RESEARCH ARTICLE

Prevalence of Methicillin-Resistant *Staphylococcus aureus* (MRSA) in Dairy Farms of Pokhara, Nepal

Lok Raj Joshi[1]*, Ananta Tiwari[1], Shiva Prasad Devkota[2], Sushil Khatiwada[1], Sushil Paudyal[1] and Kedar Raj Pande[2]

[1]Institute of Agriculture and Animal Science, Rampur, Chitwan, Nepal
[2]Regional Veterinary Laboratory, Pokhara, Kaski, Nepal

ARTICLE INFO

*Corresponding Author
Lok Raj Joshi
lokrajvet@gmail.com

ABSTRACT

MRSA (Methicillin-resistant *Staphylococcus aureus*) are the strains of *S. aureus* that are resistant to all the β-lactam antibiotics. MRSA has received a lot of attention in recent years as a zoonotic organism when studies suggested the possibility of animals serving as reservoirs for human MRSA infection. A cross-sectional study was carried out from October, 2012 to January, 2013 to determine the prevalence of MRSA in dairy farms of Pokhara. Milk samples were collected from 10 dairy farms of Pokhara selecting 10 cattle from each farm thereby making a sample size of 100 cattle (400 quarters). *Staphylococus aureus* was isolated from milk samples using bacterial culture and biochemical tests. MRSA was identified using cefoxitin disk diffusion method. All the *S. aureus* isolates were subjected to antimicrobial susceptibility test. Out of 400 milk samples, *S. aureus* were isolated from 119 (29.7%) samples. MRSA were found in 45 (11.25%) milk samples. *S. aureus* isolates were found sensitive to ciprofloxacin (97.47%), gentamicin (94.95%), ceftriaxone (91.59%) and tetracycline (89.91%) in descending order while they were found least sensitive to cefoxitin (62.18%). Results clearly suggest the increasing resistance of *S. aureus* to β-lactam antibiotics causing emergence of MRSA.

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) are the strains of *S. aureus* that are resistant to all available penicillins and other β-lactam antimicrobial drugs (David and Daum, 2010; Kumar *et al*., 2011). The remarkable ability of *S. aureus* to acquire drug resistance has led to the emergence of MRSA. This resistance is caused by an alternative penicillin-binding protein, called PBP2a. PBP2a is encoded by the *mec*A gene located in the mobile genetic element called Staphylococcal cassette chromosome (SCC*mec*) (Vanderhaeghen *et al*., 2010).

MRSA is the leading cause of nosocomial infection in hospital settings thus referred to as HA-MRSA (Healthcare-associated MRSA). Some strains of MRSA were found in people who never had contact with hospital and these strains were named as CA-MRSA (Community - associated MRSA) (Faccioli-Martins and Souza da Cunha, 2012). MRSA was reported in veterinary setting after detection of drug resistant isolates from cattle in Belgium in 1970s (Devriese *et al*., 1972). Later, resistant strains were isolated from different animals like dogs, pigs, horses, poultry along with the cattle (Leonard and Markey, 2008). Strains of MRSA that affect animals were thus named as LA-MRSA (Livestock-associated MRSA) (Vanderhaegen *et al.*, 2010). LA-MRSA has emerged as an organism of zoonotic importance when scientists proclaimed the transmission of animal MRSA strains to humans (Lee, 2003). Juhasz-Kaszanyitzky *et al.* (2007) first reported the evidence of direct MRSA transmission between cattle and humans.

MRSA causes intra-mammary infections in cattle leading to mastitis. Other clinical presentation of MRSA infection includes pyoderma, arthritis, osteomyelitis, abscesses, pneumonia and bacteremia (McCarthay and Loneragan, 2009). MRSA are often multi-drug resistant, therefore, infection is either impossible to treat or requires prolonged duration of treatment.

To our knowledge, no research has been done in Nepal till now to determine the prevalence of MRSA in animals. This study aims to determine the prevalence of MRSA in the dairy farms of Pokhara for the first time in Nepal.

MATERIALS AND METHODS

Study population and sampling

A cross-sectional study was carried out in dairy farms of Pokhara valley of Nepal from October, 2012 to January, 2013. The cattle breeds included were Holstein and Jersey raised under intensive management system. Ten dairy farms having milking cattle number greater than 10 were selected. From each of the selected farms, milk samples were collected from each of the four quarters of 10 cattle thereby making sample size of 100 cattle (400 quarters). All the laboratory works were carried out at Regional Veterinary Laboratory, Pokhara.

Collection of milk samples

Udder and teats were cleaned with water and then allowed to dry. Then teats were swabbed with 70% ethyl alcohol. The first five squirts of milk were discarded. Then, 8-10 ml milk was collected in sterile containers. The milk samples were transported to Regional Veterinary Laboratory in thermocol ice box within three hours of collection.

Isolation of *S. aureus*

Isolation of *S. aureus* was done following the guidelines provided by National Mastitis Council (Bramley *et al*., 1996). Milk samples were cultured on blood agar base (HiMedia™) enriched with defibrinated sheep blood and incubated at 37°C for 24 hours. The plates producing creamy or golden yellow colonies with beta-hemolysis and the colonies that were seen as gram-positive cocci under microscope were further sub-cultured on Nutrient Agar (HiMedia™) for pure culture isolation. Bacterial smear was prepared taking a pure colony from nutrient agar and stained with gram's stain. Gram-positive samples were subjected to catalase test. Catalase-negative isolates were identified as *Streptococcus spp*. Catalase-positive samples were further subjected to plate coagulase test. All the isolates that tested positive for coagulase test were finally confirmed to be *S. aureus* (Figure 1).

Identification of MRSA

All identified *S. aureus* isolated were subjected to antimicrobial susceptibility test according to protocol from Clinical Laboratory Standards Institute (CLSI, 2007). *S. aureus* isolates were suspended in peptone water and incubated for 4 hours at 37°C. Turbidity standard of the inoculums was maintained to 0.5 McFarland units by addition of normal saline if turbidity was higher. This inoculum was plated evenly on Muller Hilton Agar (HiMedia™) plates and antibiotics disc were placed. The plates were incubated at 37°C for 18 hours.

For identification of MRSA, cefoxitin (30 μg) disk was used. *S. aureus* isolates exhibiting resistance to cefoxitin (having zone of inhibition ≤21 mm) were identified as MRSA as suggested by CLSI (2007). Along with cefoxitin, other commercially available antibiotics discs were also used to assess the antibiotic sensitivity pattern of *S. aureus*. Other discs used were: ceftriaxone (30 μg), ciprofloxacin (30 μg), gentamicin (30 μg), tetracycline (30) and cotrimoxazole (25 μg).

RESULTS

Proportion of Gram-positive and Gram-negative bacteria in milk

Out of 400 samples, gram-positive bacteria were seen in 256 samples and gram-negative bacteria were seen in 132 samples. No growth was seen in 12 samples. Of the grams-positive samples, *Staphylococcus spp* was most frequently isolated. Out of 256 grams-positive samples, *Staphylococcus spp* was isolated from 202 samples. It was followed by *Streptococcus spp* which were isolated from 42 samples. Other Gram-positive organisms like Gram-positive rod, Gram-positive diplococci were seen in 12 samples. The proportion of different Grams-positive and negative bacteria is shown in the Table 1.

Table 1: Proportion of Grams-positive and Gram-negative bacteria

Type of bacteria	Number	Percentage
Grams' Positive	256	64
Staphylococcus spp	202	50.5
Streptococcus spp	42	10.5
Other Gm +ve	12	3
Grams' Negative	132	33
No Growth	12	3

Prevalence of *S. aureus* and Methicillin-resistant Staphylococcus aureus (MRSA)

Out of 400 milk samples, *S. aureus* was isolated from 119 milk samples. Thus, overall prevalence of *S. aureus* in dairy farms of Pokhara was 29.75%. Farm-wise prevalence of *S. aureus* has been shown in Table 2. Prevalence of *S. aureus* in different farms ranges from 22.5% to 42.5%.

Out of 400 samples, 45 samples tested positive for MRSA. Thus, overall prevalence of MRSA was 11.25%. Prevalence of MRSA ranges from 5% to 15% as shown in the Table 2.

Result of antimicrobial susceptibility test

All the *S. aureus* isolates were subjected to antimicrobial susceptibility test (Figure 2). Number of isolates sensitive to different antibiotics is shown in Table 3. All the intermediate samples were included in the same category as sensitive ones on the basis of fact that intermediate samples are susceptible at higher dose of antibiotics. Most of the isolates were found to be sensitive to ciprofloxacin (97.47%) followed by gentamicin (94.95%), ceftriaxone (91.59%), tetracycline (89.91%). Lowest sensitivity was to cefoxitin (62.18%) followed by cotrimoxazole (73.10%).

DISCUSSION

The prevalence of *S. aureus* was found to be 29.7%. This signifies higher risk of *Staphylococcal* mastitis in the dairy farms of Pokhara. This finding has been supported by many authors. Pradhan *et al.* (2011) found 34.01% prevalence of *S. aureus* in cattle milk in India. Similarly, our finding is in agreement with the finding of Abera *et al.* (2012) who found 28.1% *S. aureus* in Ethopia. Our finding contradicts with the finding of Suddan *et al.* (2005), Shrestha and Bindari (2012) who found 56% and 50% prevalence of *S. aureus* in India and Bhaktapur

Table 2: Number and proportion of *S. aureus* and MRSA

Name of the farm	Samples taken	Number of *S. aureus*	Percentage of *S. aureus*	Number of MRSA	Percentage of MRSA
Farm 1	40	14	35	6	15
Farm 2	40	9	22.5	5	12.5
Farm 3	40	9	22.5	6	15
Farm 4	40	13	32.5	3	7.5
Farm 5	40	13	32.5	3	7.5
Farm 6	40	9	22.5	6	15
Farm 7	40	17	42.5	5	12.5
Farm 8	40	12	30	4	10
Farm 9	40	11	27.5	2	5
Farm 10	40	12	30	5	12.5
Total	400	119	29.75	45	11.25

Table 3: Number of resistant and sensitive isolates of *S. aureus* against different antibiotics (Value in parenthesis shows the percentage)

Name of antibiotics	Cefoxitin	Ceftriaxone	Ciprofloxacin	Gentamicin	Tetracycline	Cotrimoxazole
Resistant	45 (37.82)	10 (8.40)	3 (2.52)	6 (5.04)	12 (10.08)	32 (26.89)
Sensitive	74 (62.18)	109 (91.59)	116 (97.47)	113 (94.95)	107 (89.91)	87 (73.10)
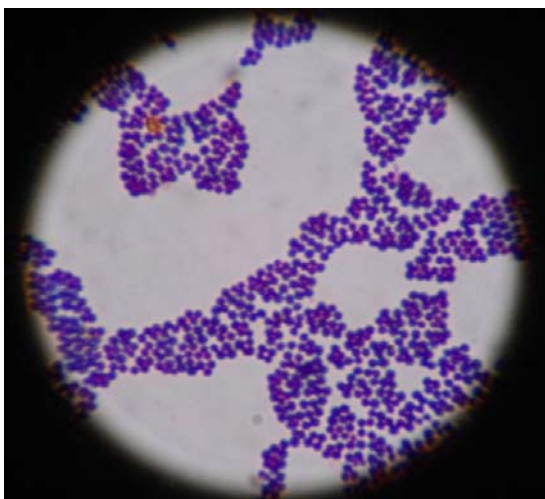
Fig. 1: *Staphylococcus aureus* seen under microscope after Gram's staining

Fig. 2: *S. aureus* showing resistant to cefoxitin (centre disc) and other antibiotics

(Nepal) respectively. Similarly, Rana (2009) reported 53% *S. aureus* from Pokhara. This difference may be due to use of only mastitis-positive milk samples by aforementioned authors, while we analyzed all the collected milk samples without prior screening for mastitis.

Prevalence of MRSA in Pokhara valley was found to be 11.25%. This finding is consistent with the finding of Kumar *et al.* (2011) who found 13.1% prevalence of MRSA in Sahiwal cattle in India. Suleiman *et al* (2012) found 8% prevalence of MRSA in Ethopia. But, our finding contradicts with the finding of many authors who reported lower prevalence of MRSA. Juhasz-Kaszanyitzky *et al.* (2007) reported 4.53% prevalence of MRSA in Hungary, Huber *et al.* (2010) reported 1.4% MRSA in Switzerland and Haran *et al.* (2012) reported 4% herd prevalence of MRSA in Minnesota, USA. Lower prevalence reported by these authors may be due to use of PCR (Polymerase Chain Reaction) method for MRSA detection instead of disk diffusion method used in this study. PCR has high sensitivity and specificity thus giving lower detection rate of *S. aureus* than culture method (Khakpoor *et al*., 2011). Higher prevalence of MRSA in Pokhara may be due to indiscriminate use of beta-lactam antibiotics as the drug of choice for the treatment of mastitis.

Antibiotic susceptibility test reveals higher sensitivity of *S. aureus* to ciprofloxacin, gentamicin, ceftriaxone and tetracycline in descending order, while cefoxitin and cotrimoxazole has been found less sensitive to *S. aureus*. This finding has been supported by Sekhan *et al.* (2011) who found ciprofloxacin (91.97%) most sensitive to *S. aureus*. Results show increasing resistance of *S. aureus* to beta-lactam antibiotics. This may have resulted due to high use of beta-lactam antibiotics to treat the mastitis cases usually administered by charlatan practitioners without performing antibiotic sensitivity test.

Conclusion

There is high prevalence of *S. aureus* (29.7%) in dairy farms of Pokhara. Prevalence of MRSA in dairy farms of Pokhara is 11.25%. This prevalence rate is comparatively higher than those reported by many other countries. Most of the *S. aureus* isolates are resistant to beta-lactam antibiotics, which indicate rising MRSA problem in dairy farms of Pokhara. Ciprofloxacin is most effective against *S. aureus* followed by gentamicin.

REFERENCES

Abera M, B Elias, K Aragaw, Y Denberga, K Amenu and D Sheferaw, 2012. Major causes of mastitis and associated risk factors in smallholder dairy cows in Shashemene, southern Ethiopia. Afric J Agric Res, 7: 3513-3518.

Bramley AJ, JS Cullor, RJ Erskine, LK Fox, RJ Harmon, JS Hogan, SC Nickerson, SP Oliver, KL Smith and LM Sordillo, 1996. Current concepts of bovine mastitis, 4th Ed. National Mastitis Council, Madison, WI.

CLSI, 2007. Performance standards for antimicrobial susceptibility testing; Seventh Information Supplement , 27, Clinical and Laboratory Standard Institute, Wayne, PA.

David MZ and RS Daum, 2010. Community-associated methicillin-resistant *Staphylococcus aureus:* epidemiology and clinical consequences of an emerging epidemic. Clin Microbiol Rev, 23: 616-687.

Devriese LA, LR Van Damme and L Fameree, 1972. Methicillin-(cloxacillin)-resistant *Staphylococcus aureus* 3468 strains isolated from bovine mastitis cases. Zentralbl. Veterinärmed, 19: 598-605. DOI: 10.1111/j.1439-0450.1972.tb00439.x

Faccioli-Martins PY and ML Souza da Cunha, 2012. MRSA epidemiology in animals. Epidemiology Insights, Available from: http://www.intechopen. com/books/epidemiologyinsights/mrsa-epidemiology-in-animals

Haran KP, SM Godden, D Boxrud and S Jawahir, 2012. Prevalence and characterization of *Staphylococcus aureus*, including Methicillin-Resistant *Staphylococcus aureus*, isolated from bulk tank milk from Minnesota dairy farms. J Clin Microbiol, 50: 688-695.

Huber H, S Koller, N Giezendanner, R Stephan and C Zweifel, 2009. Prevalence and characteristics of meticillin-resistant *Staphylococcus aureus* in humans in contact with farm animals, in livestock, and in food of animal origin. Euro Surveill, 15: p: 19542, Available online: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19542

Juhasz-Kaszanyitzky E, S Janosi and P Somogyi, 2007. MRSA transmission between cows and humans. Emerg Infect Dis, 13: 630-632.

Khakpoor M, S Safarmashaei and R Jafary, 2011. Study of milk extracted from cows related to *Staphylococcus aureus* by culturing and PCR. Glob Vet, 7: 572-575.

Kumar R, BR Yadav and RS Singh, 2011. Antibiotic resistance and pathogenicity factors in *Staphylococcus aureus* isolated from mastitic Sahiwal cattle. J Biosci, 36: 175-188.

Lee JH, 2003. Methicillin (oxacillin)-resistant *Staphylococcus aureus* strains isolated from major food animals and their potential transmission to humans. Appl Environ Microbiol, 69: 6489-6494.

Leonard FC and BK Markey, 2008. Methicillin-resistant *Staphylococcus aureus* in animals: a review. Vet J, 175: 27-36.

McCarthy RM and GH Loneragan, 2009. Methicillin-Resistant Staphylococcus aureus, the beef industry, and public health. White Paper Beef Safety Research, Cattlemen's Beef Board and National Cattlemen's Beef Association, Available on: http://www.beefresearch.org/CMDocs/BeefResearch/Safety_Fact_Sheets/MRSA.pdf

Pradhan P, SM Gopinath, MGR Reddy, HJ Dechamma and VVS Suryanarayana, 2011. Detection of major pathogens in bovine sub-clinical mastitis by multiplex PCR directly from milk samples in presence of an internal control. Indian J Fund Appl Life Sci, 1: 209-218.

Rana CB, 2009. Prevalence of subclinical mastitis in bovines of Pokhara valley. BVSc and AH Internship Report, IAAS, Rampur.

Shekhan MI, MA Al-Rodhan and JK AL-Janabi, 2011. Isolation and identification of *Staphylococcus spp.* from bovine mastitic milk and their sensitivity to some Antibiotics at Al-Qadissiya Province. AL-Qadisiya J Vet Med Sci, 10: 12-20.

Shrestha S and YR Bindari, 2012. Prevalence of sub-clinical mastitis among dairy cattle in Bhaktapur district, Nepal. Inter J Agri Biosci, 1: 16-19.

Sudhan NA, R Singh, M Singh and JS Soodan, 2005. Studies on prevalence, etiology and diagnosis of subclinical mastitis among crossbred cows. Indian J Anim Res, 39: 127-130.

Suleiman AB, VJ Umoh, JKP Kwaga and SJ Shaibu, 2012. Prevalence and antibiotic resistance profiles of Methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from bovine mastitic milk in Plateau State, Nigeria. Inter Res J Microbiol, 2: 264-270.

Vanderhaeghen W, K Hermans, F Haesebrouck and P Butaye P, 2010. Methicillin- resistant *Staphylococcus aureus* (MRSA) in food production animals. Epidemiol Infect, 138: 606-625.