JOURNAL OF PURE AND APPLIED MICROBIOLOGY, June 2015. Vol. **9**(2), p. 1539-1544

Profiling of Antibiotic Resistance of *Staphylococcus aureus* Obtained from Mastitic Milk of Cattle and Buffalo

Rahul Yadav[1]*, Sandeep Kumar Sharma[2], Jyotika Yadav[3], Sneha Choudhary[1] and Anil Kumar Kataria[1]

[1]Department of Veterinary Microbiology and Biotechnology. College of Veterinary and Animal Sciences, Bikaner. Rajasthan University of Veterinary and Animal science, Bikaner-334001 (Rajasthan), India.
[2]Department of Veterinary Microbiology and Biotechnology. Post graduate institute of veterinary education and research, Jaipur. Rajasthan University of Veterinary and Animal science, Bikaner-334001 (Rajasthan), India.
[3]College of Veterinary and Animal Sciences, Hisar. Lala Lajpat Rai University of Veterinary & Animal Sciences, Hisar-125001 (Haryana), India.

(Received: 02 February 2015; accepted: 13 March 2015)

The present study was carried out to evaluate antibiotic susceptibility and beta-lactam antibiotic resistance of *Staphylococcus aureus* isolates from mastitic milk of cattle and buffalo.In the present investigation *S. aureus* isolates were subjected to antibiogram studies using 33 antibiotics belonging to different categories and generations. Antibiotics such as doxycycline, gentamicin, methicillin, netiline, rifampisin and tobramycin were 100% effective against all isolates from both cattle and buffalo. In case of resistance pattern, maximum resistance was exhibited against nalidixic, cephalexin, polymixin B, cefexime, cephalexin and amoxycillin from both species respectively. The detection of beta-lactam antibiotic resistance was carried out by acidimetric method resulting that only two (12.5%) out of 16 isolates from cattle mastitic milk and nine (56.2%) out of 16 isolates from buffalo mastitic milk were detected as beta-lactamase producer. The analysis of the antibiogram revealed that the susceptibility and resistance shown by the isolates was dependent on use of the antibiotics. The antibiotics of initial generations showed lower efficacies than the antibiotics of latest generations.

Key words: *Staphylococcus aureus*, Cattle, Buffalo, Mastitis, Antibiogram.

Bovine mastitis is a multifactorial disease and is one of the most difficult to control. It can be caused by many different bacterial species, the most common of which is *Staphylococcus aureus* (Forsman *et al.*, 1997). Mastitis is a well-known threat in dairy sector due to its massive economic loss (Kumar *et al.*, 2010). Clinical mastitis among cattle and buffalo is usually characterized by swelling or pain in the udder, milk with an abnormal appearance and in some cases, increased rectal temperature, lethargy, anorexia and even death. Antimicrobial agents play an important role in the treatment and control of mastitis (Oliveira *et al.*, 2011). Over the last few decades, there was a sudden increase in the use of antibiotics in veterinary as well as medical science not only to control disease but also as prophylactic measure for bacterial infections secondary to viral infections (Lindeman *et al.*, 2013). The use of antibiotic in a frequent manner leads to the resistance in different diseases causing bacterial species. So it is very important to know about the resistance of the bacteria prior to administration of the treatment (Wang *et al.*, 2008). *Staphylococcus aureus* is able to produce a host of structural changes in udder and keeps on developing resistance against the most commonly used antibiotics. These resistant microorganisms become part of the environment and are transmitted from animals to humans or vice-versa. The evolution of antibiotic resistance in *Staphylococcus aureus* strains is a serious cause

* To whom all correspondence should be addressed.
Tel.: 09466930987, 09694065689;
E-mail: drrahul16889@gmail.com

of concern in dairy animals as it acquires antibiotic resistance with remarkable proficiency (Booth *et al.,* 2001). An increased resistance of *S. aureus* isolated from dairy cattle with mastitis against antibiotics has been reported by many workers (Wang *et al.,* 2008; Jeykumar *et al.,* 2013). Beta-lactams such as penicillin are the most widely used antibiotics, and beta-lactamases are the greatest source of resistance to penicillins. An understanding of beta-lactamase detection is therefore valuable (Kilic and Cirak, 2006). Hence, most effective antibiotics are sorely needed to control the infection. Presently there is growing concern among scientists in regards to increasing resistance in pathogens. The concerns are multifaceted *viz.* inaccurate diagnosis, defective dosage, indiscriminate use, development of new drugs etc.

MATERIALS AND METHODS

Sample collection, Isolation and identification

In the present investigation, total 89 mastitic milk samples were collected (41 were from cattle and 48 were from buffalo) and proceeded phenotypic identification as per standard procedures [9]. All phenotypically identified *Staphylococci* isolates were further confirmed to be *Staphylococcus aureus* based on 23S rRNA gene ribotyping (Straub *et al.,* 1999).

Beta-lactamase activity (Acidimetric method)

Hydrolysis of the ²-lactam ring generates a carboxyl group, acidifying unbuffered systems. The resulting acidity can be tested in tubes. The method described by Livermore and Brown (2001) by which 2 ml of 0.5% (w/v) aqueous phenol red solution is diluted with 16.6ml distilled water, and 1.2g of benzylpenicillin is added. The pH is adjusted to 8.5 with 1M NaOH. The resulting solution, which should be violet in color, can be stored at -20°C. Before use, 100µl portions are distributed into tubes or microtitre wells and inoculated with bacteria from culture paltes (not broth) to produce dense suspensions. A yellow color with in 5 min indicates ²-lactamase activity. Positive and negative controls must be run in parallel.

Antibiotic sensitivity test

To determine the antibiogram of the isolates against different antibiotics the method of Bauer *et al*. (1966) was followed. In brief, the isolates were inoculated in sterile 5 ml nutrient broth tube, incubated for 18 h at 37°C and then the opacity was adjusted to 0.5 McFarland opacity standards (Quinn *et al*., 1994). The inoculum was well spread over the agar surface with the help of sterilized swab. Plates were allowed to dry for 10 min at 37°C and then antibiotic discs were carefully placed on the surface with enough space around each disc for diffusion of the antibiotic. Plates were incubated for 24 h at 37°C and the zone of inhibition of growth of the organism around each disc was measured in millimeters.

RESULTS AND DISCUSSION

The ribotyping produced an amplicon of 1250 bp in 32 isolates (16 from cattle and 16 from buffalo) confirming them to be *Staphylococcus aureus.* Indiscriminate antibiotic use in dairy animals leads to treatment failure, escalated treatment costs and development of resistance to antimicrobials. The multiplicity of the cause and emergence of resistance due to indiscriminate and prolonged use of antibiotics in absence of antibiogram is a major hurdle in physical, chemical and microbiological control of mastitis. In our present investigation, acidimetric method was used for detection of beta-lactamase resistance for all the isolates and found that only two (12.5%) out of 16 isolates from cattle mastitic milk were beta-lactamase producer, while in case of buffalo isolates, nine (56.2%) out of 16 isolates were beta-lactamase producer. Our study revealed much lower percentage of beta-lactamase producers than that reported by Kilic and Cirak (2006). Using acidimetric method, they found beta-lactamase production rates of coagulase positive isolates as 84.3%-85.5%, which is much higher than our observations. Similarly, in a study conducted by Oberhofer and Towle (1982), 44 (73.3%) out of 60 *S. aureus* isolates showed as beta-lactamase producers by acidimetric method.

Further, *S. aureus* isolates were subjected to antibiogram studies using 33 antibiotics belonging to different categories and generations. Seven antibiotics (Table 1) namely chloramphenicol, doxycycline, gentamicin, methicillin, netiline, rifampisin and tobramycin were 100% effective

against all the 16 isolates from cattle followed by ceftriaxone (93.75%), neomycin, cefalor, erythromycin, azithromycin, cefdinir, novobiocin (87.50%), ofloxacin, linezolid, cotrimoxazole, bacitracin (81.25%) and amoxyclav (75%). More than 75% of the isolates were susceptible to 19 antibiotics. The highest resistance was recorded for amoxycillin (81.25%). Eight antibiotics namely cefdinir, doxycycline, gentamicin, linezolid, methicillin, netiline, rifampisin and tobramycin showed 100% efficacy against buffalo isolates (Table 2). A number of 17 antibiotics were effective against more than 75% of the isolates from buffalo origin. The maximum resistance was exhibited against nalidixic acid (93.75%). A Slight difference was recorded for the susceptibility against doxycycline, gentamicin, methicillin, netiline, rifampisin and tobramycin antibiotics among both cattle and buffalo isolates. Likewise in case of resistance pattern, cephalexin, polymixin B, cefexime, cephalexin and amoxycillin were ineffective for both cattle and buffalo isolates. The extensive variability in the antibiogram patterns exhibited by *S. aureus* from different localities and at different time intervals suggested that this organism is changing its response to different antibiotics very frequently hence, it is imperative to use right antibiotic in control of *S. aureus* infections. In the present investigation the resistance towards cefixime was unexpected

Table 1. Antibiogram of *S. aureus* isolates from cattle with clinical mastitis

S. No.	Antibiotic Disc	Per cent		
		Sensitive	Intermediate	Resistant
1	Chloramphenicol	100.00	-	-
2	Doxycycline	100.00	-	-
3	Gentamicin	100.00	-	-
4	Methicillin	100.00	-	-
5	Netiline	100.00	-	-
6	Rifampisin	100.00	-	-
7	Tobramycin	100.00	-	-
8	Ceftriaxone	93.75	6.25	-
9	Neomycin	87.50	12.50	-
10	Cefalor	87.50	6.25	6.25
11	Erythromycin	87.50	6.25	6.25
12	Azithromycin	87.50	-	12.50
13	Cefdinir	87.50	-	12.50
14	Novobiocin	87.50	-	12.50
15	Ofloxacin	81.25	18.75	-
16	Linezolid	81.25	12.50	6.25
17	Cotrimoxazole	81.25	6.25	12.50
18	Bacitracin	81.25	-	18.75
19	Amoxyclav	75.00	-	25.00
20	Cefotaxime	68.75	25.00	6.25
21	Sparfloxacin	68.75	18.75	12.50
22	Vancomycin	68.75	-	31.25
23	Moxifloxacin	62.50	12.50	25.00
24	Trimethoprim	62.50	6.25	31.25
25	Polymixin B	50.00	-	50.00
26	Nitrofurazone	37.50	50.00	12.50
27	Oxytetracycline	25.00	56.25	18.75
28	Cefexime	18.75	56.25	25.00
29	Nalidixic acid	18.75	43.75	37.50
30	Enrofloxacin	6.25	56.25	37.50
31	Cloxacillin	-	62.50	37.50
32	Cephalexin	-	50.00	50.00
33	Amoxycillin	-	18.75	81.25

because this antibiotic is not being used in the veterinary care thereby chances of development of resistance towards this antibiotic by udder *S. aureus* are very rare. This antibiotic is a third generation cephalosporin which is being used extensively in human medicine. The possible explanation for resistance occurrence may the fact that these isolates might have come from human sources (Khichar, 2011; Rathore and Kataria (2012) and Bhati *et al.* (2013). The susceptibility of all the isolates towards gentamicin in present investigation is almost similar to observations of those of Stephan *et al.* (2001), Aires-de-Sousa (2007), Ebrahimi *et al.* (2007), Moon *et al.* (2007), Ebrahimi and Taheri (2009) and Nathawat *et al.* (2013).

Vancomycin sensitivity among cattle isolates (68.75%) in the present study is in agreement to that of Khichar, 2011 (57.14%); El-Jakee *et al.* (2011) (95%); Upadhyay (2009) (60%); and Alian *et al.* (2012) (100 %). Tobramycin was one of the most effective antibiotics against all the isolates in our study was contrary with the study of El-Jakee *et al.* (2011), where 20 % resistance was also reported. Khichar, (2011) used 27 different antibiotics against 28 *S. aureus* isolates of cattle mastitis origin wherein they recorded that most effective antibiotic was azithromycin (92.86%) followed by cloxacillin and methicillin (89.29%). He also

Table 1. Antibiogram of *S. aureus* isolates from cattle with clinical mastitis

S. No.	Antibiotic Disc	Per cent		
		Sensitive	Intermediate	Resistant
1	Cefdinir	100.00	-	-
2	Doxycycline	100.00	-	-
3	Gentamicin	100.00	-	-
4	Linezolid	100.00	-	-
5	Methicillin	100.00	-	-
6	Netiline	100.00	-	-
7	Rifampisin	100.00	-	-
8	Tobramycin	100.00	-	-
9	Azithromycin	93.75	6.25	-
10	Bacitracin	93.75	6.25	-
11	Cefalor	93.75	6.25	-
12	Ceftriaxone	93.75	6.25	-
13	Ofloxacin	93.75	-	6.25
14	Moxifloxacin	87.50	6.25	6.25
15	Sparfloxacin	87.50	6.25	6.25
16	Cefotaxime	81.25	18.75	-
17	Chloramphenicol	81.25	6.25	12.50
18	Neomycin	62.50	25.00	12.50
19	Novobiocin	62.50	25.00	12.50
20	Erythromycin	56.25	43.75	-
21	Cotrimoxazole	56.25	18.75	25.00
22	Trimethoprim	56.25	-	43.75
23	Amoxyclav	50.00	-	50.00
24	Vancomycin	43.75	-	56.25
25	Nitrofurazone	12.50	81.20	6.25
26	Amoxycillin	12.50	18.75	68.75
27	Polymixin B	12.50	-	87.50
28	Oxytetracycline	6.25	81.20	12.50
29	Cloxacillin	6.25	68.75	25.00
30	Enrofloxacin	-	68.75	31.25
31	Cephalexin	-	31.25	68.75
32	Cefexime	-	18.75	81.25
33	Nalidixic acid	-	6.25	93.75

recorded resistance towards ampicillin, cefixime, metronidazole and nalidixic acid for all the isolates. In the present investigation the highest number (75%) of the total isolates were resistant towards beta-lactam antibiotics such as amoxycillin and amoxyclav and is in complete agreement with Ebrahimi *et al.* (2009), Pereira *et al.* (2009), Turutoglu *et al.* (2006) and Hussain *et al.* (2012).

In the present investigation difference in the antibiogram pattern for cattle and buffalo isolates was observed only for some antibiotics. Susceptibility to chloramphenicol was shown by 100% isolates from cattle whereas on the other hand 81.25% buffalo isolates were sensitive to chloramphenicol. In the area of study cephalexin, cloxacillin, amoxycillin and tetracycline are being used for a long time in the treatment of mastitis and other infections in animals. Hence, these antibiotics were recorded to be either ineffective or very less effective. For some time now amoxyclav is also being used which has also shown lesser efficacy in comparison to earlier reports by other workers from the same study area (Bhati *et al.,* 2013). In our investigation all the three drugs of amino-glycosides group namely gentamicin, netiline and tobramycin were effective against all the 32 isolates of *S. aureus*. Similarly, rifampisin group was effective against all the isolates. However, two drugs namely oxytetracycline and doxycycline belonging to same antibiotic group i.e. tetracycline showed opposite susceptibility patterns. Likewise, the antibiotics of initial generations showed lower efficacies than the antibiotics of latest generations. The analysis of the antibiogram revealed that the susceptibility and resistance shown by the isolates was dependent on use of the antibiotics i.e. lesser the use more the susceptibility of the isolates was detected. Many workers have worked with *S. aureus* of various origins in regards to their antibiogram patterns and found that *S. aureus* are endowed with capability of developing resistance towards an antibiotic against which isolates are exposed even for shorter periods. Further if exposure to same antibiotics is removed, the isolates become susceptible to that antibiotic against which they were previously resistant.

CONCLUSION

The antibiotics from aminoglycosides were most effective against all isolates from both species. However, two drugs namely oxytetracycline and doxycycline belonging to same antibiotic group i.e. tetracycline showed opposite susceptibility patterns. Likewise, the antibiotics of initial generations showed lower efficacies than the antibiotics of latest generations. Cephalexin, cloxacillin, amoxycillin and tetracycline were less effective as are being used for a long time in the treating animals in the study area.

REFERENCES

1. Forsman, P.; Tilsala-Timisjarvi, A. and Alatossava, T. Identification of Staphylococcal and Streptococcal causes of bovine mastitis using 16S–23S rRNA spacer regions. *Microbiol*, 1997; **143** (Pt 11), 3491 – 3500.
2. Kumar, R.; Yadav, B.R. and Singh, R.S. (2010). Genetic Determinants of antibiotic resistance in *Staphylococcus aureus* isolates from milk of mastitic crossbred cattle. *Curr. Microbiol.* 2010; **60**:379–386.
3. Oliveira, A.A.F.; Pinheiro, J.W.; Jr.; Mota, R.A.; Cunha, M.L.R.S.; Lopes, C.A.M. and Rocha, N.S. Phenotype characterization of Staphylococcus species strains isolated from buffalo (*Bubalus bubalis*) milk. *J. Vet. Diagn. Invest.*, 2011; **23**: 1208.
4. Lindeman, C.J.; Portis,E.; Johansen, L.; Mullins, L.M. and Stoltman, G.A. Susceptibility to antimicrobial agents among bovine mastitis pathogens isolated from North American dairy cattle, 2002–2010. *J. Vet. Diagn. Invest*, 2013; doi: 10.1177/1040638713498085.
5. Wang, Y.; Wu, C.M.; Lu, L.M.; Ren, G.W.N.; Cao, X.Y. and Shen, J.Z. Macrolide-lincosamide resistant phenotypes and genotypes of *Staphylococcus aureus* isolated from bovine clinical mastitis. *Vet. Microbiol*, 2008; **130** (2008) 118–125.
6. Booth, M.C.; Pence, L.M.; Mahasreshti, P.; Callegan, M. and Gilmore, M. Clonal associations among *Staphylococcus aureus* isolates from various sites of infections. *Infect. Immun*, 2001; **69**(1): 345–352.
7. Jeykumar, M.; Vinodkumar, G.; Bashir, B.P. and Krovvidi, S. Antibiogram of mastitis pathogens in the milk of crossbred cows in Namakkal district, Tamil Nadu. *Vet. World*, 2013; **6**(6):354-

356.

8. Kilic, E. and Cirak, M.Y. Comparison of Staphylococcal Beta-lactamase detection methods. *FABAD J. Pharm. Sci*., 2006; **31**: 79-84, 2006.
9. Quinn, P.J.; Carter, M.E.; Markey, B.K. and Carter, G.R. Clinical Veterinary Microbiology. Wolfe Publishing, Mosby-Year Book Europe Ltd. Lynton House, 7-12. Tavistock Square, London WCH 9LB, England 1994.
10. Straub, J.A.; Hertel, C. and Hammes, W.P. A 23S rRNA target polymerase chain reaction based system for detection of *Staphylococcus aureus* in meat starter cultures and dairy products. *J. Food. Protect,* 1999; **62** (10): 1150-1156.
11. Livermore, D.M. and Brown, D.F.J. Detection of ²-lactamase mediated resistance. *J. Antimicrob. Chemother,* 2001 **48**, *Suppl. S1, 59-64*.
12. Bauer, A.W.; Kirby, W.M.; Sherris, J.C. and Turck, M. Antibiotic susceptibility testing by a standardized single disc method. *Am. J. Clin. Pathol,* 1966; **45**(4): 493-496.
13. Oberhofer, T.R. and Towle, D.W. Evaluation of the rapid pencillinase paper strip test for detection of beta-lactamase. *J. Clin. Microbiol,* 1982; **15**(2) p. 196-199.
14. Khichar, V. Genotypic characterization of *Staphylococcus aureus* from clinical mastitis in cattle in relation to some virulence factors. M.V.Sc. Thesis submitted to Rajasthan University of Veterinary and Animal Sciences, Bikaner 2011.
15. Rathore, P. and Kataria, A.K. Antimicrobial susceptibility profiling of *Staphylococcus aureus* of camel (Camelus dromedarius) skin origin. *ABAH Int. J. Bioflux,* 2012, **4**(2).
16. Bhati, T.; Kataria, A.K.; Nathawat, P.; Sharma, S.K.; Mohammed, N. and Mathur, M. Antimicrobial susceptibility profiling of *Staphylococcus aureus* isolates from bovine subclinical mastitis. *Vet. Res,* 2013; **6**(2): 39-42, 2013.
17. Stephan, R.; Annemuller, C.; Hassan, A. and Lammler, C. Characterization of enterotoxigenic *Staphylococcus aureus* strains isolated from bovine mastitis in north-east Switzerland. *Vet. Microbiol,* 2001; **78** (2001) 373–382.
18. Aires-de-Sousa, M.; Parente, C.E.S.R.; Vieira-da-Motta, O.; Bonna, I.C.F.; Silva, D.A. and de Lencastre, H. Characterization of *Staphylococcus aureus* isolates from buffalo, bovine, ovine, and caprine milk samples collected in Rio de Janeiro state, Brazil. *Appl. Environ. Microbiol.* June 2007, p. 3845–3849, Vol. 73, No. 12,0099-2240/07/$08.00+0.
19. Ebrahimi, A.; Khirabadi, K.H.P. and Nikookhak, F. Antimicrobials susceptibility of envirnmental bovine mastitis pathogen in west central Iran. *Pak. J. Biol. Sci,* 2007; **10**(17): 3014-3016, 2007.
20. Moon, J.S.; Lee, A.R.; Kang, H.M.; Lee, E.S.; Joo, Y.S.; Park, Y.H.; Kim, M.N and Koo, H.C. Antibiogram and coagulase diversity in Staphylococcal enterotoxin producing *Stapylococcus aureus* from bovine mastitis. *J. Dairy. Sci,* 2007; **90**: 1716-1724.
21. Ebrahimi, A. and Taheri, A.M. Characteristics of Staphylococci isolated from clinical and subclinical mastitis cows in Shahrekord, Iran. *Iran. J. Vet. Res*. Shiraz University, 2009; **10**(3),Ser. No. 28, 2009**.**
22. Nathawat, P.; Bhati, T.; Sharma, S.K.; Mohammed, N. and Kataria, A.K. Prevalence of *Staphylococcus aureus* in lactating goats with clinical mastitis and their antibiogram studies. *ABAH Int. J. Bioflux*, 2013; **5**(1): 32-37.
23. El-Jakee, J.K.; Atta, N.S.; Samy, A.A.; Bakry, A.A.; Elgabry, E.A.; Kandil, M.M. and El-Said, W.A.G. Antimicrobial resistance in clinical isolates of *Staphylococcus aureus* from bovine and human sources in Egypt. *Global Veterinaria*, 2011; **7** (6): 581-586.
24. Upadhaya A. Characterization of Staphylococcus aureus with special reference to capsular polysaccharide genotyping isolated from clinically mastitic cattle and goats. M.V.Sc. thesis submitted to the Rajasthan Agriculture University, Bikaner, Rajasthan 2009.
25. Alian, F.; Rahimi, E.; Shakerian, A.; Momtaz, H.; Riahi, M. and Momeni, M. Antimicrobial resistance of *Staphylococcus aureus* isolated from bovine, sheep and goat raw milk. *Global Veterinaria*, 2012; **8**(2): 111-114.
26. Pereira, V.; Lopes, C.; Castro, A.; Silva, J.; Gibbs, P. and Teixeira, P. Characterization for enterotoxin production, virulence factors, and antibiotic susceptibility of *Staphylococcus aureus* isolates from various foods in Portugal. *Food. Microbiol,* 2009; **26**; 278–282.
27. Turutoglu, H.; Ercelik, S. and Ozturk, D. Antibiotic resistance of *Staphylococcus aureus* and coagulase-negative Staphylococci isolated from bovine mastitis. *Bull. Vet. Inst. Pulawy,* 2006; **50**, 41-45, 2006.
28. Hussain, A.; Shakoor, A.; Yousaf, A.; Rehman, S.U. and Zaman, M.A. Clinical and subclinical *Staphylococcus aureus* in dairy buffaloes: disease characteristics and antibiotic susceptibility profiles of isolates. *J. Anim. Pl. Sci,* 2012; **22**(3 Suppl.): 2012, Page: 217-220.