs of QC strains were in the acceptable range with both media used, although the QC ranges have been established in media other than TMB or supplemented CAMHB,

and therefore, it was not expected to have all MICs in the published ranges. It should be noted that the QC strain *A. pleuropneumoniae* is also a fastidious bacterium, and susceptibility testing in VFM medium according to CLSI document VET01-A4 is recommended (17). This could be the reason for the deviation of a few MICs from the acceptable range of the QC strain *A. pleuropneumoniae*. However, the results from QC strains indicate that there is no effect of medium components on the activity of antimicrobial agents.

A new medium was established for routine broth microdilution susceptibility testing of *H. parasuis*, based on CAMHB and enriched with essential supplements (7, 21). CAMHB was used since it is a validated and agreed medium for susceptibility testing with an optimal rate of cations, showing high reproducibility of MIC data and being recommended in CLSI document VET01-A4 for MIC determination of rapidly replicating bacteria. It is also low in inhibitors for sulfonamides, trimethoprim and tetracycline. Since supplementation of CAMHB with 2.5% LHB resulted in the absence of growth of *H. parasuis* field isolates, the essential supplements (NADH and sterile filtered inactivated chicken serum) from TMB were chosen to enrich CAMHB in the same concentrations as in TMB (7). Importantly, NADH-containing HTM is recommended in CLSI document M07-A9 (18) for susceptibility testing of fastidious organism such as *Haemophilus influenzae* and is therefore a CLSI-verified supplement.

Statistical analysis revealed that both incubation times tested had no influence on the homogeneity of MICs. Due to the low growth rate of *H. parasuis* compared to other rapidly replicating bacteria and few isolates showing weak growth after 16 to 20 h of incubation, an incubation time of 24 h is suggested, which might be extended to 48 h for isolates which show no growth after 24 h.

The supplemented CAMHB appears to be a suitable medium for broth microdilution susceptibility testing of the majority of *H. parasuis* isolates from Germany. As the medium had no significant influence on the homogeneity of the MICs and although TMB seems to support the growth of *H. parasuis* isolates slightly better, CAMHB provides the advantage of a validated basic broth medium (17). Preparation of this medium is less challenging and sensitive and could be used in routine diagnostic laboratories.

Due to the lack of specific breakpoints for *H. parasuis*, a classification of the 46 field isolates into the categories susceptible, intermediate, and resistant could not be obtained, and percentages of resistances could therefore not be calculated. Nevertheless, bimodal distributions of MICs were indicative of the presence of nonwild type isolates. In a study by Dayao et al., MIC_{50}s and MIC_{90}s of 97 *H. parasuis* isolates from Australia were tested in TMB, and it was seen that comparable values with a maximum of one dilution step difference were obtained for ampicillin, florfenicol, penicillin, tetracycline, and trimethoprim-sulfamethoxazole. However, for tilmicosin, the MIC_{90} was four dilution steps higher than in the present study, indicating a difference in MIC distribution of Australian and German isolates tested (7).

Conclusion. For broth microdilution susceptibility testing of *H. parasuis*, supplemented CAMBH and TMB have been shown to be most suitable. The use of both media resulted in reproducible and comparable MICs, with MICs of quality control strains being in the acceptable range for nearly all antimicrobial agents tested. Supplemented CAMHB is recommended for routine diagnostics since it is based on an agreed-upon and validated base medium, which is commercially available. For *H. parasuis* susceptibility testing, an incubation time of 24 h is recommended. The proposed method enables broth microdilution susceptibility testing of *H. parasuis* isolates in routine diagnostics and might contribute to establishment of a standardized method for this important veterinary pathogen.

MATERIALS AND METHODS

Bacterial isolates. *H. parasuis* type strain DSM 21448 (Leibniz-Institute DSMZ, Braunschweig, Germany) and 46 *H. parasuis* field isolates were used for the present study. The isolates originated from diseased pigs and were collected at pig farms from different geographic regions in Germany between 2011 and 2013. All field isolates were provided by diagnostic laboratories in Germany and included 25

TABLE 5 Test ranges of antimicrobial agents included in this study

Antimicrobial agent	Range (μg/ml)
Ampicillin	0.03–64
Amoxicillin-clavulanic acid (2:1 ratio)	0.03/0.015–64/32
Ceftiofur	0.03–64
Cefquinome	0.015–32
Cefoperazone	0.06–32
Cefotaxime	0.015–32
Ciprofloxacin	0.006–16
Colistin	0.03–16
Doxycycline	0.06–128
Enrofloxacin	0.008–16
Florfenicol	0.12–256
Gentamicin	0.12–256
Imipenem	0.015–32
Nalidixic acid	0.06–128
Neomycin	0.03–128
Penicillin	0.015–32
Streptomycin	0.25–256
Tiamulin	0.03–64
Tetracycline	0.12–128
Tilmicosin	0.06–64
Trimethoprim-sulfamethoxazole (1:19 ratio)	0.015/0.3–32/608

serotyped isolates of serovars 1 ($n = 2$), 2 ($n = 2$), 4 ($n = 2$), 5 ($n = 2$), 6 ($n = 2$), 8 ($n = 2$), 9 ($n = 1$), 10 ($n = 2$), 11 ($n = 2$), 12 ($n = 2$), 13 ($n = 2$), 14 ($n = 2$), and 15 ($n = 2$). Isolates were cultured on chocolate agar with 10% defibrinated horse blood in ambient air at an incubation temperature of 35°C ± 2°C for 24 to 48 h. To confirm that the field isolates were *H. parasuis*, a PCR assay amplifying an 821-bp internal fragment of the 16S small subunit rRNA gene was used as described earlier (20).

Growth curves. To compile growth curves, type strain DSM 21448 of *H. parasuis* and three field isolates of serovars 5 (2 isolates) and 14 (1 isolate) were used. Growth curves were performed in four different broth media (HTM, TMB, VFM, and CAMHB plus 2.5% LHB), as these media have been proven suitable for susceptibility testing of fastidious organisms (7, 10, 11, 21–23). In brief, isolates were grown on chocolate agar for 24 to 48 h at 35°C ± 2°C in ambient air, colonies were suspended in 0.9% saline, and the suspension was adjusted to an initial concentration of approximately 10^3 to 10^4 CFU/ml. Isolates were cultured in a volume of 5 ml for 48 h at 35°C ± 2°C in ambient air without shaking, and the CFU count per milliliter was determined by culture enumeration every 4 h (up to h 24) and 8 h (up to h 48), respectively. The weighted average was calculated from agar plates containing between 5 and 200 CFU/ml.

Development of a modified broth medium. As TMB is not commercially available and to provide an easier-to-prepare medium for routine diagnostic laboratories, a new medium based on CAMHB (Oxoid, Wesel, Germany) was developed for broth microdilution susceptibility testing of *H. parasuis*. For this purpose, which of the supplements of TMB were essential to provide sufficient growth of preferably all *H. parasuis* isolates and serovars was evaluated. CAMHB was taken as a basis since it is the CLSI-approved medium of choice for susceptibility testing of commonly isolated pathogens. For supplementation of CAMHB, the medium was first tested with all TMB supplements, and then one supplement at a time was removed. A list of all formulations tested is given in Table 1. Supplements were added to CAMHB aseptically after autoclaving, and concentrations of supplements were equal to concentrations used in TMB. As pH measurements showed values between pH 7.2 and 7.4, a pH adjustment of the supplemented CAMHB was not necessary.

The necessity of the supplements was tested against a set of 15 *H. parasuis* isolates in 96-well microtiter plates, and visible growth in the wells was determined (Table 1). NADH (β-NAD, reduced form, disodium salt) (Armin Baack, Schwerin, Germany, and Sigma-Aldrich, Seelze, Germany) and sterile filtered chicken serum (Bio&Sell, Feucht, Germany, and Pan-Biotech, Aidenbach, Germany) of two different producers were verified by broth microdilution susceptibility testing with type strain DSM 21448 and the three field isolates with 21 antimicrobial agents.

Susceptibility testing. For broth microdilution susceptibility testing, customized 50-μl/well microtiter plates (Sensititre, East Grinstead, UK) coated with vacuum-dried antibiotics were used. Testing included 21 antimicrobial agents and antibiotic combinations (Table 5), four of which are currently not licensed for food-producing animals and were included for comparison reasons (cefotaxime, ciprofloxacin, imipenem, and nalidixic acid). Inoculum preparation and density, incubation conditions, and MIC endpoint determinations for *H. parasuis* followed the recommendations given in CLSI documents M07-A9 and VET01-A4 for rapidly replicating bacteria (17, 18). The direct colony suspension method was used for inoculum preparation. In brief, colonies were selected from a 24-h agar plate, inoculated into 0.9% saline, and adjusted to a turbidity comparable to that of a 0.5 McFarland standard. The saline suspension was diluted 1:200 in broth medium (e.g., CAMHB with 0.0025% NADH and 1% sterile filtered heat-inactivated chicken serum) to yield an inoculum density of approximately 5×10^5 CFU/ml. A volume of 50 μl of this suspension was added to each well of the microtiter plates. Reading of microtiter

plates was done visually after 16 to 20 h and 24 h of incubation of plates in an ambient-air incubator. An inoculum density of approximately 5×10^5 CFU/ml was verified by inoculum control as described in document M07-A9. To assess the homogeneity of MICs, broth microdilution susceptibility testing was performed in five replicates. The quality control strains *Escherichia coli* (ATCC 25922), *Enterococcus faecalis* (ATCC 29212), *Staphylococcus aureus* (ATCC 29213), and *A. pleuropneumoniae* (ATCC 27090) served for quality control procedures along with their CLSI-recommended media (17, 24).

When comparing MICs generated in TMB and supplemented CAMHB, all 47 *H. parasuis* isolates were used.

Data evaluation and statistical analysis. The primary aims of this study were the exploration of MICs and the investigation of differences in MICs when using two different media (supplemented CAMHB and TMB). Of secondary interest was a comparison of the stability of MICs after 16 to 20 h and 24 h of incubation.

To investigate whether the stability of the MICs differed between the two incubation times, for each combination of isolate, medium, antibiotic agent and incubation time, MIC data received from five measurements were summarized to a dichotomous outcome. Evaluation was done by taking one model with two variables (precondition of five identical MICs, here referred to as exact MIC agreement, and MIC mode accepting a deviation of ± 1 dilution step, referred to as essential MIC agreement, respectively) into consideration. The outcome was set to 1 if preconditions were fulfilled according to the variable and 0 otherwise. Stability of measurements was analyzed based on this outcome with a multifactorial logistic regression model depending on isolate, medium, antibiotic agent, and incubation time. Differences in MICs depending on incubation time were investigated with an analysis of variance (ANOVA) model, modeling the log-transformed MICs depending on the four factors. Due to the distribution of MICs being skewed to the right and a 2-fold dilution series being used, data were log transformed to the base 2. The media were compared separately for antibiotic agents using the two-sided one-sample *t* test for the differences between the two media. Due to the explorative nature of the experiments, no multiple adjustments were performed and comparison-wise *P* values were reported. When analyzing the data, antimicrobial agents were omitted from statistical analysis when more than 50% of isolates had a MIC at the lowest concentration tested. Statistical analyses were carried out using SAS software, version 9.3 (SAS Institute, Cary, NC) (25).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JCM.01403-16.

TEXT S1, PDF file, 0.2 MB.

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