A Biosecurity measures application with proper treatment to overcome the risk factors that limit effective control of subclinical mastitis in dairy buffalo farms-A field study

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Abstract: Subclinical mastitis is one of the most costly diseases of dairy animals. This field study was carried out on 100 milking buffalo-cow aged from 2 to 7 years old in a herd group and housed at a private farm in El-Fayoum governorate, Egypt, from July, 2012 to March, 2013, (whereas decreased in milk yield, old infection by mastitis was treated systemically, workers don't check animals for mastitis periodically and had knowledge about clinical mastitis but none of them knew about subclinical mastitis plus low hygienic measures), aiming to determine: the prevalence rate of subclinical form of mastitis (SCM), monthly incidence of the disease post calving, isolation of caused pathogens and assessment of their susceptibility to commonly used antibiotics, cure rate after proper treatment in conjunction of improvement of both environmental and hygienic measures surrounded the animals to understand the constraints that limit effective control of the disease. A total of 400 quarter milk samples were collected from 100 milking buffaloes and screened for subclinical mastitis by the aid of California Mastitis Test (CMT), 108 positive CMT quarters related to 58 % of farm's animals suffering from SCM , hind quarters were most affected (66.7%).The CMT positive quarter samples, bulk milk samples, swabs of milk containers and tanks, water pipes and tanks, bedding and milker's hands were cultured for isolation of the causative bacterial agents. *Escherichia coli* was the most frequently isolated pathogen (25.92%) followed by *Staph aureus* (22.23%), coagulase negative Staphylococci (CNS) (6.48%)) and *Clostridium perfringens* type A (1.85%). The mixed growth was (43.52%) between *S. aureus , E.coli* and streptococcus spp. and staphylococcus spp.. Antibiogram analysis was carried out for bacterial isolates where Enrofloxacin, Cefotaxime, Amoxicillin and clauvilinic acid; were found the most effective drugs against the major of isolated strains in our study. Resistance of *S. aureus* to penicillin is more prevalent. It could be concluded that both environmental and hygienic measures surrounded the animals constitute a major risk factors in the occurrence of mastitis. So, continuous bacteriological investigation together with treatment, and increase hygienic measures were done in the present study in order to identify potential mastitis control measures.

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1. Introduction:

Buffaloes are the main dairy animals in some developing countries worldwide despite this species tends to have relatively slow rate of reproduction and more reproductive problems such as inactive ovaries, long calving intervals and mastitis **(Hussein 2002; Piccinini *et al*. 2006 and Abd El-Razika *et al.* 2010).**

Mastitis is an important disease of dairy animal and a threat for dairy farmers in most parts of the world **(Getahun *et al.* 2008 and Bachaya *et al.* 2011**). It has two forms, the clinical mastitis that usually has all the five cardinal signs of udder inflammation (redness, hotness, swelling, painfulness and loss of milk production) and hence can be detected without any laboratory test and even by the laymen. Whereas the subclinical form of mastitis is hidden and needs laboratory aid for diagnosis. Moreover, abnormal milk is readily detected in clinical mastitis but there is no apparent change in milk in subclinical mastitis. Among all the mastitis infections, subclinical mastitis has been reported to cause 60-70% of total economic losses in the advance country like USA **(Merrill and Galton, 1989 and Bhatt *et al.* 2012**). Moreover, quarter-wise prevalence of intra-mammary infection (IMI) in buffalo was 66%, especially during the per-parturient period, whereas the incidence is highest during the 30 days after calving **(Moroni *et al.* 2006).** These losses might be higher because of poor management and least prevention practices **(Arshad, 1999)** as prevalence of subclinical mastitis is influenced by many factors such as husbandry, management, genetics and nutrition **(Elbers *et al.* 1998 and Bielfeldt *et al*. 2004).**

Subclinical mastitis can be diagnosed by somatic cell counts (SCC), California Mastitis Test (CMT), White side test (WST) or Surf field mastitis test (SFMT) **(Muhammad *et al.* 2010).** The main causative bacteria include: *S. aureus*, *St. agalactiae* (both of which are contagious), coliforms, *Streptococci* and *Enterococci*. All of these pathogens are found in the environment of the animals (water, feed, bedding, manure and soil). Several other pathogens have been isolated from infected mammary glands which include *Actinomyces pyrogenes*, *Cl.perfringens* and other coliforms, such as *Pseudomonas aeruginosa*, *Klebsiella pneumonia* and *Pasteurella haemolytica*, among others **(Conington *et al.* 2005).**

Treating infection with antimicrobials in conjunction with good farming practices, assist in this endeavor to eliminate, or at least decrease, the incidence of mastitis infection within a dairy herd **(Pieterse and Todorov 2010).** Ceftiofur is a new broad-spectrum third generation cephalosporin antibiotics for veterinary use. It inhibits bacterial cell wall synthesis by interfering with enzymes essential for peptidoglycan synthesis **(Hornish and Kotarski 2002).**

Enrofloxacin is a fluoroquinolone developed exclusively for veterinary use and exhibit high bactericidal activity against a broad spectrum of aerobic Gram negative, some Gram-positive bacteria and *Mycoplasma* spp. (**Baroni *et al.* 2007**). A combination of enrofloxacin and levamisole as an immunomodulators were found to clear 100 % of the infection due to *Strep. agalactiae*, *disgalactiae* and *Micrococcus* spp. (**Saluja *et al.* 2005**).

Health status of mammary gland in milking animals contributes greatly in the economic importance of the farm animals. Despite susceptibility to mastitis is low in buffaloes when compared to cattle **(Saleh, 2005),** the poor management conditions practiced by small buffalo holders in rural areas may anticipate in increased percentage of subclinical mastitis. To avoid udder infections and following mastitis, it may be beneficial to find ways to stimulate the animal's immune defense for more efficient resistance against and/or elimination of infection **(Zecconi and Smith, 2000 and Hase *et al.* 2013).**

Earlier studies have reported high prevalence levels and variations among risk factors including poor hygiene, management practices as well as buffalo factors **(Okello-Uma and Gibson 1976; Nakavuma *et al.* 1994; Barkema *et al.* 1998 ; van Schaik *et al.* 2005 and Kivaria *et al.* 2006).** Effective control therefore requires understanding of the farming system, the constraints that limit milk production efficiency and the risk factors under each particular farming system and different farm circumstances and adoption of possible and affordable options for control **(Brown *et al.* 1998)**.

The main strength with this field study is its' focus not only on the prevalence of SCM, but also on the gathered information of environmental factors that could provide information of factors causing a high prevalence of SCM through combination of the CMT with bacteriological cultures to determine: (a) The actual prevalence rate of subclinical mastitis of 100 hand milked lactating buffalo aged from 2 to 7 years old in a herd group in El-Fayum governorate, from July 2012 to March, 2013. (b)Monthly incidence of SCM post calving. (c) Bacteriological examination of CMT positive quarter samples, bulk milk samples, bulk milk tanks and containers, water pipes and tanks, milker's hands and soil to identify the causative bacterial agents. (e) Susceptibility of isolated strains to commonly used antibiotics and. (d) change in the infection rate after several control options including improvement in management by application of bio-security measures together with proper treatment aiming at reducing the rates of new infections and or the disease. (f) The cure rate of animals after different lines of treatments. (g) The change in prevalence rate of SCM and milk yield by re-examination of the last samples in step number (c) to put our hands in the way of the effective control.

2. Material and Method

Animals and farm:

A total of 100 animals aged from 2-7 years old were used in This study and housed in a private farm at El-Fayum governorate, in a separate free yard on straw bedding floor whereas low hygienic measures were prevailed and decrease in milk yield. They were milked manually once daily and the milk was collected in large milk tank. They receive their needs of water through a common water trough and the houses were mainly cleaned using spade only. All buffaloes were examined at buffalo and quarter level with CMT to expel subclinical mastitis whereas buffalo without any sign of clinical mastitis.

Farmer's knowledge, practices and perceptions related to subclinical mastitis:

The characteristics of the farm workers are summarized as follow: Most of the workers were males, peasant farmers without formal employment, had primary level of education, don't check animals for mastitis periodically and had knowledge about clinical mastitis but none of them knew about subclinical mastitis. They were milking animals manually once daily in the morning without following any particular order of milking .All housing and management decisions were the

responsibility of the workers due to lack of veterinary services.

Preparation of samples:-

A-Milk Samples: Milk samples were collected using standard procedures described by **Harmon *et al.* (1990)**. Briefly, after discarding the first few milk drops, milk samples were taken from all farm's animals by wiping the teats with 70 % ethyl alcohol with paying extra attention to teat orifice. Each milk sample was collected in a sterile screw capped bottle; also bulk milk tank sample was taken aseptically in a sterile flask. Both Milk samples and tank milk samples were sent directly to the laboratory with minimum delay for the routine cultural identification after incubated aerobically at 37ºC for 24 hrs , centrifuged at 3000 rpm for 20 minutes and discarded the supernatant fluid.

B-Other environmental samples: A ten sterile cotton swabs removed from a nutrient broth tubes were rubbed onto the hands of milkers at different sites then returned back to nutrient broth tubes. Another sterile cotton swabs moisted with sterile saline were used to swab the milk containers, water tanks and pipes, water and bulk milk tank (ten of each). Also under complete aseptic condition, approximately 100 gram of the soil and bedding materials were collected from the places in which the udder of the recumbent animals was resting (ten samples). All samples were kept at 4 ºC and transported immediately to the laboratory for bacteriological examination as described by **National Mastitis Council Inc. (1987).**

California Mastitis Test (CMT): A total of 400 quarters milk samples corresponding to 100 buffaloes suspected to harbor subclinical mastitis based on decreased in milk yield and absence of visible abnormalities of milk secretions or any sign of clinical mastitis were indirectly screened for somatic cell count level by means of CMT**.** 50 ml of milk samples from each quarter were collected in a sterile McCartney bottle. The CMT result was scored basing on the gel formation, buffaloes with a CMT score ≥3 in any quarter were considered positive for subclinical mastitis and categorized as negative if there was no gel. Milk samples were collected from each affected quarter to divulge bacterial presence and indentify the pathogens, as described by **Hogan *et al.* (1999) and Hase *et al.* (2013).**

Bacteriological examination:-

Isolation and identification of bacterial pathogens: was carried out according to **Cruickshank *et al.* 1975; Koneman *et al.* 1992; and Quinn *et al.* 1994).A** loopfuls from the milk sediments,swabs from milk tanks and containers, milker's hands, water pipes and tanks as well as the bedding materials were inoculated into a brain heart infusion broth, then incubated aerobically at 37°C for 24-48 hours for enhancement of aerobic bacterial growth, subcultures were streaked on 10 % sheep blood agar, MacConkey, Edwards media, EMB media, mannitol salt agar plates. Also loopfuls was cultured in cooked meat broth then subcultures onto blood agar supplemented with Neomycin antibiotic and incubated anaerobically at 37°C for up to 48 hrs for enhancement of anaerobic bacterial growth. Suspected colonies were identified on the basis of their cultural, morphological characteristics, biochemical reactions and serological tests. *Clostridium perfringens* toxins were typing by dermonecrotic test according to **Oakley and Warrack (1953) and Quinn *et al*. (2002).**

Antimicrobial susceptibility testing: All bacteria isolated through microbiological procedures were subjected to antimicrobial susceptibility testing by disc diffusion method **(Anonymous, 2004).** The sensitivity against Gentamycin, Trimeth/sulfa, Spectinomycin, Streptomycin, Penicillin, Enrofloxacin, Cefotaxime, Amoxicillin and Clauvilinic acid and Tetracycline was determined on Muellar-Hinton agar as described by **National Mastitis Council Inc. (1999).**

Control:

Three parallel lines of control options were done in the present study including improvement in management, proper treatment and animal's immune defense stimulating for more efficient resistance against and/or elimination of infection aiming at reducing the rates of new infections and/ or the disease as follow:-

A-Treatment: Treatment schedule was carried according to **Saluja *et al.* (2005):** CMT positive animals (58) were classified into 3 groups (18 animals of each). The first group received local treatment by intra-mammary infusion with 125 mg of Cefotaxime. The second group received systematic treatment by I / M injection of 5mg/kg body weight Enrofloxacin and orally Rovimix as a source of vitamin E. The third group received a combination of both local and systematic lines of treatment. The lasted forth animals were served as untreated control. The treatment applied once daily and for 5 successive days. The efficiency of treatment cure was judged by CMT seven days post treatment and clinical cure was defined by the return to normal milk yield in the farm's animals as shown in Table (7).

B-Animal's immune defense stimulating: According to **Byarugaba *et al*. 2003),** the immune response of the animals in the present farm was fallow up by sufficient nutritional requirements

specially green rations, and by orally Rovimix as a source of vitamin E for more efficient resistance against and/or elimination of infection as shown in Tables(1&7).

C-Follow up the biosecurity measures on the studying farm: The overall hygiene and especially the hygiene routines around milking time are the main reasons of the high prevalence **(Hase *et al.* 2013).** Most of the farmers in this study did not follow any order of milking and therefore there was a risk of spreading infection from sick animals to healthy ones. One farmer started with stubborn ones (including diseased) and milks the normal one afterwards without washing hands in between milking of each buffalo. Many others used the same towel for all buffalo and such practices have been reported to spread and sustain mastitis in the herd. Major constraints (risk factors) associated with subclinical mastitis control in the present study specially during the milk time were improved to prevent or at least limit the spreading infection from sick animals to healthy ones in our field study as shown in Table (1)**.**

Table (1): Follow up the bio-security measures on the studying farm according to Giesecke *et al*. (1994) and Hase *et al*. (2013): aiming to improve the major farm risk factors.

Farm and animals risk factors	Owner and workers risk factors
• **good hygiene was carried as follow:** *The numbers of water supplies and milk tanks were increased with periodic bacteriological examination to detect any pathogens as early as possible. * The animal's house and milking place were cleaned after milking using a spade and soup. *Animals were supplied by sufficient nutritional requirements specially the green rations as mastitis control alone doesn't result in increased milk production if other issues such as sufficient nutritional demands are not met. *Bedding was dried and cleaned as the disease arose from contaminated beddings or environment through milker's hand. *The animal's immune defense was stimulated in general for more efficient resistance against and/or elimination of infection. *Buffalo with chronic subclinical mastitis were sold off, slaughtered or were treated further with advice from a veterinarian	• **Orders of milking were followed as follow:** * The milking procedure was occurred by the workers twice daily starting with the normal one. *All milker's hands and animal's udder were cleaned before and after milking each buffalo using water and soap or using disinfection. *The high somatic cell animal was separated and milked after the low-cell ones. *Increased the numbers of milking workers and equipments. *Equipments pasteurization was done daily with efficiently to inactivate different pathogenic microorganisms. *Increased the owner and workers knowledge about subclinical mastitis and aware of the losses caused by it which include reduced milk production, treatment costs, reduced income, low milk quality and deformed udder. *Illustrated the major constraints to mastitis control included high treatment costs, insufficient or lack of veterinary services, difficulty in diagnosing the disease, low income, poor hygiene especially during the rainy season and lack of equipment for controlling the disease. * Periodic check for mastitis by its two forms to early discover and control.

N.B: Milk production level and animals cure rate based on the number of CMT positive quarter samples were judged after different lines of control at the starting, after two months and in the ending of the study to noticed the changes in prevalence rate of SCM in the farm .Also bulk milk sample, milk tanks and containers, water sources (pipes and tanks), milker's hands, soil and bedding were re-examined after increased the bio-security measures to show the change of pathogens numbers and understand the risk factors that limit effective control of subclinical mastitis in dairy buffalo farms.

3. Results

Table (2): Incidence of subclinical mastitis in dairy buffaloes based on CMT and percentage of different quarter milk samples (QMS):

NO of animals studied	Total affected animals	%	NO of quarters Studied	Total affected quarters	%	Involvement of the quarters							
						Right fore		Right hind		Left fore		Left hind	
						NO	%	NO	%	NO	%	NO	%
100	58	58	400	108	27	16	14.81	32	29.63	20	18.52	40	37.44

%: Percentage NO: number

Table (3): Monthly incidence of post calving subclinical mastitis based on CMT

Months post calving	Incidence of subclinical mastitis		% %
	Tested animals(100)	Number of corresponding quarters Studied (400)	
	NO *of positive CMT(58)*	NO *of positive CMT(108)*	
1st month	22	41	37.94
2nd month	12	21	20.69

3rd month	7	13	12. 06
4th month	7	14	12.06
5th month	5	10	8.63
6th month	2	3	3.45
7th month	1	1	1.72
8th month	2	5	3.45
Total	58	108	100

CMT: California mastitis test NO: number %: Percentage calculated according to the number of affected animals

Table (4): Bacterial isolates from subclinical mastitic milk and quarter wise prevalence of different microorganism in buffalo milk samples (n=400):

Subclinical mastitic milk (n=108)			**Quarter wise prevalence**			
Bacteria	**Frequency (NO)**	**Percentage (%)**	**Right fore**	**Right hind**	**Left fore**	**Left hind**
E. coli	28	25.92	4	8	6	10
S.aureus	24	22.23	3	8	4	9
S.aureus+C.N.S	21	19.44	3	6	4	8
E.coli+St.dysgalactia	14	12.96	2	4	2	6
C.N.S + St. agalactia	12	11.12	2	3	3	4
C.N.S	7	6.48	2	2	1	2
***Cl. Perfringens* Type A**	2	1.85	0	1	0	1

CNS: Coagulase negative *Staphylococci.* N0: number

Table (5): Bacteriological examination of different environmental samples before controlling plan:

Samples											
Type		**NO**	***E.coli***	***%***	***Staphylo-coccus* spp.**	***%***	***Streptococcus* spp.**	***%***	***Cl.perfringenes* TypeA**	***%***
Bulk milk		10	8	80	4	40	3	30	3	30
Milk tank		10	7	70	7	70	6	60	2	20
Milk containers		10	8	80	9	90	5	50	2	30
Water tank		10	7	70	6	60	3	30	_	_
Water pipes		10	5	50	5	50	2	20	_	_
Bedding		10	10	100	9	90	7	70	6	60
Milker's hands		10	8	80	9	90	4	40	_	_
Total samples	***NO***	***70***	***53***		***49***		***30***		***13***	
	%	***100***	75.7		70		42.9		18.6	

%: Percentage----------No: number----------spp.: species

Table (6): Antimicrobial susceptibility testing of bacterial isolates:

Antimicrobial agent	***45* *S.aureus* (24single+21mixed isolates)**		***42* *E. coli* (28 single +14 mixed isolates)**		***40* *CNS(7 single+3 Mixed isolates)***		***12* Mixed isolates of *St.agalacia***		***14* Mixed isolates of *St.dysgalactia***		***2* *Cl.Perfringes* type A**	
	NO	**%**	**NO**	***%***	**NO**	**%**	**NO**	**%**	**NO**	***%***	**NO**	**%**
Penicillin	5	12	-	-	2	5	7	55	6	40	-	-
Enrofloxacin	43	95.2	35	83.3	36	90	11	95	14	100	1	50%
Cefotaxime	40	88	38	91.1	32	80	12	100	12	90	1	50%
Amoxicillin and clauvilinic acid	22	48	32	77.7	19	47.5	9	75	9	60	1	50%
Tetracycline	5	11.2	24	56.6	3	7.5	5	40	5	30	-	-
Gentamycin	-	-	26	62.2	-	-	-	-	-	-	-	-
Trimeth/sulfa	3	7.2	20	47.7	1	2.5	4	30	2	10	-	-
Spectinomycin	-	-	21	50	-	-	-	-	-	-	-	-
Streptomycin	-	-	15	36.6	-	-	-	-	-	-	-	-

No=Number of sensitive strains **%= Percentage of sensitive strains**

Table (7): The cure percentages after 7 days and two months of local and systemic lines of treatment and bio-security measures follow-up based on CMT:

Different lines of treatment		Total number of tested animals (58)which corresponding to(108) QMS							
		Tested animals		Cured animals after 7			Cured animalsafter2months		
		NO	QMS	NO	%	QMS	NO	%	QMS
First group(local I/mammary infusion with cefotaxime		18	21	10	55.5	12	16	88.8	19
Second group (I/M injectionof enrofloxacin and orally Rovimix(source of vitamin E)		18	35	15	83.33	29	17	94.4	29
Third group (both lines of treatment).		18	44	17	94.44	42	18	100	44
Forth group(served as a control)		4	8	0	0	8	3	75	6
TOTAL	NO of animals	58		42			54		
	%	100		72.4			93.1		

QMS: quarters milk sample I/M: intramascular %: percentage NO:number

Table (8): Records of bacteriological examination of different environmental samples after two months from controlling plan:

Samples		*E.coli*	%	*Staphylo-coccus* spp.	%	*Strepto-coccus* spp.	%	*Cl.perfringes* typeA	%
Type	No								
Bulk milk	10	3	30	2	20	1	10	-	-
Milk tank	10	3	30	4	40	3	30	1	10
Milkcontainers	10	2	20	2	20	1	10	-	-
Water tank	10	3	30	-	-	-	-	-	-
Water pipes	10	2	20	-	-	-	-	-	-
Bedding	10	5	50	3	30	2	20	2	20
Milker's hands	10	-	-	-	-	-	-	-	-
Total samples NO	*70*	*17*		*11*		*7*		*3*	
Total samples %	*100*	*24.3*		*15.7*		*10*		*4.3*	

%: percentage NO: number

Table (9): Incidence of subclinical mastitis in the end of our dairy buffalo farm study and percentage of different quarter milk samples (QMS) based on CMT:

Number of animals studied	Total affected animals	%	Number of quarters Studied	Total affected quarters	%	Involvement of the quarters							
						Right fore		Right hind		Left fore		Left hind	
						NO	%	NO	%	NO	%	NO	%
100	3	3	400	8	2	1	12.5	2	25	2	25	3	37.5

CMT: California mastitis test was carried out after 4 months of treatment and Bio-security measures application.

4. Discussion

Subclinical mastitis is the most serious type of mastitis as the infected animal shows no obvious symptoms and secrets apparently normal milk for a long time, during which causative organisms spread infection in herd, so it is an important feature of the epidemiology of many forms of bovine mastitis **(Bakken and Gudding 1982).**

The bacteriological studies applied in this field study were applied through combination of the CMT with bacteriological cultures, why? Because subclinical mastitis was defined as when mammary glands without clinical abnormalities giving apparently normal milk but was bacteriologically positive and with positive CMT **(Stefanakis *et al.* 1995). Pyorala (2003)** concluded the CMT is still the superior screening diagnostic aid for subclinical mastitis, while bacteriological examination is still the most suitable, accurate and reliable method to confirm the causative organisms.

The present study investigated the occurrence of subclinical mastitis and associated constraints faced by farmers in controlling the disease in dairy farming system. From the results had been presented in **Table (2)**, the recorded overall quarter incidence of subclinical mastitis by the CMT was 27% (108 quarters out of totally 400 and corresponding to 58 animals), it means that the prevalence rate in the present study was 58% from total animal's farm. Our results partially agree with to **Pathak and Sharma (1988)** who recorded the incidence of subclinical mastitis in buffalo ranges from 8 to 60%. Higher incidences were obtained by **Coni *et al.* (1983); Alexandrova (1986); Mahmoud (1988) and Ismail**

and Hatem (1998), in Italy, Bulgaria, Egypt and Saudi Arabia, respectively. **Table (2)** also demonstrated that, out of 108, 16 (14.81%) in right fore, 32 (29.63%) in right hind, 20 (18.52%) in left fore and 40 (37.04%) in left hind were recorded. So there was higher incidence in hind quarters than fore quarters and were found to be more susceptible. Those results agreed with **Saini *et al.* (1994)** who reported the same results.

Table (3), explains the incidence difference of subclinical mastitis post calving. 1st and 2nd months post calving were the highest incidence of subclinical mastitis in percentages of 37.94%, 20.69% respectively. While the incidence were 12.06%, 12.06%, 8.63%, 3.45%, 1.72% and 3.54% in 3rd, 4th, 5th, 6th, 7th and 8th months respectively, our results in accordance with **Corbett (2009)** who suggests that the highest number of subclinical mastitis cases occurs during the first week of lactation, and that the lactating buffalo is more likely to develop subclinical mastitis during the first three months of lactation than the remainder of the lactating period, and **Lakshmi *et al.* (2009)** who found that the buffaloes in the first stage of lactation (1- 4 months) and the last part of dry period (10-12 months) were more prone to mastitis.

Table (4), showed that *E. coli, S. aureus,* CNS, *St. agalactia, St. dysgalactia* and *Clostridium perfringens* were the most common isolates in subclinical mastitis. Subclinical mastitis caused by single infection or mixed infection. *E. coli* was the most common single cause of subclinical mastitis (25.92%), followed by *S.aureus* (22.23%), CNS (6.48%) and *Cl. Perfringens* typeA (1.85%). *S. aureus* and C.N.S was the most common mixed cause of subclinical mastitis (19.44%) followed by *E. coli* and *St. dysgalactia* (12.96%) and C.N.S and *St. agalactia* (11.12%).Our present studies were agree with **Hallén-Sandgren (2000),** who found that the most important isolations from subclinical cases were *S. aureus* (37%), CNS (31%) and *Str. uberis* (14%) in Sweden, and agree with **Gianneechini *et al.* (2002)** who isolated pathogens from subclinical cases and their relative frequencies were: *S. aureus* (62.8%), *St. agalactiae* (11.3%), *Enterococcus spp.* (8%), CNS (7.4%), *St. uberis* (6.4%), *St. dysgalactiae* (1.8%), *E. coli* (1.5%) and *S. hyicus*- positive (0.6%).

The main strength with this field study is its' focus not only on the prevalence of SCM, but also on the gathered information of environmental factors that could provide information of factors causing a high prevalence of SCM. Our mind in accordance with **Swartz *et al.* (1984)** who pointed out that if resources to diagnose SCM are poor; there is a large risk that the problem with the invisible SCM will continue to cause both big production and economical losses, even if the problems with CM are solved.

In the beginning of our present field study, the isolated strains were present in high levels in the housed animal's environments as shown in **Table (5)**, especially in milker's hands milk containers and bulk milk, and also present in bedding materials and water and milk tank as they act as primary reservoir for these environmental pathogens. Our results are completely agree with **OZ *et al.* (1985) and Sayed (1996)** who suggested that **t**he remarkable increase was may be due to passage of milk through the milking equipment which gets contaminated from the polluted water during rinsing with cold water. While in the end of our present field study, the isolated strains were present in low levels in the same previously tested housed animal's environments as shown in **Table (8)**.

The high treatment costs of last clinical cases affected our farm which can partly be related to the high resistance of the common and cheap antibiotics like penicillin and tetracycline that was observed in the bacteria isolated from the samples, similar high resistance patterns among mastitis pathogens have been reported by **Nakavuma *et al*. 1994 and Kambarage *et al.* (1996).** So Table (6) explains sensitivity of different subclinical mastitis pathogens isolated during the studying period to different antibiotics. Enrofloxacin, Cefotaxime and Amoxicillin and clauvilinic acid were found most effective drugs against 45 *S. aureus* isolates. Cefotaxime, amoxicillin and clauvilinic acid, enrofloxacin, gentamycin, tetracycline and spectinomycin were found most effective drugs against 42 *E. coli* isolates. Cefotaxime, amoxicillin and clauvilinic acid, enrofloxacin and penicillin were found most effective drugs against 12 *St. agalactia* isolates. Amoxicillin and clauvilinic acid, Cefotaxime and enrofloxacin were found most effective drugs against 14 *St. dysgalactia* isolates and 2 isolates of *Cl. perfringens*. Resistance of *S. aureus* to penicillin is more prevalent (88.8)and this findings of are in accordance with those of **Iqbal *et al.* (1984)** who found that 92.86 % of *S. aureus* isolates from buffalo milk were resistant to penicillin. Meanwhile **Costa *et al.* (2000)** found high sensitivity of *S.aureus* to gentamycin (80%), which is disagree with the findings of the present study. **Dhakal and Thapa (2002)** found that enrofloxacin had the highest average sensitivity (91%) and less effectiveness of amoxicillin to all the isolates may be due to the resistance produced in the bacteria due to extensive use of this antibiotic in cattle and buffaloes. **Farooq *et al.* (2008)** recorded that Norfloxacin and Gentamycine were found most effective antibiotics

tested *in vitro* against *S. aureus, St. agalactiae, E.coli*, bacillus spp. and mixed growth.

The goal of antibacterial therapy is to attain effective concentrations of the drug at the site of infection. Table (7) explained the difference in cure rate after 7 days of using different lines of treatments of subclinical mastitis in conjunction with improvement of biosecurity measures in the farm. The best results obtained in combination of local Enrofloxacin and orally Rovimix (vitamin E) with systemic Ceftiofur groups by cure rate 94.44%. In Enrofloxacin group, cure rate 83.33%. In Ceftiofur group, cure rate 55.55%. This table also showed the fallow up of the cure rate after two months of treatment and improvement of management in the studying farm which were 88.8%, 94.4 % and 100% in Cefotaxime group, Enrofloxacin group and the group of combination of them respectively. In general the cure rate was reached to 93.1% from the total number of previously treated animals. On the other hand, **El-Khodery and Osman (2008)** evaluate the efficacy of Ceftiofur in the treatment of buffaloes with acute coliform mastitis. Parenteral Ceftiofur neither improved clinical signs nor returned milk to pre-infection production level, whereas intra-mammary Ceftiofur and combination of intra-mammary with parenteral Ceftiofur improved the clinical signs in 10/15 and 12/15 buffaloes, respectively. **Kopcha *et al*. (1992**) concluded that Rovimix used as immune-modulator to increase the functional capabilities of neutrophills, macrophages and plasma cells. It also, increases the phagocytic and bactericidal activity of neutrophils at the mammary glands, inhibits the biochemical reactions of the most bacterial pathogens and shortens the severity of mastitis. The present treatment schedule is in agreement with **Akhtar *et al*. 2003,** who used enrofloxacin and 3-D Vet for treatment of subclinical mastitis, the differences is that in the present study, Rovimix was used as anti-inflammatory and immune-potentiator instead of Diclofenace sodium (3-D Vet).

The high SCM prevalence obtained in this field study may be attributed to a group of shared factors including bad habitat, lack of hygiene, unbalanced diet and bad draft. This group of defective conditions played a role in rendering the udder more susceptible to intra-mammary infection, this results similar high prevalence rate among subclinical mastitis have been reported by **Ghazi and Niar (2006).** My own reflection is that the overall hygiene and especially the hygiene routines around milking time are the main reasons of the high prevalence. Most of the farmers in this study did not follow any order of hygienic milking and therefore there was a risk of spreading infection from sick animals to healthy ones. One farmer indicated that he starts with stubborn ones (including diseased) and milks the normal one afterwards without washing hands in between milking of each buffalo. Many others used the same towel for all buffaloes and such practices have been reported to spread and sustain mastitis in the herd and becomes very difficult to eliminate **(Kassa *et al*. 1999; Mdegela *et al.* 2004 and Kivaria *et al.* 2006).**

The present study provide new information and will hopefully contribute to a possibly lower prevalence of SCM in the future as shown in **Table (1)** which illustrated our plan to overcome the major constraints that limit effective control of subclinical mastitis in dairy farms, this constraints included farm and animals risk factors, and owners and workers risk factors. **Among** farm and animals risk factors were treated by following the good hygiene as followed: The numbers of water supplies and milk tanks were increased with periodic bacteriological examination to detect any pathogens as early as possible. The animal's house and milking place were cleaned after milking using a spade and soup. Bedding was dried and cleaned as the disease arose from contaminated beddings or environment through milker's hand. Our feature was agree with **Andersson *et al*. (2011),** who concluded that the most important way to reduce high SCC levels is to work with preventive udder health in order to reduce the prevalence of SCM and CM in the herd.

On the other hand owner and workers risk factors were treated by following the orders of milking as follow: The milking procedure was occurred by the workers twice daily starting with the normal one, all milker's hands and animal's udder were cleaned before and after milking each buffalo using water and soap or using disinfection, the high somatic cell animal was milked after the low cell ones, increased the numbers of milking workers and equipments, equipments pasteurization was done daily with efficiently to inactivate different pathogenic microorganisms, increased the owner and workers knowledge about subclinical mastitis and aware of the losses caused by it which include reduced milk production, treatment costs, reduced income, low milk quality and deformed udder and periodic check for mastitis by its two forms was carried to early discover and control. Our feature was agree with **Nickerson and Boddie (1995)** who pointed to hygienic milking routines are also decreasing the exposure to bacteria.

Several other factors such as inadequate nutrition has been suggested to be the most serious constraints to improved milk production efficiency (**Byarugaba *et al*. 2003),** For this reason animals in the present study were supplied by sufficient nutritional requirements specially green rations as

mastitis control alone doesn't result in increased milk production if other issues such as sufficient nutritional demands are not met. The animal's immune defense was stimulated in general by orally Rovimix as a source of vitamin E for more efficient resistance against and/or elimination of infection.

Hogeveen (2005) concludes that mastitis still continues to cause significant losses to farmers, despite the availability of extensive knowledge on mastitis and its control strategies. In this study, the major constraints to mastitis control was primarily farmers' lack of good daily practice routines such as hygiene, and much of this was caused by lack of knowledge about the two forms of mastitis which is very important and plays an important role in controlling of the disease. the farmers had only knowledge about clinical mastitis which may explain the lack of adequate preventive measures as reported among smallholder farmers by **Karimuribo *et al.* (2008) and Byarugaba *et al.* (2008)** who also found that almost none of the farmers seemed to have the knowledge of methods to control mastitis: udder washing, good hygiene, culling of chronic cases, following a predetermined milking order or teat dipping were all unusual measures. Also **Bell *et al.* (2005)** examined the effects of different knowledge dissemination methods for mastitis control in smallholder dairy farmers in Tanzania and found that a combination of methods were more effective. They also noted association of knowledge uptake with the level of education. In the present study, whereas the most of workers were of secondary level education, there was no difference on the levels of mastitis on the various farms with different education background.

The milk production was decreased at the start of the study before intervention was done and therefore farmer had no motivation for investment in mastitis control. It has also been suggested that in low yielding buffalo, mastitis is hardly associated with decreased milk yield and mastitis control alone does not result in increased milk production if other issues such as sufficient nutritional demands are not met **(Omore *et al*. 1997)** which further confirms the observation that farmers were not putting much input in mastitis control except when the buffalo came down with clinical disease. So, interventions such as improved overall hygiene, especially milking hygiene, identification of buffalo with high SCC in order to separate them from healthy one (grouping), introduction of milk order (i.e. milking of the high-cells animal after the low-cell ones), practice of good dry period routines and dry period treatment were be done in the present study. By the aids of these interventions and treatment, the milk production was returned to its normal pre-infection levels.

From the results had been presented in **Table (9)**, the recorded overall quarter incidence of subclinical mastitis by the aid of CMT was 2% after 4 months of control plan (8 quarters out of totally 400 and corresponding to 100 animals) and it means that the incidence of SCM in our studying farm was dropped to 3% from total farm animals. Also the table demonstrated that, out of 8 QMS, 1 (12.5%) in right fore, 2(25%) in right hind, 2 (25%) in left fore and 3 (37.5%) in left hind were recorded. Those results were agree with **Blood and Radostitis (1989) and Byarugaba e*t al.* (2008)** who have even stated that it may be impossible to completely eradicate SCM from dairy farms and stated that its occurrence can only be minimized to acceptable levels**.**

Conclusion

The SCM prevalence obtained in this field study may be attributed to a group of shared factors including bad habitat, lack of hygiene, unbalanced diet and bad draft. This group of defective conditions played a role in rendering the udder more susceptible to intra-mammary infection**.** *E. coli*, *Staph aureus* and *streptococcuo* spp. are the main environmental pathogens that isolated from subclinical mastitis in dairy buffalo farm, good management practices such as milk hygiene, sanitization of milker's hands and udder healthy environment as well as dry off treatment and controlling other predisposing diseases should be considered among the major prophylactic measures to minimize the occurrence of the disease. Furthermore, early detection of intra mammary infection (IMI) is important for selecting and implementing proper therapy. Animals should receive immunomodulators or diets supplemented with vitamin E or selenium to reduce incidence of disease. Combination of local and systemic treatment together with immunemodulators were found to be highly efficacious against environmental pathogens causing mastitis.

Recommendation

Subclinical mastitis in the studying farm was controlled by (A) Teat disinfection after milking by wiping the teats with 70 % ethyl alcohol with paying extra attention to teat orifice; (B) Proper hygiene and follow milking procedures and adequate milking equipments; (C) Identification of buffalo with high SCC in order to separate them from healthy one (grouping). (D)Prompt treatment of subclinical mastitis during dry and lactation period and Proper treatment of subclinical as well as clinical mastitis. (E) Pasteurization equipments should be available and efficient to inactivate different pathogenic microorganisms. (F) More interest has been directed towards ways to stimulate the innate immune

mechanisms of the animal in general and /or locally in the udder, for more efficient resistance against and/or elimination of infection to avoid udder infections and following mastitis.

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