USE OF A COMMERCIAL BROTH MICRODILUTION TECHNIQUE FOR TESTING THE SUSCEPTIBILITY OF *HAEMOPHILUS SOMNUS* TO ANTIMICROBIALS

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Introduction

H. somnus has been recognized as an important opportunistic pathogen associated with bovine morbidity worldwide. While data are available on the antimicrobial susceptibility of *H. somnus* these have been generated using the agar dilution (6,7) or disc diffusion (1) techniques. A broth medium is available for the testing of some *Haemophilus* species (4) although it has not been recommended for testing *H. somnus*. The broth microdilution technique offers advantages of economy, convenience and reproducibility over more traditional methods of susceptibility testing (3). In particular, the Sensititre® system, using commercially prepared antimicrobial plates with extended room temperature storage, is well suited to veterinary diagnostic laboratories. The objective of this study was to adapt the Sensititre broth microdilution technique for determination of the antimicrobial susceptibility of *H. somnus* field isolates.

Materials and Methods

Bacterial strains

Eleven strains of *H. somnus*, isolated from cattle with bovine respiratory disease, were used during this study. *H. somnus* strains were grown on Brain Heart Infusion agar (Difco: code 0418-01-5), supplemented with 70 ml/l defibrinated bovine blood (BBL: code 12379) and 10 ml/l IsoVitalex (BBL: code 11876). Following overnight incubation at 37°C, in a 5% (v/v) CO_2-in-air atmosphere, the *H. somnus* were visible as small, grey, butyric colonies, with a distinct lemon-yellow pigment when picked from the agar surface with an inoculating loop.

Antimicrobial plates

Round-bottom 96-well microtitre plates containing the stabilized freeze-dried antimicrobials were prepared by Sensititre Ltd. Each antimicrobial was incorporated into one or more columns of the plate in a doubling dilution pattern over an appropriate range of concentrations. The antibiotics included amoxycillin, apramycin, erythromycin, furaltadone, lincomycin/spectinomycin (1:2 ratio), oxytetracycline and tylosin. An antimicrobial-free well was included as a growth control.

Growth medium

The *H. somnus* basal broth medium contained (g/l): proteose peptone, 15 (Difco: code 0120-01-4); dextrose, 2 (Difco: code 0155-17-4); soluble starch, 10 (Difco: code 0178-17-7); sodium chloride, 5 (Fisher Scientific: code S-671), autolyzed yeast extract, 5 (Sigma Chemicals: code Y-0375). The ingredients were dissolved in 940 ml warm distilled water, the pH adjusted to 7.1±0.1 units, then the medium dispensed into 9.4 ml aliquots in glass, screw capped tubes. Sterilization was by autoclaving at 115°C for 10 minutes. The basal medium could be stored at 4°C for up to two weeks, prior to use.

When required for use, 0.5 ml defined equine serum (Hyclone:code A-3311; filter sterilized through 0.1µ filter) and 0.1 ml thiamine monophosphate stock solution (Sigma Chemicals: code T-8637; stock solution containing 200 µg/ml in distilled water, filter sterilized through 0.22µ filter) were added aseptically to each tube of basal medium.

MIC determination

Using a sterile cotton-tipped swab, 3 to 5 discrete colonies were removed from an overnight agar culture of *H. somnus* and were emulsified in 4 ml of sterile distilled water. The cell density was then adjusted to approximate a 0.5 MacFarland standard. Ten ml of growth medium was inoculated with 100 µl of the standardized cell suspension and homogenized, resulting in a cell density of approximately 10^6 cfu/ml. Fifty µl of inoculated medium was placed into each well of a Sensititre® plate using an eight-channel pipette. The plate was firmly covered with a transparent adhesive seal, and incubated at 36°C ±1°for 18-24 hours. Following incubation, plates were examined using an inverted mirror reader and an oblique light source. Growth of *H. somnus* in this medium was visible as a light granular pattern, adhering to the well. If growth occurred in the growth control well, the MIC was recorded as the lowest concentration of each antimicrobial tested, that inhibited visible growth. Each *H. somnus* isolate was tested on four consecutive days and the results examined for reproducibility.

Results

In the test system used, two types of variation in MIC value were possible: the reproducibility inherent in the technique used and the variation that resulted from susceptibility differences between individual *H. somnus* isolates. Table 1 shows the reproducibility of the technique over four replications. Table 2 shows the distribution of MIC values for the seven antimicrobials against the eleven *H. somnus* isolates.

Reproducibility

The MIC values obtained for a given isolate and antimicrobial combination, when repeated four times allowed an estimate of the reproducibility of the technique (Table 1). For the majority of antimicrobials tested, replication gave an identical result or differed by one of two adjacent $\log_2$ dilutions. Tylosin and furaltadone showed greater variation, with a small proportion of replications giving results within three adjacent $\log_2$ dilution steps. None of the replicate results showed variation in excess of this.

Table 1: Reproducibility of individual *H. somnus* MIC values over four replications.

Antimicrobial	Percentage isolates with variability of: zero	one well	two wells
amoxycillin	100	-	-
apramycin	82	18	-
erythromycin	100	-	-
furaltadone	73	25	2
linco/spect* (1:2 ratio)	82	18	-
oxytetracycline	71	29	-
tylosin	91	7	2

Table 2: Distribution of MIC values for seven antimicrobials against eleven *H. somnus* strains.

	Percentage* distribution for each value tested (concentration μg/ml):							
Antimicrobial	0.5	1	2	4	8	16	32	64
amoxycillin	100	-	-	-	-	-	-	-
apramycin	-	-	-	7	32	61	-	-
erythromycin	100	-	-	-	-	-	-	-
furaltadone	-	2	11	73	14	-	-	-
linco/spect+	-	32	68	-	-	-	-	-
oxytetracycline	-	-	43	57	-	-	-	-
tylosin	-	2	16	82	-	-	-	-

*Percent of results obtained at each MIC value tested. +lincomycin:spectinomycin in ratio of 1:2, concentration of lincomycin shown.

Antimicrobial susceptibility

When the range of susceptibility against each of the antimicrobials was considered (Table 2), all isolates could be regarded as susceptible to amoxycillin, erythromycin, lincomycin/spectinomycin, oxytetracycline and tylosin, with MIC values of ≤ 4 μg/ml. The majority of isolates were sensitive to furaltadone, but with apramycin, 93% of isolates had an MIC of ≥ 8 μg/ml.

The greatest susceptibility differences between the individual isolates for a particular antimicrobial were measured with furaltadone, where MIC values over a range of 1-8 μg/ml were obtained. The least variation was with amoxycillin and erythromycin where all MIC values were 0.5 μg/ml, the lowest concentration of each tested.

Discussion

Few guidelines are available for the interpretation of veterinary MIC data, however the broth microdilution method of antimicrobial susceptibility testing has been extensively validated for human pathogens (5). In the resulting standards it is recommended that MIC values for reference isolates should be within ± one $\log_2$ dilution of the expected value. No reference isolates were available, but the reproducibility of this technique when used with field isolates meets these guidelines.

It has been demonstrated by Gavan *et al* (3) that the use of commercially available Sensititre® plates, accurately predosed with antimicrobials, can significantly improve the reproducibility of the broth microdilution technique by removing the variation associated with the lengthy preparation of antimicrobial dilutions. The extended room temperature stability of Sensititre® plates offers a further advantage for diagnostic laboratories.

The MIC results obtained here for amoxycillin are in agreement with previously reported data (7) which showed beta-lactam susceptibility in all 29 strains of *H. somnus* tested. Sugimoto *et al* (6), in a broader survey of *H. somnus* isolates, showed similar results to ours for oxytetracycline and tylosin but lower susceptibility to erythromycin and lincomycin. This is not unexpected, since they used an agar dilution technique incubated under 10% carbon dioxide, and, it has been well documented that macrolide activity decreases with decreasing pH (2). In addition, we did not test lincomycin alone, but rather in combination with spectinomycin. Corboz (1) in a study of 115 *H. somnus* isolates, obtained similar results to ours using the disc diffusion test, with all isolates susceptible to ampicillin, erythromycin and spectinomycin, and some 74% susceptible to lincomycin.

In this exploratory study, only eleven isolates were tested, however the reproducibility observed, together with agreement between these and existing data on the antimicrobial sensitivity of *H. somnus*, suggests that this Sensititre technique can be considered as an alternative to previously described methods for the testing of *H. somnus* field isolates.

References

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Summary

A broth medium capable of supporting growth of *Haemophilus somnus* is described. This medium was used in the Sensititre® broth microdilution system to determine the antimicrobial susceptibility of *H. somnus* field isolates. Eleven isolates were tested four times to assess reproducibility of the technique. Replicate results were generally identical or differed by only one of two adjacent dilutions. This technique offers a reproducible, convenient method for determining the antimicrobial susceptibility of *H. somnus*, without the need for the lengthy preparation of antimicrobial dilutions associated with existing methods. By this method, all isolates were susceptible to amoxycillin, erythromycin, lincospectin, oxytetracycline and tylosin. Most were susceptible to furaltadone, but resistant to apramycin.