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

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**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). *Staphylococcus 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 mecA gene located in the mobile genetic element called *Staphylococcal cassette chromosome (SCCmec)* (Vanderhaegen 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 thermostatic 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 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. The milk 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 isolates 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 gram-positive samples, Staphylococcus spp was most frequently isolated. Out of 256 gram-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.

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%.

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

<table>
<thead>
<tr>
<th>Type of bacteria</th>
<th>Number</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grams' Positive</td>
<td>256</td>
<td>64</td>
</tr>
<tr>
<td>Staphylococcus spp</td>
<td>202</td>
<td>50.5</td>
</tr>
<tr>
<td>Streptococcus spp</td>
<td>42</td>
<td>10.5</td>
</tr>
<tr>
<td>Other Gm +ve</td>
<td>12</td>
<td>3</td>
</tr>
<tr>
<td>Grams' Negative</td>
<td>132</td>
<td>33</td>
</tr>
<tr>
<td>No Growth</td>
<td>12</td>
<td>3</td>
</tr>
</tbody>
</table>

Prevalence of S. aureus ranges from 5% to 15% as shown in Table 2. 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 Ethiopia. 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

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

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

<table>
<thead>
<tr>
<th>Name of antibiotics</th>
<th>Cefoxitin</th>
<th>Ceftriaxone</th>
<th>Ciprofloxacin</th>
<th>Gentamicin</th>
<th>Tetracycline</th>
<th>Cotrimoxazole</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resistant</td>
<td>45 (37.82)</td>
<td>10 (8.40)</td>
<td>3 (2.52)</td>
<td>6 (5.04)</td>
<td>12 (10.08)</td>
<td>32 (26.89)</td>
</tr>
<tr>
<td>Sensitive</td>
<td>74 (62.18)</td>
<td>109 (91.59)</td>
<td>116 (97.47)</td>
<td>113 (94.95)</td>
<td>107 (89.91)</td>
<td>87 (73.10)</td>
</tr>
</tbody>
</table>

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. Saleem et al. (2012) found 8% prevalence of MRSA in Ethiopia. But, our finding contradicts with the finding of many authors who reported lower prevalence of MRSA. Juhasz-Kaszanitzky 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.
Acknowledgement

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