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Research Article

STUDIES ON THE SIGNIFICANCE OF MICROBIOLOGICAL ASSESSMENT OF MILK FROM BOVINE MASTITIS IN RELATION TO ITS ANTIMICROBIAL RESIDUE

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ABSTRACT
The study was conducted on 30 nos. of milk samples of bovine depending on the history of ailing from clinical mastitis and scrutiny of same herd for subclinical mastitis. This study was cross sectional case control study. The 30 samples were chosen on the basis of positive reaction for White Side Test (WST), California Mastitis Test (CMT) and pH of milk. On the basis of field experience four (04) types of antibiotics has been taken into consideration such as Amoxycillin, Ceftriaxone, Tetracycline, Enrofloxacin. All the 30 samples were subjected to drug sensitivity test. The most effective antibiotic was Tetracycline (86.66%) followed by Enrofloxacin (66.66%), Amoxycillin (56.67%), Doxycycline (46.67%), Gentamicin (43.33%) and Ampicillin (33.33%). Microorganisms were mostly resistant to drug like Doxycycline, Gentamicin, Ampicillin, Ciprofloxacin, Ceftriaxone in increasing order of resistance. Here, drug sensitivity tests were done abiding the criteria of CLSI-2014. Hence, it is suggested that the line of treatment should be based on antibiogram study of various isolates of bovine milk acquired from bovine mastitis. But, if the use of antibiotics is necessary as in prevention and treatment of animal disease like mastitis, a withholding period must be observed until the residues are negligible or no longer detected. Here, this study also target the residue of four (04) antibiotics like Tetracycline, Amoxycillin, Ceftriaxone and Enrofloxacin through High Performance Liquid Chromatography (HPLC), which yields a significant outcome, i.e., all milk samples show the significant level of residue of Ceftriaxone (100%) followed by Amoxycillin (40%), Enrofloxacin (30%) and Tetracycline (13.33%). Milk pathology tested on the basis of Papanicolaou staining technique reveals lipid vacuolation and fibrosis. This result may be due to indiscriminate and irrational use of such drugs, which may out of count in scientific point of view. Besides, it is observed that the drugs which are resistant to the micro organisms are found as residue in milk, may be due to its un utilization on micro organism biochemically. Selection of antibiotics and its rational use may cure this mastitis like problem and resist evolving of resistant micro-organisms and reduce the existence of toxic level antibiotic residues in the milk and milk products. Though this is a pilot study which warrants long term prospective study to strengthen this view.

Keyword: *Drug Sensitivity, HPLC, Mastitis, Drug Residue, Papanicolaou Staining Technique*

INTRODUCTION
Mastitis is a multi aetiological complex disease, which is defined as inflammation of parenchyma of mammary glands and is characterized by physical, chemical and usually bacteriological changes in milk and pathological changes in glandular tissues (Radostitis *et al.*, 2000). An annual economic loss of over Rs.6000 cores due to mastitis has been recorded, of this, Rs.1700 cores are lost due to clinical mastitis (Financial Daily, 2002). Today, it is second to Foot and Mouth Disease (FMD) as a most challenging disease in high yielding dairy animals in India (Varshney and Mukherjee, 2002) as documentary but present scenario has been changed. As per reports of occurrence of mastitis in dairy animals, it stands at

first position because prevalence of mastitis had been reported more than 90% in high yielder crossbred dry cows (Sharma, 2003). The situation has been complicated by continued indiscriminate and irrational use of antibiotics by defying the scientific approach of selection of suitable antibiotics after culture and antibiotic sensitivity test of milk. This may be due to insensitive approach of dairy farmers, who instead of consulting qualified veterinarians, prefer to take over the counter supply of medicines by drug retailers or quacks. Veterinarian who do not utilize the available diagnostic tests are also responsible for increase in the incidence of mastitis. Besides antibiotic sensitivity test pathological parameters like presence of lipid vacuole and reticulin fibres in milk are also very considerable (Ranjan, 2010). The use of antimicrobial drugs to treat food animals has the potential effect on human health through 02(two) mechanisms: i) increase the risk of antibiotic residues, and ii) influencing the generation or selection of antimicrobial resistant food borne pathogens. The risk of antimicrobial residue in meat and milk is well known and is the focus of intensive regulatory processes. However, there is increasing public health concern about the impact of antimicrobial usage in food animals on the development of antimicrobial resistance. Traditional methods of pasteurization reduce the quantity of bacteria present in milk to negligible levels but will not appreciably reduce the level of antibiotic residue (Moats, 1999).

Therefore, the present study was conducted to study drug sensitivity, detection of antibiotic residues in milk for selection of suitable drugs for treatment in reference to milk pathology and to investigate the relationship between antibiogram picture and residue of antibiotics of those milk sample obtained from animals having history of ailing from clinical mastitis but already elapsed the withholding period of antibiotic therapy and scrutiny of the animals for the same herd for subclinical mastitis.

MATERIALS AND METHODS

The specimen for the present research work consists of milk samples having the history of ailing from clinical cure of bovine mastitis and scrutiny of same herd for subclinical mastitis. In this context, sample is collected strictly abiding the norms of atleast 30 days withdrawal period of drugs used for treatment of the stated disease i.e. mastitis. The place chosen the adjacent village near Kalyani and Haringhata where there is a trend of dairy farming. The study design is cross sectional case control study. So, the samples collected were collected on the basis of positive reaction for White Side Test (WST), California Mastitis Test (CMT) and pH of milk (Chauhan, 1995). Those samples were subjected to isolation of micro-organism and all the isolates were subjected to in vitro drug sensitivity test as per method described by Bauer et al and abiding the criteria of CLSI-2014.

Antimicrobials in the form of antibiotic disc (Hi-media, Mumbai, India) are commercially available from the market are chosen for in vitro antibiogram on the basis of history of treatment and trend of use of antibiotics in the area of study under consideration. Both old and new generations of antimicrobials were taken under purview like Amoxycillin, Gentamicin, Ampicillin, Doxycycline, Ciprofloxacin, Tetracyclin, Enrofloxacin, Ceftriaxone for testing the in vitro efficacy. The milk sample was subjected to culture by following the standard norms to describe the growth of micro organism.

The Antibiotic discs are placed on the surface of a Muler Hinton Agar plate seeded previously with a standard amount of organism to be tested. The plates were incubated at 37^{o}C for 18 to 24 hrs. Subsequently, the plates were tested for development of zone of inhibition around the discs. That diameter of the zone of inhibition was measured in mm and compared with the values listed in CLSI-(2014), on the basis of which the isolates are categorized as sensitive (S), Intermediate (I) and Resistant (R) to the antimicrobial contained in that particular disc.

On the basis of field experience same milk samples were subjected to High Performance Liquid Chromatography (HPLC) following the standard method (Schenck and Callery, 1998) for defection of antimicrobial residue in milk samples. Each 2ml of milk samples were treated with 10 ml Acetonitryl, 3 gm Magnesium Sulphate and 1 gm Sodium Chloride then mixture was stirred, vortexed and centrifuged at cold centrifuge at-5^{o}C in 6000 to 7000 RPM for 20 minutes. Clear alliquote was prepared by filtering those processed samples by 2µ filter. The mobile phase was prepared from Acetonitrile and milipore distilled water after filtration ad sonication. Then those processed samples are subjected to HPLC

following standard protocol utilizing "LC Real Time Analysis" software. Result was analyzed on the basis of comparison between curve obtained from samples with that of standard one.

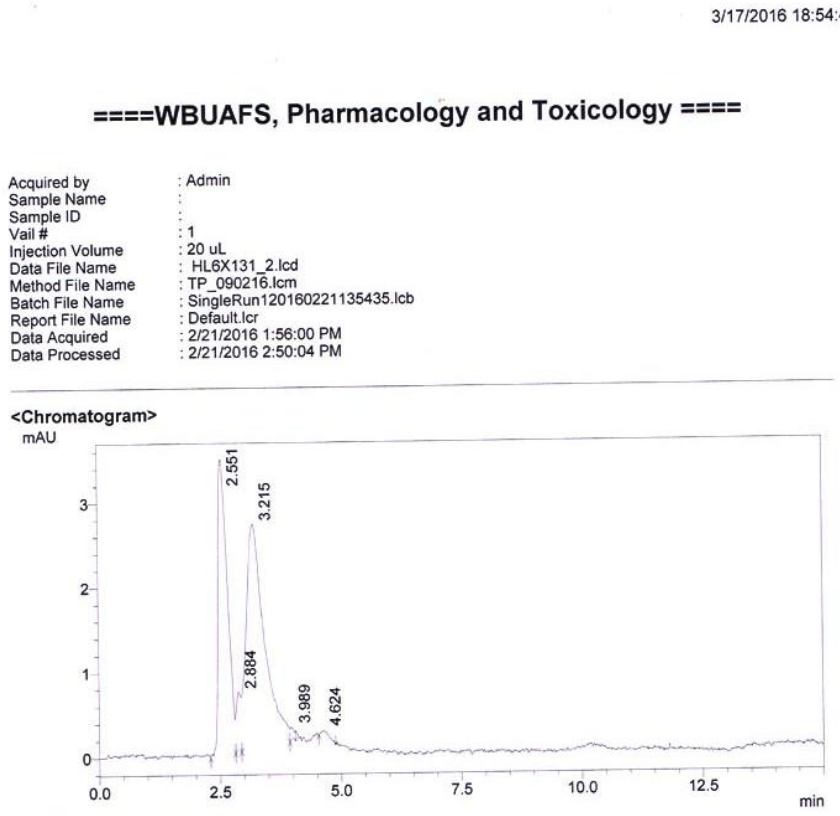
For microscopic examination of milk pathology 10ml of properly mixed milk samples were centrifuged at 1500 rpm for 5 minutes. Supernatant was discarded and smears were prepared in duplicate from the sediment of each sample. Both smears each were stained by Papanicolaou technique (Sengupta, 2011) to determine the presence of degree of lipid vacuolation and reticulin fibres.

10 samples as control were collected on the basis of negative reaction towards CMT, WST and pH detection of milk and all those are subjected to microbiological, pathological and HPLC studies following same standard techniques.

RESULTS AND DISCUSSON

Result

Total 30 nos. of milk samples were collected on the basis of positive reaction towards CMT, WST and pH. Growth of micro organism was observed in all the samples under consideration. Micro-organisms was identified from the isolates by morphological and biochemical study abiding the standard protocol (CLSI, 2014). Isolates was identified as *staphylococcus aureus* (coagulae positive) in 24 samples (80%) and *E. Coli* in 6 samples (20%).



PeakTable

254nm 4nm

	Ret. Time	Area	Height	Area %	Height %
1	2.551283	46718	3487	38.031	49.017
2	2.884283	4834	713	3.935	10.021
3	3.214700	69190	2657	56.325	37.349
4	3.989333	1023	168	0.833	2.369
5	4.624283	1076	89	0.876	1.246
otal		122842	7113	100.000	100.000

Figure 1: Diagram of Result of HPLC of One Milk Sample

All the isolates obtained were subjected to antibiogram assay. The most effective antibiotic was Tetracycline (86.66%) followed by Enrofloxacin (66.66%), Amoxycillin (56.67%), Doxycycline (46.67%), Gentamicin (43.33%) and Ampicillin (33.33%). Micro-organisms were mostly resistant to drugs like Doxicycline, Gentamicin, Ampicillin, Ciprofloxacin, Ceftriaxone in increasing order of resistance (Table-1).

On HPLC study, it yields significant outcome, i.e. all milk samples show the significant level of residue (figure 1) of Ceftriaxone (100%) followed by Amoxycillin (40%), Enrofloxacin (30%) and Tetracycline (13.33%) (Table-2).

By Papanicolaou staining technique all samples (100%) shows reticulin fibre and 85% samples show lipid vacuolation (figure 2).

All 10 samples collected as control were subjected to micro biological HPLC and pathological study and show no isolate, no drug residue and no lipid vacuolation and reticulin fibre. So, the result was statistically significant as statistical calculation is done by t-test ($p < 0.001$).

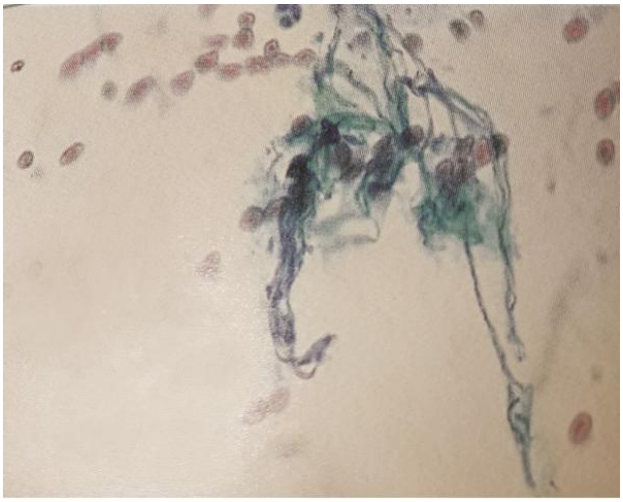


Figure 2: Papanicolaou Staining Shows Reticulin Fibre and Lipid Cell Vacuolation in Milk Sample

Discussion

From the result of Antibiotic sensitivity test, it is suggested that the line of treatment should be based on antibiogram study of various isolates of bovine milk acquired from bovine mastitis. Though, it is difficult to choose the antimicrobial agent exclusively on the basis of *in vitro* sensitivity test, because of several factors like type of organisms, drug response variation among and within herds, site of infection, stage of infection, udder pathology, physico-chemical properties and kinetic behavior of antibiotics in udder and milk, pH of milk (Rajan *et al.*, 2010). Additionally, study of milk pH and cytological study helps in determination of proper medicine. Such as, where there is a presence of lipid vacuolation lipophilic drugs would be the choice e.g. Amoxicillin. Again, presence of reticulin fibre shows the threat of chronic mastitis which needs administration of fibrinolytic agents like hyluronidase, streptokinase as an adjunct therapy (Chakraborti, 2003). On the other hand, drug acting on higher pH would be the choice like that of Gentamicin when milk show high pH value, where as milk with acidic pH needs the administration of drugs have an good efficacy in acidic pH like Ampicillin, Amoxycillin, Cephalosporin etc.

But, if the use of antibiotics is necessary as in prevention and treatment of animal disease like mastitis, a withholding period must be observed until the residues are negligible or no longer detected. Therefore, result of HPLC reveals the indiscriminate and irrational use of such antimicrobial agents, which may out of count in scientific point of view. Besides, it is observed that the drugs which are resistant to the micro-organisms were found as residue in milk may be due to its un utilization on micro-organisms biochemically.

Table 1: Analytic Report on Microbiological Test Done on Milk Sample

Sl. No.	Sample Taken	DATE	CMT	WST	pH	Zone Diameter (Nearest Whole mm)								
						Amx	Ceft	Amp	Ofl	Cip	Gen	Tetra	Dcx	Enr
1	Milk sample of cow	18-05-15	R++	R++	8.40	30/S	20/R	31/S	11/R	13/R	10/R	17/I	21/S	16/I
2	Milk sample of cow	18-05-15	R+	R+	8.00	27/R	18/R	30/S	12/R	14/R	16/S	11/R	18/S	15/I
3	Milk sample of cow	18-05-15	R+	R+	7.60	26/R	20/R	24/R	13/R	14/R	13/I	15/I	17/S	19/S
4	Milk sample of cow	18-05-15	R+	R+	8.50	25/R	18/R	26/R	12/R	13/R	11/R	17/I	14/I	11/R
5	Milk sample of cow	18-05-15	R++ +	R++ +	8.00	32/S	20/R	29/S	16/I	17/I	13/I	15/I	11/R	12/R
6	Milk sample of cow	18-05-15	R+	R+	6.50	26/R	20/R	31/S	12/R	14/R	10/R	18/I	10/R	16/I
7	Milk sample of cow	18-05-15	R++	R++	7.50	28/R	19/R	31/S	13/R	12/R	13/I	20/S	14/I	19/S
8	Milk sample of cow	18-05-15	R+	R+	8.50	27/R	20/R	28/R	10/R	11/R	15/S	12/R	12/R	10/R
9	Milk sample of cow	18-05-15	R+	R+	8.50	25/R	18/R	26/R	12/R	12/R	13/I	17/I	11/R	15/I
10	Milk	18-05-15	R+	R+	8.40	34/S	20/R	27/R	9/R	13/R	10/R	21/S	13/I	16/I

	sample of cow													
11	Milk sample of cow	18-05-15	R++	R++	8.30	26/R	19/I	30/S	17/I	15/I	10/R	16/I	16/S	18/S
12	Milk sample of cow	18-05-15	R++ +	R++ +	7.60	32/S	17/R	20/R	14/R	12/R	12/R	17/I	14/I	16/I
13	Milk sample of cow	18-05-15	R+	R+	8.00	24/R	15/R	21/R	10/R	12/R	9/R	17/I	11/R	15/I
14	Milk sample of cow	20-6-15	R+	R+	7.60	29/S	16/R	27/R	15/I	17/I	11/R	15/I	10/R	19/S
15	Milk sample of cow	20-6-15	R+	R+	8.50	31/S	18/R	25/R	13/R	10/R	12/R	19/S	12/R	17/I
16	Milk sample of cow	20-6-15	R+	R+	7.50	29/S	16/R	18/R	13/R	11/R	15/S	18/I	13/I	13/R
17	Milk sample of cow	20-6-15	R+	R+	7.60	19/R	20/R	15/R	11/R	11/R	13/I	21/S	10/R	11/R
18	Milk sample of cow	20-6-15	R+	R+	8.00	30/S	19/R	11/R	14/R	12/R	14/I	18/I	12/R	10/R
19	Milk sample of cow	16-7-15	R+	R+	7.8	19/R	18/R	29/S	15/I	15/I	16/S	20/S	14/I	22/S
20	Milk sample of cow	16-7-15	R++	R++	8.6	30/S	20/R	25/R	12/R	14/R	15/S	22/S	12/R	17/I

21	Milk sample of cow	16-7-15	R+	R+	7.8	29/S	21/R	29/S	10/R	10/R	10/R	18/I	10/R	12/R
22	Milk sample of cow	16-7-15	R+	R+	7.5	31/S	17/R	19/R	16/I	15/I	11/R	14/R	10/R	14/R
23	Milk sample of cow	16-7-15	R++	R++	9.00	33/S	16/R	23/R	11/R	13/R	10/R	15/I	11/R	10/R
24	Milk sample of cow	16-7-15	R+	R+	8.5	31/S	13/R	21/R	10/R	12/R	9/R	17/I	14/I	16/I
25	Milk sample of cow	16-7-15	R+	R+	8	30/S	20/R	35/S	14/R	12/R	12/R	15/I	16/S	15/I
26	Milk sample of cow	16-7-15	R+	R+	7.6	29/S	14/R	19/R	11/R	13/R	11/R	17/I	11/R	16/I
27	Milk sample of cow	18-05-15	R++	R++	8.50	30/S	18/R	31/S	16/I	17/I	13/I	15/I	11/R	12/R
28	Milk sample of cow	18-05-15	R++	R++	8.00	34/S	16/R	29/S	15/I	16/I	14/I	17/I	12/R	11/R
29	Milk sample of cow	18-05-15	R++	R++	7.90	31/S	20/R	30/S	16/I	19/I	14/I	16/I	10/R	14/R
30	Milk sample of cow	18-05-15	R++	R++	8.00	32/S	17/R	29/S	17/I	20/I	13/I	15/I	11/R	12/R

Table 2: Analytical Report of HPLC of Cow Milk

SL.NO.	Analytical Report of HPLC														
	Vol. of Sample Taken (V1) in ml.	Final Vol. of Sample after Processing (V2) in ml.	Concentration of Standard (C) (in ppm)	Area of Standard Chromatogram of -				For Sample -	Area of Sample Chromatogram of -				Concentration of drugs (in ppm)	MRL (ppm)	Multiple of concentration exceeding MRL
				Ceftriaxone (a1Cf)	Tetracyclin (a1T)	Enroflox acin (a1En)	Amoxycillin (a1Ax)	Retension Time (RT)	Ceftriaxone (a2Cf)	Tetracyclin (a2T)	Enroflox acin (a2En)	Amoxycillin (a2Ax)			
1	2	10	2.00	58720	0	0	0	3.216	57750	0	0	0	9.83480926	0.1	98.34809264
	2	10	2.00	58720	0	0	0	3.177	56352	0	0	0	9.59673025	0.1	95.96730245
2	2	10	2.00	0	0	0	241957	3.797	0	0	0	2770	0.11448315	0.004	28.62078799
	2	10	2.00	0	10407	0	0	4.427	0	2416	0	0	2.32151437	0.1	23.21514365
3	2	10	2.00	58720	0	0	0	3.214	58081	0	0	0	9.89117847	0.1	98.91178474
	2	10	2.00	0	0	0	241957	3.765	0	0	0	4061	0.16783974	0.004	41.95993503
4	2	10	2.00	58720	0	0	0	3.226	61453	0	0	0	10.4654292	0.1	104.6542916
	2	10	2.00	0	0	0	241957	3.808	0	0	0	3676	0.15192782	0.004	37.98195547
5	2	10	2.00	58720	0	0	0	3.178	29622	0	0	0	5.04461853	0.1	50.44618529

	2	10	2.00	0	0	37214	0	5.344	0	0	753	0	0.2023432	0.075	2.697909389
6	2	10	2.00	58720	0	0	0	3.202	48431	0	0	0	8.2477861	0.1	82.47786104
	2	10	2.00	0	0	0	241957	3.819	0	0	0	2648	0.10944093	0.004	27.36023343
7	2	10	2.00	58720	0	0	0	3.185	51030	0	0	0	8.6903951	0.1	86.90395095
	2	10	2.00	0	0	0	241957	3.819	0	0	0	2982	0.12324504	0.004	30.81125985
	2	10	2.00	58720	0	0	0	3.172	74776	0	0	0	12.7343324	0.1	127.3433243
	2	10	2.00	0	0	0	241957	3.776	0	0	0	4997	0.2065243	0.004	51.63107494
8	2	10	2.00	0	10407	0	0	4.448	0	12795	0	0	12.2946094	0.1	122.946094
	2	10	2.00	0	0	37214	0	5.291	0	0	5451	0	1.46477132	0.075	19.530283
	2	10	2.00	58720	0	0	0	3.169	58429	0	0	0	9.95044278	0.1	99.50442779
9	2	10	2.00	0	0	0	241957	3.797	0	0	0	3906	0.16143364	0.004	40.35841079
10	2	10	2.00	58720	0	0	0	3.237	49993	0	0	0	8.51379428	0.1	85.13794278
11	2	10	2.00	0	0	0	241957	3.989	0	0	0	1023	0.04228024	0.004	10.57005997
12	2	10	2.00	58720	0	0	0	3.224	70629	0	0	0	12.0280995	0.1	120.2809946
13	2	10	2.00	58720	0	0	0	3.185	56757	0	0	0	9.66570163	0.1	96.65701635

	2	10	2.00	0	0	0	241957	3.861	0	0	0	4822	0.19929161	0.004	49.82290242
14	2	10	2.00	58720	0	0	0	3.149	29490	0	0	0	5.02213896	0.1	50.22138965
15	2	10	2.00	58720	0	0	0	3.170	102276	0	0	0	17.4175749	0.1	174.1757493
16	2	10	2.00	58720	0	0	0	3.169	108067	0	0	0	18.4037807	0.1	184.0378065
	2	10	2.00	0	0	37214	0	5.311	0	0	20168	0	5.41946579	0.075	72.259543 9
	2	10	2.00	58720	0	0	0	3.166	64086	0	0	0	10.9138283	0.1	109.1382834
17	2	10	2.00	0	0	0	241957	3.829	0	0	0	9812	0.4055266	0.004	101.3816505
	2	10	2.00	0	0	37214	0	5.307	0	0	5084	0	1.36615252	0.075	18.21536698
18	2	10	2.00	58720	0	0	0	3.164	68980	0	0	0	11.7472752	0.1	117.472752
	2	10	2.00	0	0	37214	0	5.329	0	0	1375	0	0.3694846	0.075	4.926461368
	2	10	2.00	45520	0	0	0	3.176	47855	0	0	0	10.5129613	0.1	105.1296134
19	2	10	2.00	0	0	0	241957	3.797	0	0	0	2955	0.12212914	0.004	30.53228466
	2	10	2.00	0	7942	0	0	4.075	0	2631	0	0	3.31276756	0.1	33.12767565
	2	10	2.00	0	0	45446	0	4.924	0	0	5528	0	1.21638868	0.075	16.21851575
20	2	10	2.00	45520	0	0	0	3.128	641495	0	0	0	140.925967	0.1	1409.259666

	2	10	2.00	0	0	0	241957	3.897	0	0	0	10526	0.43503598	0.004	108.7589944
21	2	10	2.00	45520	0	0	0	3.119	3067524	0	0	0	673.884886	0.1	6738.848858
22	2	10	2.00	45520	0	0	0	3.116	2131023	0	0	0	468.150923	0.1	4681.509227
	2	10	2.00	0	0	45446	0	4.913	0	0	858	0	0.18879549	0.004	47.19887339
	2	10	2.00	45520	0	0	0	3.133	123396	0	0	0	27.1080844	0.1	271.0808436
23	2	10	2.00	0	7942	0	0	3.926	0	24017	0	0	30.2404936	0.1	302.4049358
	2	10	2.00	0	0	45446	0	4.940	0	0	3459	0	0.7611230	0.075	10.14830788
24	2	10	2.00	45520	0	0	0	3.128	3624003	0	0	0	796.134227	0.1	7961.342267
	2	10	2.00	0	0	45446	0	5.013	0	0	4330	0	0.9527791	0.075	12.70372163
25	2	10	2.00	45520	0	0	0	3.119	2302662	0	0	0	505.857206	0.1	5058.572056
26	2	10	2.00	45520	0	0	0	3.117	2724586	0	0	0	598.547012	0.1	5985.470123
27	2	10	2.00	45520	0	0	0	3.121	5211140	0	0	0	1144.80228	0.1	11448.02285
28	2	10	2.00	58720	0	0	0	3.216	57550	0	0	0	9.8007493	0.1	98.00749319
29	2	10	2.00	58720	0	0	0	3.216	55750	0	0	0	9.4942098	0.1	94.94209809
30	2	10	2.00	58720	0	0	0	3.216	50750	0	0	0	8.6427111	0.1	86.42711172

Recent concern has focused on the potential for antibiotic residue in milk to contribute to the development and transmission of resistant bacteria (Mitchell *et al.*, 1988), but traditional methods of pasteurization reduce the quantity of bacteria present in the milk to negligible levels but will not appreciably reduce the level of antibiotic residue (Moats, 1999), which will be a threat regarding public health hazards point of view.

Conclusion

Selection of antibiotics and its rational use may cure this mastitis like problem and resist the colonization of resistant micro-organisms in the environment and reduce the existence of toxic level antibiotic residues in the milk and milk products. Though this is a pilot study which warrants long term prospective study to strengthen this view.

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