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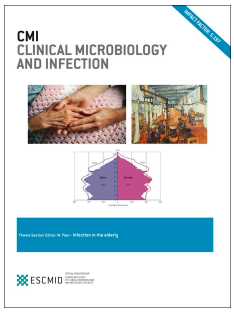

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EUCAST Disk Diffusion Criteria for the Detection of *mecA*-Mediated β-Lactam Resistance in *Staphylococcus pseudintermedius*: Oxacillin Versus Cefoxitin

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Keywords: *Staphylococcus pseudintermedius,* susceptibility testing, oxacillin, cefoxitin, methicillin resistance, MRSP

Objectives: Until recently, the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommended the cefoxitin disk to screen for *mecA*-mediated betalactam resistance in *Staphylococcus pseudintermedius.* A recent study indicated that cefoxitin was inferior to oxacillin in this respect. We have re-evaluated cefoxitin and oxacillin disks for screening for methicillin resistance in *S. pseudintermedius.* **Methods:** We included 224 animal and human *S. pseudintermedius* isolates from Europe (n=108) and North America (n=116), of which 109 were *mecA*-positive. Disk diffusion was performed per EUCAST recommendations using 30 µg cefoxitin and 1 µg oxacillin disks from three manufacturers and Mueller-Hinton agar from two manufacturers. **Results:** Cefoxitin inhibition zones ranged from 6-33 mm for *mecA*-positive *S. pseudintermedius* (MRSP) and from 29-41 mm for *mecA*-negative *S. pseudintermedius* (MSSP). The corresponding oxacillin zone intervals were 6-20 mm and 19 – 30 mm. For cefoxitin 16% (14.8%-18.0%, 95% CI) of the isolates were in the area where positive and negative results overlapped. For oxacillin the corresponding number was 2% (1.6%-2.9%). For oxacillin a breakpoint of S, ≥20 mm and R,<20 mm resulted in only 0.4% and 1.1% VME and ME rates respectively.
Conclusions: This investigation confirms that the 1 µg oxacillin disk predicts *mecA*-mediated methicillin resistance in *S. pseudintermedius* better than the 30 µg cefoxitin disk. For a 1 µg oxacillin disk we propose that 20 mm should be used as cut off for resistance i.e. isolates with a zone diameter <20 mm are resistant to all beta- lactam antibiotics except those with effect against methicillin resistant staphylococci.

Introduction

Staphylococcus pseudintermedius is a coagulase-positive *Staphylococcus* species adapted to *Canidae* and one of the most important bacterial pathogens in dogs but also causes infections in humans including serious infections (1-4). The introduction of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) for bacterial

identification has shown that the incidence of *S. pseudintermedius* infections in humans is probably underestimated due to mis-identification as *Staphylococcus aureus* (4-6). Methicillin (β-lactam)-resistant *S. pseudintermedius* (MRSP) was first reported in 1999 in North America (7) and in 2006 in Europe (8). Since then, five MRSP lineages (CC45, 68, 71, 112, 258) with specific traits regarding antimicrobial resistance, genetic diversity and geographical distribution have spread globally (1, 9). Hitherto, according to our knowledge, only *mecA*-based resistance have been reported in *S. pseudintermedius*. Variable MRSP prevalence among clinical isolates (1-33%) has been reported by recent studies from different geographical areas and study populations (2, 10-15). A study in the United States (US) showed that the prevalence of methicillin resistance in canine clinical isolates increased from <5% in 2001 to nearly 30% in 2007 . Some MRSP clones such as sequence type (ST) 71 display resistance to virtually all antimicrobial agents licensed for veterinary use, posing one of the most challenging problems so far encountered in the antimicrobial management of veterinary infectious diseases. According to a recent review, approximately two thirds of MRSP isolates submitted to the multilocus sequence typing (MLST) database originate from skin samples associated with pyoderma, surgical site and wound infections (1).

Cefoxitin is endorsed by both the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Clinical and Laboratory Standards Institute (CLSI) as the preferred agent for detecting methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-resistant coagulase-negative *Staphylococcus* (MRCoNS) isolates by disk diffusion (16-18). In contrast, there has been divergence between EUCAST and CLSI on the antimicrobial agent to use for the detection of MRSP by disk diffusion. EUCAST has

advocated for the use of cefoxitin, whereas CLSI recommends oxacillin for detection of MRSP (17, 18). Previous studies have shown that cefoxitin growth inhibition zone diameter breakpoints recommended for detection of MRSA (susceptible, ≥22 mm; resistant, <22 mm) and MRCoNS (S, ≥25 mm; R, <25 mm) are not reliable for MRSP (19). In 2012, based on a study of 1,146 *S. pseudintermedius* isolates originating from different regions in the US, Bemis *et al.* proposed an epidemiological cut-off value for non-wildtype of ≤30 mm to maximize sensitivity (97%) and specificity (92%) for predicting methicillin resistance by cefoxitin disk diffusion (20). Our group further investigated 243 *S. pseudintermedius* isolates to identify the most suitable cefoxitin breakpoint to distinguish between MSSP and MRSP. The isolates were predominantly of European origin and the results indicated a breakpoint of S, ≥35 mm and R, <35 mm with only two (0.4%) major errors (ME) and one (0.2%) very major error (VME) (unpublished own data). On the basis of these data, these breakpoints were added to the EUCAST breakpoint table 4.0 published January 2014 (21).However, in a subsequent study Wu *et al.* showed that the EUCAST breakpoint produced a significant number of major errors (ME) in a study using 115 human and veterinary "*Staphylococcus intermedius* group" isolates (111 *S. pseudintermedius* and four *Staphylococcus delphini* isolates) from the US. The authors concluded that cefoxitin disk diffusion is not reliable for MRSP detection and that laboratories should perform oxacillin disk diffusion or broth-based minimum inhibitory concentration tests (22). This was confirmed by Yarbrough *et al*. who found that none of 12 MRSP isolates were detected by cefoxitin disk diffusion whereas all 12 were detected using oxacillin disk diffusion (4).

The current study was conducted to re-evaluate disk diffusion breakpoints using cefoxitin (30 µg disk) and oxacillin (1 µg disk) disk diffusion to detect *mecA*-mediated β-lactam resistance in *S. pseudintermedius* using disks from three manufacturers and Mueller-Hinton agar (MHA) from two manufacturers. For the present evaluation, our strain collection included strains from both Europe and North America to take the marked differences in the distribution of clonal lineages existing between these two geographical regions into account (1).

Materials and Methods

Bacterial isolates

A total of 224 clinical *S. pseudintermedius* isolates were tested, including 115 *mecA*-negative (MSSP) isolates and 109 *mecA*-positive (MRSP) isolates. The isolates were obtained from colleagues in Europe and North America representing a convenience sampling and included the 111 *S. pseudintermedius* isolates described by Wu and colleagues. Sixty-seven isolates from dogs and six from cats isolated between 2006 and 2011 were from a strain collection at the National Veterinary Institute in Sweden (SVA). Forty-nine of these isolates were from different European countries, three from Canada and two from the US (23). Forty canine isolates isolated between 2008 and 2011 were from the Norwegian Veterinary Institute (NVI). The remaining 111 isolates described by Wu *et al.* were obtained and included in this present study (the four *S. delphini* isolates were not included) (22) to investigate if the difference between the data published by Wu *et al*. and those obtained in our previous investigation were explained by differences between isolates from Europe and isolates from North America. The isolates originated

from humans (n=45) and animals (n=66), including dogs, cats, birds and pigs. MLST data were available for 76 of the 78 MRSP isolates from the SVA and NVI collections using the MLST_5 scheme for 52 isolates (SVA) and the MLST_7 scheme for 24 isolates (NVI) (24, 25). A total of 18 different MLST types including world epidemic lineages such as ST68, ST71 and ST258 were represented in the study. While no MLST data were available for the isolates described by Wu and co-workers, repetitive-sequence PCR (rep-PCR) demonstrated the collection was composed of six different rep-PCR clonal lineages (designated A to F) (22). No correlation between rep-PCR clonal type and antimicrobial susceptibility data was encountered, implying results were not due to a specific *S. pseudintermedius* lineage. All isolates were identified in the laboratory at Växjö to the species level with MALDI-TOF MS using the Microflex system with the MALDI Biotyper 3.1 software and MBT 6903 Library (Bruker Daltonics, Bremen, Germany) per the manufacturer's instructions. *mec* status was determined by the contributing laboratories: SVA (*mecA*) (26) and NVI (*mecA*) (27), or as described in Wu *et al*. (*mecA* and *mecC*) (22). In case of discrepancy between the *mec* status and the phenotypic results obtained in this study, the *mec* status were confirmed by a real-time PCR assay that tested for both *mecA* and *mecC* (28). The study did not require patient consent or ethical approval since isolates were not associated with any identifiable patient information.

Antimicrobial susceptibility testing

Disk diffusion was performed according to EUCAST recommendations (29) using 30 µg cefoxitin and 1 µg oxacillin disks from Oxoid/ThermoFisher Scientific (Basingstoke, UK), Mast Diagnostics (Bootle, UK) and Becton Dickinson (Heidelberg, Germany). All isolates

were tested in parallel from the same inoculum on in-house prepared MHA plates using pre-formulated powder from ThermoFisher Scientific (Oxoid agar) and Becton Dickinson (BBL agar), and commercial plates from Becton Dickinson (BBL agar). *Staphylococcus aureus* ATCC® 29213 was used as quality control.

Data analysis

The ability of cefoxitin (30 µg) and oxacillin (1 µg) disks to predict the presence of *mecA*-mediated β-lactam resistance in *S. pseudintermedius* was evaluated by 1) comparing the degree of measurements placed in the interval where both *mecA*-negative and *mecA*-positive isolates presented values (disregarding the measurements of the aberrant strain, and 2) the number of major Errors (ME) and very major errors (VME) for the present EUCAST breakpoint for cefoxitin (S, ≥35 mm and R, <35 mm) and for oxacillin using the CLSI breakpoint (S, ≥18 mm and R, ≤17 mm) as well as an alternative breakpoint (S, ≥20 mm and R, <20 mm) based on the present study (total isolate set). ME and VME were calculated based on the number of susceptible and the number of resistant tests, respectively.

Analyses on performance were done disregarding the clearly aberrant *mecA*-negative isolate (see results) for a) the total aggregated set of measurements:2,007 data points (223 isolates × 3 different disk manufacturers × 3 different MHAs), b) for isolates from Europe vs isolates from North America and c) for each of the individual combinations of MHAs and disk brands. Comparison of the distributions of zone diameters were performed using the Mann-Whitney U test, $p>0.05$ were used as significance level

Results

The results for the cefoxitin 30 µg and oxacillin 1 µg disk screening tests are shown in Table 1/Figure 1 and Table 2/Figure 2, respectively. One *mecA*- (and *mecC*) negative isolate was clearly aberrant by oxacillin testing with an inhibition zone size between 14-16 mm for oxacillin and 28-29 mm for cefoxitin. This isolate was also clearly resistant in the investigation by Wu *et al*. (22), the mechanism of resistance for this has not been elucidated. Disregarding this isolate, the inhibition zone sizes of isolates from Europe and North America spanned over similar ranges; *i.e.*, a maximum difference of 2 mm for both cefoxitin and oxacillin except for *mecA*-positive isolates tested against cefoxitin where isolates from Europe ranged from 6-33 mm versus 21-32 mm for isolates from North America (Tables 1 and 2). Nevertheless, comparison of isolates from Europe and North America for each of the four distributions; cefoxitin *mecA*-negative, cefoxitin *mecA*-positive, oxacillin *mecA*-negative and oxacillin *mecA*-positive were significantly different ($p<0.0001$, $p<0.01$, $p<0.002$ and, $p<0.0001$). Measurements from the individual disk and MHA combinations only showed minor differences (*i.e.*, maximum difference in minimum or maximum values of 1-2 mm [Tables 1 and 2]).

For the aggregated dataset for the 30 µg cefoxitin disks, 16% (14.8%-18.0%, 95% CI) of the zone size measurements were in the region (29 -33 mm) where both *mecA*-negative and *mecA*-positive isolates tested (Table 1). For the 1 µg oxacillin disks, only 2% (1.6%-2.9%, 95% CI) of the measurements were in the region (19-20 mm) where both *mecA*-negative and *mecA*-positive isolates tested (Table 2). Furthermore, the vast majority of the *mecA*-positive isolates displayed no zone of inhibition with the 1 µg oxacillin disk which provides

much better separation between the *mecA*-negative and the *mecA*-positive populations compared to the 30 µg cefoxitin disk (Figure 1 and Figure 2).

For the 1 ug oxacillin disk the number of MEs and VMEs using both the CLSI breakpoint (S, ≥18 mm and R, ≤17 mm) and the breakpoint suggested on the data in this publication (S, ≥20 mm and R,<20 mm) are shown in Table 1 and 2 both for the total dataset as well as for the individual datasets (excluding the aberrant *mecA*-negative isolate). The CLSI breakpoint resulted in a total of nine *mecA*-positive isolates (six European and three North American isolates, 40 data points) being reported as susceptible resulting in a VME rate of 4.1%, and one *mecA*-negative isolate (one North American isolate, 9 data points) would be reported as resistant; *i.e.*, 0.9% ME. In contrast, changing the breakpoint to S, ≥20 mm and R,<20 mm the corresponding VME and ME rates were 0.4% (one European isolate, 4 data points) and 1.1% (2 North American isolates, 11 data points), respectively.

Discussion

Detection of *mecA*-based methicillin resistance using cefoxitin or oxacillin disks is in fact a dichotomous screening test where the ideal substance has a cutoff that clearly distinguishes between *mecA*-positive and *mecA*-negative isolates with no or very little overlap. In this study, where *S. pseudintermedius* isolates from Europe and North America were tested by using disks from three different manufacturers and MHA from two different manufacturers, oxacillin was markedly better than cefoxitin in separating *mecA*-negative from-positive isolates. By the 1 µg oxacillin disk, only 2% of the total number of data points were in the interval where zone sizes for *mecA*-negative and *mecA*-positive isolates overlapped (it was not possible to classify an isolate as either susceptible or

209 resistant) in comparison to 16% of the data points for the 30 µg cefoxitin disk diffusion.
210 Thus, our previous finding that cefoxitin disk diffusion can reliably differentiate between
211 *mecA*-negative and *mecA*-positive isolates of *S. pseudintermedius* has been modified
212 based upon our current data where a greater variety of strains, disks and media were
213 assayed. Furthermore, the oxacillin disk had the advantage that the majority of *mecA*-
214 positive isolates did not exhibit any zone of growth inhibition (they grew up to the edge of
215 the disk), permitting good separation of MSSP and MRSP.
216 Our data confirm the recommendation made by Wu *et al*. in favour of using oxacillin disk
217 diffusion for detection of methicillin resistance in *S. pseudintermedius* (22). However,
218 using the breakpoint suggested by Wu *et al* (the breakpoint adopted by CLSI) nine (8%) of
219 the *mecA*-positive isolates, would be classified as false susceptible in comparison to one
220 isolate (0.9%) using a breakpoint of S, ≥20 mm and R, <20 mm. In a previous study, Bemis
221 *et al*. also found two PBP2a-positive isolates that displayed zone sizes greater than 17 mm
222 (18 mm and 23 mm) (19), (Bemis personal communication).
223 Interestingly six of the nine isolates were of European origin and none of the three North
224 American isolates were false susceptible in all tested variants, providing a possible
225 explanation for the difference found in this evaluation compared to the evaluation by Wu
226 *et al* (22). Accordingly, for both cefoxitin and oxacillin the zone size distribution of isolates
227 from Europe were significantly different from the North American isolates possibly
228 reflecting differences in clonal distribution between Europe and North America.
229 The findings in this study stresses the need for testing isolates from different clonotypes
230 and to use disks and media from more manufacturers when setting breakpoints. Thus, for
231 the 1 µg oxacillin disks, we propose that 20 mm is a more appropriate breakpoint to

distinguish between *mecA*-negative (zone diameter ≥20 mm) and *mecA*-positive (zone diameter <20 mm) isolates. This new breakpoint should reduce the frequency of VME (resistant isolates that test as susceptible) compared to the current CLSI breakpoint. The breakpoints generated by this study are now accepted by the EUCAST (EUCAST breakpoint table v 7.1, 2017 (30).

The inclusion of media and disks from different manufacturers which is an integrated part of EUCAST method development is a strength and demonstrates study originality since it incorporates the unavoidable variation in materials between manufacturers. An important limitation of the study is that the strain collection does not include isolates from Africa, Asia, or Australia which potentially could affect the proposed breakpoints. We did not test all isolates for *mecC,* however, isolates resistant for cefoxitin or oxacillin by disk diffusion, but negative for *mecA* were tested for *mecC*. Nevertheless, we cannot exclude that among the phenotypically susceptible isolates there were *mecC*-positive isolates, why our findings only apply for *mecA*-mediated β-lactam resistance (as reflected in the title).

In conclusion, the present investigation confirms the findings from previous studies that oxacillin is better than cefoxitin for detection of *mecA*-mediated β-lactam resistance in *S. pseudintermedius*. As a result of this study, oxacillin is now recommended by CLSI and EUCAST for detecting *mecA*-mediated β-lactam resistance in *S. pseudintermedius.* This outcome contributes to optimize MRSP detection in both veterinary and human diagnostic laboratories and has therefore important implications for antimicrobial treatment in both populations.

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Conflicts of Interest: Drs. Skov, Varga, Matuschek, Åhman, Bemis, Bengtsson, Sunde, Westblade, Guardabassi and Kahlmeter report nothing to disclose. Dr. Humphries reports employment by Accelerate Diagnostics, Inc. and stocks with Accelerate Diagnostics.

Table 1. Cefoxitin 30 µg disk inhibition zone sizes (mm), Major errors (ME) and Very Major errors (VME) using a breakpoint for S, ≥34 mm and R, <35 mm for *S. pseudintermedius* isolates (n=223*) obtained from Europe and North America for the total number measurements and for individual subgroups.

MHA Manufacturer	Disk Manufacturer	Number of measurements	Zone diameter, mm		Interval (mm) with measurements from both *mecA* negative and *mecA* positive isolates (% of total values)	Number (%) of ME (Breakpoint R<35 mm)	Number (%) of VME (Breakpoint S≥34 mm)
			mecA-positive	*mecA*-negative*			
All	All	2007 (223x3x3)	6-33	29-41	29-33 (16.3)**	376 (36.3%)	0 (0%)
Europe	All	972 (108 x3x3)	6-33	31-40	31-33 (6.9%)	68 (18.4%)	0 (0%)
North America	All	1035 (115 x3x3)	14-32	29-41	29-32 (12.2%)	308 (46.2%)	0 (0%)
	BD	223 (223x1x1)	10-31	29-40	29-31 (8.0%)	64 (55.7%)	0 (0%)
BBL commercial	Mast	223 (223x1x1)	12-32	29-40	29-32 (9.8%)	59 (51.3%)	0 (0%)
	Oxoid	223 (223x1x1)	9-32	30-40	30-32 (6.7%)	45 (39.1%)	0 (0%)
BBL prepared in-house	BD	223 (223x1x1)	9-32	31-40	31-32 (6.3%)	34 (29.6%)	0 (0%)
	Mast	223 (223x1x1)	10-32	31-41	31-32 (5.8%)	26 (22.6%)	0 (0%)
	Oxoid	223 (223x1x1)	6-33	31-41	31-33 (7.1%)	24 (20.9%)	0 (0%)
Oxoid prepared in-house	BD	223 (223x1x1)	6-32	30-40	31-32 (6.3%)	51 (44.3%)	0 (0%)
	Mast	223 (223x1x1)	6-33	30-40	30-33 (14.7%)	41 (35.7%)	0 (0%)
	Oxoid	223 (223x1x1)	6-33	31-41	31-33 (9.4%)	32 (27.8%)	0 (0%)

* Data for the aberrant *mecA/C*-negative strain with cefoxitin readings of 28-29 mm and oxacillin readings of 14-16 mm are omitted.

** Percentage of measurements that overlap between the zone sizes for *mecA*-negative and *mecA*-positive isolates. The interval is greater for all media/disks combined than for each individual media as the overlapping zones differ for the individual media/disks

280

281 Table 2. Oxacillin 1 µg disk inhibition zone sizes (mm), Major errors (ME) and Very Major errors (VME) using breakpoint for S, ≥20 mm and R, <20 mm
282 and S, ≥18 mm and R, ≤17 mm for *S. pseudintermedius* isolates (n=223*) obtained from Europe and North America for the total number measurements
283 and for individual subgroups.

MH Agar Manufacturer	Disk Manufacturer	Number of measurements	Zone diameter, mm		Interval (mm) with measurements from both *mecA* negative and *mecA* positive isolates (Pct of total values)	Number (%) of ME (Breakpoint R≤17 mm)	Number (%) VME (Breakpoint S≥18 mm)	Number (%) of ME (Breakpoint R<20mm)	Number (%) VME (Breakpoint S≥20 mm)
			mecA-positive	*mecA*-negative*					
All	All	2007 (223x3x3)	6-20	19-30	19-20 (2.2%)**	9 (0.9%)	40 (4.1%)	11 (1.1%)	4 (0.4%)
Europe	All	972 (108 x3x3)	6-20	20-29	20 (0.9%)	0 (0.0%)	32 (4.9%)	0 (0.0%)	4 (0.6%)
North America	All	1035 (115 x3x3)	6-19	19-30	19 (0.2%)	9 (1.4%)	8 (2.4%)	11 (1.7%)	0 (0.0%)
BBL commercial	BD	223 (223x1x1)	6-19	19-28	19 (1.3%)	1 (0.9%)	4 (3.7%)	2 (1.8%)	0 (0.0%)
	Mast	223 (223x1x1)	6-18	19-28	-	1 (0.9%)	3 (2.8%)	2 (1.8%)	0 (0.0%)
	Oxoid	223 (223x1x1)	6-20	20-29	20 (1.3%)	1 (0.9%)	4 (3.7%)	1 (0.9%)	1 (0.9%)
BBL prepared in-house	BD	223 (223x1x1)	6-19	20-28	-	1 (0.9%)	5 (4.6%)	1 (0.9%)	0 (0.0%)
	Mast	223 (223x1x1)	6-19	20-29	-	1 (0.9%)	5 (4.6%)	1 (0.9%)	0 (0.0%)
	Oxoid	223 (223x1x1)	6-19	20-28	-	1 (0.9%)	4 (3.7%)	1 (0.9%)	0 (0.0%)
Oxoid prepared in-house	BD	223 (223x1x1)	6-20	20-29	20 (0.9%)	1 (0.9%)	4 (3.7%)	1 (0.9%)	1 (0.9%)
	Mast	223 (223x1x1)	6-20	20-29	20 (1.8%)	1 (0.9%)	6 (5.5%)	1 (0.9%)	1 (0.9%)
	Oxoid	223 (223x1x1)	6-20	21-30	-	1 (0.9%)	5 (4.6%)	1 (0.9%)	1 (0.9%)

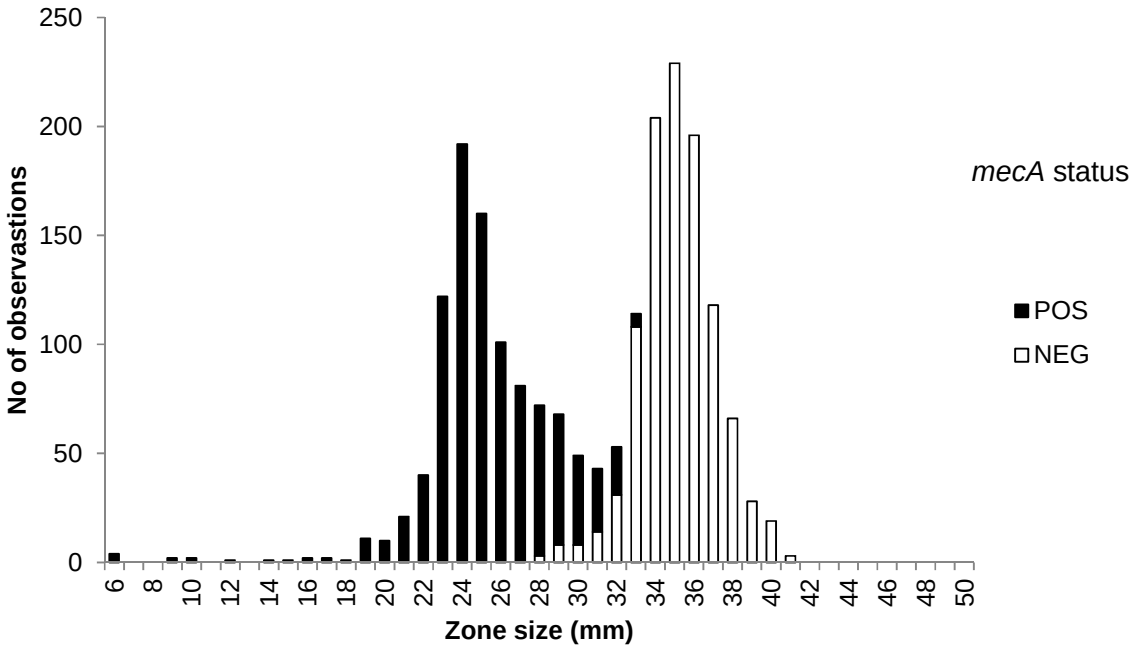
284 *Data for the aberrant *mecA/C*-negative strain with cefoxitin readings of 28-29 mm and oxacillin readings of 14-16 mm are omitted

285 ** Percentage of measurements that overlap between the zone sizes for *mecA*-negative and *mecA*-positive isolates. The interval is greater for all
286 media/disks combined than for each individual media as the overlapping zones differ for the individual media/disks

287 **Figure 1.** Cefoxitin 30 µg disk inhibition zone sizes versus *mecA* status for the 224 *S.*

288 *pseudintermedius* isolates from Europe and North America (2,016 data points, each isolate

289 tested using disk and media from three manufacturers [3 ×3 ×224 = 2,016]).



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292 **Figure 2.** Oxacillin 1 µg disk inhibition zone sizes versus *mecA* status for 224 *S.*
293 *pseudintermedius* isolates obtained from Europe and North America (2,016 data points,
294 each isolate tested using disk and media from three manufacturers [3 ×3 ×224 = 2,016]).

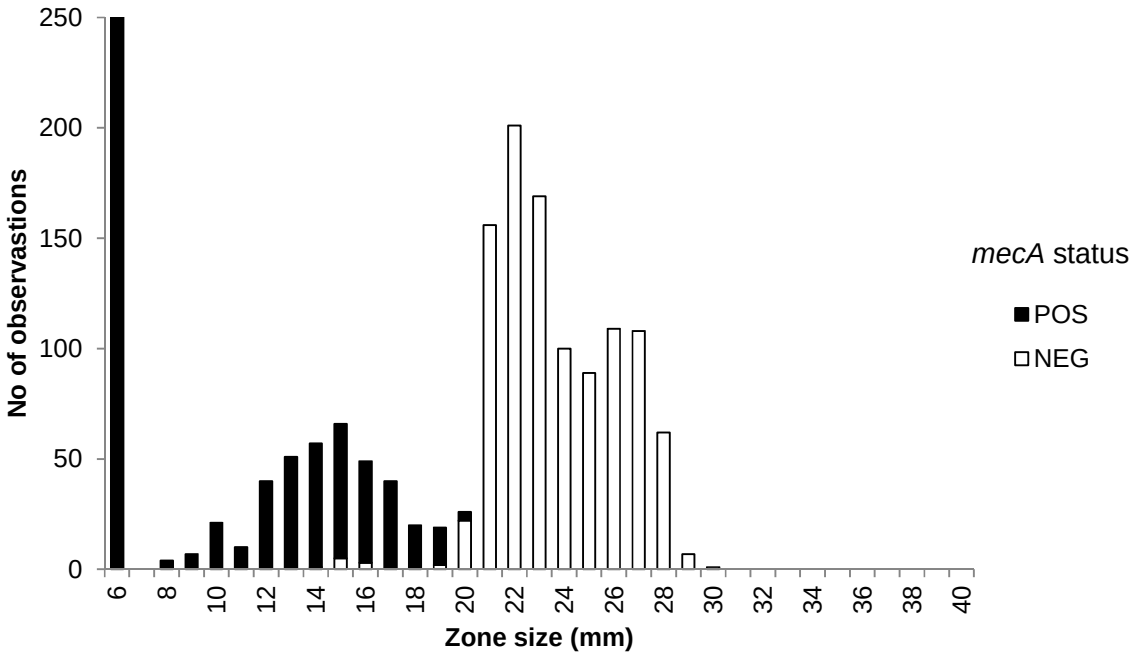

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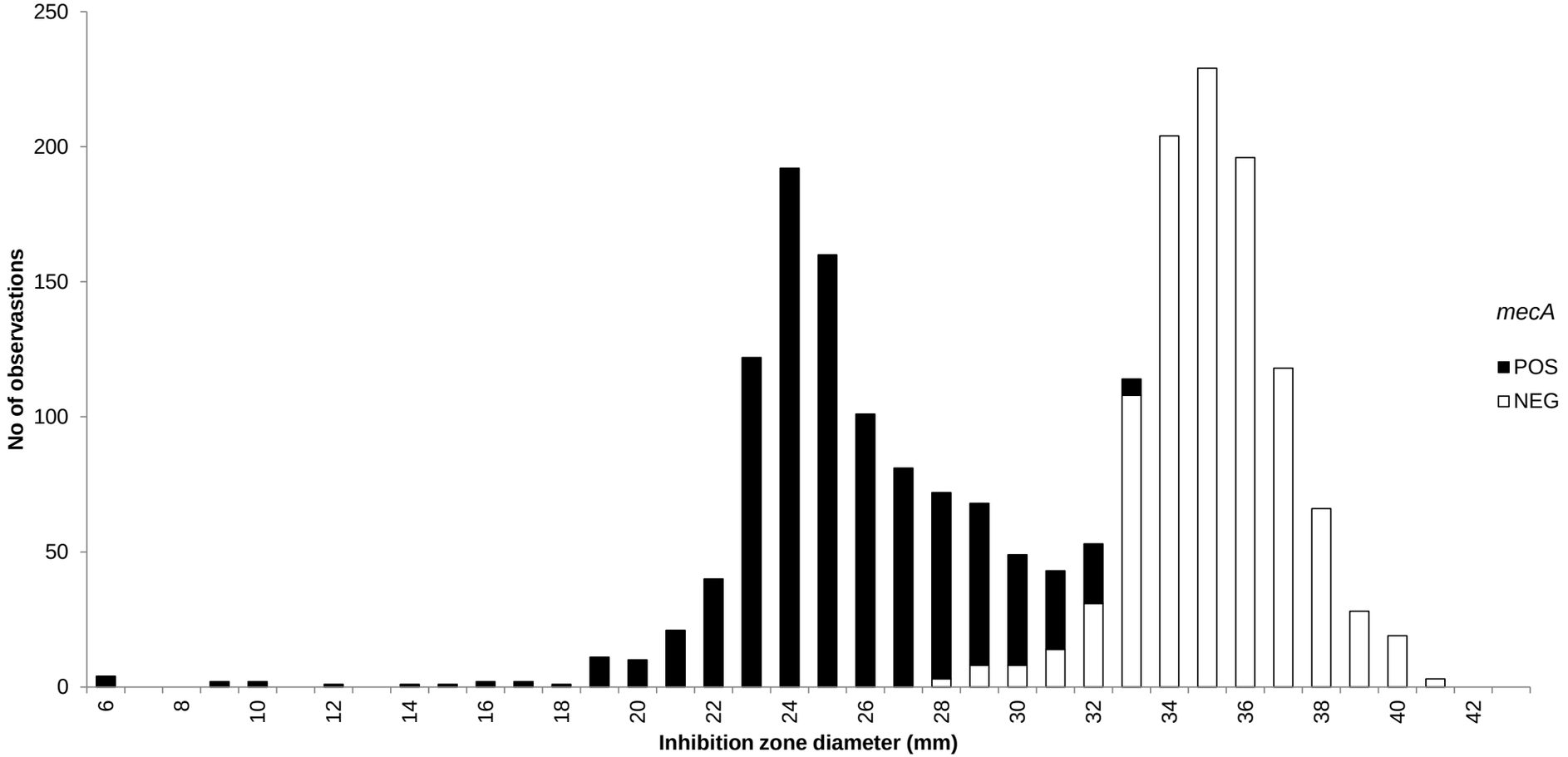
Figure 1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No of observastions
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Inhibition zone diameter (mm)
mecA
POS
NEG