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THERAPEUTIC EVALUATION OF AN OUT OF THE BOX ORAL NON-ANTIBIOTIC FORMULATION AGAINST CLINICAL MASTITIS IN BUFFALOS

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Mastitis is regarded as most economically important disease of dairy animals in Pakistan. The cure rate of mastitis with use of antibiotics/antibacterials is often quite poor. In addition, use of these agents leads to undesirable residues in milk and poses several problems to milk processing industry. Therefore, measures other than the use of antibiotics/antibacterials are being investigated to improve mastitis cure rate and to circumvent the problem. Keeping these considerations in view, the current study was planned to evaluate the therapeutic efficacy of a non-antibiotic, non-proprietary antibacterial preparation for the treatment of clinical bubaline mastitis. For this purpose, buffaloes (n=40) suffering with clinical mastitis were selected and randomly divided into two equal groups viz. group I and group II. The animals of group I (n=20) were treated with combination of trisodium citrate (TSC; 40 g), vitamin C (10 g), zinc sulphate (3 g) and copper sulphate (1 g) for 7 days. Group II (n=20) was treated with routine mastitis therapy i.e. tylosin at dose rate of 10 mg/kg, I/M along with amoxicillin & clavulanic acid combination at total dosage of 600 mg I/mm infusion daily for 5 days. The efficacy of the treatment was evaluated based on grading of improvement, surf field mastitis test (SFMT) scores and microbiological examination. Regarding grading of improvement, 50% animals of group I recovered clinically while corresponding value for group II was 45%. The SFMT based cure rates for clinical mastitis in groups I and II were 70.21% and 72.09%, respectively. Administration of allocated treatments to the buffaloes affected with clinical mastitis led to a quarter based bacteriological cure rate of 66.67% in group I and 65.12% in group II. It was concluded that administration of non-antibiotic, non-proprietary formulation containing trisodium citrate, vitamin C, zinc sulphate and copper sulphate resulted in almost equivalent therapeutic results in buffaloes affected with clinical mastitis compared to antibiotic therapy.

Keywords: Bubaline, clinical mastitis, oral therapy, trisodium citrate, antioxidants.

INTRODUCTION

Mastitis is considered as mostly costly disease among all other infectious diseases in dairy animals. In Punjab, clinical mastitis accounts for losses of approximately 240 million rupees per year (Khan and Khan, 2006). Antibiotics are intensively used as an integral part of mastitis therapy. This makes mastitis as the most important reason for using antibiotics in dairy animals. However, this intensive use of antibiotics leads to the development of resistance in bacteria. Moreover, undesirable residues of antibiotics make the milk and meat of treated animals unfit for human consumption (Deluyker *et al*.,2005). Furthermore, the cure rates for mastitis therapy are appallingly low regardless of the antibiotics used (Sears and McCarthy, 2003). Antibiotic residues in milk may impede the fermentation process required to produce fermented dairy products like yogurt and cheese. This poses a great threat to the milk processing industries. Therefore, alternatives to antibiotics for the treatment of mastitis is strongly urged in dairy farming.

A variety of non-antibiotic agents for the treatment of mastitis in dairy animals is now used worldwide (Yousaf, 2009). Several studies have demonstrated that citrate level reduces to very low in milk of mastitis infected animals and the use of trisodium citrate (TSC) may result in restoration of milk pH as well as citrate level (Dhillon and Singh, 2013; Rai *et al*., 2013). Citrate is responsible for maintenance of milk pH as well as for the sequestration of soluble Ca^{2+} in milk (Shennan and Peaker, 2000). Thus, its reduction may result in high alkalinity of mastitic milk and clumping of Ca^{2+} manifested as flakes in milk. Therefore, bringing the pH to acidic may not only create hostile milieu for the growth of mastitis pathogens but would also render some antibiotics more effective.

Mastitis is also associated with increased total oxidant capacity of milk because of release of free radicals, whereas, the total antioxidant level becomes low. This necessitates the use of antioxidants which inhibit the activity of oxidizing enzymes by hunting the free radicals. Therefore, supplementation of antioxidants such as vitamin A, C, E, selenium, zinc (Zn) and copper (Cu) to the mastitic dairy animals may be helpful in mastitis treatment. In clinical and

sub clinical mastitis, serum level of ascorbic acid (Vit. C) is significantly reduced (Kleczkowski *et al*., 2005; Ranjan *et al*., 2005). It has been suggested that clinical severity of mastitis is directly associated with reduced vitamin C levels (Weiss *et al*., 2004).

Zinc (Zn^+) is an important trace mineral. It is the essential constituent of approximately 300 enzymes which are involved in metabolism (Yang *et al*., 2011). It has a significant role in conserving the lining of streak canal of the mammary glands which is made up of keratin. Reduction in Zn^+ levels may result in poor quality of milk with increased somatic cell count (SCC) and higher mastitis incidence in dairy animals (Gaafar *et al*., 2010). Copper (Cu^+) is also a trace mineral that confers robust antioxidant properties. It is the key component of super oxide dismutase (SOD) and ceruloplasmin and thus has an important role in mastitis treatment and control. It has been reported that supplementation with Cu^+ alleviated the severity of clinical signs in coliform mastitis (Scaletti *et al*., 2003).

The above mentioned attributes of TSC, Vit. C, Zn^+ and Cu^+ will stimulate anyone to look into the beneficial effects of this oral treatment with a combination of these non-antibiotics which may potentially prove effective in treating clinical mastitis in buffaloes. The present study was, therefore, planned to evaluate a non-proprietary (non-antibiotic) mixture containing TSC, Vit. C, Zn^+ and Cu^+ in the cure of clinical mastitis in dairy buffaloes (*Bubalus bubalis*).

MATERIALS AND METHODS

***Experimental animals*:** Forty clinically mastitic buffaloes were included in this study. These buffaloes were equally and randomly distributed into 2 groups I and II. Clinically mastitic buffaloes were included based on clinical signs, changes in udder and milk and Surf Field Mastitis Test (SFMT).

***Treatment protocol*:**

Group-I: Animals received combination of TSC (40 g), vitamin C (10 g), zinc sulphate (3 g) and copper sulphate (1 g) orally for 7 days (Modified from Dhillon and Singh, 2013).

Group II: Buffaloes were treated with Tylosin I/M and amoxicillin plus clavulanic acid IMM for 5 days. (Adopted from Ahmad, 2009).

Evaluation criteria:

Grading of improvement: It was assessed based on severity of clinical signs (Rai *et al*., 2013). No improvement in clinical picture of the animal was designated as a negative (-). The presence of blood in milk and flakes was considered as mild recovery (+). Absence of blood from milk with a few traces of flakes with a marked decrease in udder swelling was considered as moderate recovery (++). The complete absence of blood and flakes in the milk and udder swelling disappeared completely was designated as complete recovery (+++). This grading was done at the end of study period.

Surf field mastitis test (SFMT) scores: SFMT scores were noted after performing SFMT at each sampling time point as described by Muhammad *et al*. (2010)

***Quarter based bacteriological cure rates*:** Bacteriological examination was carried out by following the guidelines of National Mastitis Council (Hogan *et al.*, 1999).

These parameters were evaluated at baseline, day 7^{th}, 14^{th} and 28^{th} post initiation of treatment.

RESULTS

***Grading of improvement*:** The buffaloes suffering from clinical mastitis were subjected to assessment of grading of improvement. It was based on observation of severity of clinical signs displayed by the animals suffering from clinical mastitis and decline in the severity after the institution of treatment (Table 1 and 2). A complete recovery of 50% (10

Table 1. Grading of improvement in individual buffaloes of group I affected with clinical mastitis.

Buffalos suffering from clinical mastitis	Grading Score		Buffalos suffering from clinical mastitis	Grading Score	
Identification	Pre-treatment (Rx)	At day 28^{th} Post initiation of Rx	Identification	Pre Rx	At day 28^{th} Post initiation of Rx
Meeni	—*	+++****	Chorry sir wali	—	+++
Bhoori	—	+**	Sheeday wali	—	+
Lamby seengh wali	—	++***	Chandni	—	+++
Ashraf wali	—	++	Roop	—	+
Naseebo	—	++	Sadru wali	—	+
Taray wali	—	+	Tillari	—	++
Choti kabootri	—	++	Lundi	—	+
Bakhto	—	++	Saima	—	+++
Raisham	—	+++	Bhoori (2)	—	+
Nili	—	+	Meeni (2)	—	+

Group I=Daily oral institution of TSC 40 g, Vit. C10 g, Zinc Sulphate 3 g and Copper Sulphate 1 g x 7 days. * - No improvement; **+ Blood in milk became nil but flakes visible (mild healing); ***++ No blood flakes occasional, swelling reduced significantly (moderate healing); ****+++ No blood, no flakes, swelling disappeared (full recovery)

Table 2. Grading of improvement in individual buffaloes of group II affected with clinical mastitis.

Buffalos suffering from clinical mastitis	Grading Score		Buffalos suffering from clinical mastitis	Grading Score	
Identification	Pre Rx	At day 28th Post initiation of Rx	Identification	Pre Rx	At day 28th Post initiation of Rx
Nayan wali	—*	++	Malko	—	+++
Aslam Wali	—	+**	Gulabo BB	—	+
Moty Singh wali	—	+++****	8/12	—	+++
80 Chack wali	—	++***	Safena	—	+
Tillary	—	+++	Pinki	—	+
Khushbu	—	++	2 than wali	—	++
Choti chotti	—	+++	Saaqy wali	—	+++
Sapna	—	++	84 wali	—	+++
Masto	—	+++	Ashraf wali	—	+
Sudo	—	+++	12/14	—	+++

Group II= Daily institution of antibiotics IM + IMM x 5 days. * - No improvement; **+ Blood in milk became nil but flakes visible (mild healing); ***++ No blood flakes occasional, swelling reduced significantly (moderate healing); ****+++ No blood, no flakes, swelling disappeared (full recovery)

Table 3. Dynamics of SFMT based quarter cure rate at different days after initiation of treatment in buffaloes of group I and II affected with clinical mastitis.

Treatment groups with no. of animals	Total +ve quarters on the basis of SFMT	SFMT based quarter cure rate at day 7 after start of the therapy	SFMT based quarter cure rate at day 14 after start of the therapy	SFMT based quarter cure rate at day 28 after start of the therapy
I (20)	47	20 (42.55%)	25 (53.19%)	33 (70.21%)
II (20)	43	25 (58.14%)	30 (69.77%)	31 (72.09%)

Group I=Daily oral institution of TSC 40 g, Vit. C10 g, Zinc Sulphate 3 g and Copper Sulphate 1 g x 7 days
Group II= Daily institution of antibiotics IM + IMM x 5 days

Table 4. Dynamics of quarter based bacteriological cure rate at different days after initiation of treatment in buffaloes of group I affected with clinical mastitis.

Bacterial spp.	Days following start of therapy			
	0	7	14	28
Total bacteriologically +ve quarters	47	22 (46.81%)	18 (38.30%)	16 (34.04%)
Staphylococcal spp.	33 (70.21%)	14 (63.64%)	13 (72.22%)	13 (81.25%)
a. *Staph. aureus*	26 (78.79%)	13 (92.86%)	12 (92.31%)	12 (92.31%)
b. Coagulase Negative Staph.	7 (21.21%)	1 (7.14%)	1 (7.69%)	1 (7.69%)
Streptococcal spp.	9 (19.15%)	5 (22.73%)	2 (11.11%)	
a. *Str. agalactiae*	9 (100%)	5 (100%)	2 (100%)	
b. *Environmental streptococci (esculin +ve)*	-	-	-	
E. coli	3 (6.38%)	1 (4.55%)	1 (5.56%)	1 (6.25%)
Others	2 (4.26%)	2 (9.09%)	2 (11.11%)	2 (12.5%)

Group I=Daily oral institution of TSC 40 g, Vit. C10 g, Zinc Sulphate 3 g and Copper Sulphate 1 g x 7 days

out 20 buffaloes) was evident after the institution of combination of TSC, vit. C, zinc sulphate and copper sulphate to buffaloes of group I. The corresponding values for group II were 45% (9 out 20 buffaloes).

***SFMT based cure rate*:** Overall, a higher SFMT based cure rate was observed in buffaloes of group II (72.09%) which were dosed with rational antibiotic therapy followed by those of group I (70.21%, Table 3). In the buffaloes of group I, 47 quarters were positive for clinical mastitis initially and administration of treatment led to cure (SFMT –ve) of 42.55% (20 quarters) on day 7 after start of therapy. The SFMT based cure rate observed at day 14 and 28 after institution of treatment in this group was 53.19% and 70.21%, respectively. A total of 43 quarters were found positive for clinical mastitis in buffaloes of group II which were treated with routine antibiotic mastitis therapy (IM and IMM infusion of antibiotics). The treatment resulted in 58.14% (25 quarters) SFMT based cure rate at day 7 of the trial that increased to 72.09% (31 quarters) at the end of the trial.

Table 5. Dynamics of quarter based bacteriological cure rate at different days after initiation of treatment in buffaloes of group II affected with clinical mastitis.

Bacterial spp.	Days following start of therapy			
	0	**7**	**14**	**28**
Total bacteriologically +ve quarters	43	19 (44.19%)	15 (34.88%)	15 (34.88%)
Staphylococcal spp.	26 (60.47%)	8 (42.11%)	8 (53.33%)	8 (53.33%)
a. *Staph. aureus*	24 (92.31%)	7 (87.5%)	7 (87.50%)	7 (85.50%)
b. Coagulase Negative Staph.	2 (7.69%)	1 (12.50%)	1 (12.50%)	1 (14.29%)
Streptococcal spp.	9 (20.93%)	5 (26.32%)	3 (20%)	3 (20%)
a. *Str. agalactiae*	7 (77.78%)	4 (80%)	3 (100%)	3 (100%)
b. *Environmental streptococci (esculin +ve)*	2 (22.22%)	1 (20%)	-	-
E. coli	5 (11.63%)	3 (15.79%)	1 (6.67%)	1 (6.67%)
Others	3 (6.98%)	3 (15.79%)	3 (20%)	3 (20%)

Group II= Daily institution of antibiotics IM + IMM x 5 days

***Quarter based bacteriological cure rate*:** At the beginning of trial, in buffaloes of group I, out of 47 clinically positive quarters, 33 (70.21%) quarters were yielded the growth of *Staphylococcal spp*., 9 (19.15%) were carrying *Streptococcal spp*. and 3 (6.38%) were infected with *Escherichia coli*. The administration of the allocated treatment led to a bacteriological clearance of 25 (53.19%) quarters at day 7 after the start of therapy which further improved to a bacteriological clearance of 31 (65.96%) at the termination of trial (Table 4). In group II which was dosed with antibiotics, a total of 43 quarters were found positive of which 26 (60.47%) quarters yielded growth of *Staphylococci*, 9 (20.93%) were carrying *Streptococci* and 5 (11.63%) were infected with *E. coli* infection. At day 7 after the start of treatment, 19 quarters were positive on bacteriological examination displaying 55.81% quarter based bacteriological cure rate. The bacteriological load at day 14 and 28 after start of therapy in the group was 15 (34.88%) bacteriologically positive quarters (Table 5).

DISCUSSION

Efficacy of the treatment protocols in buffaloes suffering from clinical mastitis was evaluated by different parameters out of which first was grading of improvement. It was observed through severity of clinical signs and decrease in severity afterwards after institution of respective treatments. Significant improvement was observed in animals of group I which received combination of TSC, vit. C, zinc sulphate and copper sulphate, where 50% buffaloes recovered completely while corresponding value for group II was 45%. These outcomes are in line with the results of Rai *et al*. (2013). In terms of SFMT based cure rate, the higher cure rate was recorded in group II treated with rational antibiotic therapy during the study period. These findings are in broad agreement with the findings of the previously reported studies (Dhillon *et al*., 1995; Kumar *et al.,* 2007; Dhillon and Singh, 1989; Sarfraz *et al.,* 2009; Dhillon and Singh, 2013).

An inverse trend was observed in quarter based bacteriological examination of clinically mastitic buffaloes where the lower number of bacteriologically positive quarters were found in group I at the end of study. These results endorsed the results of previous reports by Latif *et al*. (2014) and Dhillon *et al.* (2013).

Previously, it has been reported that there is an inverse relationship between mastitis and milk citrate levels as the citrate levels drop in mastitis (Peaker and Linzel, 1975; Dhillon *et al*., 1995). Citrate is believed to be the "harbinger of milk synthesis", so, this suggests that citrate plays an important role in mastitis treatment as indicated by an association between decreases in milk citrate levels due to mastitis. The decrease in citrate level results in impaired sequestration of Ca^{++} from casein micelles leading to clumping of calcium which ultimately appears as flakes in mastitic milk. These calcium clumps are also believed to be responsible for injury to udder tissue along with many other factors, hence, exaggerating the mastitis. Furthermore, these injuries to udder tissue destroy the udder blood barrier resulting in the free movement of ions. The ions like bicarbonates tend to concentrate in the udder resulting in alkalinity (higher pH) of the milk. This alkaline pH is considered favorable environment for bacterial growth (Kumar *et al.,* 2007; Dhillon and Singh, 1989; Dhillon *et al*., 2013).

Trisodium citrate helps to reduce the milk pH, hence, helps in clearance of udder infection. As is evident from the findings of present study, replenishing the citrate levels in the form of combination (group I) therapy results in significant reduction of mastitis, decrease in milk pH, quarter based infection, milk somatic cell counts and increment in milk quality and quantity. Similarly, a direct relationship has been reported for vit. C concentration with mastitis in dairy animals (Antila and Antila, 1979; Steffert, 1993; Naresh *et al*., 2002; Weiss *et al*., 2004; Kleczkowski *et al*., 2005; Ranjan *et al*., 2005). Vitamin C is believed to be helpful in recovery from injury by contributing the synthesis of collagen through hydroxylation, which depends on ascorbate-dependent enzyme required for

recovery. As such, provision of vit. C could be helpful in a rapid recovery in mastitis. In addition, Vit. C has immuno-modulatory and anti-oxidant effects which may also play vital role in recovery process. Basu and Schorah (1982) reported that approximately 40-60 times higher concentration of ascorbic acid is found in the immune cells, and infection or trauma can lead to depletion of these high levels.

Vitamin C alone or in combination with cupric ions was found to be beneficial in treating mastitis (Naresh *et al*., 2002; Ranjan *et al*., 2005). Recently, Yang and Li (2015) have also reported many beneficial effects of vit. C in mastitis control in animals, as it has been recognized as an oxidative stress biomarker in bovine mastitis. Vitamin C being acid in nature also has the tendency to lower the increased pH of the milk which results in cessation of microbial growth and clearing the udder infection.

Zinc is an essential trace mineral and is component of more than 300 metabolic enzymes and is considered vital to sustain the integrity of the linings of the mammary glands (Gropper *et al*., 2005; Yang *et al*., 2011). Zinc is also considered as an important anti-oxidant as cytosolic and extracellular SOD is a zinc dependent enzyme. It also aids in synthesis of other substances like vitamin A in liver which helps in fighting oxidative stress/mastitis (Gropper *et al*., 2005). Gaafar *et al*. (2010) have reported that decrease in zinc concentration results in low quality milk having high somatic cell count. Popovic (2004) and Kellog *et al*. (2004) reported in their studies that provision of zinc resulted in significant decrease in milk somatic cell count.

Ceruloplasmin is a copper dependent acute phase protein and is helpful in reducing mastitis (Gropper *et al*., 2005; Tomlinson *et al*., 2008). Additionally, SOD is copper dependent as well, along with zinc dependency. Reduction in severity of clinical signs in *E. coli* mastitis in cows have been reported by Scaletti *et al*. (2003) and O'Rourke (2009) after use of copper as mastitis therapy. Ranjan *et al*. (2005) have described the valuable effects of cupric ions and vit. C in treatment of mastitis in dairy animals.

***Conclusion*:** In short, the administration of non-antibiotic, non-proprietary formulation containing TSC (40 g), vitamin C (10 g), zinc sulphate (3 g) and copper sulphate (1 g) resulted in almost equivalent therapeutic results in buffaloes affected with clinical mastitis compared to antibiotic therapy. Furthermore, facile mode of administration along with no threat of antibiotic residues tainted milk than antibiotics administration makes this therapy superior to later.

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