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CURING OF SUBCLINICAL MASTITIS BY USING AN ORAL NON-ANTIBIOTIC PREPARATION IN COWS

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Mastitis is one of the most significant problems to the dairy all over the world. In addition to production losses, mastitis also deteriorates the chemical, and physical properties of milk which ultimately leads to poor shelf life of processed milk. The present study was designed to evaluate a non-proprietary, non-antibiotic preparation containing trisodium citrate, Vitamin C, Zinc sulphate and Copper sulphate in treating sub-clinical mastitis in dairy cows. For this purpose, cows suffering from sub-clinical mastitis (n=40) were selected and randomly divided into two groups. The animals of group A (n=20) were treated orally with a combination of tri-sodium citrate (40 gm), vitamin C (10 g), zinc sulphate (3 g) and copper sulphate (1 g) for 7 days. Group B was treated with standard mastitis therapy (Tylosin at a dose rate of 10 mg/kg for three days intramuscular and amoxicillin + clavulanic acid at 600 mg intramammary infusion daily for five days). The efficacy of the treatment was evaluated through surf field mastitis test (SFMT) scores and microbiological examination. The findings of the study revealed that administration of oral non-antibiotic formulation resulted in a better quarter based SFMT cure rate. The values observed for SFMT based cure rates in sub-clinically mastitic cows at the termination of study were 90.90% and 88.46% for groups A and B, respectively. The observed bacteriological cure rate for group A (tri-sodium citrate, vit. C, $ZnSO_4$ and $CuSO_4$) was 72.73%, and 69.23% for group B (antibiotic). The findings of the current study showed that the use of a combination therapy of non-antibiotic chemicals has potential impact on treating sub-clinical mastitis in dairy animals.
Keywords: Sub-clinical mastitis, non-antibiotic antibacterial, trisodium citrate, bovine, bacteriological cure rate

INTRODUCTION

Mastitis is one of the most significant problems bedeviling the dairy and milk processing industry all over the world. Similarly, in Pakistan, it is the most important disease of the livestock (Latif *et al*., 2014). Reduced milk production (up to 30%) is one of the major economic impacts of mastitis in dairy animals (Prakash *et al*., 2009). The economic losses due to subclinical/ asymptomatic mastitis prevail over the impacts of clinical mastitis. As compared to clinical mastitis, the prevalence of subclinical form is 30-40 times higher (Dua, 2005).

In addition to causing production losses, mastitis also deteriorates the chemical and physical properties of milk which ultimately leads to poor shelf life of processed milk (Barbano *et al*., 2006). For the treatment of mastitis, antibiotics are considered as an integral part of any treatment regimen (Deluyker *et al*., 2005). Despite of extensive use of antibiotic for the treatment of mastitis, therapy often remains unrewarding. In addition, issue of antibiotic resistance is also a big snag in successful treatment of infectious diseases. Keeping the nemesis of antibiotic usage in perspective, a wide range of non-antibiotic agents have now been trialed in mastitis therapy as alternatives of antibiotics in dairy animals (Yousaf, 2009).

Mastitis renders alkalinity to milk of affected animals. In healthy animals, milk pH is maintained in a narrow range (6.5 to 6.8) owing to its buffer system with citrate as a major component of this system. Reduction in udder citrate level would result in the clumping of Ca^{2+} manifested by flakes in milk. It has been revealed that milk citrate level significantly decreases in mastitic milk. Milk pH and citrate levels can be effectively restored by giving trisodium citrate as treatment in mastitis (Dhillon and Singh, 2013; Rai *et al*., 2013; Gupta *et al*., 2016). In addition, total oxidant capacity of milk becomes high in mastitis due to release of free radicals, and hence, total antioxidant capacity is reduced. Vitamin C (Vit. C) is a potent water-soluble antioxidant (Sauberlich, 1994). It has been reported that dairy animals suffering from mastitis (clinical or sub clinical) show a significant reduction in serum vitamin C levels (Kleczkowski *et al*., 2005; Ranjan *et al*., 2005). Copper being a vital constituent of Super oxide dismutase (SOD) also possesses solid antioxidant properties. Regarding the mammary glands, Zn is very important in maintaining the integrity of streak canal lining composed of keratin. Lower Zn levels lead to low quality milk with higher somatic cell count (SCC) and increased incidence of mastitis (Gaafar *et al*., 2010).

Milk processing industry faces a great problem due to the presence of antibiotic residues in milk because these residues

may interfere with the fermentation process necessary to produce yogurt and cheese. It has been observed that several tankers of antibiotic contaminated milk are discarded every now and then by the milk processing plants in Pakistan (personal observations by the field veterinarians). Therefore, alternatives to antibiotic use are desperately looked for by dairy industry. The desirable traits of trisodium citrate, Vit. C, Zn and Cu may galvanize one to investigate the efficacy of these non-antibiotics in treating of subclinical mastitis. However, there are no reports available describing the use of these agents as a therapeutic regimen for mastitis. Therefore, the present study was designed to evaluate a non-proprietary, non-antibiotic preparation containing trisodium citrate, Vitamin C, Zinc sulphate and Copper sulphate in the treatment of sub-clinical mastitis in dairy cows.

MATERIALS AND METHODS

***Experimental animals, grouping and treatment protocols*:** Forty cows of Sahiwal breed suffering from subclinical mastitis were selected based on positive results of surf field mastitis test (Muhammad *et al*., 2010). These cows were randomly divided into two groups A (non-antibiotic, oral formulation) and B (routine antibiotic therapy) and were treated as given below:

Animal groups	No. of animals	Treatment	Duration of treatment
A	20	Oral administration of extemporaneously prepared product containing tri-sodium citrate (40 g), Vitamin C (10 g), Zinc sulphate (3 g) and Copper sulphate (1 g) (Modified from Dhillon and Singh, 2013)	7 days
B	20	Intra muscular administration of tylosin (Inj. Tylosel® Selmore Pharmaceuticals (Pvt.) Ltd. at the rate of 0.05 ml/kg + Intramammary infusion of amoxicillin and clavulanic acid (Combimox® Norbrook Laboratories Ltd. daily intramammary infusion of 1 syringe per affected quarter (Adopted from Ahmad, 2009)	5 days

Evaluation Parameters:

***Surf field mastitis test (SFMT) reactions*:** Surf field mastitis test reactions as per Muhammad *et al.* (2010) were determined at day 0 and day 7, 14 and 28 post initiation of treatment. A negative reaction at either of 3 post treatment sampling time points (day 7, 14 and 28) was interpreted as cure at the respective sampling time point whereas as positive SFMT reaction as non-cured.

***Bacteriological cure rate*:** Quarter foremilk samples were collected aseptically at day 0 (before administration of treatment) and at day 7, 14 and 28 post initiation of treatment (Hogan *et al.*, 1999). The collected milk samples were transported on ice to the Mastitis Research Lab. Department of Clinical Medicine and Surgery, University of Agriculture, Faisalabad. The samples were then cultured on blood agar and MacConkey's agar plates (Hogan *et al.*, 1999) within one hour of arrival.

RESULTS

***Effects of oral non-antibiotic formulation on SFMT based cure rates*:** In general, higher SFMT based cure rate was recorded in group A treated with trisodium citrate, vit. C, $ZnSO_4$ and $CuSO_4$ combination as compared to by group B (Table 1). Out of the 44 subclinically mastitis positive quarters treated with non-antibiotic preparation, 28 tested negative in SFMT on day 7 post initiation of treatment. This translates into a cure rate of 63.63%. At day 14 and 28 post initiation of treatment, the SFMT based quarter cure rates in this group were 77.27 and 90.90%, respectively. Out of the 52 subclinically mastitic quarters treated with standard antibiotic therapy, 34 tested negative in SFMT on day 7 post initiation of treatment. This resulted into a cure rate of 65.38 %. At day 14 and 28 post initiation of treatment, the SFMT based quarter cure rates in this group were 80.77 and 88.46%, respectively.

***Efficacy of oral non-antibiotic formulation on quarter based bacteriological cure rates*:** Laboratory testing showed that the highest clearance of infection in the quarters was recorded in group A treated with non-antibiotic preparation followed by group B (Table 2 and 3). At the start of the study, in cows of group A out of 44 sub-clinically positive quarters, 32 (72.72%) quarters were having staphylococcal infection, 8

Table 1. Surf field mastitis test (SFMT) based quarter cure rates on different days (post treatment) in subclinically mastitic cows treated with combination of trisodium citrate, vit. C, $ZnSO_4$ and $CuSO_4$ *vis-à-vis* antibiotic therapy.

Group (n=20 cows)	Total SFMT +ve quarters at day 0	SFMT based cured quarters on day 7 post initiation of treatment	SFMT based cured quarters on day 14 post initiation of treatment	SFMT based cured quarters on day 28 post initiation of treatment
A	44	28 (63.63%)	34 (77.27%)	40 (90.90%)
B	52	34 (65.38%)	42 (80.77%)	46 (88.46%)

Table 2. Effects of tri-sodium citrate, vit. C, $ZnSO_4$ and $CuSO_4$ treatment on quarter based bacteriological cure rates in subclinically mastitic cows.

Isolates	Post treatment days			
	0	7	14	28
Total bacteriologically +ve quarters	44	22 (50%)	12 (27.27%)	12 (27.27%)
Staphylococci	32 (72.72%)	14 (63.63%)	8 (66.67%)	8 (66.67%)
a. *S. aureus*	28 (87.5%)	11 (78.57%)	8 (100%)	8 (100%)
b. CNS	4 (12.5%)	3 (21.43%)		
Streptococci	8 (18.18%)	5 (22.72%)	1 (8.33%)	1 (8.33%)
a. *Str. agalactiae*	4 (50%)	3 (60%)	1 (100%)	1 (100%)
b. Esculin +ve Strep.	4 (50%)	2 (40%)	-	-
Escherichia coli	2 (4.54%)	1 (4.54%)	1 (8.33%)	1 (8.33%)
Others	2 (4.54%)	2 (9.08%)	2 (16.66%)	2 (16.66%)

Table 3. Effects of rational antibiotic therapy on quarter based bacteriological cure rate in subclinically mastitic cows at different post treatment days.

Isolates	Post treatment days			
	0	7	14	28
Total bacteriologically +ve quarters	52	22 (42.30 %)	20 (38.46%)	16 (30.77%)
Staphylococci	36 (69.23%)	15 (68.18%)	14 (70%)	12 (75%)
a. *S. aureus*	30 (83.33%)	13 (86.67%)	12 (85.71%)	12 (100%)
b. CNS	6 (16.67%)	2 (13.33%)	2 (14.29%)	
Streptococci	9 (17.30%)	3 (13.64%)	3 (15%)	2 (12.5%)
a. *Str. agalactiae*	9 (100%)	3 (100%)	3 (100%)	2 (100%)
b. Esculin +ve Strep.	-	-	-	-
Escherichia coli	6 (11.54 %)	3 (13.64%)	2 (10%)	1 (6.25%)
Others	1 (1.92%)	1 (4.54%)	1 (5%)	1 (6.25%)

(18.18%) had streptococcal infection and 2 (4.54%) had *Escherichia coli* infection. The provision of the treatment resulted in clearance of 22 (50%) quarters microbiologically at day 7 which further improved to 72.73% quarter clearance at the end of study period (Table 2). In group B, out of 52 positive quarters, 36 (69.23%) quarters were found to be infected with staphylococci, 9 (17.30%) with streptococci and 6 (12%) with *E. coli*, which decreased to 22 quarters resulting in clearance of 42.30% of the affected quarters microbiologically at day 7 post initiation of treatment. At day 14 and 28 post initiation of treatment in this group, 20 (38.46%) and 16 (28.57%) quarters were positive for isolates, respectively (Table 3) hence the cure rate at these sampling time points was 61.54% and 71.43%, respectively.

DISCUSSION

Out of 44 SFMT positive quarters in group A, 40 quarters (90.90%) tested negative at the end of study period. The standard antibiotic treatment (group B) showed 88.46% (46 of 52 quarters) SFMT based cure rate in subclinically mastitic cows. Considering the quarter based microbiological cure rates, the non-antibiotic formulation resulted in 72.73% cure rate in subclinically mastitic cows. The quarter based microbiological cure rate afforded by routine antibiotic treatment was 69.23% (36 quarters out of 52 quarters).

Similar findings have been reported by Prakash *et al*. (2013) who determined CMT based cure rates in mastitic cows treated with trisodium citrate. Similarly, Sarfraz *et al*. (2009) recorded a better SFMT based cure rates in the animals which were dosed with trisodium citrate and levamisole HCl as mastitis therapy. Beneficial role of trisodium citrate in reducing udder infections has been reported by many workers in the past (Prakash *et al.*, 2013; Dhillon *et al*., 2013).

In the past, an inverse relationship has been reported between milk citrate levels and mastitis, as mastitis results in fall in milk citrate levels (Peaker and Linzel, 1975; Dhillon *et al*., 1995; Gupta *et al*., 2016). As citrate is considered essential for milk synthesis and its decrease in milk during mastitis indicates that provision of citrate to animal can play important role in curing the mastitis. The Ca^{++} sequestration from casein micelles is severely affected by this reduced milk citrate concentration resulting in Ca^{++} clumping. These clumps constitute the flakes apparent in mastitic milk and are considered one of the many reasons inflicting injury to mammary tissue, worsening the mastitis. These injuries result in destruction of barrier between the udder and the blood which leads to free ionic movements. The alkaline nature (elevated pH) of the mastitic milk is believed to be the product

of this free ionic movement as bicarbonate concentration increases in mastitic udder. This alkaline environment is most suitable for growth of the micro-organisms, which is again a contributing factor in worsening the situation (Kumar *et al.*, 2007; Dhillon and Singh, 1989; Dhillon *et al*., 2013). Trisodium citrate alleviates udder infection by reducing the milk pH. The findings of the present study manifest that instituting a treatment containing citrate in sub-clinically mastitic cows leads to a significant reduction in quarter-based incidence of micro-organisms and SFMT based cure rate.

In dairy animals, a direct relationship between vit. C concentration and mastitis has been reported by Antila and Antila (1979), Steffert (1993), Naresh *et al*. (2002), Weiss *et al*. (2004), Kleczkowski *et al*. (2005) and Ranjan *et al*. (2005). It is believed that ascorbic acid dependent enzyme is required for hydroxylation process to synthesize collagen which is required for recovery from the damage. Therefore, it can be assumed that a quick recovery from mastitis can be achieved by administering vit. C. Furthermore, immuno-modulatory and anti-oxidant effects of vit. C can also play an indirect role in the recovery process. Approximately, 40-60 times higher concentration of vit. C in the immune cells as compared to other blood cells was reported by Basu and Schorah (1982) and a decrease in these high levels results from infection or trauma. Naresh *et al.* (2002) and Ranjan *et al.* (2005) have reported beneficial effects of vit. C alone or in combination with cupric ions in curing mastitis as a significant recovery from mastitis was reported by the authors. As an oxidative stress biomarker in bovine mastitis, many valuable properties of vit. C (like immune enhancer, anti-oxidant, epithelial regeneration) in controlling mastitis have been reported by Yang and Li (2015). It is also of value in reducing milk pH due to its acidic nature and this reduction in milk pH is considered to be helpful to stop the growth and hence reducing the burden of udder infection.

Zinc acts as co-factor in biological reactions for approximately 300 enzymes involved in metabolism, and it is categorized as an essential trace mineral. The maintenance of integrity of udder tissue lining is associated with zinc (Gropper *et al*., 2005; Yang *et al*., 2011). As super oxide dismutase (SOD) is a zinc dependent enzyme, zinc is considered as an important anti-oxidant. Gropper *et al.* (2005) have reported that zinc also helps in synthesis of many other substances e.g. vit. A in liver that helps in combating oxidative stress/mastitis. Increase in SCC of milk culminating in deterioration of milk quality is resultant of decrease in zinc concentration (Gaafar *et al*., 2010). A significant reduction in milk SCC after provision of zinc has been reported in previous studies (Popovic, 2004; Kellog *et al*., 2004).

Ceruloplasmin is an acute phase protein considered to be helpful in eliminating mastitis, which is copper dependent in nature (Gropper *et al*., 2005; Tomlinson *et al*., 2008). Moreover, SOD is also dependent on Cu along with Zn for its functioning. Scaletti *et al*. (2003) and O'Rourke (2009) have reported that decrease in severity of *E. coli* mastitis can be achieved by using Cu as treatment of mastitis. Beneficial effects of cupric ions along with vit. C to treat the mastitis in dairy animals have been reported by Ranjan *et al*. (2005).

***Conclusions*:** The findings of the current study showed that use of a combination therapy of non-antibiotic chemicals has potential impact on treating sub-clinical mastitis in dairy animals. Moreover, farmer friendly mode of administration and no discarding of milk due to antibiotic residues makes the test formulation superior to routine antibiotic therapy.

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