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Comparison of phenotypic tests for detecting penicillin G resistance with presence of *blaZ* gene in *Staphylococcus aureus* isolated from bovine intramammary infections

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Few studies have described the relationship between genotypic and phenotypic methods for detecting penicillin resistance in *Staphylococcus aureus* isolated from bovine intramammary infection (IMI). Six phenotypic methods for penicillinase detection were compared with a genotypic method testing the presence of the β-lactamase gene *blaZ* in *Staph. aureus* ($n = 150$) isolated from bovine IMI. Highest sensitivities and specificities were observed for disk diffusion (DD) (93 and 97·4%), minimum inhibitory concentration (MIC) (90·3 and 97·4%), Cefinase™ (85·9 and 97·4%) and Diatabs™ (85·7 and 98·7%). The estimated cut-off points estimated in the present study can be considered close to the ones indicated by CLSI (2013). The molecular detection of *blaZ* gene is the only method that may indicate the real or potential capacity of producing β-lactamase in *Staph. aureus*. Considering that from a clinical standpoint a false negative result from a phenotypic test is the most unfavourable situation, a combination of standard DD with Diatabs™ or Cefinase™ should be performed by routine mastitis laboratories to minimise false negative results.

Keywords: *Staphylococcus aureus*, intramammary infections, penicillin resistance, *bla*Z.

Staphylococcus aureus is one of the most prevalent major mastitis pathogens in dairy herds worldwide (Tenhagen et al. 2006; Zecconi et al. 2006; Persson et al. 2011; Dieser et al. 2014). Most authors agree that, albeit with limitations to predict the outcome of therapy, susceptibility testing should precede antibiotic treatment, mainly in case of subclinical mastitis (Haveri et al. 2005; Barkema et al. 2006). Penicillin G has been considered the drug of choice for treating *Staph. aureus* IMI; however, resistant isolates were early detected and its prevalence reported to vary widely between countries from 1·8 to 100% (Aarestrup & Jensen, 1998; De Oliveira et al. 2000; Russi et al. 2008; Persson et al. 2011). Production of β-lactamase is considered the most frequent mechanism of penicillin resistance among *Staph. aureus* isolated from bovine IMI (Watts & Salmon, 1997; Haveri et al. 2005, Olsen et al. 2006). Mastitis diagnostic laboratories usually perform standard disk diffusion (DD) or dilution tests to determine *Staph. aureus* susceptibility to penicillin. However, β-lactamase producing *Staph. aureus* isolated from bovine IMI had penicillin minimum inhibitory concentrations (MIC) near or below the breakpoint currently recommended by CLSI (2013); suggesting that this breakpoint could be too high to detect penicillin resistance, mainly for those isolates

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yielding test results close to the detection limit (Watts & Salmon, 1997; Haveri et al. 2005; Klement et al. 2005; Russi et al. 2008). In such cases, additional testing should be required to correctly identify β-lactamase producing isolates. Detection of the *blaZ* gene by PCR is considered the reference method, since it correlates well with β lactamase production (Olsen et al. 2006). Few published studies describe the relationship between genotypic and phenotypic methods for detecting penicillin resistance in *Staph. aureus* isolated from bovine IMI (Haveri et al. 2005; Pitkälä et al. 2007). However, there is no information about comparison of DD test, the most widely used in routine laboratories, with presence of *blaZ* gene. In addition, the clover leaf is an easy, cost-effective and sensitive method for detection of β-lactamase production in staphylococci (Jarløv & Rosdahl, 1986); however, there are only few reports about its use in *Staph. aureus* isolated from bovine IMI (Gianneechini et al. 2002; Persson et al. 2011) and only one compares its performance with presence of *blaZ* gene (Pitkälä et al. 2007). The aim of this study was to compare phenotypic methods for penicillinase detection currently used by laboratories that carry out routine mastitis diagnosis with detection of *blaZ*.

Materials and methods

Bacterial isolates

One hundred and fifty *Staph. aureus* isolates were obtained from either quarter or composite milk samples from lactating cows; ninety-nine were from subclinical cases and fifty-one from clinical cases. Isolates belonged to 95 dairy farms located in 5 Argentinian provinces that concentrate more than 90% of the country dairy production: Santa Fe ($n = 48$), Buenos Aires ($n = 40$), Córdoba ($n = 55$), Entre Ríos ($n = 3$) and La Pampa ($n = 4$). A maximum number of 3 isolates from the same dairy herd were included. Samples were collected and cultured according to standard methodology (Oliver et al. 2004). Isolates were tentatively identified as *Staph. aureus* on the basis of conventional biochemical reactions. Briefly, colonies were tested for cell morphology after Gram staining, catalase production, clumping factor, coagulase production using rabbit plasma, acetoin production and selective growth on P Agar added with 7 µg/ml acriflavin (Roberson et al. 1992). Isolates characterised as *Staph. aureus* by biochemical reactions were further identified by PCR amplification of a specific genomic DNA fragment as previously described (Martineau et al. 1998). Following identification, isolates were kept as frozen stocks in BHI/glycerol 15% at −70 °C.

Determination of MIC and agar diffusion test

Minimal inhibitory concentration (MIC) of penicillin was determined by an agar dilution procedure according to CLSI (2008) recommendations, using *Staph. aureus* ATCC 29213 as control strain. Prior to susceptibility testing, bacteria were activated from frozen stocks by overnight culture at 35 °C on Columbia agar base (Laboratorios Britania, Buenos Aires) supplemented with 5% bovine defibrinated blood. Muller-Hinton agar (Merck & Co., Inc. Whitehouse Station NJ, USA) plates with different penicillin concentrations (dilution range 0·015–2 µg/ml) were inoculated with bacterial suspensions by a multipoint inoculator. The breakpoint to consider isolates as resistant was ≥0·25 µg/ml (CLSI, 2008). The agar disk diffusion method was performed according to CLSI (2008) guidelines using the following disks: penicillin (10 U) and oxacillin (1 µg) (Laboratorios Britannia). Interpretive criteria for penicillin and oxacillin were those adopted by the CLSI (2008). Oxacillin disks were used to rule out presence of methicillin-resistant *Staph. aureus*.

Penicillinase detection

The following tests were performed: (i) chromogenic cephalosporin (nitrocefin) disk method (Cefinase™ Paper Disc BBL™, Sparks MD, USA) carried out according to the manufacturers' directions; (ii) acidimetric method (Diatabs™ beta-lactamase diagnostic tablet, Rosco Diagnostica, Taastrup, Denmark) performed and interpreted according to the manufacturer's directions; (iii) iodometric method, a penicillin starch paper strip, performed and interpreted according to previous descriptions (Oberhofer & Towle, 1982); (iv) clover leaf test was performed as described by Bergan et al. (1997) using *Staph. aureus* Oxford strain (ATCC 9144), kindly provided by Dr R.E. Gianneechini, as an indicator on Mueller-Hinton agar (Merck & Co.). The test was interpreted as positive if the indicator strain grew with the test isolate into the inhibition zone and as negative if the inhibition zone was circular with no shape of a cloverleaf. For every test, *Staph. aureus* ATCC 29213 and 25923 were included as positive and negative controls for β-lactamase production, respectively.

Detection of blaZ gene

Whole genomic DNA was isolated as described by Pospiech & Neumann (1995). PCR amplification of the internal region of the *blaZ* gene was carried out using primers designed by Vesterholm-Nielsen et al. (1999) with the following sequences: *blaZ* primer1: AAG AGA TTT GCC TAT GCT TC and *blaZ* primer2: GCT TGA CCA CTT TTA TCA GC. The PCR reaction mixture (25 µl) contained 1-µM of primer 1 and 2, 0·2-mKm of dNTP, 0·25 µl of Taq buffer 10x, 0·25 U of Taq polymerase (Invitrogen CA, USA) and 25 ng of DNA template. Amplification was carried out on thermal cycler Techne TC 3000 G (Techne Inc. NJ, USA) using a program as follows: an initial 5-min denaturation step at 94 °C, followed by 35 cycles of 30 s of denaturation at 94 °C, 30 s of annealing at 55 °C, and 1 min of extension at 72 °C; with a final extension step at 72 °C for

Table 1. Performance of seven phenotypic tests for detection of penicillin resistance or production of β-lactamase compared with presence of *blaZ* gene detected by PCR

Test	*N*	True positive†		False negative‡		Kappa (IC 95%)	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)
		+	−	+	−			
Cefinase™	149	61	10	2	76	0·83 (0·75–0·92)	85·9 (77·8–94·0)	97·4 (93·9–100)
Diatabs™	148	60	10	1	77	0·85 (0·76–0·93)	85·7 (77·5–93·9)	98·7 (96·2–100)
Iodometric	150	50	22	11	67	0·55 (0·42–0·69)	69·4 (58·8–80·1)	85·9 (78·2–93·6)
Clover leaf	149	57	14	2	76	0·78 (0·62–0·92)	80·3 (71·0–89·5)	97·4 (93·9–100)
MIC	150	65	7	2	76	0·87 (0·80–0·95)	90·3 (83·4–97·7)	97·4 (93·9–100)
Disk diffusion	150	67	5	2	76	0·90 (0·83–0·97)	93·0 (87·2–98·9)	97·4 (93·9–100)

†Presence of *bla*Z gene
‡Absence of *bla*Z gene

10 min (Haveri et al. 2005). PCR products were analysed by electrophoresis on ethidium bromide-stained 2% agarose gels (Biodynamics, Buenos Aires, Argentina). A positive (*Staph. aureus* ATCC 29213) and a negative control (without DNA template) were included in each PCR run.

Statistical analysis

Kappa coefficient was used to determine rate of agreement of qualitative tests with detection of *blaZ* gene by PCR. In the case of MIC and DD methods the above mentioned breakpoints were used to define positive and negative results. In addition, for these later tests, analysis of the receiver operating characteristic curve (ROC) was performed to define the relationship between sensitivity and specificity of the test based on the cut-off value used to define a result as positive or negative. Area under the curve (AUC) values near to 1 indicate a higher discriminative power of the test and can estimate the best cutoff point for a given test. A Student's *t* test was carried out to determine variation of MIC and DD test values among isolates with respect to presence or absence of *blaZ* gene. Linear correlation between MIC and DD test was evaluated using Spearman's correlation coefficient and the relationship between MIC and size of inhibition zone determined by the DD test was determined by an error-rate bounded classification scheme using one MIC breakpoint (Metzler & DeHaan, 1974).

Results

All isolates were confirmed as being *Staph. aureus* based on genotypic typing. No oxacillin-resistant isolates were detected. Performance of the six phenotypic methods compared with detection of *blaZ* gene by PCR is shown in Table 1.

Most isolates with MICs ≥0·25 µg/ml and inhibition zone diameters ≤28 mm (CLSI breakpoints) carried the *blaZ* gene, although two PCR-negative isolates with MICs ≥0·25 µg/ml and inhibition zone diameters ≤28 mm were observed (2/67). Phenotypic expression of β-lactamase was not detected for these isolates, except for one isolate that yielded a positive result by the iodometric method. Conversely, 8 isolates that carried the *blaZ* gene showed MICs in the range 0·12–0·03 µg/ml; six of those isolates showed inhibition zone diameters ≥29 mm, while the remainder showed inhibition zone diameters of 26 and 27. Three of those 8 isolates produced β-lactamase detected by Diatabs™ and Cefinase™ methods. Combining simultaneously DD with Diatabs™ or Cefinase™ and considering as positive the result of either test, sensitivity increased to 99%, reducing false negative results.

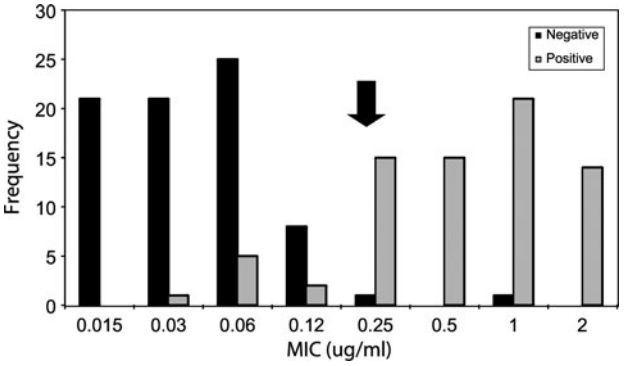

Fig. 1. Distribution of minimum inhibitory concentrations (MIC) of penicillin G for 150 *Staphylococcus aureus* isolates from bovine intramammary infections. Arrow indicates MIC breakpoint. Bars depict presence (positive) or absence (negative) of *blaZ* gene.

MIC_{50} and MIC_{90} were 0·12 and 1 µg/ml, respectively. MIC_S and inhibition zone diameters distributions of isolates according to the presence or absence of *blaZ* gene are shown in Figs. 1 & 2.

For those tests yielding quantitative results (MIC and DD test) a ROC curve analysis was performed to define the relationship between sensitivity and specificity of each test according to the cut-off point used to define the test as positive or negative. For MIC the calculated AUC was 0·969 and the cutoff value ≥0·185 µg/ml; while for DD test AUC was 0·961 and the cut-off value was 28·5 mm. Correlation

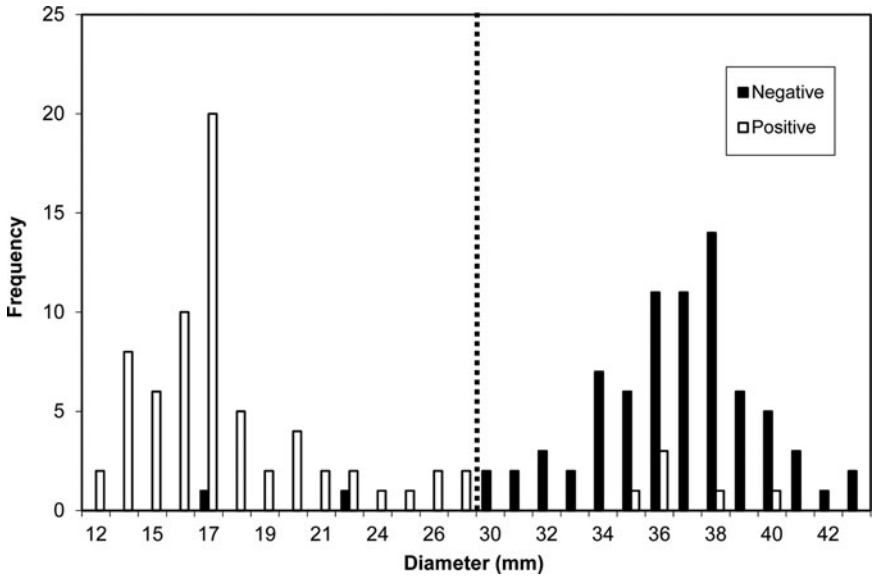

Fig. 2. Distribution of inhibition zone diameters of penicillin G disks for 150 isolates from bovine intramammary infections. Dotted line indicates breakpoint. Bars depict presence (positive) or absence (negative) of *blaZ*gene.

coefficient between the two tests was 0·87. Two major errors (false resistant) and no very major errors (false susceptible) were detected. One isolate could not be evaluated by the clover leaf method since an inhibition zone against the indicator strain precluded test interpretation.

Discussion

In the present study, the highest sensitivity was observed for DD (93%) while the highest specificity for Diatabs™ (98·7%). Among the colour-based tests, sensitivity and specificity for Cefinase™ was similar to that obtained by Haveri et al. (2005) using liquid nitrocefin (92·3 and 96·5%, respectively). In addition Pitkälä et al. (2007) observed sensitivities ranging from 70 to 100% using Cefinase and nitrocefin disks, respectively. The proportion of false negative and false positive results for iodometric method in the present study was high. False positive results observed for this test have been attributed to nonspecific reactions of iodine with bacterial proteins (Livermore, 1995). Only one previous report evaluated detection of penicillin resistance by clover leaf method compared with presence of *blaZ* gene (Pitkälä et al. 2007). Proportion of false positive results obtained in the present study was similar to that reported by Pitkälä et al. (2007); however a high percentage of false negative results (14/71) was observed which disagrees with previous findings (Pitkälä et al. 2007).

A good correlation between MIC determination and DD test was found in the present investigation which agrees with results from previous studies (Gianneechini et al. 2002; Pengov & Ceru, 2003). Conversely, Schlegelova et al. (2001) reported an accordance of 84·11% between standard broth microdilution and DD methods and Klement et al. (2005) reported a substantially lower susceptibility to penicillin G by DD test (42·1%) than by MIC (59·5%) with a correlation between methods of 0·7; indicating that discrepancies in the classification of isolates as susceptible or resistant were mainly related to inadequacy of interpretive criteria. In an early study, 23·4% of *Staph. aureus* isolates with MICs of 0·06–0·125 µg/ml were shown to possess the *blaZ* gene and most of them produced β-lactamase when tested by nitrocefin (Haveri et al. 2005). In addition, in a previous study we have shown that two (4%) *Staph. aureus* isolates with MICs of 0·19 µg/ml produced β-lactamase when tested by nitrocefin (Russi et al. 2008). Taken together, these data support the evidence that CLSI proposed breakpoint to identify penicillin-resistant isolates may be too high. In this regard, Klement et al. (2005) obtained an estimated cut-off point of 21 mm for *Staph. aureus* susceptibility to penicillin G instead of 29 mm recommended by CLSI for DD test. The nature of the differences found between Klement et al. (2005) study and the present study can be explained not only by the higher correlation between MIC and DD test observed in our study leading to a lower percentage of false resistant (major errors) and lack of false susceptible (very major errors), but also by the use of *blaZ* detection as a reference method. The ≥0·185 µg/ml and 28·5 mm resistant cut-off points estimated in the present study can be considered close to the ones indicated by CLSI (2013). However, it has to be taken in account that, albeit low, a number of false negative isolates potentially capable of producing β-lactamase with 35–40 mm inhibition diameters and a MICs 0·03–0·12 µg/ml were detected and less than half of them were positive to β-lactamase by Cefinase™ or Diatabs™. Current CLSI recommendations to perform β-lactamase testing when diameter zones are >29 mm or

MIC < 0·12 µg/ml can certainly minimise these potential false negative outcomes.

In conclusion, the DD method used for routine clinical practice in veterinary laboratories yielded the least number of false negative results compared with genotypic detection of *blaZ* gene. Considering that from a clinical standpoint a false negative result is the most unfavourable situation, for routine laboratories a combination of standard DD test with Diatabs™ or Cefinase™ should be advisable to minimise false negative results.

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