Drug Resistance in Chronically Affected Mastitic Cows in Two Districts of Indian State of Kashmir

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Abstract: The milk from 300 different cross bred cows suffering from the chronic clinical and subclinical mastitis was collected from the two districts of Indian Kashmir. The microbes from the milk were isolated using different culture media and the microorganisms were identified using biochemical methods and genetic characterization of 16S rRNA genes of the microbes. The tests revealed that the majority of the cows were affected with mixed bacterial infections comprising mainly of Staphylococcus aureus, Streptococcus, E. coli, Klebsiella along with yeasts and molds. The highest resistance against antibiotics was shown by coliforms and Staphylococcus aureus. The two antibiotics viz., cefixime, cloramphenicol were most effective against almost all pathogens. The axenic cultures derived from the milk were subjected to antimicrobial sensitivity tests with thirteen different types of antibiotics. It was found that about 40% of the cultures obtained from the affected cows were resistant to multiple antibiotics that are normally affective against them. This resistance of antibiotics is mainly due to the indiscriminate use of antibiotics in the veterinary medications which finds its way in soil due to their excretion in urine and feces. The presence of antibiotics in soil results in the evolution of resistant genes in soil microbiota which finds its way back to the animal microbes through various horizontal gene transfer mechanisms.

Keywords: mastitis; antibiotics; drug resistance; gene; cow.
1. Introduction

Antibiotics have been used over the last fifty plus years for treatment of various diseases of the animals effectively. Mastitis is one of the most prevalent diseases occurring in the dairy cows. It has been a disease of cattle for probably as long as cows were domesticated for the purpose of milk by mankind. In order to contain it antibiotics are liberally used in bovine species. According to one estimate, bovine mastitis is the single most common cause for antibacterial use in lactating dairy cattle (Moore and Heider, 1984; Kaneene et al., 1992). Treatment of this disease is also the most common cause of illegal antibacterial residues in marketed milk (Erskine, 1996). Antibacterial therapy of bacterial-induced diseases in cattle has been incriminated as a catalyst for resistance in bacteria isolated from treated animals, other animals within the herd, and food derived from cattle for human consumption (Berghash et al., 1983; Griggs et al., 1984; Singh et al., 1992; Piddock, 1996). In more recent times this type of treatment has resulted in resistance rather than the destruction of the pathogenic microbes in the dairy cattle. Concerns have been raised that the use of antimicrobials has caused resistance in bacteria isolated from treated animals, other animals in the population, and from food derived from animals for human consumption. The emergence of antibacterial resistance among microorganisms that impact domestic animal health has been a growing concern in veterinary medicine. Increased resistance of microbial isolates recovered from mastitic domestic ruminants to different antimicrobial agents has been reported by several authors (Myllys et al., 1998; Pitkala et al., 2004). Although antibiotic treatment of mastitis leads to significant increase in milk quantity and quality, lower somatic cell count and is likely associated with reduction in prevalence of clinical mastitis among herds, which is economically beneficial but it also results in the formation of multiple antibiotic resistant superbugs (Ndieyira et al., 2008). Additionally, antibacterial use has been suggested as a selective force in determining the bacterial ecology of bovine mastitis (Myllys et al., 1994). The judicious use of antibiotics and following the proper dosage regimen are important factors which can negate the menace of antibiotic resistance in the pathogenic microbes as lower doses than normal more often than not favours the development of antibiotic resistant strains.

Bacterial identification and susceptibility tests are important for selecting the appropriate antimicrobial agent when treating mastitis and the antimicrobial susceptibility of various mastitis causing organisms isolated from intramammary infected ruminants have been previously published (Erskine, 2002). Drugs most commonly used are β-lactams, aminoglycosides, macrolides, cephalosporin and lincosamides (Gentilini et al., 2000; Goni et al., 2004). These drugs are generally effective against various kinds of pathogenic microbes causing the mastitis. The common mastitis causing microbes are Staphylococcus aureus, Streptococcus uberis, S. Agalactiae, S. dysagalactiae, Escherichia coli, Klebsiella and various species of yeast and molds. However, growing number of
mastitis affected cows are getting resistant against various antibiotics in the recent times. This resistance is due to the indiscriminate use of antibiotics in the veterinary practice. To fully understand the development and dissemination of resistance, we need to study the antibiotics and their resistance genes not just in clinics but in natural (nonclinical) environments as well, as little is known about the diversity, distribution and origins of resistance genes, especially for the unculturable majority of environmental bacteria. This approach is important due to the fact that the bacteria can develop resistance to antibiotics by not only mutating existing genes (vertical evolution) (Alekshun and Levy, 2007) but also by acquiring new genes from other unculturable strains or species present in the environment (horizontal gene transfer) by many different mechanisms (Partridge et al., 2009) as the presence of antibiotics in the environment creates an environmental reservoirs of resistance determinants. Antimicrobial resistance in food animals poses a risk not only for animal health but consequently threatens the public health, where these pathogens get transmitted to humans as food borne contaminants. The purpose of this study was to determine the percentage of microbes which are resistant to the commonly used veterinary antimicrobial in dairy cows in the state of Kashmir and possibly predict the reasons for development of such resistance in the microbes.

2. Material and Methods

The study was undertaken in dairy cattle suffering from the chronic form of mastitis in the Indian Kashmir during the period starting from March 2010 to December 2012.

2.1. Sample Collection

The milk samples were collected aseptically directly from the affected cows in the pre-sterile sampling bottles. The sampling bottles were kept in the icepack and sent to the laboratory for analyses on the same day. The samples of milk were serially diluted and different dilution were inoculated in different growth media using first pour plate methods followed by streaking till individual colonies (axenic cultures) were obtained in different cultures.

2.2. Identification

For identification of the microflora in milk from mastitic cows, biochemical and PCR techniques were used.

2.2.1. Biochemical tests

These are used for the identification of microorganisms’ up to species level. For this purpose Analytical Profile Index (API 20E, API staph, API 20strep) kit (bioMérieux’s API) were employed. It
has 20 different biochemical tests for identification of Gram positive and Gram negative bacteria and yeast.

2.2.2. PCR

For the microbial assay, real time PCR was carried out in a Smart Cycler II (Cepheid, Sunnyvale, California, USA) real time PCR thermal cycler. The PCR products for the end point assays were visualized under UV light by ethidium bromide staining after agarose gel electrophoresis for detection of strains of microbes using the band patterns of DNA using the method of Chakravorty et al. (2007).

2.3. Antibiotic Sensitivity Tests

For preparation of a suspension of the bacteria in the saline tube, a large colony (2-3 mm diameter) of the bacterium (pure culture) was inoculated into the 0.85% NaCl solution, making sure that the suspension was homogenous and without clumps of floating bacteria. This solution was standardized using McFarland barium sulfate standard 0.5 to quantitate the suspension for inoculation into nutrient agar petri-plates. Antimicrobial susceptibility was determined by means of the Kirby-Bauer disk diffusion method. The 0.1 mL of this bacterial suspension was used to inoculate the growth media. The different antibiotic discs (Oxoid) were placed in center of each plate. Petri-plates were inoculated at 25-35 ºC for 24-48 h dependent upon the nature of inoculating microorganisms. The cultures were examined for any zone of inhibition, if found, was recorded after 24 and 48 h. The diameter of inhibition zones were measured for each plate and the average reading of the three replicates for each antibiotic are shown in Table 1. Zones of inhibition were determined in accordance with the National Committee for Clinical Laboratory Standard (Kirby et al., 1966), isolates were categorized as susceptible and resistant while intermediate were considered as resistant.

3. Result and Discussion

The milk samples collected from various mastitic cows were used for the isolation and identification of microbes and isolated microbes were consequently subjected to the antimicrobial sensitivity tests for determination of drug resistant microbial species in the mastitic milk. A diffusion disk method was used to assay 13 kinds of antibiotics. The results of various antibiotic sensitivity tests are presented in Table 1. Greater resistance to different classes of antibiotics was exhibited by three main microorganisms of mastitis in cows viz., Staphylococcus aureus, E. coli and Klebsiella. These three microbes have also shown higher amount of the multiple drug resistance against almost all the common used veterinary antibiotics used for the study (Fig. 1). The highest resistance was observed in the cases of chronic clinical mastitis caused by Staphylococcus aureus, E. coli and Klebsiella ranging
from 10 to 71%, 3 to 79% and 13 to 99%, respectively. Resistance significantly decreased in 13 kinds of antibiotics in the group of cases with latent mastitis.

**Table 1.** Resistance of different microorganisms against common antibiotics

<table>
<thead>
<tr>
<th>Drug Conc.</th>
<th>Staph. aureus</th>
<th>Staph. epidermidis</th>
<th>Str. uberus</th>
<th>Str. agalactiae</th>
<th>Str. dysgalactiae</th>
<th>E. coli</th>
<th>Klebsiella</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin 10 IU</td>
<td>47</td>
<td>38</td>
<td>40</td>
<td>3</td>
<td>7</td>
<td>79</td>
<td>95</td>
</tr>
<tr>
<td>Ampicillin 10 µg</td>
<td>46</td>
<td>40</td>
<td>15</td>
<td>3</td>
<td>8</td>
<td>23</td>
<td>89</td>
</tr>
<tr>
<td>Amoxicillin 10 µg</td>
<td>31</td>
<td>32</td>
<td>13</td>
<td>21</td>
<td>21</td>
<td>29</td>
<td>86</td>
</tr>
<tr>
<td>Tetracycline 10 µg</td>
<td>14</td>
<td>10</td>
<td>23</td>
<td>30</td>
<td>36</td>
<td>37</td>
<td>40</td>
</tr>
<tr>
<td>Erythromycin 10 µg</td>
<td>56</td>
<td>50</td>
<td>9</td>
<td>7</td>
<td>10</td>
<td>65</td>
<td>99</td>
</tr>
<tr>
<td>Neomycin 20 µg</td>
<td>45</td>
<td>41</td>
<td>13</td>
<td>34</td>
<td>35</td>
<td>74</td>
<td>70</td>
</tr>
<tr>
<td>Cefixime 10 µg</td>
<td>15</td>
<td>7</td>
<td>6</td>
<td>7</td>
<td>21</td>
<td>6</td>
<td>15</td>
</tr>
<tr>
<td>Cloxacillin 10 µg</td>
<td>17</td>
<td>10</td>
<td>8</td>
<td>9</td>
<td>12</td>
<td>35</td>
<td>21</td>
</tr>
<tr>
<td>Gentamicin 10 µg</td>
<td>71</td>
<td>60</td>
<td>34</td>
<td>34</td>
<td>36</td>
<td>12</td>
<td>23</td>
</tr>
<tr>
<td>Streptomycin 10 µg</td>
<td>45</td>
<td>35</td>
<td>35</td>
<td>41</td>
<td>43</td>
<td>43</td>
<td>52</td>
</tr>
<tr>
<td>Oxacillin 10 µg</td>
<td>25</td>
<td>30</td>
<td>41</td>
<td>21</td>
<td>23</td>
<td>15</td>
<td>59</td>
</tr>
<tr>
<td>Chlormephicol 10 µg</td>
<td>10</td>
<td>4</td>
<td>14</td>
<td>14</td>
<td>20</td>
<td>3</td>
<td>19</td>
</tr>
<tr>
<td>Sulfadimidine 10 µg</td>
<td>36</td>
<td>21</td>
<td>26</td>
<td>12</td>
<td>29</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

Note: Total numbers of isolates for each microorganism tested for different antibiotic were between 50 and 200.

The cause of resistance is the use of antibiotics on the regular basis which tend to evolve microbes in such a way to negate the effect of the inhibiting agent. This evolution of microbes against the antimicrobial agents can be substantiated from the fact that park et al. (2012) found that *Staphylococci* were significantly less resistant to β-lactam antibiotics when isolated from milk after the herd transitioned to organic management. Cessation of the use of antimicrobial therapies in dairies in combination with organic management could lead to a reduction in the antimicrobial resistance of mastitis pathogens.
From the current study of 300 samples of milk taken between March 2010 to December 2012, it has led to the identification of 250 bacterial isolates. *Streptococcus uberis* (23.1%), *Escherichia coli* (19%), and *Staphylococci aureus* (17.6%) were identified as the major causative agents of clinical mastitis, whereas *Staphylococci epidermidis* (30.2%) and *Streptococcus dysgalactiae* (9.3%), *Klebsiella* (8.1%) and *Str. dysgalactiae* (8%) were predominantly implicated in subclinical mastitis. Yet, in both types of mastitis, about 25% of all cases were due to a large number of different bacterial species that were isolated at a low frequency i.e. less than 5%.

The highest rate of resistance was observed in *Klebsiella* against erythromycin (99%), penicillin (95%) and the highest rate of sensitivity to cefixime followed by chloramphenicol, cloxacillin and gentamycin. The widespread use of antibiotics has led to the increased presence of pathogens that are less susceptible to their antibacterial effect (Lacasse et al., 2008).

### 3.1. *Staphylococcus* (*S. aureus, S. epidermidis*)

The infection with the *Staphylococcus* shows moderately high level of resistance against different classes of antibiotics. The resistance against β-lactam type of antibiotics like penicillin, ampicillin is on higher side of 47% and more. These results were consistent with a previous report from Poland, where 68.9% of *S. aureus* isolates were resistant to ampicillin (Malinowski et al., 2002). The higher values are due to the ability of some strains of *Staphylococcus* to produce β-lactamase which renders the β-lactam antibiotics ineffective. Penicillin was initially used in late 1940’s as the important treatment agent against antibiotics. Since then, β-lactamase producing *Staphylococcus aureus* has emerged in a number of countries, and the level of resistance have remained fairly constant at around 10-15% (Stephan et al., 1999). *Staphylococcus aureus* has been extensively studied for the
mastitic study with respect to antibiotic resistance. S. aureus isolates had relatively high MIC values for penicillin and ampicillin, and implied this was because of β-lactamase inactivation of the drugs. β-lactamase production is induced in some bacteria when exposed to β-lactam drugs. The importance of continued β-lactamase related resistance in S. aureus was underscored by the Watts and Salmon report of higher MIC values for isolates that produced this enzyme as compared with isolates that did not.

In our study both isolates of S. aureus and S. epidermidis demonstrated high level of resistance to ampicillin. These results were consistent with previous several reports from the world wherein it was found that the microbes in mastitis are growing effective resistance against commonly used antibiotics especially from ampicillin and penicillin groups (Malinowsk et al., 2002). Our data showed that our Staphylococcal isolates are less resistant to tetracycline compared with penicillin group of antibiotics as β-lactamases are only effective against lactam type of antibiotics. There are variable resistances from 8.5% to 69% in the current study with use of different antibiotics. The climate and local microbiota also plays its role on the resistance development. The differences in strains, resistant genes, geography, numbers of isolates used within a study, and inconsistencies in laboratory methods which have significantly effect on the outcome of the study. As the studies by previous researchers were from the different regions so variation in the results of the resistant is quite obvious. As an example, two studies performed in the same year by Costa et al. (2000) and Gentilini et al. (2000) reported the proportion of oxacillin resistant strains of S. aureus as 42.0 and 0%, respectively. On the other hand, we found that most Staphylococcal collection is sensitive to tetracycline, cephalosporins and chloramphenicol and this is in accordance with the previous study (Yoshimura et al., 2002), in which it was found that all isolates were susceptible to chloramphenicol. This may be due to that the fact that these antibiotics are now used rarely for the treatment of domestic ruminants in this country. In general our Staphylococcal isolates showed high level of resistance particularly to ampicillin, amoxicillin, neomycin, gentamycin, streptomycin and erythromycin which are commonly used in treatment of animals. This high rate of resistance to these antibiotics in our Staphylococcal collection is likely due, in part, to selective pressure resulting from misuse of these antibiotics as drug or as antimicrobial growth promoters (Witte, 2000). This observation may provide the rational for alternative therapy that could be used in mastitic animals likely to be colonized with multidrug resistant Staphylococcal isolates. Multidrug resistance to at least 3 drugs was found in 47% and 42.4 % of S. aureus and S. epidermidis isolates, respectively. The presence of multidrug resistance in Staphylococcal isolates has been reported by several investigators. The other mechanisms for the resistance is the acquisition of the resistant genes which makes them resistant to the particular antibiotics viz., methicillin-resistance in Staphylococcus aureus (MRSA) strains were characterized by presence of the mecA gene (Normanno et al., 2007).
3.2. Streptococci (S. uberis, S. agalactiae, S. dysgalactiae)

It is interesting to note that in the field of human medicine, resistance in *Streptococcus pneumoniae* to penicillin, tetracycline and macrolides has been observed in Europe and in some areas, and multiple antimicrobial resistances have limited the number of antimicrobials that can be used to treat infections with this microorganism. It is also noteworthy that *Str. dysgalactiae*, with its wider range of habitats on the bovine host than *Str. agalactiae*, shows greater resistance than the *Str. agalactiae* isolates. The reason for this is not entirely clear and could be due to intrinsic bacterial factors or increased exposure to commensals carrying transferable resistance factors in *Str. dysgalactiae* by horizontal gene transfer mechanisms viz., transposons, plasmids etc.

Erythromycin resistance was detected in a low number of *Str. dysgalactiae* and in *Str. agalactiae* isolates. Erythromycin and the other macrolides act by preventing bacterial protein synthesis. A common resistance mechanism, encoded by erm (erythromycin ribosome methylation) genes, involves methylation of a single adenine in the peptidyltransferase 23S rRNA. More than 30 erm genes have been described which all methylate this same adenine residue. The result is a reduction in binding of the 50S ribosomal subunit to macrolide-lincosamide-streptogramin antimicrobials. *Streptococcus agalactiae*, *Str. dysgalactiae* and *Str. uberis* can all carry the erm F gene, usually in conjunction with other erm genes B, C or Q (Costa et al., 2000). Some 71 streptococci from cases of clinical mastitis in dairy cows in the USA were examined by Roberts and Brown (1994) who found that 7.1% were resistant to erythromycin and/or lincomycin and nine of the isolates hybridised with erm gene probes.

Resistance to streptomycin and tetracycline’s in *Str. agalactiae*, *Str. dysgalactiae* and *Str. uberis* was found to be 41, 43 and 35% respectively. The higher resistance of these microorganisms against the streptomycin was reported by Jayarao and Oliver (1990) also. The multiple antimicrobial resistances have also been observed in the mastitis streptococci (Brown and Scassera, 1990). Although no penicillin resistance was detected in the American study on *Streptococal* isolates (Roberts and Brown, 1994), Polish workers found using a disc diffusion method that 86.7% of *Str. agalactiae*, 84.6% of *Str. dysgalactiae* and 88.5% of *Str. uberis* strains were sensitive to penicillin (Malinowski et al., 1992). In a Canadian study, none of 68 strains of *Str. agalactiae* of bovine origin showed resistance to penicillin or erythromycin (Meissier et al., 1994). In New Zealand, resistance to streptomycin in *Str. uberis* and *Str. dysgalactiae* has been described (Carman and Gardner, 1997). The higher resistance in our and other studies could be due to the mutation in the ribosomal protein of streptococci which may have resulted in high-level streptomycin resistance and confers complete resistance to any synergy from combination with penicillin (Whittem and Hanlon, 1997).
3.3. *Coliforms* (*E. coli and Klebsiella*)

Coliform organisms were divided into *Klebsiella* and *Escherichia coli*, a marked variability in resistance to antibacterial drugs was observed between these genera, but there was a consistent pattern of resistance within species.

The *E. coli* that infect the bovine udder tend to belong to a wide range of strains with different somatic antigen types. The major source of *E. coli* in the cow’s environment is presumed to be the adult bovine alimentary tract. One would therefore expect that the resistance of mastitis *E. coli* strains would be comparable with levels in strains from the adult alimentary tract.

The amoxicillin resistance levels are higher in the mastitis isolates due to widespread dispersal of the coliform in the soil in which it is more likely to exchange genes with the other resistant faecal and soil microbes.

For the Gram-positive mastitis pathogens, percentages of isolates resistant to various β-lactam antimicrobials exhibited very high resistance ranging from 79 to 95%. The coliforms in general showed higher resistance to β-lactam group, tetracycline, erythromycin, neomycin and streptomycin while as cephalosporin’s and chloramphenicol along with sulpha drugs which were only tested against coliform exhibited very low resistance indicating there efficacy against these kinds of pathogenic microbes.

4. Conclusions

In summary, before going for the treatment of the mastitis or any other infection, it is important that the antimicrobial sensitivity tests should be done in order to find the best antibiotic or their combination for the control of the disease. This will limit indiscriminate and unnecessary use of different antibiotics in the veterinary medication. The lower and more effective use of antibiotics would lead to better control of pathogens on one side and less development of resistant genes in the environment on the other side. These ultimately will lead to lesser accumulation of resistance in the environment. The presence of mastitis bacteria that are resistant to antimicrobials has obvious implications for the treatment of infected animals as well as have the potential implications for the consumer if raw, unpasteurised milk or milk products contained such resistant bacteria is consumed. Most milk is pasteurised and further safeguards include the quality standards regarding the maximum total bacterial content of milk and regulations preventing mastitic milk going for human consumption.
References


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