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ANTIMICROBIAL SENSITIVITY Vs ANTIMICROBIAL RESIDUE IN MILK IN CASE OF BOVINE MASTITIS -A STUDY.

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Abstract

The study was conducted on 26 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 26 samples was 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 Amoxicillin, Ceftriaxone, Tetracycline, Enrofloxacin. All the 26 samples are subjected to drug sensitivity test. The most effective antibiotic was Tetracycline(88.46%) followed by Enrofloxacin (65.38%), Amoxicillin (57.69%), Doxycycline (46.15%),Gentamicin (42.30%) and Ampicillin (34.61%). 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 with holding 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, Amoxicillin, Ceftriaxone and Enrofloxacin through HPLC, which yields a significant outcome , i.e., all milk samples show the significant level of residue of Ceftriaxone(100%) followed by Amoxicillin(46.15%), Enrofloxacin(34.61%) and Tetracycline(15.38%). This result may be due to indiscriminate and irrational use of such drugs, which may out of

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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 unutilization 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.

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Introduction:-

Mastitis 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). Mastitis causes heavy economic losses to dairy industry world wide.The economic losses reported in India due to mastitis was about ` 6053.21crore annually (Dua, 2001). 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). Due to indiscriminate and irrational use of antibiotics by defying the scientific approach of selection of suitable antibiotics after culture and antibiotic sensitivity test of milk, the situation has been complicated. Insensitive approach of cattle owners, who by avoiding qualified veterinarians, prefer to take over the supply of medicines by drug retailers or quacks, leads to this complicated senario. The increased incidence of mastitis is due to those Veterinarians who do not utilize the available diagnostic tests for mastitis. The use of antimicrobial drugs to treat food animals has the potential effect on human health through several mechanisms, i.e., increase the risk of antibiotic residues and 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 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 with holding 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 adjusten 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, Doxicycline, Ciprofloxacin, Tetracyclin, Enrofloxacin, Ceftriaxone for testing the in vitro efficacy. The milk samples 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 an Muler Hinton Agar plate seeded previously with a standard amount of organism to be tested. The plates were incubated at 37°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 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°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.

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.

Result:-

Total 26 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-organsims 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 22 samples (80%) and *E. Coli* in 4 samples (20%).

All the isolates obtained were subjected to antibiogram assay. The most effective antibiotic was Tetracycline(88.46%) followed by Enrofloxacin (65.38%), Amoxicillin (57.69%), Doxycycline (46.15%),Gentamicin (42.30%) and Ampicillin (34.61%). 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 of Ceftriaxone(100%) followed by Amoxicillin(46.15%), Enrofloxacin(34.61%) and Tetracycline(15.38%)(Table-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 ($p < 0.001$).

Discussion:-

From the result of Antibiotic sensitivity test, it in 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 behaviour of antibiotics in udder and milk, pH of milk (Rajan *et al*, 2010).
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, Amoxicillin, Cephalosporin etc.

But, if the use of antibiotics is necessary as in prevention and treatment of animal disease like mastitis, a with holding 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.

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.

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	Cefit	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 sample of cow	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
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
SL.NO.	SAMPLE TAKEN	DATE	CMT	WST	pH	Zone Diameter(nearest whole mm)								
						Amx	Cefit	Amp	Ofl	Cip	Gen	Tetra	Dcx	Enr
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	16-7-	R++	R++	9.0	33/	16/	23/	11/	13/	10/	15/I	11/	10/

	cow	15	+	+	0	S	R	R	R	R	R		R	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

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 (a1 Cf)	**Tetracyclin (a1 T)**	**Enrofloxacin (a1En)**	**Amoxycillin (a1 Ax)**	**Retension Time (RT)**	**Ceftriaxone (a2 Cf)**	**Tetracyclin (a2 T)**	**Enrofloxacin (a2En)**	**Amoxycillin (a2 Ax)**			
1	2	10	2.00	58720	0	0	0	3.216	57750	0	0	0	9.8348092 6	0.1	98.348092 64
2	2	10	2.00	58720	0	0	0	3.177	56352	0	0	0	9.5967302 5	0.1	95.967302 45
	2	10	2.00	0	0	0	241957	3.797	0	0	0	2770	0.1144831 5	0.0004	28.620787 99
	2	10	2.00	0	10407	0	0	4.427	0	2416	0	0	2.3215143 7	0.1	23.215143 65
3	2	10	2.00	58720	0	0	0	3.214	58081	0	0	0	9.8911784 7	0.1	98.911784 74
	2	10	2.00	0	0	0	241957	3.765	0	0	0	4061	0.1678397 4	0.0004	41.959935 03
4	2	10	2.00	58720	0	0	0	3.226	61453	0	0	0	10.465429 2	0.1	104.65429 16
	2	10	2.00	0	0	0	241957	3.808	0	0	0	3676	0.1519278 2	0.0004	37.981955 47
5	2	10	2.00	58720	0	0	0	3.178	29622	0	0	0	5.0446185 3	0.1	50.446185 29

	2	10	2.00	0	0	37214	0	5.344	0	0	753	0	0.2023432	0.075	2.69790938 9
6	2	10	2.00	58720	0	0	0	3.202	48431	0	0	0	8.2477861	0.1	82.477861 04
	2	10	2.00	0	0	0	241957	3.819	0	0	0	2648	0.1094409 3	0.0004	27.360233 43
7	2	10	2.00	58720	0	0	0	3.185	51030	0	0	0	8.6903951	0.1	86.903950 95
	2	10	2.00	0	0	0	241957	3.819	0	0	0	2982	0.1232450 4	0.0004	30.811259 85
8	2	10	2.00	58720	0	0	0	3.172	74776	0	0	0	12.734332 4	0.1	127.34332 43
	2	10	2.00	0	0	0	241957	3.776	0	0	0	4997	0.2065243	0.0004	51.631074 94
	2	10	2.00	0	10407	0	0	4.448	0	12795	0	0	12.294609 4	0.1	122.94609 4
	2	10	2.00	0	0	37214	0	5.291	0	0	5451	0	1.4647713 2	0.075	19.530284 3

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 (a1 Cf)	**Tetracyclin (a1 T)**	**Enrofloxacin (a1En)**	**Amoxycillin (a1 Ax)**	**Retension Time (RT)**	**Ceftriaxone (a2 Cf)**	**Tetracyclin (a2 T)**	**Enrofloxacin (a2En)**	**Amoxycillin (a2 Ax)**			
9	2	10	2.00	58720	0	0	0	3.169	58429	0	0	0	9.9504427 8	0.1	99.504427 79
	2	10	2.00	0	0	0	241957	3.797	0	0	0	3906	0.1614336 4	0.000	40.358410 79

														4	
10	2	10	2.00	58720	0	0	0	3.237	49993	0	0	0	8.5137942 8	0.1	85.137942 78
11	2	10	2.00	0	0	0	241957	3.989	0	0	0	1023	0.0422802 4	0.0004	10.570059 97
12	2	10	2.00	58720	0	0	0	3.224	70629	0	0	0	12.028099 5	0.1	120.28099 46
13	2	10	2.00	58720	0	0	0	3.185	56757	0	0	0	9.6657016 3	0.1	96.657016 35
	2	10	2.00	0	0	0	241957	3.861	0	0	0	4822	0.1992916 1	0.0004	49.822902 42
14	2	10	2.00	58720	0	0	0	3.149	29490	0	0	0	5.0221389 6	0.1	50.221389 65
15	2	10	2.00	58720	0	0	0	3.170	102276	0	0	0	17.417574 9	0.1	174.17574 93
16	2	10	2.00	58720	0	0	0	3.169	108067	0	0	0	18.403780 7	0.1	184.03780 65
	2	10	2.00	0	0	37214	0	5.311	0	0	20168	0	5.4194657 9	0.0075	72.259543 9
17	2	10	2.00	58720	0	0	0	3.166	64086	0	0	0	10.913828 3	0.1	109.13828 34
	2	10	2.00	0	0	0	241957	3.829	0	0	0	9812	0.4055266	0.0004	101.38165 05
	2	10	2.00	0	0	37214	0	5.307	0	0	5084	0	1.3661525 2	0.0075	18.215366 98
18	2	10	2.00	58720	0	0	0	3.164	68980	0	0	0	11.747275 2	0.1	117.47275 2
	2	10	2.00	0	0	37214	0	5.329	0	0	1375	0	0.3694846	0.0075	4.926461 368
19	2	10	2.00	45520	0	0	0	3.176	47855	0	0	0	10.512961 3	0.1	105.12961 34
	2	10	2.00	0	0	0	241957	3.797	0	0	0	2955	0.1221291 4	0.000	30.532284 66

														4	
	2	10	2.00	0	7942	0	0	4.075	0	2631	0	0	3.3127675 6	0.1	33.127675 65
	2	10	2.00	0	0	45446	0	4.924	0	0	5528	0	1.2163886 8	0.0 7 5	16.218515 75

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 (a1 Cf)	**Tetracyclin (a1 T)**	**Enrofloxacin (a1En)**	**Amoxycillin (a1 Ax)**	**Retension Time (RT)**	**Ceftriaxone (a2 Cf)**	**Tetracyclin (a2 T)**	**Enrofloxacin (a2En)**	**Amoxycillin (a2 Ax)**			
20	2	10	2.00	45520	0	0	0	3.128	641495	0	0	0	140.92596 7	0.1	1409.259666
	2	10	2.00	0	0	0	241957	3.897	0	0	0	10526	0.4350359 8	0.0 0 0 4	108.7589944
21	2	10	2.00	45520	0	0	0	3.119	3067524	0	0	0	673.88488 6	0.1	6738.848858
22	2	10	2.00	45520	0	0	0	3.116	2131023	0	0	0	468.15092 3	0.1	4681.509227
	2	10	2.00	0	0	45446	0	4.913	0	0	858	0	0.1887954 9	0.0 0 0 4	47.19887339
23	2	10	2.00	45520	0	0	0	3.133	123396	0	0	0	27.108084 4	0.1	271.0808436
	2	10	2.00	0	7942	0	0	3.926	0	24017	0	0	30.240493 6	0.1	302.4049358
	2	10	2.00	0	0	45446	0	4.940	0	0	3459	0	0.7611230 9	0.0 7 5	10.148307 88
24	2	10	2.00	45520	0	0	0	3.128	3624003	0	0	0	796.13422 7	0.1	7961.342267

	2	10	2.00	0	0	45446	0	5.013	0	0	4330	0	0.9527791 2	0.0075	12.703721 63
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

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. Through this is a pilot study which warrants long term prospective study to strengthen this view.

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