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Comparison of culture methodology for the detection of methicillin-resistant *Staphylococcus pseudintermedius* in clinical specimens collected from dogs

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Abstract. Methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) has emerged as a major pathogen in dogs and has been implicated as a hospital-acquired pathogen in veterinary hospitals. We attempted to determine if selective culture methods will detect more MRSP when compared to the traditional culture methods in clinical samples from dogs in Atlantic Canada with a high risk for MRSP infection. Each sample was tested using 4 culture methods: traditional culture; mannitol salt agar with 2 μg/mL of oxacillin (MSAox); enrichment broth (EB) with MSAox; and EB with traditional culture. Detection of penicillin-binding protein 2', via latex agglutination, was used as a confirmatory test for oxacillin resistance. We analyzed 741 samples from 556 dogs between February 2013 and April 2014. The prevalence of MRSP in samples detected by any method was estimated at 13.4% (95% CI: 11.1–16.0%). When the prevalence of MRSP was determined according to culture method, EB with MSAox detected the highest prevalence (11.2% [9.1–13.7%]), followed by EB with traditional (10.8% [8.8–13.2%]), traditional (10.1% [8.1–12.5%]), and MSAox (8.9% [7.1–11.2%]). The prevalence using the traditional culture method did not differ significantly from any of the 3 selective culture methods. Culture with MSAox detected significantly fewer MRSP than either of the EB methods. The addition of EB to current methodology is recommended, particularly for patients considered at high risk for MRSP infection.

Key words: Antimicrobial resistance; dogs; prevalence; selective culture; sensitivity and specificity.

Introduction

Staphylococcus pseudintermedius is the organism isolated most frequently from clinical specimens collected from dogs. Methicillin-resistant *S. pseudintermedius* (MRSP) has emerged as a major pathogen in dogs, primarily associated with skin, surgical site, and wound infections.[1,20,27] The potential for MRSP to be involved in veterinary hospital-acquired infections has been reported, and the epidemiology in hospital environments continues to be investigated.[12,20,29] The accurate and rapid culture and antimicrobial susceptibility testing of MRSP is essential for delivering effective antimicrobial therapy and implementation of appropriate infection control measures in a timely manner.[15]

The prevalence of MRSP in subclinical or colonized patients (carriers) as well as in clinical specimens has been reported previously. The carriage of MRSP in dogs has been estimated to be 0–5%[7,11,19,21] except in one study reporting a 45% prevalence.[5] Reports of MRSP prevalence in dogs with clinical infections is 0–41%.[3,13,18,26] Direct comparisons of prevalence estimates between studies are difficult because of differences in culture methodology. Specifically, some studies used a selective staphylococcal enrichment broth (EB) or solid medium with or without oxacillin, the recommended surrogate for methicillin-resistance testing in *S. pseudintermedius*.[6] EBs have been used in studies investigating subclinical carriage of methicillin-resistant *Staphylococcus aureus* (MRSA) because they have been shown to increase the sensitivity of screening.[4] To our knowledge, differences between selective and nonselective culture methods for the recovery of MRSP from clinical specimens in dogs have not been reported.

Selective culture methodologies have the potential to be beneficial as rapid screening tools for the identification of MRSP in clinical specimens. Selective culture media would allow presumptive identification of MRSP as early as 24 h after receipt of a specimen, compared to at least 48 or 72 h using traditional culture methods. Molecular assays have been designed for rapid detection of *mecA*, which is considered the

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gold standard for methicillin-resistance testing.[4,15] However, these assays cannot readily differentiate between staphylococcal species (e.g. MRSP vs. MRSA) without bacterial culture and are not practical in smaller diagnostic laboratories. A culture-based assay for the rapid detection of MRSP in clinical specimens would be simple to implement and use in most veterinary diagnostic laboratories.

Numerous commercial media are available for detecting MRSA in humans,[8] but none are commercially available for MRSP detection in dogs. One study compared 6 commercial MRSA selective media for the detection and isolation of MRSP.[14] The authors of the study concluded that a commercial oxacillin resistance screening agar with a selective supplement (Oxacillin resistance agar screening base and ORSAB selective supplement, Oxoid, Nepean, Canada) or a chromogenic MRSA agar (Brilliance MRSA Agar, Oxoid, Basingstoke, UK) could be used routinely for culture of diagnostic samples obtained from canine patients.[14] However, the study used stored isolates of MRSP, a situation that is not representative of clinical sample testing. The commercial oxacillin resistance screening agar used in this prior study[14] was a modified mannitol salt agar with 2 μg/mL of oxacillin and 50,000 IU/L of polymyxin B (Oxoid).

Mannitol salt agar with 2 μg/mL of oxacillin (MSAox; Oxoid) was chosen as the selective medium in our study because is it similar to the oxacillin resistance screening agar used in the previous study on MRSP isolates.[14] As well, MSAox has been used in previous studies investigating MRSP in dogs,[3,19] and the medium is simple to prepare and has a low cost. In our study, we 1) compared different culture methods for detection of MRSP, 2) evaluated the utility of a selective culture medium for rapid culture-based detection and/or presumptive identification of MRSP in clinical specimens, and 3) estimated the prevalence of MRSP in clinical samples from canine patients in Atlantic Canada deemed at higher risk for MRSP infections.

Materials and methods

Study population

Samples analyzed in our study were submitted to the Atlantic Veterinary College Diagnostic Services Bacteriology Laboratory (AVC-DSBL, Charlottetown, PEI, Canada) for routine culture and susceptibility testing. Specimens from dogs at higher risk of having MRSP infection were included in the study. Specimens defined as being at higher risk for MRSP infections included samples primarily associated with MRSP infection in dogs: abscesses, surgical site infections (SSIs), wounds, or the ears, nose, eyes, and skin.[1,20,27] Any canine patient with a previous history of MRSP was also included in this study, regardless of specimen site.

Bacterial culture

A single swab (sample) was analyzed using 4 culture methods, in the following order: 1) traditional culture, 2) mannitol salt agar with 2 μg/mL of oxacillin (MSAox), 3) EB with MSAox, and 4) EB with traditional culture. The MSAox contained 75 g of NaCl (BDH sodium chloride, VWR, Radnor, PA), 10 g of peptone (Bacto peptone, Becton Dickinson, Sparks, MD), 10 g of mannitol (D-mannitol, Sigma-Aldrich, St. Louis, MO), 1 g of beef extract (BBL beef extract, Becton Dickinson), 0.03 g of phenol red (BDH phenol red, VWR), 15 g of agar (Acumedia, Lansing, MI), and 0.002 g of oxacillin (Sigma-Aldrich) per liter of water. The EB contained 10 g of tryptone (Becton Dickinson), 75 g of NaCl (Becton Dickinson), 10 g of mannitol (Sigma-Aldrich), and 25 g of yeast extract (Oxoid) per liter of water.

Traditional cultures were performed by AVC-DSBL and involved plating the sample to Columbia agar (Oxoid) with 5% defibrinated sheep blood (Quad Five, Ryegate, MT), MacConkey agar (Acumedia), and phenylethyl alcohol agar (Becton Dickinson) with 5% sheep blood (PEA). For samples collected from abscesses, SSIs, and wounds, a thioglycollate broth and smear for Gram staining were also included. The latter 3 culture methods were conducted in a research laboratory no more than 4 h later on the same day. Samples were plated to the MSAox, and placed into the EB. After 24-h incubation at 35°C, 10 μL of EB was plated to both the MSAox and PEA. All agar plates were incubated for 48 h, and growth was checked at 24 h. Any growth on MSAox was purified on PEA. Isolates with hemolysis that was characteristic of MRSP were further identified with tube coagulase and mannitol fermentation.

A multiplex PCR reaction was used to identify coagulase-positive staphylococci to the species level based on partial amplification of the *nuc* gene locus.[22] Methicillin resistance was detected following the Clinical Laboratory and Standards Institute standard[6] by testing antimicrobial susceptibility to oxacillin (1-μg disk, Oxoid) using disk diffusion and confirmed by detecting the penicillin-binding protein 2´ (PBP2´) by latex agglutination (Oxoid). Isolates were classified as methicillin-resistant if they were resistant to oxacillin and positive for PBP2´.

Statistical analyses

The data structure was hierarchical, in which individual samples were clustered by patients. The apparent prevalence of MRSP was estimated at sample and patient levels for each of the testing methods. At the sample level, MRSP detected by any 1 of the 4 culture methods was classified as a MRSP-positive sample. This sample level result was used as the overall MRSP result (i.e., the reference standard) in further analyses. At the patient level, a patient was considered MRSP-positive if any 1 of the samples submitted over the study period was positive for MRSP using any of the 4 culture methods. Descriptive statistics were applied to describe sample level factors, such as specimen source, and patient-level factors, including age, breed, sex, province of residence, and previous antimicrobial therapy. Patient information on the laboratory requisition form was recorded, although not all

fields on the requisition form were completed by all veterinarians submitting samples. Breeds were categorized using the Canadian Kennel Club Guidelines (https://goo.gl/bJuSJ7).

Logistic generalized estimating equations (GEEs) were used to estimate the sensitivity and specificity of each of the culture methods (model 1). The overall MRSP result was used as the reference standard for estimating test characteristics. Model 1 was restricted to true-positive or true-negative samples for sensitivity and specificity, respectively, based on the reference standard. Similarly, logistic GEEs were used to determine discordance between the culture methods (model 2). Model 2 included only samples with disagreement between 2 test methods, an extension of the McNemar test to account for clustering. In this model, the intercept represented the log-odds of the proportion of one disagreement scenario among all samples with test results that disagreed. Based on this model, we tested whether the probability of 1 disagreement scenario was equal to 50%, or equivalently that the 2 tests had equal apparent prevalence. Significant discordance was detected if $p \leq 0.05$, corresponding to systematic overrepresentation of one disagreement scenario over the other. Both GEE models included clustering on patients. The effect of test order was investigated by evaluating the sequence of positive results obtained. For all culture methods, the sensitivity was estimated including MRSP confirmation steps; specificity was not estimated because confirmed samples cannot have a false-positive result. To evaluate selective culture methods as a rapid screening tool, the sensitivity and specificity was estimated for MSAox alone and EB with MSAox at 24 h and 48 h based on growth or no growth without confirmatory steps. The true prevalence of MRSP in all submissions from dogs was estimated by adjusting the apparent prevalence obtained by the traditional method using the sensitivity of the traditional method relative to the 4 test methods (using equation 5.7).[10] Confidence intervals for the estimate of true prevalence were calculated using equations 4 and 5 as described previously.[16] All statistical computations were performed using commercial software (Stata/IC 13.1 for Mac, StataCorp, College Station, TX).

Results

Study population

We collected 741 samples from 556 individual dogs between February 2013 and April 2014. The majority of samples were from ears (48.9%), followed by skin (21.7%), SSIs (10.4%), wounds (8.4%), abscesses (7.4%), and other body sites (3.2%). Other body sites included anal glands (2), eyes (4), nose (12), peritoneal cavity (1), urine (1), uterus (1), and vagina (1). The specimens from other body sites were included and considered high risk for being MRSP positive because of a history of previous MRSP infection or antimicrobial treatment failure.

The median age of dogs in our study was 6.7 y with a range of 0.13–15.8 y. The ages of 40 dogs were not recorded on the submission form. The study samples included 82 breeds that were then subdivided into 8 breed categories. Breed was not recorded for 30 dogs. The study samples included 300 male patients and 242 female patients; sex was not recorded for 14 patients. The majority of patients visited veterinary clinics in Nova Scotia (256), followed by New Brunswick (136), Prince Edward Island (105), Newfoundland and Labrador (57), and Ontario (2).

Table 1. Estimates of prevalence and sensitivity from model 1 at the sample level, using 4 testing methods for the isolation of methicillin-resistant *Staphylococcus pseudintermedius* from clinical specimens (n = 741).

	Prevalence (%)	Sensitivity (%)
Traditional	10.1 (8.1–12.5)	74.9 (64.2–83.2)
MSAox	8.9 (7.1–11.2)	66.4 (56.1–75.3)
EB with MSAox	11.2 (9.1–13.7)	84.0 (75.0–90.1)
EB with traditional	10.8 (8.8–13.2)	80.8 (71.6–87.6)
Overall	13.4 (11.1–16.0)	100.0*

Numbers in parentheses are 95% confidence intervals. EB with MSAox = enrichment broth with mannitol salt agar with 2 μg/mL of oxacillin; EB with traditional = EB with routine diagnostic method; MSAox = mannitol salt agar with 2 μg/mL of oxacillin; Overall = a positive test result on any 1 of the 4 test methods; Traditional = routine diagnostic method.
* The overall method was used as the reference for positive samples.

Table 2. Discordance assessment between each test method, where the value listed is the p value for a statistical test where the 2 test methods have an equal prevalence.

	Traditional	MSAox	EB with MSAox
MSAox	0.208		
EB with MSAox	0.223	0.003*	
EB with traditional	0.322	0.015*	0.547

EB with MSAox = enrichment broth with mannitol salt agar with 2 μg/mL of oxacillin; EB with traditional = EB with routine diagnostic method; MSAox = mannitol salt agar with 2 μg/mL of oxacillin.
* Significant difference ($p \leq 0.05$).

Isolation of MRSP

MRSP was detected in 13.4% (95% confidence interval [CI]: 11.1–16.0%) of samples; 12.3% (95% CI: 9.6–15.2%) of patients were MRSP positive. SSI submissions had the highest prevalence of MRSP (33.8%), followed by skin (22.4%), wounds (14.5%), ears (7.2%), other (4.2%), and abscesses (1.8%). Differences in prevalence estimates between testing methods were observed, and the prevalence estimates for each testing method at the sample level were determined (Table 1). Evaluation of discordance between testing methods revealed significant differences in the recovery of MRSP between MSAox alone and EB with MSAox (p = 0.003) and MSAox alone and EB with the traditional method (p = 0.015; Table 2). MRSP was not detected in 24 samples (41.7% ears, 37.5% skin, 8.3% surgical, 8.3% wounds, and 4.2% nasal

Table 3. Estimates of sensitivity and specificity at the sample level, using mannitol salt agar with 2 µg/mL of oxacillin (MSAox) and enrichment broth (EB) with MSAox as a rapid screening tool for the detection of methicillin-resistant *Staphylococcus pseudintermedius* from clinical specimens (n = 741).

	Sensitivity (%)	Specificity (%)
MSAox at 24 h	36.6 (26.5–48.3)	97.9 (96.6–98.8)
MSAox at 48 h	69.9 (59.4–78.7)	82.6 (79.2–85.6)
EB with MSAox at 24 h	80.7 (70.6–87.9)	83.2 (79.9–86.1)
EB with MSAox at 48 h	89.7 (81.4–94.6)	72.9 (69.0–76.5)

Numbers in parentheses are 95% confidence intervals.

flush) using the traditional culture method: 16 samples had methicillin-susceptible *S. pseudintermedius*, 5 samples had non-staphylococci, whereas 3 samples had no microbial growth. MRSP was not detected in 16 samples (37.5% ears, 18.8% skin, 31.3% surgical, 6.2% wound, and 6.2% other) using EB and MSAox, and, in 6 of those samples, MRSP growth was scant-to-light on traditional culture.

Test characteristics

The test methods that included EB had the highest sensitivities compared to the other test methods evaluated in our study, and were significantly higher than the selective medium MSAox alone (Table 1). The EB with MSAox at both 24 h and 48 h had the highest sensitivity and lowest specificity, whereas MSAox at both 24 h and 48 h had the lowest sensitivity and highest specificity (Table 3). For MSAox alone and EB with MSAox, an additional 24 h of incubation increased the sensitivity but decreased the specificity.

True prevalence of MRSP in all canine submissions

During the study period, 2,037 canine samples were submitted to the AVC-DSBL for testing, which included samples from canine patients not considered to be at high risk for MRSP (n = 1,296) as well as the study population considered to be at high risk for MRSP (n = 741). Urine specimens accounted for 49% (n = 998) of the canine sample submissions to this laboratory. Most urine specimens (n = 647, 64.8%) yielded no microbial growth. Urine specimens that were culture-positive (n = 352) grew mainly *Escherichia coli* (n = 228, 43.6%), whereas MRSP was isolated from 5 (1.4%) culture-positive urine specimens based on traditional culture methods. Fecal specimens accounted for <1% of total canine sample submissions. MRSP was not isolated from fecal specimens during the study period. In total, MRSP was isolated from 13 (1.0%) of the samples not included in the study (n = 1,296) and from 99 (13.4%) of the high-risk samples included in the study (n = 741). The traditional method detected 75 of the MRSP-positive study samples (n = 741) and only 13 MRSP-positive samples not included in the study (n = 1296). The estimated true prevalence of MRSP in all submissions from dogs was 5.8% (95% CI: 4.6%–7.0%).

Discussion

MRSP was detected in 13.4% of higher-risk clinical samples from dogs submitted to the AVC-DSBL during the time period of our study. Samples were submitted from 556 individual dogs; 12.3% of the dogs had at least 1 MRSP-positive sample. The estimated true prevalence of MRSP in all submissions from dogs at this laboratory was 5.8%. The prevalence estimates in our study are similar to previous reports of prevalence in samples from clinically ill dogs.[2,3,9,17,26] The cumulative prevalence in our study may have biased this comparison, as most studies in the literature are point-prevalence studies. The estimates of MRSP prevalence reported for each test method by our study overestimate the prevalence of MRSP in canine submissions at this laboratory as certain sample types considered low risk were excluded.

MRSP has been reported to be the leading cause of SSIs and skin disease.[20] This trend was observed in our study, wherein MRSP was isolated from 33.8% of SSIs and 22.4% of skin infections. In a recent publication by the Ontario Veterinary College Health Sciences Centre, MRSP was isolated the most frequently from SSIs (47.4%, n = 26).[25] A high prevalence of methicillin-susceptible *S. pseudintermedius* in SSIs was reported in a study from Sweden, with only 4 of those isolates being methicillin-resistant.[28] Many studies have investigated the prevalence of MRSP in dogs with skin disease, with prevalence estimates of 0–40.5%.[2,3,13,23,26] The study reporting the highest prevalence of 40.5% involved dogs being evaluated by a dermatology referral service. The high prevalence could be explained by the patient population, which included dogs with chronic skin infections that were not responding to antimicrobial therapy.[3]

Although the discordance between the traditional method and any other culture method was not statistically significant, both testing methods that included an EB (EB with MSAox and EB with traditional culture) detected the most MRSP cases. The MSAox selective medium alone detected significantly less MRSP when compared to either method containing the EB. This shows that the addition of EB plays a key role in enhancing the detection of MRSP in clinical specimens from dogs. The prevalence of MRSP in studies that only used a selective agar plate without EB could therefore have underestimated prevalence, and caution should be exercised when making comparisons to such studies.[2,26,28]

When compared to each sample's overall MRSP result (if positive by any 4 test methods), EB with MSAox had the highest sensitivity of 84%. MSAox alone did not perform as well, detecting MRSP in only 8.9% of samples, achieving the lowest sensitivity of the tested methods (66.4%). Studies investigating MRSA culture methods using human isolates found that mannitol salt agar without oxacillin had the lowest sensitivity.[15,24] The design of our study likely underestimated

the sensitivity of the selective culture methods. The convenience samples used in our study were from submissions to the diagnostic laboratory only. In order to not interfere with the routine process, samples were first set up in the laboratory, which includes plating on up to 4 different culture media as well as preparation of a smear for Gram staining. This could have affected the sensitivity of subsequent testing, but upon inspection of the sequences of positive results obtained using the specified test order and the sensitivities of each of the 4 tests, no substantial effect of testing order was noted. It is possible that all viable bacterial organisms present in the specimen were inoculated to traditional culture media and thioglycollate broth, with insufficient numbers remaining for the selective culture methods. This could explain the cases where the selective culture methods did not detect MRSP in 16 samples that were positive by traditional culture methods.

When MSAox alone and EB with MSAox were evaluated as rapid screening tests, MSAox was out-performed by methods that included an EB step. The MSAox selective medium would not be ideal for use as a rapid screening test, as a large proportion of positive samples would not be detected at either 24 h or 48 h of incubation. Addition of EB does increase the sensitivity of MSAox; however, the extra day of incubation required slows the reporting time of the MRSP result. When the sensitivity of MSAox as a screening test was estimated for critical specimen types, such as SSIs, the sensitivity did not change (data not shown.)

In our study, there was an intrinsic selection bias that overestimated the reported prevalence of MRSP. Samples were conveniently collected from routine submissions to the main veterinary bacteriology laboratory for the Atlantic region. Samples are usually submitted to this laboratory because previous empirical antimicrobial therapy failed (e.g., non-response to antimicrobials), therefore, it can be assumed that such samples would have a higher likelihood of being positive for antimicrobial-resistant organisms, including MRSP. Also, we chose to include only high-risk samples, so the samples used in our study represent those samples with a potentially higher likelihood of being MRSP positive. This resulted in the inclusion of the majority of canine sample submissions to the laboratory except for urine and fecal specimens.

The estimated MRSP prevalence in our study is similar to findings in other parts of Canada and the rest of the world. The traditional culture method did not differ significantly from any of the test methods; however, both test methods that included EB detected more cases of MRSP. The MSAox selective agar should not be used alone because of its low sensitivity. This suggests that differences in prevalence estimates from different studies could be attributed to culture methodology. The isolation of MRSP from any clinical sample could impact therapeutic decisions, thus incorporating EB into the routine diagnostic testing method of high-risk specimens should be considered. Further work evaluating alternative methods for the detection of MRSP in clinical specimens should be explored, particularly for those laboratories in which molecular tools are unavailable.

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