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A study on treatment of resistant mastitis in dairy cows

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Abstract: The study was undertaken to determine the prevalence and treatment of antibiotic resistant mastitis in dairy cows. The predominant resistant causative pathogen was *Escherichia coli* (50.64 %) followed by *S. aureus* (44.25 %) and Methicillin resistant *Staphylococcal aureus* (5.11%).These isolates were found sensitive to gentamicin, enrofloxcain, amoxicillin+sulbactam, ceftriaxone and resistant to amoxicillin, oxytetracycline, penicillin G and oxacillin. In all the treatment groups of *E. coli*, *S. aureus* and MRSA mastitis, the post treatment pH, SCC was significantly ($P < 0.01$) decreased when compared to pre treatment pH, SCC values and the post treatment electrical conductivity was significantly ($P < 0.01$) increased when compared to pre treatment electrical conductivity value. In *E. coli* mastitis, treated with amoxicillin+sulbactam, ceftriaxone, enrofloxacin and gentamicin showed 74.1%, 67.75 %, 76.67 % and 64.52 % clinical recovery and in *S. aureus* mastitis, showed 65.25 %, 65.25 %, 72.43 % and 68.98 % clinical recovery. In MRSA mastitis, enrofloxacin was found to be highly effective in comparison to amoxicillin+sulbcactam.

Keywords: Antimicrobial sensitivity, Bovine mastitis, *E. coli,* Electrical conductivity, *S. aureus*

INTRODUCTION

Mastitis is considered as most important disease affecting the productive performance of cattle world-side contributing to the economic losses (Kumar *et al.*, 2010). In the control of mastitis, the improper use of antimicrobial agents on dairy farm animals is a major concern as it leads to the emergence of resistant zoonotic bacterial pathogens (Piddock, 1996). The emergence of antibiotic-resistance in *S. aureus* from mastitic dairy animals has been shown in recent years. Beta–lactam antibiotics are frequently used in mastitis therapy and the resistance is due to the production of beta-lactamases and low–affinity penicillin-binding protein, PBP2A (Olsen *et al.*, 2006).

Acquired antimicrobial resistance in bacteria is an increasing threat in human as well as in veterinary medicine. Among the various antibiotic-resistant strains the Methicillin-resistant *S. aureus* (MRSA) is a serious cause because of its public health significance. All MRSA were resistant to members of the penicillin family, such as ampicillin, oxacillin and penicillin. In India, high prevalence of MRSA (13.1 %) and the isolates were resistant to streptomycin, oxytetracycline, gentamicin and chloramphenicol, pristinomycin and ciprofloxacin (Kumar *et al.*, 2011). As on date β-lactamase resistant penicillins such as methicillin and oxacillin are not used in dairy cows except for cloxacillin in some of the products used for intramammary administration (Turutoglu *et al.*, 2006). Comprehensive information on the prevalence of antimicrobial resistance in bovine mastitis pathogens in milk and their management is lacking in India. Enrofloaxacin was found to be most effective against MRSA (Kenar *et al.*, 2012). Keeping this in view, the present study, an attempt has been made to find out the treatment of resistant mastitis in dairy cows.

MATERIALS AND METHODS

Sampling and bacterial culture: 401 milk samples from acute mastitis cases from Large Animal Clinic Medicine Unit of Madras Veterinary College Teaching Hospital and six dairy farms in Coimbatore district were collected, transported, cultured. The *Escherichia coli* and Staphylococci were isolated as per the guidelines of National Mastitis Council (NMC). The *S. aureus* isolates were also characterized by their growth on blood agar and mannitol salt agar, the positive results for catalase and coagulase while the *E. coli* isolates were identified on eosin methylene blue

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agar, a negative oxidase test. Based on incidence of common causative pathogen and sensitivity tests, isolates were categorized as resistant i.e exhibiting *in vitro* resistance to 1 or 2 antimicrobials and multidrug-resistant i.e exhibiting *in vitro* resistance to 3 or more antimicrobials. Cows with resistant mastitis were grouped as follows, Group I *E. coli* (n=119), Group II *Staphylococcus aureus* (n=104) and Group III Methicillin resistant *S. aureus* (n=12).

Antibiotic sensitivity test: Antimicrobial susceptibility testing was carried out at equivalent to 0.5 McFarland turbidity standard by agar disc diffusion method on Mueller-Hinton agar plates following the guidelines of CLSI (2008). All the bacteria isolated were tested *in vitro* for their sensitivity to 8 antibiotics viz., Enrofloxacin, Amoxicillin+sulbactam, Amoxicillin, Gentamicin, Ceftriaxone, Oxytetracycline, Penicillin G and Oxacillin that are commonly used in veterinary practice.

PCR for identification of the mastitis causing bacteria: The isolates were confirmed to be *E. coli* and *S. aureus* by targeting the specific gene 16s-23s r RNA and *gap* gene as described by Riffon *et al.*, (2001) and Yugueros *et al.*, (2001). The *Staphylococci* isolates were confirmed to be MRSA by amplifying the MRSA specific genes *mec*A and *bla*Z as described by Lee, (2003) and Martineau *et al.* (2000), respectively.

Clinical signs: The cows with acute clinical mastitis exhibiting clinical signs such as inappetance, anorexia, pyrexia, reduced rumen motility, swelling of udder, hot and painful udder. Milk colour changes include dirty white, yellowish colour, flakes, serous/ watery and blood mixed. Clinical examination was carried out as described by Boddie, (2000).

pH, electrical conductivity and somatic cell count (SCC): The collected milk samples were further subjected to pH (pH strips), Electrical conductivity (DRAMINSKI mastitis detector) and somatic cell count (De Laval somatic cell counter). The SCC value > 5,00,000 cells / ml of milk with clinical signs were taken as criteria to declare the animal as clinically mastitic. The results were statistically analyzed, utilizing SPSS – Version 14 statistical software package.

Treatment: Based on culture, antibiotic sensitivity test, cows were allotted the following four treatment trial.

1. Gentamicin @ 4mg / kg body weight IM once daily for 7 days in *E. coli* (n=31) group and *S. aureus* (n=29) group as well as 100 mg per quarter intramammary infusion once daily for 7 days in *S. aureus* group.
2. Ceftriaxone @ 5 mg / kg body weight IM once daily for 7 days in *E. coli* (n=31) group and *S. aureus* (n=23) group as well as Ceftriaxone 250 mg per quarter intramammary infusion once daily for 7 days in *S. aureus* group.
3. Enrofloxacin @ 5 mg / kg body weight IM once daily for 7 days in *E. coli* (n=30) group, *S. aureus* (n=29) group and MRSA (n=6) group as well as 100 mg per quarter intramammary infusion once daily for 7 days in *S. aureus* and Methicillin resistant *S. aureus* (MRSA) group.
4. Amoxycillin + Sulbactam @ 10 mg / kg body weight IM twice daily for 7 days in *E. coli* (n=27) group, *S. aureus* (n=23) group and MRSA (n=6) group as well as 300 mg per quarter intramammary infusion once daily for 7 days in *S. aureus* and Methicillin resistant *S. aureus* (MRSA) group.

Depending on the severity, cows in all groups were treated with non-steroidal anti-inflammatory drug meloxicam IV @ 0.5 mg/kg body wt daily for 1-5 days and chlorpheniramine maleate @ 0.5 mg /kg body wt IM daily for 5 days. In *E. coli* group Normal Saline was administered @10 ml/kg body weight IV daily for 1-5 days depending on the severity. Post treatment assessment was carried out after 7 days, based on milk pH, electrical conductivity, somatic cell count and clinical improvement.

RESULTS

Antibiotic resistant mastitis was detected in 235 out of 401 cows accounting to 56.1 %. The predominant resistant causative pathogen was *E. coli* (50.64 %) followed by *S. aureus* (44.25 %) and MRSA (5.11 %).

Antibiotic sensitivity test: *E. coli* showed more sensitivity to enrofloxacin (79 %) followed by amoxicillin and sulbactam (74 %), gentamicin (73.1 %) and ceftriaxone (69 %). The isolates had highest resistance to penicillin (63 %) followed by amoxicillin (52.1 %), oxytetracycline (47.9 %) and methicillin (45.4 %). Most of the *E. coli* isolates (86.55 %) were found to be resistant i.e resistance to 1 or 2 of antimicrobials and few *E. coli* isolates (13.45 %) were found to be multi-drug resistant i.e resistance to 3 or more of antimicrobials.

S. aureus isolates were most sensitive to enrofloxacin (79.8 %) followed by gentamicin (71.2 %), amoxicillin and sulbactam (69.2 %) and ceftriaxone (69.2 %). The isolates showed highest resistance to penicillin (63.5 %) followed by amoxicillin (61.5 %), oxytetracycline (49 %) and methicillin (52.9 %). Most of the *S. aure*us isolates (80.77 %) were found to be resistant i.e reistance to 1 or 2 of antimicrobials and few *S. aureus* isolates (19.23 %) were found to be multi-drug resistant i.e resistance to 3 or more of antimicrobials.

MRSA showed maximum sensitivity to enrofloxacin (75 %), amoxicillin and sulbactam (75 %) followed by gentamicin (66.7 %) and ceftriaxone (58.3 %). The isolates showed highest resistance to methicillin (100 %), amoxicillin (91.7 %), followed by penicillin (83.3 %) and oxytetracycline (41.7 %). Few MRSA isolates (8.33 %) were found to be resistant i.e resistance to 1 or 2 of antimicrobials and most of the MRSA isolates (91.67 %) were found to be multi-drug resistant i.e resistance to 3 or more of antimicrobials.

PCR for identification of the mastitis causing bacteria : Out of 235 milk samples, the specific target gene 16s-23s

r RNA (*E. coli*) could be amplified from 119 isolates with a %age of positivity as 50.64 (119/235), *gap* gene (*S. aureus*) could be amplified from 104 isolates with a %age of positivity as 44.25 (104/235). Screening for the specific target gene for both *mec*A (MRSA) and *bla*Z (MRSA) resulted in positivity in 12 samples with a %age of positivity as 10.34 (12/116) among the *S. aureus* isolates.

pH, electrical conductivity and somatic cell count (SCC): The post treatment mean ± S.E values of milk pH, Electrical conductivity and SCC in *E. coli*, *S. aureus* and MRSA isolates are as given in tables 1- 3**.** In *E. coli*, *S. aureus* and MRSA groups, a highly significant ($P < 0.01$) decrease in post treatment milk pH values was observed in all treatment groups when compared to pre treatment group. In *E. coli* and *S. aureus* groups, post treatment pH value (Tables 1 and 2) of treatment groups showed lowering trend towards control group. However, In MRSA group, the post treatment pH value (Table 3) of enrofloxacin group was comparable to the control value and amoxicillin + sulbactam group was slightly above the control value.

In *E. coli, S. aureus* and MRSA groups, a highly significant ($P < 0.01$) increase in post treatment milk electrical conductivity values (Tables 1, 2 and 3) was observed in all treatment groups when compared to pre treatment values. However, in *E. coli, S. aureus* groups, there was no significant difference between different treatment groups in post treatment values and post treatment electrical conductivity value of treatment groups showed increasing trend towards control group.

In MRSA group, post treatment electrical conductivity value of enrofloxacin group showed increasing trend when compared to control group. Even though significant increase in post treatment electrical conductivity was observed in amoxicillin+sulbactam group, it did not reach the values towards control group.

In *E. coli, S. aureus* and MRSA groups, a highly significant ($P < 0.01$) decrease in post treatment milk SCC values (Tables 1, 2 and 3) was observed in all treatment groups when compared to pre treatment values. However, in *E. coli, S. aureus* groups, there was no significant difference between different treatment groups in post treatment value and post treatment SCC value of treatment groups showed lowering trend towards control group.

In MRSA group, post treatment SCC value of enrofloxacin group showed lowering trend towards control group. Whereas post treatment SCC value of amoxicillin+sulbactam group was slightly above the control value.

Clinical signs: In *E. coli* group, 74.1 %, 67.75 %, 76.67 % and 64.52 % of cases treated with amoxicillin+sulbactam, ceftriaxone, enrofloxacin and gentamicin respectively showed normal milk colour.

In *S. aureus* group, 65.25 %, 65.25 %, 72.43 % and 68.98 % of cases treated with amoxicillin+sulbactam, ceftriaxone, enrofloxacin and gentamicin respectively

Table 1. Comparison of post treatment values of pH, EC and SCC in *E. coli* mastitis among different groups of antibiotics.

S. N.	Parameters	Control	Amoxicillin + sulbactam (n=27)		Ceftriaxone (n=31)		Enrofloxacin (n=30)		Gentamicin (n=31)		F - value
			Pre	Post	Pre	Post	Pre	Post	Pre	Post	
1	pH	$6.45^{a} \pm 0.05$	7.61^{b} ±0.08	6.43^{a} ±0.07	7.59^{b} ±0.09	6.48^{a} ±0.07	7.60^{b} ±0.10	6.43^{a} ±0.06	7.70^{b} ±0.09	6.48^{a} ±0.07	56.976 **
2	Electrical conductivity (Units)	$356.00^{b} \pm 6.42$	246.66^{c} ±2.72	334.00^{a} ±7.07	242.58^{c} ±3.65	320.32^{a} ±6.17	242.66^{c} ±3.45	330.74^{a} ±7.33	241.61^{c} ±3.53	321.25^{a} ±6.39	75.383**
3	SCC x 10^3	$60.95^{a} \pm 8.52$	2038.22^{c} ±126.51	166.85^{b} ±23.71	2128.58^{c} ±166.16	201.16^{b} ±24.30	2216.53^{c} ±144.67	191.93^{b} ±22.09	2198.87^{c} ±146.30	199.64^{b} ±36.82	100.709**

Mean bearing the same superscript in the same row do not differ significantly; ** Highly significant ($P<0.01$)

Table 2. Comparison of post treatment values of pH, EC and SCC in *S. aureus* mastitis among different groups of antibiotics.

S. N.	Parameters	Control	Amoxicillin + sulbactam (n=23)		Ceftriaxone (n=23)		Enrofloxacin (n=29)		Gentamicin (n=29)		F - value
			Pre	Post	Pre	Post	Pre	Post	Pre	Post	
1	pH	6.45^{a} ± 0.05	7.69^{b} ±0.10	6.50^{a} ±0.06	7.76^{b} ±0.13	6.56^{a} ±0.08	7.63^{b} ±0.04	6.53^{a} ±0.08	7.51^{b} ±0.09	6.53^{a} ±0.07	42.640**
2	Electrical conductivity (Units)	356.00^{c}± 6.42	235.21^{a} ±3.71	322.41^{b} ±8.95	234.78^{a} ±3.96	319.56^{b} ±9.14	241.72^{a} ±3.94	322.06^{b} ±8.64	241.37^{a} ±4.31	291.73^{b} ±11.06	41.184**
3	SCC x 10^3	60.95^{a} ± 8.52	2316.21^{c} ±146.72	203.17^{b} ±24.33	2378.39^{c} ±222.62	223.44^{b} ±29.60	2346.48^{c} ±158.6	212.60^{b} ±32.44	2298.20^{c} ±126.41	218.27^{b} ±28.7	97.127**

Mean bearing the same superscript in the same row do not differ significantly; ** Highly significant (P<0.01)

showed normal milk colour.

In MRSA group, 50 %, and 50 % of cases treated with amoxicillin+sulbactam and enrofloxacin respectively showed normal milk colour.

DISCUSSION

Mastitis is the most common cause for antibiotic use in dairy herds. However, improper use of antibiotics creates problems such as the emergence of bacterial resistance to antibiotics. The present study has demonstrated the existence of alarming levels of resistance of *E .coli, S. aureus* and MRSA to commonly used antimicrobial agents in the study farms and the results are in accordance with reports from earlier studies in other countries. Edward *et al*., (2002) suggesting a possible development of resistance from prolonged and indiscriminate usage of some antimicrobials. Systemic application of an *in vitro* antibiotic susceptibility test prior to the use of antibiotics in the treatment of intra-mammary infections will prevent the antibiotic resistance.

Based on the antibiotic susceptibility test *E. coli, S. aureus* and MRSA showed maximum sensitivity to enrofloxacin, amoxicillin + sulbactam followed by gentamicin and ceftriaxone. These susceptible antibiotic drugs will be used as the effective drugs against *E. coli, S. aureus* and MRSA resistant isolates.

In the present study, in all the treatment groups of *E. coli* and *S. aureus* mastitis, the post treatment pH, SCC was significantly decreased when compared to pre treatment pH, SCC values indicated that the treatment was effective in controlling the inflammation. The post treatment electrical conductivity was significantly increased when compared to pre treatment electrical conductivity value.

In the present study, cows affected with *E. coli* and *S. aureus* mastitis treated with amoxycillin+sulbactam, ceftriaxone, enrofloxacin and gentamicin showed uniform improvement in clinical mastitis. In *E. coli* mastitis, treated with amoxicillin+sulbactam, ceftriaxone, enrofloxacin and gentamicin showed 74.1 %, 67.75 %, 76.67 % and 64.52 % clinical recovery and in *S. aureus* mastitis, showed 65.25 %, 65.25 %, 72.43 % and 68.98 % clinical recovery and this might be due to isolates were found to be resistant i.e. resistance to 1 or 2 of antimicrobials. The present observation was in agreement with Karthikeyan, (2003) who reported that the most sensitive antimicrobial agent against gram negative pathogens (*E. coli*) was found to be enrofloxacin (100 %) followed by ciprofloxacin and gentamicin and sensitive antimicrobial agent against gram positive pathogens were found to be gentamicin followed by ciprofloxacin and enrofloxacin.

Evira (2009) recommended fluroquinolone as the first antibiotic of choice for *E. coli* mastitis because of pharmacokinetic and pharmacodynamic properties.

Highest clinical recovery rate was recorded in the bovine mastitis treated with gentamicin (84.21 %) followed by enrofloxacin (80.77 %) within 3.32 and 3.46 days

Table 3. Comparison of post treatment values of pH, EC and SCC in MRSA mastitis among different groups of antibiotics.

S. N.	Parameters	Control	Amoxicillin + sulbactam (n=6)		Enrofloxacin (n=6)		F - value
			Pre	Post	Pre	Post	
1	pH	6.45^{a} ± 0.05	8.08^{c} ±0.08	6.83^{b} ±0.10	8.16^{c} ±0.10	6.50^{a} ±0.18	100.105**
2	Electrical conductivity (Units)	356.00^{b} ± 6.42	230.00^{a} ±3.65	247.34^{c} ±17.4	236.66^{a} ±2.10	352.66^{b} ±17.63	32.110**
3	SCC x 10^3	60.95^{a} ± 8.52	2394.50^{c} ±170.27	324.16^{e}± 58.15	2434.16^{c} ±100.76	216.85^{d} ±46.64	325.848**

Mean bearing the same superscript in the same row do not differ significantly; ** Highly significant (P<0.01)

(average) respectively (Tufani *et al*., 2012).

Ceftriaxone is a third generation cephalosporin and has remarkable activity against Enterobacteriaceae (Prescott and Baggot, 1994) and *Staphylococcus* Spp. (Sumathi *et al.*, 2008).

In MRSA mastitis, treated with amoxicillin+sulbactam and enrofloxacin showed 50 % clinical recovery and lower clinical recovery compared to *E. coli* and *S. aureus* mastitis might be due to multi-drug resistant i.e resistance to 3 or more of antimicrobials.

Based on the post treatment pH, EC and SCC values, enrofloaxacin was found most effective antibiotic against MRSA (Kenar *et al.*, 2012). This might be due to immunomodulatory (Hoeben *et al.*, 1997), concentration dependent and post antibiotic effect. Hui *et al.* (2013) also reported that a good bactericidal activity *in vitro* was achieved for AMX/SUL (4:1) combination against common mastitis pathogens in cows. In the present study, Amoxicillin+ sulbactam was sensitive *in vitro* (6 out of 6 cases) but it had a poor efficacy *in vivo* (3 out of 6 cases). The lower efficacy of amoxicillin + sulbactam noticed in the current study might be due to development of resistance by bacterial strains which was confirmed by PCR by targeting specific gene *mec*A and *bla*Z. Loeffler and Lloyd, (2010) opined that detection of *mec*A and *bla*Z gene by PCR was gold standard test for confirmation of methicillin resistance. Local clinical signs, such as swelling, pain and firmness of the inflamed mammary quarters, were less severe in the treated cows (Hoeben *et al.*, 2000).

Conclusion

It was concluded that systemic application of an *in vitro* antibiotic susceptibility test prior to the use of antibiotics in the treatment of intra-mammary infections will prevent the antibiotic resistance. Cows affected with *E. coli* and *S. aureus* mastitis treated with amoxycillin+sulbactam, ceftriaxone, enrofloxacin and gentamicin showed uniform improvement. In MRSA mastitis, treated with amoxicillin+sulbactam and enrofloxacin showed 50 % clinical recovery.

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