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Standardization of Broth Microdilution and Disk Diffusion Susceptibility Tests for *Actinobacillus pleuropneumoniae* and *Haemophilus somnus*: Quality Control Standards for Ceftiofur, Enrofloxacin, Florfenicol, Gentamicin, Penicillin, Tetracycline, Tilmicosin, and Trimethoprim-Sulfamethoxazole

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Quality control (QC) standards for the in vitro antimicrobial susceptibility testing of two fastidious veterinary pathogens, *Actinobacillus pleuropneumoniae* and *Haemophilus somnus*, were developed in a multilaboratory study according to procedures established by the National Committee for Clinical Laboratory Standards for broth microdilution and disk diffusion testing. The medium recommended for the broth microdilution testing is cation-adjusted Mueller-Hinton broth supplemented with 2% lysed horse blood, 2% yeast extract, and 2% supplement C. This medium has been designated veterinary fastidious medium. The medium recommended for the disk diffusion testing is chocolate Mueller-Hinton agar. The recommended QC organisms are *A. pleuropneumoniae* ATCC 27090 and *H. somnus* ATCC 700025. The QC MICs of ceftiofur, enrofloxacin, florfenicol, gentamicin, penicillin, tetracycline, tilmicosin, and trimethoprim-sulfamethoxazole were determined for each isolate, as were the zone size ranges. Of the results from the participating laboratories, 94.0% of the zone diameter results and 97.0% of the MIC results fell within the suggested QC ranges for all compounds. These QC guidelines should allow greater accuracy in interpreting results when testing these antimicrobial agents against fastidious pathogens.

Actinobacillus pleuropneumoniae and *Haemophilus somnus* are fastidious bacteria commonly encountered in clinical specimens from swine and cattle, respectively. *A. pleuropneumoniae* is isolated primarily from the respiratory tracts of diseased pigs (2, 5) with acute pneumonia and/or chronic pleuritis. *H. somnus* has been associated with acute respiratory disease, meningoencephalitis, myocarditis, arthritis, and reproductive tract infections in cattle and with pneumonia, mastitis, polyarthritis, meningitis, epididymitis, orchitis, and septicemia in sheep (2, 8). Because diseases caused by these organisms may be life-threatening, it is essential that an appropriate antibacterial therapy be initiated as quickly and accurately as possible. Traditionally, veterinary practitioners have been able to rely on clinical experience or published information (e.g., package inserts) to empirically choose an effective antibacterial agent. However, the widespread trends toward decreased susceptibility in numerous animal (12, 13) and human (9) bacterial pathogens make accurate antimicrobial selection more important. In order to determine an anti-infective agent to which these organisms may be susceptible in vivo, clinical isolates must be subjected to in vitro antimicrobial susceptibility testing by using procedures standardized for the bacterial pathogen to be tested. Due to the fastidious growth requirements of *A. pleuropneumoniae* and *H. somnus*, they cannot be tested accurately by use of the methods described by the National Committee for Clinical Laboratory Standards (NCCLS) for the in vitro antimicrobial susceptibility testing of rapidly growing organisms such as the *Enterobacteriaceae* and other pathogens isolated from clinical specimens.

To address the need for a reliable and reproducible susceptibility testing procedure for these organisms, the NCCLS Subcommittee on Veterinary Antimicrobial Susceptibility Testing established a working group. A multiphase project was developed that would lead to a recommended method for testing these bacteria and provide quality control information. The objectives of this study were threefold: first, to identify a medium for the in vitro testing of clinical isolates of *A. pleuropneumoniae* and *H. somnus* using broth microdilution and disk diffusion testing procedures; second, to identify reference strains of these organisms; and third, to establish quality control (QC) ranges for the reference strains when testing ceftiofur, enrofloxacin, florfenicol, gentamicin, penicillin, tetracycline, tilmicosin, and trimethoprim-sulfamethoxazole.

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MATERIALS AND METHODS

Participating laboratories. Initial studies to identify QC strains of *A. pleuropneumoniae* and *H. somnus* were conducted in three laboratories: Microbiology

Reference Laboratories, Franklin, Tenn.; University of Iowa College of Medicine, Iowa City; and College of Veterinary Medicine, Michigan State University, East Lansing. Studies to determine the QC limits for broth microdilution and disk diffusion testing of *A. pleuropneumoniae* and *H. somnus* were conducted in six collaborating laboratories. The six laboratories included the three sites mentioned above as well as the Clinical Microbiology Institute, Wilsonville, Oreg.; Animal Disease Diagnostic Laboratory, Purdue University, West Lafayette, Ind.; and Infectious Disease Research Laboratory, College of Medicine, Michigan State University, East Lansing.

Bacterial strains and growth conditions. Initial testing to select the QC strains involved 14 strains of *A. pleuropneumoniae* and 11 strains of *H. somnus*. Three *A. pleuropneumoniae* strains were from the American Type Culture Collection (ATCC), as were two of the *H. somnus* strains. The remaining *A. pleuropneumoniae* and *H. somnus* strains were clinical isolates from specimens submitted to the Diagnostic Bacteriology-Mycology Laboratory at the College of Veterinary Medicine, Michigan State University. These organisms were selected as possible QC organisms based on their ability to survive repeated passage on artificial media while retaining their original growth characteristics and antibiograms. In an attempt to determine an agar and a broth medium that would support the rapid growth of clinical isolates of *A. pleuropneumoniae* and *H. somnus*, several different formulations were tested. These included chocolate agar, gonococcus medium, *Haemophilus* test medium (HTM), brucella agar and broth, Mueller-Hinton (MH) agar and broth, and PPLO agar, with and without various supplements, including lysed horse blood, supplement C, and yeast extract. These media were also tested for the intralaboratory reproducibility of susceptibility testing results for selected strains from each species.

From these original 25 isolates, two strains of *A. pleuropneumoniae* and two strains of *H. somnus* were chosen for subsequent testing by three laboratories (Microbiology Reference Laboratories, the Michigan State University College of Veterinary Medicine, and the University of Iowa College of Medicine) to determine inter- and intralaboratory reproducibility when media from different manufacturers were used. These studies resulted in the identification of single strains of *A. pleuropneumoniae* (ATCC 27090) and *H. somnus* (ATCC 700025). The selection criteria were intralaboratory and interlaboratory reproducibility of MICs and zone diameters on the test media from different sources. The two organisms were then sent to six laboratories for additional testing in accordance with the guidelines outlined in NCCLS document M37-A (11).

Antimicrobial agents. Ceftiofur, enrofloxacin, florfenicol, gentamicin, penicillin, tetracycline, tilmicosin, and trimethoprim-sulfamethoxazole were the agents assayed in this study. For the broth microdilution testing, the MIC trays were prepared by Prepared Media Laboratory (Wilsonville, Oreg.). Disks with enrofloxacin, florfenicol, tetracycline, and tilmicosin were obtained from Difco (Detroit, Mich.), and disks with ceftiofur, gentamicin, penicillin, tetracycline, and trimethoprim-sulfamethoxazole were obtained from Becton Dickinson Microbiology Systems (BDMS) (Cockeysville, Md.). For disk diffusion testing, two different lots of susceptibility testing disks were used for florfenicol, gentamicin, penicillin, tetracycline, and trimethoprim-sulfamethoxazole. For ceftiofur, enrofloxacin, and tilmicosin, separate lots of disks were unavailable, and thus the tests included two disks from the same lot.

Broth microdilution susceptibility testing. The MICs of each antibacterial agent were determined for each QC strain by using veterinary fastidious medium (VFM). VFM was composed of cation-adjusted MH (CAMH) broth supplemented with 2% lysed horse blood (Cleveland Scientific, Bath, Ohio), 2% supplement C, and 2% yeast extract (BDMS) in a microdilution format in accordance with NCCLS standards (10). CAMH broth base from four manufacturers (Difco; BDMS; Accumedia [now Neogen], Baltimore, Md.; and Oxoid, Basingstoke, England) was used to prepare the microdilution trays. Microdilution trays containing a common lot of CAMH broth (Difco lot 92191 JB) and a unique lot of CAMH broth from one of four manufacturers (Difco lot 69437 JB or 95642 JC, BDMS lot A8DFKF or C7DD1F, Accumedia lot 9407166, or Oxoid lot 46881) were also tested in each laboratory. The CAMH broths were prepared in accordance with the manufacturers' instructions.

The QC organisms were grown for 20 to 24 h on chocolate MH agar in a 5% CO_2 environment. Inocula were prepared by suspending each organism in sterile water to obtain a turbidity equivalent to that of a McFarland standard of 0.5. The suspension was further diluted to provide a final inoculum concentration of approximately 5×10^5 CFU/ml in the wells of the broth microdilution trays. Colony counts were performed for each inoculum to ensure appropriate cell concentrations. The microdilution trays were incubated at 35°C in a 5% CO_2 environment for 20 to 24 h prior to determination of MICs.

Disk diffusion tests. Disk diffusion tests were performed on each QC strain with each antibacterial agent according to the method described by the NCCLS (10) using 150-mm-diameter chocolate MH agar plates. The chocolate MH agar was prepared by Prepared Media Laboratory as described in NCCLS document M31-A (10) and distributed to participating laboratories. Each laboratory was provided with agar plates prepared from a common lot (Difco lot 71463 JB) and a unique lot from one of four manufacturers (Difco lot 69565 JB or 90306 JE, BDMS lot G9DGSN or G9DSP, Accumedia lot 9505124, or Oxoid lot 56573). The plates were inoculated with an organism suspension in sterile distilled water with a turbidity equivalent to that of a McFarland standard of 0.5. Each assay on both the unique lot and the common lot involved testing two different lots of disks, when available, for each antimicrobial agent. If two lots were not available, two disks of the same lot were tested for that drug. The disks used in the study contained ceftiofur (BDMS lot 605654), enrofloxacin (Difco lot 92974JA), florfenicol (Difco lots 95618JA and 91133JA), gentamicin (BDMS lots 504603 and 603595), penicillin (BDMS lots 603611 and 512600), tetracycline (Difco lot 70690JB and BDMS lot 412597), tilmicosin (Difco lot 54115JB), and trimethoprim-sulfamethoxazole (BDMS lots 603581 and 603681). The disk diffusion plates were incubated at 35°C in a 5% CO_2 environment for 20 to 24 h prior to measurement of the diameters of the zones of inhibition.

Testing protocol. The study was carried out in accordance with the guidelines in NCCLS document M37-A (11). Each laboratory performed broth microdilution tests for each QC organism with media prepared from a unique lot of media and media from a common lot. For the unique lot, each laboratory prepared 20 inocula over a period of at least 3 days. For the common lot, each laboratory prepared five inocula over a period of at least 3 days. This resulted in a total of 25 MICs being generated by each laboratory for each antimicrobial agent. For disk diffusion testing, each laboratory tested both QC organisms with media from a unique lot and with media from a common lot. For the unique lot, each laboratory prepared 20 inocula over a period of at least 3 days, whereas for the common lot, each laboratory prepared 10 inocula over a period of at least 3 days. The replicate testing was performed over a period of 3 or more days in order to assess interlaboratory reproducibility in generating MICs and zone diameters with each drug. Data from all of the laboratories were analyzed to establish acceptable QC limits for MICs and zone diameters with each drug in accordance with the guidelines described in NCCLS document M37-A (11).

RESULTS

Several different medium formulations, including GC agar base and HTM, were examined in an attempt to obtain reliable growth of clinical isolates of these fastidious organisms, both in broth medium and on an agar surface. One criterion for selecting a susceptibility testing medium was optimal growth of clinical isolates within a 24-h incubation interval. While several different formulations supported the growth of many clinical isolates, the formulation designated VFM resulted in the best growth of nearly all of the clinical isolates that were tested; therefore, it was selected as the broth growth medium for subsequent testing. This medium formulation in an agar form also provided adequate growth, growth comparable to that obtained with chocolate MH agar. Since chocolate MH agar is more widely used and is commercially available, it was selected as the growth medium for disk diffusion testing.

Selection of QC strains of *A. pleuropneumoniae* and *H. somnus* from ATCC isolates and clinical isolates was based both on rates of survival and phenotypic stability following many passages on artificial media and on inter- and intralaboratory reproducibility of in vitro antimicrobial susceptibility testing results. The *A. pleuropneumoniae* strain selected was an ATCC isolate, whereas the *H. somnus* strain was a clinical isolate that was subsequently submitted to the ATCC and designated ATCC 700025.

The participating laboratories used VFM broth lots unique to their location and a VFM broth common to all laboratories. Each isolate was tested 20 times using the unique broth lot and five times using the common lot, for a total of 150 tests per isolate per antibacterial agent. Table 1 summarizes the MICs and the QC limits for the eight antimicrobial agents tested for

TABLE 1. Broth microdilution QC results for *A. pleuropneumoniae* ATCC 27090 using VFM medium[a]

Antimicrobial agent	MIC range (μg/ml)	Mode(s) (μg/ml)	Recommended QC range (μg/ml)	% of MICs:	
				At mode	Within QC range
Ceftiofur	0.002–0.03	0.008	0.004–0.016	57	97
Enrofloxacin	0.008–0.06	0.03	0.016–0.06	68	97
Florfenicol	0.125–0.5	0.5	0.25–1	82	99
Gentamicin	4–32	16	8–32	51	97
Penicillin	0.125–0.5	0.25, 0.5	0.125–1	81	100
Tetracycline	0.125–1	0.5	0.25–2	56	99
Tilmicosin	2–16	8, 16	4–32	92	99
Trimethoprim-sulfamethoxazole[b]	0.015–0.06	0.03, 0.56[c]	0.015–0.06	79	100

[a] VFM was made using seven lots of MH broth, with six lots used uniquely by one laboratory and the seventh lot common to all six laboratories.
[b] Present in a 1:19 ratio.
[c] First value indicates mode for trimethoprim; second value indicates mode for sulfamethoxazole.

A. pleuropneumoniae. The results were highly reproducible. The recommended QC ranges include the observed modal MIC ± 1 $\log_2$ dilution for all drugs except penicillin, tetracycline, and tilmicosin. Penicillin and tilmicosin MICs had dual modes, while the upper QC limit of tetracycline was two dilutions above the observed mode. The modes for penicillin and tilmicosin were 0.25 and 0.5 μg/ml and 8 and 16 μg/ml, respectively. The MIC QC limits for ceftiofur, enrofloxacin, florfenicol, gentamicin, and trimethoprim-sulfamethoxazole encompassed more than 97% of the observed values. The MIC ranges of penicillin, tetracycline, and tilmicosin spanned 4-$\log_2$ dilution steps and encompassed more than 99% of the participant values generated for *A. pleuropneumoniae*.

For *H. somnus*, the recommended QC ranges include the observed modal MIC ± 1 $\log_2$ dilutions for enrofloxacin, florfenicol, gentamicin, and penicillin (Table 2). Ceftiofur had dual modes at 0.001 and 0.002 μg/ml, which resulted in the MIC limits spanning a 4-$\log_2$ dilution range; the MIC limits for tetracycline and tilmicosin spanned similar ranges. The accepted MIC QC ranges encompassed 99 to 100% of the observed values for all of the antibacterial agents tested, except for gentamicin and tilmicosin, for which the accepted MIC QC ranges encompassed 97 and 95% of the participant values, respectively.

Each participating laboratory used chocolate MH agar lots unique to their location and an agar lot common to all laboratories. Each isolate was tested 20 times with the unique agar lot and 10 times with the common lot. In addition, each laboratory tested two lots of disks for florfenicol, gentamicin, penicillin, tetracycline, and trimethoprim-sulfamethoxazole and a single lot in duplicate for ceftiofur, enrofloxacin, and tilmicosin. This resulted in a possible total of 60 tests by each laboratory for each antibacterial agent, for a total of 360 test values for each bacterium-drug combination. When enrofloxacin and penicillin were tested against *A. pleuropneumoniae*, one laboratory recorded zone diameters that were inconsistent with the zone diameters generated by the other participating laboratories for the common lot medium. A second laboratory recorded zone diameters that were consistently smaller than those recorded by the other five laboratories in testing ceftiofur, penicillin, and tetracycline against *H. somnus* with the common lot medium. Because these zone diameters varied relative to the zone diameters recorded by the other laboratories on the common lot media, zone diameter data recorded by these two laboratories on common and unique agar lots for those antibacterial agents were excluded from the data analysis. This resulted in a total of 300 data points for each bacterium-antibacterial agent combination.

Table 3 summarizes the zone diameter ranges of *A. pleuropneumoniae* and the QC limits of the eight antimicrobial agents tested against it. The zone diameter QC ranges for each organism-antibacterial agent combination were determined using a modification of the median method described by Gavan et al. (6). The percentage of participant zone diameters that

TABLE 2. Broth microdilution QC results for *H. somnus* ATCC 700025 using VFM[a]

Antimicrobial agent	MIC range (μg/ml)	Mode(s) (μg/ml)	Accepted QC range (μg/ml)	% of MICs:	
				At mode	Within QC range
Ceftiofur	0.001–0.004	0.001, 0.002	0.0005–0.004	95	99
Enrofloxacin	0.015–0.03	0.03	0.015–0.06	88	100
Florfenicol	0.125–0.25	0.25	0.125–0.5	87	100
Gentamicin	2–16	16	8–32	69	97
Penicillin	0.015–0.06	0.03	0.015–0.06	72	100
Tetracycline	0.25–0.5	0.5	0.125–1.0	59	100
Tilmicosin	0.25–16	8	2–16	53	95
Trimethoprim-sulfamethoxazole[b]	0.015–0.125	0.03, 0.06, 0.125[c]	0.03–0.125	99	99

[a] VFM was made with seven lots of MH broth, with six lots used uniquely by one laboratory and the seventh lot common to all six laboratories.
[b] Present in a 1:19 ratio.
[c] First and second values indicate modes for trimethoprim; third value indicates mode for sulfamethoxazole.

TABLE 3. Disk diffusion QC results for *A. pleuropneumoniae* ATCC 27090 using chocolate MH agar[a]

Antimicrobial agent	Disk content of antimicrobial	Zone diam (mm)			% of zone diam within proposed QC range
		Interlaboratory range	Median	Proposed QC range	
Ceftiofur	30 μg	30–43	37	34–42	98
Enrofloxacin	5 μg	30–40	34	31–38	96
Florfenicol	30 μg	29–42	35	31–40	95
Gentamicin	10 μg	12–20	17	15–19	96
Penicillin	10 U	29–37	32	29–36	98
Tetracycline	30 μg	17–31	26	23–30	97
Tilmicosin	15 μg	8–21	10	8–13	95
Trimethoprim-sulfamethoxazole	1.25 μg, 23.75 μg[b]	27–33	31	28–32	98

[a] Chocolate MH agar was made with six lots of media, with five lots used uniquely by one laboratory and the sixth lot common to all five laboratories.
[b] First value indicates disk content of trimethoprim; second value indicates disk content of sulfamethoxazole.

fell within the recommended QC ranges exceeded 95% for each antibacterial agent. The interlaboratory range and the QC range of the zone diameters of *H. somnus* for the eight antibacterial agents tested against it are shown in Table 4. The recommended QC ranges encompassed ≥95% of the results for each antimicrobial agent, except for ceftiofur and gentamicin, for which they encompassed 94%.

DISCUSSION

It is known that standardized in vitro antimicrobial susceptibility testing methods are reliable indicators of antibacterial effectiveness in vivo (4). The current QC parameters are important for susceptibility testing, serving as a mechanism for determining whether day-to-day testing results within and between laboratories are accurate and reliable. Due to the fastidious growth requirements of *A. pleuropneumoniae* and *H. somnus*, standardized methods for the in vitro antimicrobial susceptibility testing of clinical isolates of these species have not been previously available. The results of this multicenter study established a standardized method for the broth microdilution and disk diffusion susceptibility testing of these two organisms when tested against eight drugs (ceftiofur, enrofloxacin, florfenicol, gentamicin, penicillin, tetracycline, tilmicosin, and trimethoprim-sulfamethoxazole). These trials also identified *A. pleuropneumoniae* ATCC 27090 and *H. somnus* ATCC 700025 as the QC strains.

Several different media were assayed, including HTM and GC agar base. Supplementation of MH agar (as described above) provided optimal growth of clinical isolates in a 24-h time frame. CAMH broth was analyzed to determine interlaboratory reproducibility. Initially, CAMH broth with 5% lysed horse blood provided optimal growth of the clinical isolates being tested. However, 5% lysed horse blood could not be dispensed reliably by commercial suppliers making microtiter trays. Thus, the proportion of lysed horse blood was reduced and the medium was supplemented with supplement C. These modifications did not affect reproducibility among participating laboratories and resulted in a growth medium formulation that supported all of the isolates tested.

Isolates of *A. pleuropneumoniae* and *H. somnus* are frequently associated with life-threatening diseases that may involve high mortality rates in infected herds, resulting in substantial economic loss (3, 7). Changes in antimicrobial susceptibility patterns among many bacterial pathogens are making empiric treatment less reliable. In addition, the American Veterinary Medical Association's judicious antimicrobial use guidelines emphasize the importance of identifying the etiologic agent before the initiation of therapy and selecting the most appropriate antimicrobial (1). Selection of the most appropriate antimicrobial agent frequently requires in vitro antimicrobial susceptibility testing. To ensure intra- and interlaboratory reproducibility, QC organisms with defined control ranges for each agent tested must be established. The proposed control ranges and testing conditions described in this report, as well as the QC organisms, have been accepted by the NCCLS Subcommittee for Veterinary Antimicrobial Susceptibility Testing for susceptibility testing of *A. pleuropneumoniae* and *H. somnus* and are published in document M31-A (10). The development of these standards for determining the anti-

TABLE 4. Disk diffusion QC results for *H. somnus* with chocolate MH agar[a]

Antimicrobial agent	Disk content of antimicrobial	Zone diam (mm)			% of zone diam within proposed QC range
		Interlaboratory range	Median	Proposed QC range	
Ceftiofur	30 μg	34–46	40	36–46	94
Enrofloxacin	5 μg	30–40	36	32–38	97
Florfenicol	30 μg	33–46	39	34–44	96
Gentamicin	10 μg	13–28	17.5	14–22	94
Penicillin	10 U	35–45	40	35–44	99
Tetracycline	30 μg	27–35	30	27–33	97
Tilmicosin	15 μg	6–17	10	8–16	96
Trimethoprim-sulfamethoxazole	1.25 μg, 23.75 μg[b]	25–33	29	26–32	99

[a] Chocolate MH agar was made with six lots of media, with five lots used uniquely by one laboratory and the sixth lot common to all five laboratories.
[b] First value indicates disk content of trimethoprim; second value indicates disk content of sulfamethoxazole.

bacterial susceptibility of *A. pleuropneumoniae* and *H. somnus* will greatly aid the veterinary practitioner in choosing an appropriate agent for treating infections caused by these fastidious pathogens. In addition, the quality of the data gathered in surveillance studies and clinical trials will be improved by reducing the variability among diagnostic laboratories.

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