Comparison of Tests for Detection of β -Lactamase-Producing Staphylococci[▽]

A. Pitkälä,^{1*} L. Salmikivi,² P. Bredbacka,² A.-L. Myllyniemi,¹ and M. T. Koskinen²

Finnish Food Safety Authority Evira, Mustialankatu 3, 00790 Helsinki, Finland,¹ and Finnzymes Diagnostics, Espoo, Finland²

Received 21 March 2007/Accepted 29 March 2007

Penicillin resistance identification tests are important in veterinary medicine. Six enzyme assays and a PCR test were compared for the detection of β -lactamase production or the β -lactamase gene in 175 staphylococcal isolates. We conclude that the PCR test and two nitrocefin-based assays can be recommended for routine clinical use.

Staphylococci are common causes of a wide variety of diseases in animals. The most important staphylococcal pathogens include *Staphylococcus aureus* and coagulase-negative staphylococci (CNS), which cause bovine mastitis; *Staphylococcus intermedius*, which causes otitis externa and pyoderma in dogs; and *Staphylococcus hyicus*, which is responsible for exudative dermatitis in pigs (see, e.g., references 4, 8, and 9).

In veterinary medicine, penicillin is recommended as the first choice for bacteria that are inherently sensitive to it. In contrast to human isolates (3), the prevalence of penicillin resistance in staphylococci causing animal diseases can be relatively low (1) and is most commonly due to the *blaZ* gene, which encodes the production of β -lactamase (7). The aim of this study was to compare the performance and evaluate the practicality of various alternative methods for the determination of β -lactamase production or the β -lactamase gene in staphylococci.

A total of 175 staphylococcal isolates were used in this study, including 95 *Staphylococcus aureus*, 50 *Staphylococcus intermedius*, and 30 CNS isolates (Table 1). *S. aureus* and CNS isolates were taken from mastitis samples from cows. *S. intermedius* isolates were obtained from clinical samples of dogs. The CNS included eight *S. epidermidis*, five *S. xylosus*, four *S. chromogenes*, three *S. cohnii*, three *S. haemolyticus*, two *S. hyicus*, two *S. saprophyticus*, two *S. simulans*, and one *S. warneri* isolate. Hence, the number of CNS available for this study was relatively low, and results with them should be considered preliminary.

Detection of the *blaZ* gene by PCR was used as a reference method, as the presence of *blaZ* has been demonstrated to correlate well with β -lactamase production (5). The bacterial cells were lysed as described by Predari et al. (10). PCR amplification was performed with primers having the following sequences (5'→3'): forward, AAG AGA TTT GCC TAT GCT TC; reverse, GCT TGA CCA CTT TTA TCA GC (11). All of the PCR parameters were as described previously (5).

The staphylococcal isolates were tested with the following assays and according to the manufacturers' instructions: (i) Beta-Lactamase Identification Sticks (Oxoid Ltd., Basingstoke, Hampshire, England); (ii) Beta-Test MW980 strips (Medical

Wire Equipment Co. Ltd., Corsham, Wiltshire, England); (iii) Cefinase test (bioMérieux, Marcy-l'Etoile, France); (iv) lyophilized nitrocefin SR00112 (Oxoid, Ltd.), using a broth method and a slide method; (v) nitrocefin disk (AB Biodisk, Solna, Sweden), using bacterial suspensions in physiological saline; (vi) a microbiological clover leaf test (2); and (vii) a commercial test based on detection of the *blaZ* gene by PCR (PathoProof Mastitis PCR assay; Finnzymes Oy, Espoo, Finland).

The clover leaf test was performed as described by Bergan et al. (2), with a *Staphylococcus aureus* Oxford strain (ATCC 9144) used as an indicator on Mueller-Hinton agar (BD, Le Pont de Claix, France). The PathoProof Mastitis PCR assay was carried out with bacterial DNA extracted from blood-agar cultures by Chelex-proteinase K treatment. The fragment lengths resulting from the PCR assay were compared against a ϕ X174 DNA-HaeIII digest molecular weight marker under UV light. The PathoProof Mastitis PCR assay amplifies a 170-bp fragment for bacterial strains positive for the *blaZ* gene. In addition, the kit amplifies a 340-bp internal positive control fragment for verification of acceptable PCR conditions. The presence of the positive control was always confirmed before a given strain was scored as negative for the *blaZ* gene.

Staphylococcus aureus ATCC 29213 was used as a positive control, and *Staphylococcus aureus* ATCC 25923 was used as a negative control each time the tests were performed.

A McNemar test was used to assess the statistical significance of differences between each β -lactamase test and the detection of the *blaZ* gene with the reference method.

The results of this study indicate that the sensitivities and specificities of the different tests vary for *S. aureus* and *S. intermedius*, as well as for CNS (Table 1). The Beta-Test MW980 strip, nitrocefin SR00112 broth method, nitrocefin disks, and PathoProof Mastitis PCR assay were the only methods that did not produce any false-positive results. The PathoProof Mastitis PCR assay was the only method that did not give any false-negative results (Table 1).

In the instructions for the color-based tests (Beta-Lactamase Identification Sticks, Beta-Test MW980, Cefinase, nitrocefin SR00112, and nitrocefin disks), a positive reaction was described as a distinctive color change. A reaction was considered negative if no color appeared. However, the numbers of staphylococci with only a very weak color reaction were very high: 19 with the Beta-Lactamase Identification Sticks, 8 with Beta-Test MW980 strips, 21 with the Cefinase test, 11 with the nitrocefin

* Corresponding author. Mailing address: Finnish Food Safety Authority Evira, Mustialankatu 3, 00790 Helsinki, Finland. Phone: 358 2077 2003. Fax: 358 2077 24350. E-mail: anna.pitkala@evira.fi.

[▽] Published ahead of print on 11 April 2007.

TABLE 1. Results of five commercial β -lactamase tests, the clover leaf test, and the PCR kit test

Organism and test method	<i>n</i>				Sensitivity (%) (95% CI)	Specificity (%) (95% CI) ^a
	True positive	False positive	True negative	False negative		
<i>S. aureus</i> (n = 95, in Cefinase n = 94)						
Beta-Lactamase Identification Sticks	26	0	66	3	89.7 (81.3–94.7)	100 (95.2–99.9)
Beta-Test MW980 strip	26	0	66	3	89.7 (81.3–94.7)	100 (95.2–99.9)
Cefinase	19	2	64	9	70.0 (59.5–78.8)	97.0 (90.6–99.3)
Nitrocefin SR00112 broth method	24	0	66	5	82.8 (73.4–89.5)	100 (95.2–99.9)
Nitrocefin SR00112 slide method	18	0	66	11	62.1 (51.5–71.7)	100 (95.2–99.9)*
Nitrocefin disk	29	0	66	0	100 (95.2–99.9)	100 (95.2–99.9)
PathoProof Mastitis PCR assay	29	0	66	0	100 (95.2–99.9)	100 (95.2–99.9)
Clover leaf method	28	0	66	1	96.6 (90.0–99.1)	100 (95.2–99.9)
<i>S. intermedius</i> (n = 50)						
Beta-Lactamase Identification Sticks	31	0	11	8	79.5 (65.3–89.1)	100 (91.1–99.8)*
Beta-Test MW980 strip	24	0	11	15	61.5 (46.7–74.6)	100 (91.1–99.8)*
Cefinase	22	0	11	17	56.4 (41.7–70.1)	100 (91.1–99.8)*
Nitrocefin SR00112 broth method	20	0	11	19	51.3 (36.9–65.5)	100 (91.1–99.8)*
Nitrocefin SR00112 slide method	19	0	11	20	48.7 (34.5–63.1)	100 (91.1–99.8)*
Nitrocefin disk	24	0	11	15	61.5 (46.7–74.6)	100 (91.1–99.8)*
PathoProof Mastitis PCR assay	39	0	11	0	100 (91.1–99.8)	100 (91.1–99.8)
Clover leaf method	38	0	11	1	97.4 (87.1–99.8)	100 (91.1–99.8)
CNS (n = 30)						
Beta-Lactamase Identification Sticks	10	5	13	2	— ^b	—
Beta-Test MW980 strip	6	0	18	6	—	—
Cefinase	8	3	15	4	—	—
Nitrocefin SR00112 broth method	6	0	18	6	—	—
Nitrocefin SR00112 slide method	6	1	17	6	—	—
Nitrocefin disk	8	0	18	4	—	—
PathoProof Mastitis PCR assay	12	0	18	0	—	—
Clover leaf method	12	10	8	0	—	—

^a *, comparison with the reference method ($P < 0.05$).^b —, not calculated due to small sample size.

SR00112 broth method, 27 with the nitrocefin SR00112 slide method, and 19 with nitrocefin disks. Of these, 13, 8, 19, 11, 23, and 15 isolates, respectively, were truly *blaZ* positive based on the reference test. Different β -lactamase enzymes exhibit differences in substrate specificities, which may explain the partial color reactions (12). Nevertheless, partial color reactions resulted in an increase in false negatives for all of the color reaction-based tests.

Bergan et al. (2) considered the clover leaf method to be the most reliable alternative for investigating β -lactamase production in staphylococci. In the present study, however, the rate of false-positive CNS with the clover leaf test was very high (10 out of 30 CNS samples). Additionally, the inhibition zone caused by *S. intermedius* against the indicator strain complicated interpretation of the results. The reason for this result, which was also reported by Jarlöv and Rosdahl (6), remains unclear. Finally, the clover leaf method is rather slow and, in our estimate, more useful in research than in routine use.

The detection of antimicrobial resistance genes with PCR is an interesting possibility for complementing or replacing conventional antibiotic resistance testing. In this study, compared against the reference method, the PathoProof Mastitis PCR assay was the most reliable test (Table 1). Support for the potential of β -lactamase production by all strains positive for *blaZ* based on PCR was provided by the fact that at least one method based on β -lactamase identification always detected these strains as positive.

We conclude that the PathoProof Mastitis PCR assay, the nitrocefin SR00112 broth method, and nitrocefin disks can be recommended for routine clinical use in veterinary laboratories. However, laboratories should be aware that, with the color-based methods, the enzymatic reactions can be incomplete, leading to a potentially large number of false-positive or false-negative results.

We thank Stina Kärkkäinen and Kaisa Rouhiainen for excellent technical assistance.

REFERENCES

1. Aarestrup, F. M., and S. Schwarz. 2006. Antimicrobial resistance in staphylococci and streptococci of animal origin, p. 187–212. In F. M. Aarestrup (ed.), Antimicrobial resistance in bacteria of animal origin. ASM Press, Washington, DC.
2. Bergan, T., J. N. Bruun, A. Digraanes, E. Lingaas, K. K. Melby, and J. Sander. 1997. Susceptibility testing of bacteria and fungi. Report from the Norwegian Working Group on Antibiotics. Scand. J. Infect. Dis. **103**(Suppl.): 1–36.
3. Chambers, H. F. 2001. The changing epidemiology of *Staphylococcus aureus*? Emerg. Infect. Dis. **7**:178–182.
4. Fox, L. K., S. T. Chester, J. W. Hallenberg, S. C. Nickerson, J. W. Pankey, and L. D. Weaver. 1995. Survey of intramammary infections in dairy heifers at breeding age and first parturition. J. Dairy Sci. **78**:1619–1628.
5. Haveri, M., S. Suominen, L. Rantala, T. Honkanen-Buzalski, and S. Pyörälä. 2005. Comparison of phenotypic and genotypic detection of penicillin G resistance of *Staphylococcus aureus* isolated from bovine intramammary infection. Vet. Microbiol. **106**:97–102.
6. Jarlöv, J. O., and V. T. Rosdahl. 1986. Quantitative determination of β -lac-

- tamase production in *Staphylococcus aureus* strains compared to qualitative testing by a microbiological clover leaf test, a chromogenic cephalosporin test and a iodometric test. *Acta Pathol. Microbiol. Immunol. Scand. B* **94**:415–421.
7. Lowy, F. D. 2003. Antimicrobial resistance: the example of *Staphylococcus aureus*. *J. Clin. Investig.* **111**:1265–1273.
 8. Myllys, V., K. Asplund, E. Brofeldt, V. Hirvelä-Koski, T. Honkanen-Buzalski, J. Junttila, L. Kulkas, O. Myllykangas, M. Niskanen, H. Saloniemi, M. Sandholm, and T. Saranpää. 1998. Bovine mastitis in Finland in 1988 and 1995—changes in prevalence and antimicrobial resistance. *Acta Vet. Scand.* **39**:119–126.
 9. Østerås, O., L. Sølvørød, and O. Reksen. 2006. Milk culture results in a large Norwegian survey—effects of season, parity, days in milk, resistance, and clustering. *J. Dairy Sci.* **89**:1010–1023.
 10. Predari, S. C., M. Ligozzi, and R. Fontana. 1991. Genotypic identification of methicillin-resistant coagulase-negative staphylococci by polymerase chain reaction. *Antimicrob. Agents Chemother.* **35**:2568–2573.
 11. Vesterholm-Nielsen, M., M. Olhom Larsen, J. Elmerdahl Olsen, and F. M. Aarestrup. 1999. Occurrence of the blaZ gene in penicillin resistant *Staphylococcus aureus* isolated from bovine mastitis in Denmark. *Acta Vet. Scand.* **40**:279–286.
 12. Volardi, R. K. R., and D. S. Kernodle. 1998. Characterization of a chromosomal gene encoding type B β -lactamase in phage group II isolates of *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **42**:3163–3168.