A PhD RESEARCH THESIS ON

"STUDY ON THE IMPACT OF ETHNOVETERINARY HERBAL PREPARATIONS IN BOVINE DISEASES TO MINIMISE USE OF ANTIMICROBIAL DRUGS"

A THESIS TO BE SUBMITTED TO
THE UNIVERSITY OF TRANS-DISCIPLINARY
HEALTH SCIENCES AND TECHNOLOGY

THE UNIVERSITY OF TRANS-DISCIPLINARY
HEALTH SCIENCES & TECHNOLOGY

FOR THE AWARD OF THE DEGREE OF

Doctor of Philosophy

BY

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UNDER THE GUIDANCE OF

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DECLARATION BY THE CANDIDATE

I declare that this thesis entitled **"Study on the Impact of Ethnoveterinary Herbal Preparations in Bovine Diseases to Minimise use of Antimicrobial Drugs"** submitted for the award of Doctor of Philosophy to THE UNIVERSITY OF TRANS-DISCIPLINARY HEALTH SCIENCES AND TECHNOLOGY, Bengaluru, is my original work, conducted under the supervision of my guide Dr. N Punniamurthy and co-guides, Dr. SK Rana and Dr. MNB Nair. I also wish to inform that no part of the research has been submitted for a degree or examination at any university. References, help and material obtained from other sources have been duly acknowledged

I hereby confirm the originality of the work and that there is no plagiarism in any part of the dissertation.

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CERTIFICATE

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Place: Bengaluru

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Dedicated To

Dairy Farmers

of

India

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ABBREVIATIONS

S. No	Abbreviation	Full form
1	AMR	Antimicrobial Resistance
2	ARGs	Antimicrobial Resistance Genes
3	AST	Antibiotic Sensitivity Test
4	BA	Blood agar
5	BD	Becton Dickinson
6	BQ	Black Quarter
7	BT	Bluetongue
8	bTB	Bovine tuberculosis
9	CCHF	Crimean-Congo haemorrhagic fever
10	CLSI	Clinical and Laboratory Standards Institute
11	CM	Clinical Mastitis
12	CMT	California Mastitis Test
13	DAHD&F	Department of Animal Husbandry, Dairying and Fisheries
14	DNA	Deoxyribonucleic acid
15	dNTPS	Deoxynucleotide triphosphates
16	EMB	Eosin Methylene Blue
17	ESBL	Extended-spectrum beta-lactamases
18	EVHP	Ethno-veterinary Herbal Preparations
19	FAO	Food and Agricultural Organisation
20	FMD	Foot and Mouth Disease
21	FRLHT	Foundation for Revitalization of Local Health Tradition
22	HF	Holstein Friesian
23	HS	Haemorrhagic Septicaemia
24	IBR	Infectious Bovine Rhinotracheitis
25	IDA	International Dairy Federation
26	INAPH	Information Network for Animal Productivity and Health
27	JD	Johne's disease
28	KVK	Krishi Vigyan Kendra

S. No	Abbreviation	Full form
29	LSD	Lumpy Skin Disease
30	MAP	*Mycobacterium avium* subspecies *paratuberculosis*
31	MCF	Malignant Catarrhal Fever
32	MCPP	Mastitis Control Popularisation Project
33	MiRNA	MicroRNA
34	MIS	Management Information System
35	MPC	Milk Producer Company
36	MRSA	Methicillin-resistant *Staph aureus*
37	MRSE	Methicillin-resistant *Staph epidermidis*
38	MSA	Mannitol Salt Agar
39	MU	Milk Union
40	NAAS	National Academy of Agricultural Sciences
41	NDDB	National Dairy Development Board
42	OD	Optical Density
43	PBS	Phosphate Buffer Saline
44	PCR	Polymerase Chain Reaction
45	PPR	Peste Des Petits Ruminants
46	RNA	Ribonucleic acid
47	SCC	Somatic Cell Count
48	SCM	Sub-clinical Mastitis
49	Staph.	Staphylococcus
50	Str.	Streptococcus
51	TANUVAS	Tamil Nadu Veterinary and Animal Sciences University
52	TPC	Total Plate Count
53	TSC	Tri-sodium Citrate
54	VRSA	Vancomycin Resistant *Staph* aureus
55	WHO	World Health Organisation
56	WOAH (OIE)	World Organisation for Animal Health

CONTENTS

ABSTRACT

A study on the impact of ethnoveterinary herbal preparations (EVHP) in bovine diseases was carried out for the doctoral degree at TDU, to ascertain the possibility of reducing the use of antimicrobial drugs for the common ailments of bovine and to combat the antimicrobial resistance issues (AMR).

A total of 215 milk samples, collected from suspected cases of bovine mastitis were subjected for bacteriological isolation from which 202 bacterial isolates were recovered. Species-wise distribution of isolates revealed, Staphyloccus spp. as maximum (51 %) followed by Streptoccus spp. (21 %), E. coli (17.8 %), Klebsiella spp. (6 %) and others (14 %). A representative number of 60 bacterial isolates were further subjected to species specific PCR available for 6 different bacterial species i.e. E. coli, Kleb. pneumoniae, Staph. aureus, Str. agalactiae, Str. dysgalactiae and Str. uberis. All of the isolates had a 100% match in species identification between the BD Phoenix system and the PCR data. The BD Phoenix M50 equipment was used to perform an antibiogram on 123 isolates. Conventional disc diffusion test was performed on 17 isolates where BD Phoenix kit was not available.

Strains of Staph. aureus isolated from mastitic milk showed highest resistance to Cefazolin, Norfloxacin (18.4 % each) followed by Oxacillin (17.9 %), Erythromycin, Gentamicin (12.8 % each), Trimethoprim- Sulfamethoxazole (10.3 %), Clindamycin, Quinupristin-dalfopristin, Tetracycline (5.1 % each), Linezolid, Rifampicin, Teicoplanin and Vancomycin (2.6 % each). None of these isolates were resistant to Daptomycin and Nitrofurantoin. The strains of E coli isolated from mastitic milk showed highest resistance to Ampicillin (50 %) followed by Tetracycline, Trimethoprim- Sulfamethoxazole (37.5 % each); Amoxicillin-Clavulanate, Cefepime, Aztreonam, Cefoxitin, Ceftazidime, Ceftazidime, Piperacillin (25 % each) and Cefotaxime, Ciprofloxacin, Gentamicin, Levofloxacin (12.5 % each). No resistance was observed for Amikacin, Chloramphenicol, Imipenem and Piperacillin-Tazobactam. The strains of Kleb. pneumoniae isolated from mastitic milk showed highest resistance to Ampicillin (100 %) followed by Aztreonam, Cefepime, Cefoxitin, Ceftazidime, Trimethoprim- Sulfamethoxazole (11.11 % each). No resistance was observed for Amikacin, Amoxicillin-Clavulanate, Chloramphenicol, Cefotaxime, Ciprofloxacin, Gentamicin, Levofloxacin Imipenem, Piperacillin, Piperacillin-Tazobactam and Tetracycline.

All the 60 representative bacterial isolates belonging to 6 species were genotyped for the presence of various antimicrobial resistant genes (ARGs). In the present study, screening was carried out for bla_Z, bla_tem_all, bla_SHV, bla_CTX_M, mecA, mecC, TeT-K, TeT-M,

TeT-O and TeT-A ARGs, however presence of only bla_Z, bla_SHV, bla_CTX_M, mecA, TeT-K and TeT-M could be detected. Presence of ARGs i.e. mecA (in 4 of 32 isolates of Staph. aureus), bla-Z (in 4 of 32 isolates of Staph. aureus), bla-SHV (in 6 of 8 isolates of Kleb. pneumoniae), bla CTX-M (in 2 of 6 isolates of Escherichia coli), tet-K (in 5 of 32 isolates of Staph. aureus and tet-M (Str. agalactiae, 1 of 6 isolates; Str. uberis, 5 of 5 isolates) were obtained.

EVHP intervention in SCM, clinical mastitis, diarrhoea, pyrexia, LSD, and in repeat breeders showed very encouraging results without the use of synthetic medications. With a cost-efficient and effective alternative animal disease control strategy, EVHP has great potential in developing country like India where the resources available to stakeholders are constrained. Additionally, this reduces the amount of drug residues in milk and milk products, which stall the emergence of AMR. Limited study indicates mastitis EVHP has no adverse effects on rats.

1. INTRODUCTION

India is in the top position in world dairy production for decades, although the animal health care coverage in rural areas needed more attention due to lesser number of veterinarians available in the country. There are veterinarians from Central and State Government, Statutory bodies, Universities, Krishi Vigyan Kendras (KVKs-732 nos.) as well as from private sectors for handling animal health care and extension activities, although reaching the farmers at last mile still challebulknging in India.

In addition to that, recent issues like antimicrobial resistance (AMR), climate change etc. putting more pressure on landless, marginal, small, medium even to the large-scale farmers in making their livelihood sustainable. The requirement of repeated treatment for bovine ailment such as mastitis due to antimicrobial resistance issues, compel the farmer to bear extra medicine as well as veterinarian visit cost. Simultaneously, due to repeated treatment without result, the milk production of the animal also goes down affecting the farmers' economic condition.

Rampant use of antibiotic and other medicines without veterinarian's advice, lack of knowledge on withdrawal time after drug use and its public health significance, make the situation of presence of drug residues in milk and meat more complex in India. As per WHO reports, AMR will be taking the lives of more people than cancer by 2050.

Ethno-veterinary herbal preparations have been used in India mainly by the traditional healers through mouth-to-mouth knowledge transfer. These are cost effective, efficacious, easy to prepare and ingredients of these formulations are commonly available in the farmer's household (Punniamurthy, 2005, Rana *et al*. 2017).

1.1. Objectives:

A. To conduct a survey on awareness of farmers on the use of Ethno-veterinary Herbal Preparations (EVHP) with special reference to common diseases of dairy animals.

B. Studies on causative bacterial agents of bovine mastitis, identify and assessing antibiotic resistance pattern in these agents.

C. To study the efficacy of EVHP in the management of bovine mastitis.

D. Case studies on the recovery of clinical mastitis by using EVHP under field condition.

E. To study the effect of using EVHP in bovine disease on antibiotic residues in milk in the study villages.

Additional studies were carried out:

F. Field studies on the recovery from Pyrexia, Diarrhoea, Repeat Breeder and Lumpy Skin Disease (LSD) by using EVHP under field condition.

G. The effect of using EVHP in bovine mastitis on somatic cell counts in bulk milk samples under village conditions.

H. Dermal safety study of mastitis EVHP preparation in experimental rats.

2. LITERATURE REVIEW

India enjoys top rank in the world in milk production with 198.4 million tonnes in 2019-20 having **per capita availability of** 406 Grams**/day (Source: Basic Animal Husbendry Statistics, DAHD&F, GoI)**. Milk is an integral part of human food as well as, a major cash commodity for millions of farmers in India. Therefore, any situation that causes a drop-in milk production or quality will have a significant impact on household nutrition and livelihood. In addition, milk yield and quality are below average nationally. Mastitis, which tops the list of ailments costing dairy farmers monetarily, is a major cause of both low milk yield and poor milk quality. Its frequency in dairy animals has rapidly increased over the years. The recent trend of transformation from traditional dairying towards commercial dairying where hundreds to thousands of animals are reared together necessitates development of suitable measures to control mastitis. Mastitis has a very high negative impact on smallholder production systems. Thus, controlling mastitis becomes extremely important to reduce production losses and, more importantly, human health-related difficulties. The milk enters the milk procurement system as a result of difficulties in diagnosing subclinical mastitis, and as a result, the bacteria and their toxins in the milk may cause health issues for the consumers (NAAS, 2013).

1.1. Burden of bovine diseases:

India is having the largest livestock population in the globe with 199 million cattle, 140.5 million goat, 105.3 million buffalo and 71.5 million sheep (Hemadri and Hiremath, 2011; Biswal *et al.*, 2012; Dhama *et al*., 2014; Chand *et al*., 2015). Due to several factors such as deforestation, increased exposure of domesticated animals/human with wild animal/birds, heavy population density, lesser public awareness etc., the chances for occurrences of emerging and re-emerging diseases also surged (Depa *et al.,* 2012; Chakraborty *et al.,* 2014).

Livestock being affected with lot of diseases which have stern consequences in their productivity along with distresses on human health, livestock trades etc., resulting in significant economic losses (Gibbs, 1981; Depa *et al*., 2012; Dhama *et al.,* 2014).

1.1.1. Zoonoses

Diseases like bovine tuberculosis, bovine brucellosis, Crimean-Congo haemorrhagic fever (CCHF) virus, Rabies, Anthrax, Salmonellosis etc. are quite significant from zoonotic

point of view (McDaniel *et al*., 2014). Leptospirosis, a re-emerging zoonotic disease, initially reported from Indian cattle by Adinarayanan *et al*., (1960), also being reported in India by many authors (Srivastava *et al.,* 1991; Varma *et al*., 2001; Sivaseelan *et al*., 2003).

1.1.2. Transboundary diseases

Along with these, Foot and Mouth Disease (FMD), Infectious Bovine Rhinotracheitis (IBR), Haemorrhagic Septicaemia (HS), Black Quarter (BQ), Malignant Catarrhal Fever (MCF), Goatpox, Camelpox, Sheeppox, Peste Des Petits Ruminants (PPR), Bluetongue (BT) etc. are endemic diseases of ruminants in India and these have the probability of crossing continental boundaries (Arya & Bhatia, 1992; Benkirane and De Alwis, 2002; Bhanuprakash *et al*., 2011; Biswal *et al*., 2012; Saminathan *et al.,* 2013; Bayry, 2013; Chand *et al*., 2015; Kumar *et al.,* 2015a).

1.1.3. FMD/HS/BQ endemic diseases of India

FMD is endemic in our country since 1864 (Subramaniam *et al.,* 2013), an economically significant disease and resulting in drop in milk yield of the animal. FMD is responsible for an estimated direct loss of 20,000 crore per annum (Venkata Ramanan *et al*., 2006, Mathew & Menon, 2008) and also causes severe damage in international trade, which compelled the WOAH (OIE) and FAO to declare it as “High Priority Disease”, appealing countries for strategic FMD control through effective grouping (FAO and WOAH (OIE), 2012).

Haemorrhagic septicaemia (HS) is endemic in India and is one of the most significant bacterial diseases, showing more severity in young adults especially in buffaloes, having an estimated economic loss of Rs 5255 crore across India (Shivachandra *et al*., 2011, Singh *et al*., 2014).

Another bacterial disease, Black Quarter (BQ) in bovines caused by *Clostridium chauvoei.* It mainly affects the young animals having good body condition *(6* months to 2 years age group). Hemadri and Hiremath (2011) had documented BQ outbreak in West Bengal (42 % mortality, 95 outbreaks) and in Maharashtra (75 % mortality, 37 outbreaks) during 2009-10. Zahid *et al.* (2012) had also reported sporadic outbreak of BQ in HF crossbreds in Ludhiana.

1.1.4. Anthrax

Anthrax is one of the top five zoonotically important diseases, highly fatal and acute which is endemic in South Asia and Bangladesh. In India, endemic in states like TN, Karnataka, AP, Maharashtra, Orissa, WB, Jammu and Kashmir (Sekar *et al*., 2011, Gunaseelan *et al.,* 2011, Thapa *et al.,* 2014)

1.1.5. Chronic bovine diseases: Brucellosis/Tuberculosis/Johne's disease

Bovine brucellosis is another significant disease where the estimated annual economic losses in livestock in India is INR 9,212 crore (Bardhan *et al*., 2020). Shome *et al*. (2019) has recorded incidence of brucellosis as 8.3 % in cattle and 3.6 % in buffaloes across several Indian states, highest in Punjab (cattle 23.51 % and buffalo 10.2%). He has also reported comparative higher prevalence of brucellosis in cattle than buffaloes in many Indian states except in Manipur.

Bovine tuberculosis (bTB) is a bacterial zoonotic disease, chronic in nature, widely prevalent and responsible for 10-25 % losses in productivity with overall prevalence of 14.31 to 34.42% (Prasad *et al*., 2005, Thakur *et al*., 2010, Verma *et al*., 2014). Although, Dutta *et al.* (2016) have reported low incidence rate of tuberculosis (0-1.55 % in five years) in two bio-secure farms in India. bTB has a serious public health significance in developing countries mainly due to close contact of these animals with humans (Grange, 2001). However, many public health and epidemiological aspects of bTB infection still remains unclear (Neeraja *et al*., 2014a; Neeraja *et al*., 2014b; Baqir *et al*., 2014; Verma *et al*., 2014).

Johne's disease (JD) or *Paratuberculosis* caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP) is a chronic infectious disease, requires early detection of the same to minimise production losses as well as spread of the disease (Tripathi, 2008, Singh *et al*., 2014b). Trangadia *et al.* (2011) has recorded apparent prevalence of JD as 13.39% (true prevalence, 15.68%) in Gujarat and 16.26% (true prevalence, 19.31%) in Andhra Pradesh.

Although, Dutta *et al.* (2015) have reported low incidence rate of JD (0-1.86 % in five years) in two bio-secure herds.

1.1.6. Parasitic diseases:

Parasitic diseases like fasciolosis have been reported in cattle (10.79 %), buffaloes (13.9 %), sheep (2.78 %) and goats (2.35 %) by different authors (Sharma *et al*., 1989; Garg *et al*., 2009). A very high infestation of *Fasciola gigantica* in buffaloes (94 %) has been reported by Singh & Agarwal, 1981.

Trypanosomiasis is a haemoprotozoan infection, outbreak of the same were also reported in India by Tewari *et al*. (2013) and Pandey *et al*. (2015) in water buffaloes.

Incidence rate of bovine tropical theileriosis caused by *Theileria annulata* was reported as 14.94 % (blood smear examination), whereas seropositivity of the same was reported in a higher range of *30-60 %* in India except in Himalayan region (Singh *et al*., 1993; Naik *et al*., 2010; Kohli *et al*., 2014; Kumar *et al*., 2015b).

Another haemoprotozoan disease, bovine babesiosis is also prevalent in Indian states like Assam, Meghalaya, Kerala, Arunachal Pradesh, Orissa, Mizoram, Punjab and U.P. (Ravindran *et al.,* 2002; Wadhwa *et al*., 2008; Singh *et al*., 2009; Sharma *et al*., 2013; Saravanan *et al*., 2013).

1.1.7. Emerging Disease - LSD

Lumpy skin Disease (LSD), an economically important emerging viral disease, animals may become debilitated for up to six months (Feyisa, 2018) and was first reported in India by Sudhakar *et al.* (2020) in India and now affecting thousands of dairy animals in the country. LSD affected animals may remain incapacitated for up to six months as reported by FAO (2020).

1.1.8. Metabolic disorders of bovines:

Metabolic diseases in bovine affects the cow at parturition or within a month of parturition may cause high economic losses, may be due to milk yield reduction or with impaired reproductive performances.

Thirunavukkarasu *et al*. (2010a) have reported 13.67 % incidence of milk fever in cows and 11.99 % in buffaloes in TamilNadu.

Several authors have reported prevalence of ketosis in range of 11-12 % (Dohoo and Martin, 1984, Erb and Grohn, 1988; Rasmussen *et al*., 1999; Ostergaard and Grohn, 2000). Thirunavukkarasu *et al*. (2010b) reported 9.38% prevalence of ketosis in cows and 2.92 % in buffaloes in Tamil Nadu. Prevalence of 6.9-14.1 % subclinical ketosis within first two months of lactation has been reported by several authors (Anderson and Emanuelson, 1985; Nielen *et al*., 1994; Duffield *et al*., 1997).

1.1.9. Reproductive diseases:

Honparkhe *et al.* (2021) recorded incidence of Repeat breeding comparatively higher in cattle than in buffaloes (approximately 19 *vs.* 9%, respectively) in India. He has also listed bovine anestrus as another common reproductive issue in India, recording 12.37 to 64.66 % incidences in heifers, which is even higher in adult cattle and buffaloes. It was also observed that, around 21 % cows develop uterine infection within 10 days post calving, while 40 % of these cows unable to eliminate the infections and develop endometritis.

1.1.10. Recording of disease incidence: INAPH software-NDDB

The Animal Health module of INAPH (Information Network for Animal Productivity and Health) has been implemented in Kolhapur milk union since 2016-17. INAPH helps in recording activities on individual bovines pertaining to health, breeding and nutrition. During analysis of the treatment data captured by the veterinarians in INAPH, it was observed that clinical mastitis, agalactia, indigestion, impaction, enteritis, fever, metritis and repeat breeding cases consist around 60% of the total illness in female cattle and buffaloes. 3,67,763 nos. of cattle and 3,45,840 nos. of cases in buffaloes were included in the study (Dutta *et al.*, 2022a).

1.2. Dairying in India: A passion and a livelihood avocation

With an estimated 127.3 million tonnes of milk produced in 2011–12, India leads the globe in this category. By 2030, there would be a need for 200 million tonnes of milk, which would mean an annual rise of nearly 4 million tonnes over the following two decades. The majority of people in India include milk on their dining tables because it is an essential component of human nutrition. For millions of farmers, it is also a significant source of income. Therefore, any factor causing a drop-in milk production or quality will have a significant impact on household nutrition and livelihood. In addition, milk yield and quality are below average nationally. Mastitis, which ranks #1 among the diseases causing significant loss to

dairy farmers, is a major concern for both low output and poor quality of milk. Its frequency in dairy animals has rapidly increased over the years. An effective mastitis management strategy is required, as the recent trend of switching from traditional dairying to commercial dairying, where above hundreds of cows are raised together. Mastitis has a very high negative impact on smallholder production systems. Therefore, controlling mastitis is required for reducing production losses and, more importantly, addressing issues relating to human health. Because subclinical mastitis is difficult to detect, milk enters the milk procurement system. The bacteria and their toxins in the milk may resulting in health issues for consumers. (NAAS, 2013).

Dairy production in India is largely dependent on small and marginal dairy farmers and landless laborers with very limited animal holdings and limited resources. The livelihood of these farmers depends to a great extent on dairy farming, as it offers relatively stable income throughout the year, ensuring regular cash flow for farmers. Dairy is an instrument for rural prosperity and a major source of rural employment, particularly for women. The majority of Indian dairy farmers are associated with producer-owned institutions (Milk Cooperatives and Producer Companies), which provide a fair and transparent system of milk collection round the year at the village level for dairy farmers and process and make available milk and milk products for consumers at affordable prices. To improve the productivity of dairy animals, farmers need proper nutrition for animals enabled through feeding a balanced ration, genetic improvement through artificial insemination and improvement in the animal's health by providing required preventive and curative medications (Annual report DAHD, 2019-20). India holds about 303 million bovines (cows & buffaloes) and about 80 million rural households in India are involved in milk production (Annual report DAHD, 2019-20).

1.3. Bovine mastitis: a burning issue in dairy industry

Bovine mastitis is always a major challenge in achieving the production targets. Because there may be 30 per cent loss in productivity in affected quarter and animal wise, cow may lose about 15 per cent production (Radistitis *et al*., 2000). Mastitis is one of the leading economically important diseases of bovines in India with an annual loss of ₹ 7165.51 crore estimated in 2009. More losses were recorded in sub-clinical mastitis (₹ 4151.16 crore) than clinical mastitis (₹ 3014.35 crore) (Bansal and Gupta, 2009). Around 70 % of the avoidable productivity losses in bovine is reported to be due to mastitis alone (Sumathi *et al*., 2008). Mastitis may be due to physical trauma or infections caused by microorganisms. Mastitis in

dairy animals is considered as one of the most economically significant animal diseases resulting huge economic loss in India.

1.3.1. Incidence of bovine mastitis:

1.3.1.1. Global context:

Kossaibati and Esslemont (1997) documented that bovine mastitis is responsible for around 38 per cent of the total direct costs of the common production diseases globally. Krishnamoorthy *et al*. (2021) have performed a meta-analysis of global prevalence of sub-clinical (from 222 studies) and clinical (from 150 studies) mastitis, which revealed pooled prevalence of SCM and CM as 42% and 15% respectively.

1.3.1.2. Indian context:

According to Dua (2001), within five decades, 115 folds increase in economic losses results due to mastitis in India. Meta-analysis studies conducted by several authors have also revealed above 40% prevalence of SCM in different parts of the country (Bangar *et al*. 2015, Krishnamoorthy *et al*., 2017). Dutta *et al.* (2017) have reported SCM positivity in pooled milk samples as 60 % in cattle and 49 % in buffaloes. Krishnamoorthy *et al*., (2017) have stated prevalence of 27 % clinical mastitis in India. Krishnamoorthy *et al*. (2021), in another meta-analysis study, have reported prevalence of subclinical (from 103 studies) and clinical (from 37 studies) mastitis as 45% and 18% respectively in India.

1.3.2. Aetiology of Mastitis:

Various factors involving the host, pathogen(s) and the environment are associated with the outcome of Mastitis. Herd management and housing of animals are also crucial factor for occurrence of mastitis. Microbial species of at least 137 nos. including bacteria, yeast, fungi and algae, could be responsible for bovine mastitis. (Rinaldia *et al*., 2010)

The causal pathogens can be sourced as environmental pathogens and contagious pathogens. Important environmental pathogens include coliform organisms (*E. coli, Klebsiella* spp., Enterobacter spp. and Citrobacter spp.) and streptococcal bacteria like *Str. uberis, Str. bovis* and *Str. dysgalactiae*. The contagious pathogens involved in mastitis are *Str. agalactiae, Staph. aureus, Corynebacterium bovis* and *Mycoplasma* spp. In India, Staphylococcus spp. Is being reported as one of the major causative agents of mastitis in cattle and buffaloes. (NAAS, 2013)

Dutta *et al.* (2018) has found *Str. agalactiae* as most frequently isolated organism followed by *E. coli, Str. dysgalactiae, Staph. aureus and Klebsiella* spp. from clinical mastitis cases from Gujarat. Rana *et al.* (2019) have recorded presence of *Str. uberis, Staph. aureus, Str. dysgalactiae, Srt. agalactiae, Klebsiella spp, E. coli, Staph. xylosus,* other *non-Staph. aureus, Aerococcus viridians, Staph. epidermidis* associated with clinical and sub-clinical mastitis.

As per NAAS Policy paper, 2013, distribution of mastitis causing organisms may vary between regions and husbandry systems. For implementation of successful management, it is important to pre-establish the epidemiological pattern of mastitis pathogens.

1.3.3. Signs of Mastitis:

1.3.3.1. Acute mastitis:

The main symptom(s) of acute mastitis include abnormalities in udder such as swelling, heat, redness, hardness, or pain. Milk may become watery, having flakes, or clots. In some cases, it may contain bloody tinge. There may be decrease in milk yield. In such cases, it requires immediate treatment of the cow or buffalo by the veterinarian.

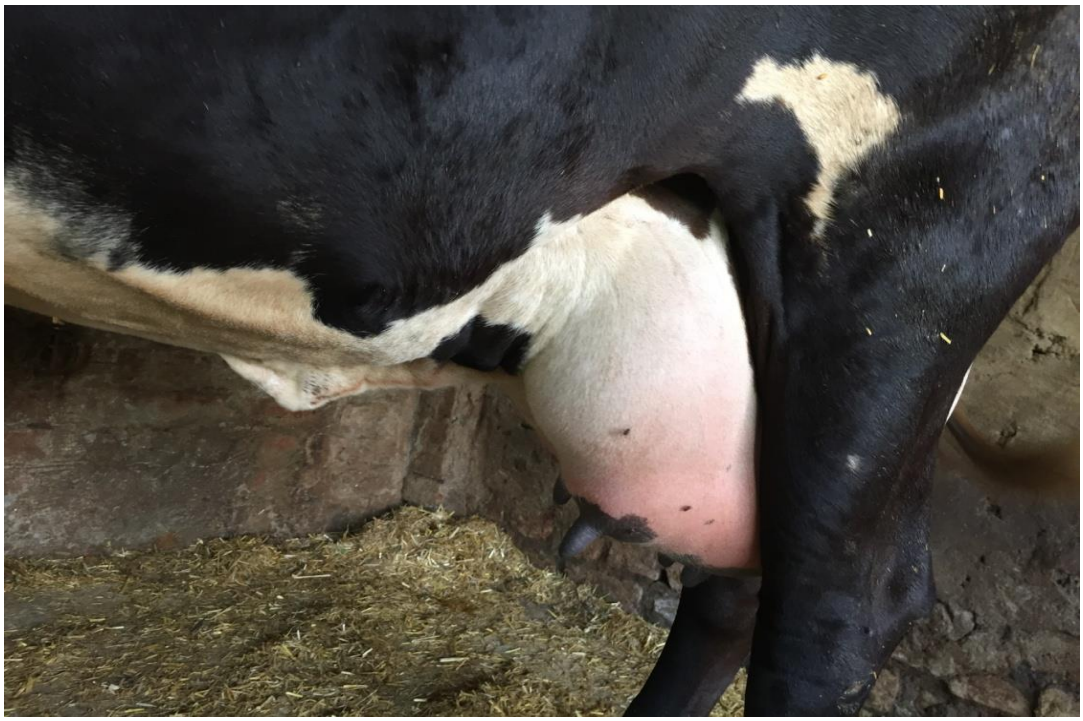


Figure 1: A cow having acute mastitis (photo credit: MCPP)

1.3.3.2. Chronic mastitis:

In Chronic mastitis, the udder becomes hard in consistency and the size of the affected quarter may be reduced. It is very difficult to treat once it becomes chronic in nature.

1.3.3.3. Sub-clinical Mastitis:

In case of sub-clinical mastitis, there will not be any visible signs on the udder. There will not be any indication of infection or abnormality in milk colour or gross consistency of milk. But there may be slight decrease in milk yield and milk may spoil quickly. Sub-clinical mastitis can be treated easily if diagnosed early. As the farmers generally not aware about this type of mastitis, the milk loss continues until the cow being treated or cured. There is every chance of spread of the microorganisms from one infected cow to the nearby healthy cow(s) during milking. Screening for sub-clinical mastitis in dairy animals at regular interval by use of California Mastitis Test (CMT) and subsequently managed with trisodium citrate oral regimen can reduce the incidence of sub-clinical mastitis in dairy animals (Dutta *et al.* 2017).

1.3.4. Impact of mastitis on Quality:

1.3.4.1. Somatic Cell Count: an indicator of sub-clinical mastitis

Nair *et al.* (2017) recorded external application of mixture of *Aloe vera* leaves, *Curcuma longa* rhizome and calcium hydroxide on udder also reduces SCC in mastitis cows. Mukherjee *et al*. (2005) observed potential of aqueous extract of *O. sanctum* in reducing SCC in sub-clinically infected cows. Mukherjee *et al*. (2010) recorded use of hydro-methanolic extract of *Tinospora cordifolia* stem can reduce SCC in sub-clinical mastitis infected cows within 15 days.

1.3.4.2. Total Bacterial Count (TBC) in milk:

Mukherjee *et al*. (2005) studied Immunotherapeutic potential of aqueous extract of O. sanctum treatment and found that it can reduce the TBC in sub-clinically infected bovine mastitis cases. Mukherjee *et al*. (2010) also investigated therapeutic and immunity enhancement potential of the diseased mammary gland by use of hydro-methanolic extract of *Tinospora cordifolia* stem in bovine subclinical mastitis and reduction in TBC was observed from day 3 onwards.

1.3.4.3. pH of mastitic milk:

Swami *et al.* (2017) recorded that the average pH value of subclinical mastitis cow milk (6.88 ± 0.015) increases significantly in comparison to normal cow milk (6.57 ± 0.021) whereas in subclinical mastitis buffalo milk (6.68±0.016) increases significantly in comparison to normal buffalo milk (6.91 ± 0.021). Modh *et al*. (2018) also documented that milk pH value in subclinical mastitis in cows (7.1 ± 0.01) being elevated in comparison to the healthy cows (6.65 ± 0.01).

1.3.4.4. Change in composition of milk components:

Swami *et al.* (2017) recorded that fat, protein, lactose, SNF and total solid percentage was decreased significantly in milk obtained from sub-clinical mastitis. Although, Abase and Kurtu (2018) reported reduction of fat and lactose, but increase of protein content in milk of sub-clinical mastitis.

1.3.4.5. Impact of mastitis milk on milk products:

Higher SCC, presence of antibiotic residues in milk having impacts on product preparation. Cheese yield efficiency become lower, increases moisture content in cheese with higher SCC. Fat and protein losses in whey also increases when milk SCC is high (Barbano *et al.,* 1991). Presence of antibiotic residues in milk may interfere production of cheese and yogurt by inhibiting the starter cultures (Sachi *et al.,* 2019).

1.3.5. Mastitis diagnosis:

1.3.5.1. California mastitis Test:

California Mastitis Test (CMT) is a simple test for detection of sub-clinical mastitis which is based on presence of Somatic cells in milk forming a gel (Middleton *et al.,* 2004; Whyte *et al.,* 2005).

1.3.5.2. Somatic cell count:

Somatic cells are body-derived cells and they normally present at low levels in milk. They are part of the defense in the immunity of the individual animal. Dairy cattle and buffaloes those having SCC more than 200,000 SCC/ml in milk, more often got infected in at least one quarter (Vishnoi *et al*., 2007, Dang *et al.* 2008). Lower SCC contributes to better milk products with a longer shelf life (Alhussien and Dang, 2018).

1.3.5.3. *In vitro* culture based diagnosis:

For mastitis detection, tt is considered as a test of gold standard (Pyorala, 2003). Although it is time consuming and isolation and Identification of few agents may be cumbersome (Koskinen *et al*., 2010).

1.3.5.4. PCR/Multiplex PCR/realtime PCR based diagnosis:

PCR based diagnosis directly from mastitic milk is less time consuming (Amin *et al*., 2011). In multiplex PCR, it is easier to detect multiple organisms as mastitis may be caused by involvement of multiple causative agents (Phuektes *et al*., 2001). Real time-based PCR can be alternative to In vitro diagnosis, but due to high cost involvement, it is not much affordable in countries like India (Koskinen *et al*., 2009, Rajeev *et al*., 2009).

1.3.5.5. Other novel diagnostic tools for mastitis detection:

Circulating miRNA (Gu *et al*., 2007), Immune assay (Fox and Adams, 2000, Hiss *et al*., 2004; Eckersall, 2007), Proteomics based detection (Lippolis and Reinhardt, 2005; Smolenski *et al*., 2007; Boehmer, 2011), Biochips (Lee *et al*., 2008) and Biosensors for detecting bovine mastitis (Pemberton *et al*., 2001 and Akerstedt *et al*., 2006) have been established for diagnosis of bovine mastitis.

1.3.6. Mastitis prevention and control:

Clean and hygienic milk production and good animal husbandry practices has been reported to reduce the chances of mastitis (Kumar *et al*., 2010). Post milk teat dip or spray has been reported to be an effective management practice for prevention of contagious mastitis (Olde *et al*., 2012). Shed management, fly control, regular screening of milk, optimal nutrition, proper bedding material, cleaning of milking equipment etc. are quite helpful in prevention of mastitis (Shkreta *et al*., 2004; Calzolari *et al*., 1997; De Vliegher *et al*., 2012; Fontaine *et al*., 2002; Chang *et al*., 2008; Nielsen, 2009; Yin *et al*., 2009).

The contemporary management of mastitis in Indian field conditions includes testing of milk samples by CMT and antibiotic treatment usually infused in the udder through teats (Dutta *et al.*, 2018). Due to fear of losing productivity and money, farmers always want to initiate mastitis therapy as early as possible (Rathod *et al*., 2017) and generally, it includes use of broad-spectrum antibiotics (Kalla *et al*., 2015, Lunden, 2015, Chauhan *et al*, 2018). Intra-mammary is the most preferable route generally practiced (Padol, 2015) followed by parenteral injections.

Due to high cost involvement and poor outcome, treating sub-clinical mastitis with antibiotics in milch animals generally not practiced. Due to antimicrobial resistance issues, treatment of mastitis cases with herbal formulations gaining more attention day by day (Sahay *et al*., 2006; Hawari and Al-Dabbas, 2008; Pyorala, 2009; Awandkar *et al*., 2009; Kumar *et al*., 2010; Vashney *et al*., 2012). Dutta *et al.* (2017) have reported higher efficacy in use of tri-sodium citrate in control of sub-clinical mastitis.

Various vaccines against bovine mastitis have been attempted or in field trial having mixed success (Fontaine *et al*., 2002, Shkreta *et al*., 2004, Chang *et al*., 2008, Yin *et al*., 2009, Camussone *et al*., 2013).

New techniques such as quorum sensing (Novick and Geisinger, 2008; Moore, 2011), Disease resistant breeding (Zhang *et al*., 2009, Chen *et al*., 2011, Deb *et al*., 2012, Deb *et al*., 2013), Bacteriophage therapy (Dias *et al*. 2013; Porter *et al*. 2016; Amiri Fahliyani *et al.* 2018; Varela-Ortiz et al. 2018), Nanoparticle-based therapy (Castelani *et al.* 2019; Kalinska *et al*. 2019; Orellano *et al*. 2019; Pinheiro Machado *et al.* 2019) Stem cell therapy (Capuco *et al.* 2012, Peralta *et al*. 2020), Acoustic pulse therapy (Leitner *et al.* 2018) have been explored for management of bovine mastitis.

1.4. Presence of antimicrobial residues in milk:

Globally, the use of antimicrobial in animals is doubled than use in human (Aarestrup *et al*, 2012) out of these, 30%–70% being released unaltered into the environment (Kummerer, 2001). The main reason of getting antibiotic residues in milk is injudicious usage of antibiotics in infectious diseases treatment in animals (Zhang *et al.*, 2009). In analysing 224 articles across globe, Sachi *et al*., (2019) found that residues of β-lactam group have been detected mostly followed by the other antibioitics viz., tetracyclines, fluoroquinolones, sulfonamides and aminoglycosides which is a serious public health concern. Antibiotic residues in milk i.e. for antibiotics like penicillin, tetracycline, oxytetracycline, beta lactam group, enrofloxacin, neomycin, streptomycin, sulphonamides, chloramphenicol, gentamicin has reported by several authors in India (Kalla *et al*., 2015, Kumarswamy *et al.*, 2018, Nirala *et al.*, 2017, Lunden, 2015, Lejaniya *et al.*, 2017, Gaurav *et al.*, 2014).

Antibiotic residues in milk is suspected to have potential effects on public health. It may cause antibiotic resistance allergic reactions, carcinogenicity, mutagenicity, teratogenicity and disturbances in the normal intestinal environment (Beyene, 2016)

1.5. Issues connected with antimicrobial use in livestock and their implication in antimicrobial resistance (AMR)

Antimicrobial resistance (AMR) in bacteria causing infection in humans and animals has been increased in last few decades. This is as a result of increase in usage of antimicrobials for different etiologies. Therapeutic as well as non-therapeutic uses of antibiotics in animal production is another reason for increasing trend of AMR. AMR is natural phenomenon in bacteria. The use of antimicrobial drugs in agriculture, health care, or industrial purposes, has been created a selection pressure, which supports survival of resistant strains (or genes) over susceptible ones. Thus, within microbial communities, there is a relative increase in resistant

bacteria. For example, those countries have banned fluoroquinolones in animal production, the AMR in fluoroquinolones is also lesser in livestock in those countries. Prolonged use of antimicrobial growth promoters in a subtherapeutic level can encourage resistance emergence. Although this practice is common in lot of countries at present. Animal to animal transmission of resistant bacteria can happen easily in intensive livestock production systems those having inadequate biosecurity protocol. (FAO, 2016)

As per reports of various authors, overuse/improper use of antimicrobials across globe is the prime drivers of development and spread of AMR (Aminov and Mackie, 2007, APUA, 2008, Aarestrup *et al.*, 2008).

For rapid growth in global livestock production, antimicrobial use for animal production has been increased. Van Boeckel *et al.*, 2015, stated that two thirds of the future growth of antimicrobial use will be for animal production. In high income countries, the use of antimicrobials for growth promotion, prophylaxis and metaphylaxis has been substantially reduced recently.

Although, the growth is still significant in low- and middle-income countries (Van Boeckel *et al.*, 2015). Recently, food-borne pathogens resistant to colistin were detected in animals, humans, vegetables and in retail meat in different countries. (Skov and Monnet, 2016, Doumith *et al.*, 2016, Kluytmans–van den Bergh *et al.*, 2016, Zurfuh *et al.*, 2016).

Due to increased contact between human, food producing animals and wildlife, AMR traits transfers to those bacterias, which are normally not exposed to selection pressure results (Cristobal-Azkarate *et al.*, 2014, Osterblad *et al.*, 2001). Smillie *et al.* (2011) reported high transfer of resistant genes through horizontal gene transfer (HGT) for human skin and oral system.

Multi-drug resistance TB is expected to drastically increase by 2040 in countries like India, Russia, the Philippines, and South Africa, which have always had high number of TB cases compared to other regions of the world. (Sharma *et al.* 2018).

1.5.1. Use of antibiotics in Livestock sector:

Use of antimicrobials in animal husbandry is a factor for emergence of antimicrobial resistance (AMR) in India (Sharma *et al*., 2018). However, India has no effective monitoring system on the use of antimicrobial drugs (Sharma *et al*., 2018) and AMR occurrence (Bhatia and Walia, 2017).

In animal feed, antimicrobials are generally added in sub-therapeutic doses as growth promoter, which may contribute in development of AMR (Chattopadhyay, 2014, Teillant, 2015). The antimicrobials used for food animals globally, as per 2010 and 2013 estimates were 63,151 and 1,31,109 tons respectively (Van *et al*., 2015, Van *et al*., 2017) which indicates sharp rise of the antimicrobial use over time. India is responsible for around 3 % growth on increased use of antimicrobials in food animals globally (Van *et al*., 2015).

Use of antibiotics is very common for treatment of diseases in livestock. When there is failure in monitoring drug withdrawing periods in food animals, there is possibility for contamination (Padol, 2015) and may have food safety concern if exceeds maximum residue levels (FAO, 2018, FAO/WHO, 2018). AMR bacteria may be transmitted via direct contact, contaminated environment or through contaminated foods (Sharma *et al*., 2018, Argudín, *et al*., 2017, Muloi, *et al*., 2018).

In India, over-the-counter availability of antibiotics without prescription is quite common (Chauhan *et al.,* 2018, Sahoo, 2008). Many farmers in India arbitrarily decide themselves which antibiotic to use (Lunden, 2015), based on availability, quicker results and past experiences (Chauhan *et al.,* 2018, Kumar and Gupta, 2018). Salesman and drug distributors even do direct marketing of these drugs to the farmers (Chauhan *et al.,* 2016,

Chauhan *et al.*, 2018), resulting in unnecessary use of antimicrobials. Even farmers many times even not aware proper milk disposal procedure from diseased as well as treated animals (Parkunan *et al.*, 2019). Antimicrobial-residue contaminated milk may trigger allergic reactions (Panigrahi *et al.*, 2017)

Inappropriate and inadequate antimicrobial use provide chances to bacteria to mutate, thereby resisting the therapy (Richardson, 2017). The resistant genes of the mutated bacteria can be acquired by other bacteria and become resistant (Jayalakshmi *et al.*, 2017). As the antimicrobials used for both livestock and human are more or less similar (Ghafur *et al*., 2013, Phillips *et al.*, 2004), these resistant microbes may result in public health implications along with problems in animal health.

In search of cheaper options or lack of awareness, farmers have tendency to consult quacks first (Rathore *et al*., 2010), unless the case become serious which may necessitate use of multiple antibiotics (Chauhan *et al.*, 2016, Garg and Mohanta, 2012, Jingar *et al*., 2017). There is also tendency among farmers to sell their animals if it does not respond to treatment (Parkunan *et al.*, 2019). It is also difficult to get treatment history from the farmers so that veterinarian can decide which antibiotic will be appropriate to mitigate issue of AMR (Kumar *et al.*, 2018). To overcome this issue, 'animal status' has been developed by NDDB as 'MIS report' for 'INAPH animal health' module where detailed animal history can be obtained by the veterinarian, transaction-wise, starting from date of animal registration in the software (Harikumar *et al.*, 2015).

Isolation of *E. coli* (ESBL producing) in bovine mastitis has been reported by different groups (Kar *et al.*, 2015; Batabyal *et al*. 2018)

ESBL producing *Kleb. pneumoniae* in milk from animals have also been documented by several workers (Koovapra *et al.*, 2016, Bandyopadhyay *et al.*, 2018). Dutta *et al.* (2018) have recorded that ampicillin/cloxacillin combination was least sensitive to isolates recovered from clinical mastitis affected milk samples.

Resistance to ampicillin, cloxacillin, penicillin, nitrofurantoin, cefotaxime, ampicillin, chloramphenicol, tetracycline, oxytetracycline, streptomycin was also reported (Sharma *et al.* 2015; Verma *et al*., 2018). Sivakumar *et al.* (2020) documented presence of ESBL *E. coli* in raw foods, environmental samples and ready to eat foods from Utter Pradesh and Delhi. Out of

total 42 ESBL isolates recovered, 85.71% were found to be multidrug-resistant, which is alarming.

Methicillin-resistant *Staph. aureus* (MRSA) has been isolated from mastitis affected milk samples and have the potential to complicate treatment of bovine mastitis (Vanderhaeghen *et al.,* 2010). Bandyopadhyaya *et al. (*2015) had documented simultaneous occurrence of Methicillin-resistant *Staph. epidermidis (*MRSE), MRSA and Extended-spectrum beta-lactamases (ESBL) producing *E. coli* in bovine mastitis that might be a major problem for dairy industry as for public health. Bhattacharyya *et al. (*2016) have reported vancomycin resistant S. aureus (VRSA) in cattle and goat milk samples.

AMR not only complicates the animal treatment with antimicrobials, but also increases the treatment cost and ultimately affecting the animal productivity. It is one of the reasons for low remedial rate in bovine Mastitis (Barkema *et al.,* 2006). AMR makes bovine mastitis challenging task for the field veterinarians. To overcome such issues, during 2016-17, mastitis control popularisation project (MCPP) was initiated by National Dairy Development Board (NDDB) in 23 milk unions in 9 states to manage SCM and CM in Indian field condition. As per IDA Animal Health Report, 2019, MCPP was found as a sustainable model for developing countries like India (Rana *et. al*, 2019)). Common ailments in bovine i.e. mastitis, seasonal diarrhea, non-specific pyrexia, where heavy quantity of antimicrobials is generally being used by field practitioners, can be managed easily with use of ethno-veterinary medicines. These efforts can minimize the use of these antimicrobials, ultimately helps to reduce antimicrobial resistance issues (Dutta *et al*., 2020).

1.6. Ethnoveterinary herbal medicine: the concept and application

According to the World Health Organisation (WHO), the term 'traditional medicine' term is used to differentiate any ancient or traditionally used health care practices from the modern medicinal sciences or Allopathy.

Traditional medicine is comprising of indigenous medicinal systems which includes

a. Popular Asian systems such as Siddha, Ayurvedha, Chinese, Unani and Tibb medical systems.

b. The less widespread, largely orally transmitted practices (ethno-medicine) are used by traditional communities across India (Punniamurthy and Udayasurian, 2010).

WHO has been encouraging study and application of traditional medicine to combine traditional and western resources which can create solutions to problems of sanitation, nutrition and health care (Punniamurthy and Udayasurian, 2010). In India, traditional medical practitioners covers majority of the population for their primary health care (Jain, 2000).

"Ethno-veterinary medicine" is the knowledge of livestock as well as herbal healing practices those evolved over generations. It can be defined as 'the holistic interdisciplinary study of locally available knowledge and its associated skills, practices, beliefs of the practitioners, social structures related to healthcare and healthful husbandry of food, work, and other income producing animals, always with an eye to practical development applications within livestock production and livelihood systems with the ultimate goal of increasing human well-being via increased benefits from stock raising" (McCorkle, 1998). In general, EVP is not restricted to treatment of animal diseases alone, but also involves various disciplines related to people's knowledge and practices in animal healthcare, productivity and performance (Lans *et al.,* 2007).

In ancient India, *Rishis* had observed the wild animals closely to learn the self-medication traits used by them and also recorded the behavior of sick animals (*Zoopharmacognosy*). *Atharvaveda* (VIII, 7. 23) mentions *'a wild boar knows the herb which will cure it as does the mongoose'*. With the use of earth, minerals, animal products and magico-religious practices, the ancient healers cured diseases. The importance of herbs in ancient Indian animal care was featured by the edict of Samrat *Ashoka* (NAVS, India). In ancient Vedic texts originated during circa 3147 BC, use of medicinal plants were described (Silver, 2007). Texts like Skanda Purana, Devi Purana etc. having discussion on animal husbandry. During 2350 BC, Acharya Shalihotra was specialised for treatment of elephants and horses. He had compiled an Indian Materia Medica, having detailed description on use of herbs (Rath *et al.* 2020). EVHP has been practiced for different livestock and zoo animals in different developing as well as developed countries. In India, it is spread out across the country (Rath *et al.* 2020).

An important social, religious, and economic legacy of rural India is the folk wisdom surrounding animal husbandry. These include convictions, expertise, methods, and abilities linked to the medical treatment of livestock. The Pashu Vaidyas, or indigenous healers, are skilled and informed in the field of conventional veterinary medicine. However, because these folk health practices and information are passed down orally across generations, they are rarely

documented. The majority of the medicinal plants used by the local healers to treat the animals are indigenous to the area and are distinctive to the eco-system and ethnic population. As a result, different people, communities, and geographical areas have very different traits, levels of sophistication, and strengths of these systems. (Nair and Punniamurthy, 2010).

1.6.1. Zoopharmacognosy-self-medication in the wild animals

Zoopharmacognosy is the study of behavior shown by the animals for self-medication. It may be for preventive or curative purpose. To know these self-medication behaviors, it is necessary to understand the animal's responses to probable threats to their health and reproductive soundness (Huffman and Vitazkova, 2007). Animal learn these self-medication approaches through struggle for survival, learning from other animals (ENGEL, 2005; Huffman, 2001). They utilize plant's secondary components or non-nutritional ingredients for self-medication (Huffman, 1997). For example, Chimpanzees consume plants containing sesquiterpene lactones particularly for therapeutic purpose (Robles *et al*. 1995). Chimpanzees, bonobos and gorillas use to swallow whole plant leaves which helps in removal of intestinal parasites by defecating intact leaves. Thus, animals can provide indication on sources of herbal medicines. Humans had observed the wild and domesticated animal's behaviour on self-medication and used them as source for herbal medicines. (Huffman and Vitazkova, 2007)

1.6.2. Legacy of Indian Knowledge system

Singh Sahana (2017) has described regarding ancient India's highly enriched knowledge centers in her book entitled "The educational heritage of ancient India". She mentioned regarding "Forest Universities" which were laid out an entire spread of subjects i.e. a holistic approach of learning (Mookerjee, 1960, Chattopadhyaya, 1996). There were "Temple Universities" in India as they became centres of knowledge dissemination and debating. Many Chinese scholars came to India during the first century CE. Many Sanskrit manuscripts were carried to China and translated to Chinese (Mookerjee, 1960). The Indian medical texts as well as information on variety of herbs were translated and knowledge transfer was spread from India to Greece, Islamic world and Europe (Royle, 1837, Philips, 2010)

1.6.3. Ethnoveterinary herbal medicine (EVHP): current status in India

Significance of ethnoveterinary herbal medicine in animal husbandry is now a days quite realising, especially in the developing countries. However, EVHP and other ethno-

knowledge systems were looked upon with disbelief and distrust by the modern practitioners for so long. This is mostly because traditional wisdom has been improperly disseminated, hidden, distorted, and lack of validatation by science. It is also contributed by non-availability of proper raw material, declining herbal resources. The usage of EVHP is declining in acute disease settings due to sluggish treatment response and adoption of an intensive livestock production system. The younger generation is less interested in raising livestock the old-fashioned way, which contributes to the EVHP techniques' waning popularity. Adding to it, the academic curriculum also overlooked the significance of the traditional healing practices, until now.

At present, different organisations/Institutions have been working on documentation, popularisation and validation of EVHP in India. ANTHRA-an Indian NGO, TDU, TANUVAS, NDDB, different agricultural/veterinary college/universities documenting ethno-veterinary works (Punniamurthy, 2002, 2005, 2008 and 2009). NDDB, India has been working on popularization of ethnoveterinary medicines to reduce the use of antibiotics in bovine with collaboration with TANUVAS, TDU, GLOHMSIWA Research Lab and different milk unions (MU) and milk producer companies (MPC) (Rana *et al*. 2017).

Rana *et al.* (2021) reported around 5.61 lakh cases of dairy cattle treatment records managed by use of EVHP with high recovery rate. These cases were compiled for 26 bovine ailments from different milk unions/producer companies. Reduction in treatment cost specially antibiotics in milk union due to extensive use of EVHP have also been mentioned.

Recently, some ethnoveterinary medications were included to the e-GOPALA APP, which was created in collaboration with the Government of India (GoI) and NDDB, to enable dairy farmers to easily and widely access them.

1.6.4. Role of EVHP in selected bovine diseases:

Ethnoveterinary herbal medicines have been used for management of common ailments for livestock. Ailments like bovine mastitis (Bhatt *et al*. 2014; Nair *et al*., 2017, Aruna *et al*. 2019; Dutta *et al*., 2020), ecto/endo parasitic infestations (Ademola *et al*. 2006; Jeyathilakan *et al*. 2012; Nyahangare *et al*. 2015; Sanhokwe *et al*. 2016, Nimbalkar *et al.* 2020a), respiratory problems (Ayrle *et al.* 2016), reproductive issues (Bettaieb *et al.*, 2011, Gopalakrishnan *et al.*, 2016, Seema, 2015, Elamaran *et al.* 2018, Dey *et al*. 2020, Satheshkumar *et al.* 2021, Dutta *et al.*, 2022b), diarrhoea (Dutta *et al.*, 2020), fever (Dutta *et al.*, 2020), fungal infections (Dikhoba

et al. 2019), foot and mouth disease (Kpodekon *et al*. 2015), african swine fever (Fasina *et al.* 2013), lumpy skin disease (Dutta *et al.,* 2022c) etc were recorded.

Nair *et al*. (2017) have evaluated the components used in mastitis EVHP i.e. Aloe vera, *Curcuma longa* and calcium hydroxide. In collaboration with TDU and TANUVAS, NDDB had initiated treatment of clinical mastitis cases in Sabar Dairy during 2016. Dairy veterinarians were trained at TDU and through a Training of Trainers (ToT) model, the concept was propagated to all the milk shed areas in Sabar Dairy and by 2020, around 67,833 mastitis cases were managed by the dairy veterinarians with EVHP alone with a high recovery rate. It was also established that the treatment cost with EVHP is much lower than the use of modern medicines. Mastitis cases having bacterial isolates with AMR and biofilm producing genes, were also recorded as recovered when managed with EVHP use. EVHP demonstration plots were also established for farmer awareness (Rath *et al*., 2020).

Using EVHP comprising of Aloe vera, *Curcuma longa* and calcium hydroxide formulated and standardised by Prof. N. Punniamurthy has been found effective in managing bovine mastitis and has also been found that this EVHP had inhibitory activity against organisms like *E. coli and S aureus* (Punniamurthy *et al.*, 2017a). In order to justify and decrease the usage of antibiotics for treating mastitis, NDDB, TDU, and TANUVAS presented the idea of EVHP to various milk unions in the beginning of 2017, under the technical supervision of Professor N. Punniamurthy. EVHP were found to be very effective remedies for treatment of mastitis and various other ailments. These were found to be incredibly affordable, environmentally friendly, simple to make and apply, and the majority of the remadies were created with items that farmers typically have on hand. Suitability for EVHP is also being assessed for other diseases/ailments like FMD, repeat breeding, non-specific fever, diarrhoea, teat obstruction, udder edema, wart, pseudocowpox, indigestion, deworming bloat, wounds, prolapse, retention of placenta, control of ecto-parasite etc.

Diarrhoea is a common ailment of varied causes in bovines, both infectious and non-infectious, which ultimately results in severe productivity losses. Pyrexia in dairy animals, primarily a symptom of the body trying to cope with causative agents, has been attributed to various reasons. During fever, animals generally becomes off-feed causing subsequent reduction in productivity. Both the diarrhoea and non-specific pyrexia cause economic losses to the dairy farmer. Verma (2014) documented the use of ethno-veterinary medicinal plants by

rural farmers and traditional herbal healers of villages at Bundelkhand region for different animal ailments including diarrhoea and fever.

A cow is considered a repeat breeder if she is cycling regularly and exhibits no clinical abnormalities but is still not conceived after at least two subsequent inseminations. Anoestrus is the condition where cow is not coming in heat. Both Repeat Breeding and anoestrus condition cause losses to the dairy farmers, economically. Dey *et al.* (2020) documented various ethno-veterinary practices (EVPs) for retention of placenta, anoestrus and repeat breeding in dairy animals of rural Punjab which were found to be very effective as per farmers' observation.

Veterinarians typically treat diseases in cows by administering antibiotics and/or other medications as needed. However, with the prevalence of drug resistance, field veterinarians are having difficulty treating a wide range of cattle illnesses. Because there is little systematic recording of traditional techniques or ethno-veterinary practices, scientists and veterinarians often dislike to depend on EVHP. It is urgently necessary to evaluate EVHP using a transdisciplinary approach. It is important to recognize that a significant portion of indigenous knowledge is, in fact, extremely modern and contemporary, as they can contribute to our current understanding of the biological, physical, and spiritual aspects of nature. Establishing epistemologically sensitive departments for transdisciplinary research and teaching—inspired by indigenous knowledge systems—can be one method to facilitate its diversity. (Darshan Shankar, ICEVP 2010 proceedings, TANUVAS-I-AIM).

3. MATERIALS AND METHODS

3.1. Awareness survey on bovine mastitis:

3.1.1. Sabarkantha Milk Union:

To learn more about the current level of knowledge among the dairy farmers about bovine mastitis, an awareness survey was carried out using a pretested structured questionnaire. The survey's questions were explained to farmers, and their feedback was recorded in person. The awareness questionnaire is enclosed as Annex.1. Data analysis was performed using Microsoft Excel analysis tools.

3.1.2. Pre and post-intervention farmer intervention study:

Another awareness survey was carried out using a pretested structured questionnaire to learn more about the current level of knowledge among dairy farmers in three milk union/producer companies, *viz.* Baani MPC, Saahaj MPC, and West Assam Milk Union Limited, regarding bovine mastitis and ethnoveterinary herbal preparations. Farmers were then made aware of the issue, and a post-awareness survey was also conducted after a year. The survey's questions were explained to farmers, and their comments were recorded using Google Forms. Prior concent from these farmers were obtained before conducting the survey. Pre and post awareness questionnaires are detailed in Annex-2a and 2b. Data analysis was performed using Microsoft Excel analysis tools.

3.2. Studies on causative bacterial agents of bovine mastitis, identification and assessing antibiotic resistance pattern in these agents:

3.2.1. Laboratory Consumables, equipment, culture media and reagents:

Required laboratory consumables, including culture media for bacteriological isolation and identification, ready-made petri plates, sterile disposable loops, *etc.* were obtained from M/s HiMedia laboratories. PCR-related reagents including dNTPS, premix, DNA ladders, Taq polymerase, etc. were of TAKARA made. Microcentrifuge tubes, plastic falcon tubes, and other laboratorywares were purchased from Corning in New York, USA.

The Annex-3 contains a thorough list of all the necessary consumables, equipmnets, culture media, reagents etc.

3.2.2. Study area:

In the current study, milk samples from cows and buffaloes having clinical or subclinical mastitis in milk unions/producer firms located various geographic regions across nine states were collected.

3.2.3. Collection of Milk Samples

Sub-clinical mastitis-affected animals were screened at farmers' doorstep using the CMT. Milk samples were collected aseptically in 50 ml falcon tubes with the addition of a Bronopol tablet as a preservative (1 tab/40 ml milk) from animals that tested positive either for clinical or sub-clinical mastitis. The animals were adequately restrained before collection of milk samples and the udder and teats were properly cleansed using disposable sterile tissue paper. Animals with udders that were egregiously unclean were cleaned with water and subsequently sample collection was then carried out following a thorough drying. After that, it was cleaned for at least 30 seconds with 70% isopropanol (70% IPA).

In case of multiple sampling, a separate swipe was used for each teat. For each sampling, a different set of gloves was utilised. After discarding an initial few strips of milk, 40 ml of milk from the affected quarter was collected. As soon as possible, milk samples were transferred in a cold box (2 to 8°C) and delivered to the lab for further microbiological investigation.

Sample collection kits were prepared beforehand containing one pair of sterile gloves, self-standing test tubes with Bromophol tablets, tissue paper, alcohol swipe and a marker pen (Figure 1).

Figure 2: Milk sample collection kits for bacteriological investigation

3.2.4. Isolation and Identification of microbes in the Milk samples

3.2.4.1.Isolation of microbes from milk samples:

For bacteriological examination and estimation of milk composition, speciman from each quarter of an animal is considered as a sample. The samples were cultured by the standard methods (Quinn *et al.*, 2000). Prior to processing, the milk samples were withdrawn from refrigeration temperature and kept outside until it reached room temperature. The samples were vortexed so that the bacteria got dispersed uniformly and being scattered from milk fat. In 15 ml falcon tube, 0.5 ml of milk sample was diluted with 4.5 ml of sterile nutritent broth. The milk samples were mixed again and then serially diluted up to the third dilution in nutrient broth. On blood (5%) agar plates, 0.1 ml of the sample from each dilution, was added and evenly disseminated using a sterilised metal spreader. The plates after inoculated were incubated for 24 hours at 37^0C. The isolated colonies on blood agar were identified on the basis of colony morphology, Grame staining, and a motility test. The plates, where no microbial growth was recorded, were further incubated at 37^0C in a CO_2 incubator (5%) for 72 hours.

3.2.4.2.Isolation and identification of microbes on basis of colony characteristics

Microbial colonies were indentified on ths basis of form, margins, shape, size, texture, and colour (Annex. 4). Each microbial colony was streaked over nutritional agar and incubated for 24 hours at 37^0C. A loopful of an isolated culture was mixed with one drop of sterile distilled water on a glass slide in order to generate a smear. After allowing the slides to air dry for fifteen to twenty minutes, they were heated, avoiding the point of overheating.

3.2.4.3.Identification of microbes by Gram's staining:

Gram's staining kit (Himedia, catalogue no. K001L-1KT) was used to strain heat-fixed smears as per the manufacturer's instructions. In brief, Gram's Crystal Violet was added and kept for a minute followed by addition of Gram's Iodine for a minute, finally Gram's Safranin (for 30 seconds) were poured one after another. Every step was followed by a wash beneath the running tap. Smears were allowed to air dry for 15 to 20 minutes before being inspected under an oil immersion light microscope (Olympus, BX 50).

3.2.4.4.Identification of microbes on differential medium:

3.2.4.4.1. Identification of bacteria on blood agar:

a. Blood agar ready-to-use plates:

Ready-to-use Blood agar plates were purchased from Himedia. The plates were kept at 2-8^0C until subsequent use.

b. Passage of culture on blood agar (BA):

On blood agar, quadrant or continuous streaking was done and the plate was incubated at 37^0C for 24 hours. Both the colony characteristics and the hemolysis pattern were recorded. After use, culture plates were either decontaminated using a liquid cycle in an autoclave for discard or preserved at 2-8^0C for further use.

3.2.4.4.2. Identification of *Staphylococcus spp.* on Mannitol Salt Agar:

a. Preparation of Mannitol Salt Agar (MSA):

111.02 gram of MSA agar powder were reconstituted in 1000 ml of sterilized distilled water and heated to completely dissolve the media powder in accordance with the manufacturer's instructions (HiMedia laboratories). The prepared medium was placed under 15 pounds of pressure and autoclaved for 15 minutes (121°C). Aseptically adding 5% by volume of egg yolk emulsion (FD045) and mixed thoroughly. The sterility of the prepared plates was examined. Plates that had no growth were carefully packed and kept at 2-8^{0}C until needed.

b. Passage of bacterial culture on Mannitol Salt Agar (MSA):

MSA was used as a selective medium to isolate *Staphylococcus* species. After streaking the MSA agar plates, they were incubated for 24 houss at 37^{0}C. It was noted when the coagulase and lipase activities of the bacteria caused a color change in the agar media. The precipitation zone surrounding the bacterial colonies were also noted. After use, culture plates were either stored at 2-8 ^{0}C for for future use or decontaminated using a liquid cycle in an autoclave.

3.2.4.4.3. Identification of Streptococcus species on Edwards Medium:

a. Preparation of Edwards Medium:

41.33 grams of Edwards Medium agar powder were reconstituted in 1000 milliliters of distilled water and heated to completely dissolve the media powder, in accordance with the manufacturer's instructions (HiMedia laboratories). The prepared media was autoclaved for fifteen minutes at fifteen lb pressure (121°C). The sterility of the prepared plates was examined. Plates that had no growth were carefully packed and kept at 2-8^{0}C until used.

b. Passage of *Streptococcus species* on Edwards Medium:

As a selective medium, Streptococcus sp. was grown on Edwards Medium agar. After streaking, the plates were incubated for 24 hours at 37°C, bacterial colonies were observed for alterations in color, shape, and other attributes. After use, culture plates were either stored at 2-8^{0}C for further bacterial culture or decontaminated using a liquid cycle in an autoclave.

3.2.4.4.4. Identification of bacteria on Eosin Methylene Blue Agar (EMB agar):

a. Preparation of Eosin Methylene Blue Agar (EMB agar):

35.96 grams of EMB agar powder were reconstituted in 1000 milliliters of distilled water and heated to fully dissolve the media powder, as per the manufacturer's instructions (HiMedia laboratories). The prepared media was autoclaved for fifteen minutes at fifteen lb pressure (121°C) and allowed to cool. The sterility of the prepared plates was examined. Plates that had no growth were carefully packed and kept at 2-8^0C until needed.

b. Passage of bacterial culture on Eosin Methylene Blue Agar (EMB agar):

As a selective medium, EMB agar was used to cultivate E. coli. After streaking, the plates were incubated for 24 hours at 37°C, bacterial colonies were observed for alterations in color, shape, and other attributes. Typical metallic green sheen, indicating vigorous lactose and/or sucrose fermentation ability typical of faecal coliforms were recorded. After use, culture plates were either stored at 2-8^0C for further bacterial culture or decontaminated using a liquid cycle in an autoclave.

3.2.4.4.5. Identification of bacteria on MacConkey agar:

a. Preparation of MacConkey agar:

An amount of 49.53 grams of MacConkey agar powder were reconstituted in 1000 milliliters of distilled water and heated to fully dissolve the media powder, as per the manufacturer's instructions (HiMedia laboratories). The prepared media was autoclaved for fifteen minutes at fifteen pounds of pressure. (121°C) and allowed to cool. The sterility of the prepared plates was examined. Plates that had no growth were carefully packed and kept at 2-8^0C until needed.

b. Passage of culture on MacConkey agar:

MacConkey agar was used as a selective medium to cultivate coliforms and non-coliforms. After streaking, the plates were incubated for 24 hours at 37°C, bacterial colonies were observed for alterations in color, shape, and other attributes. After use, culture plates were either stored at 2-8^0C for further bacterial culture or decontaminated using a liquid cycle in an autoclave.

3.2.4.4.6. Identification of Gram-negative rods on Klebsiella Selective Agar

a. Preparation of *Klebsiella* Selective Agar:

An amount of 20.4 gm of Klebsiella Selective agar powder were reconstituted in 1000 ml of distilled water and heated to completely dissolve the media powder, in accordance with the manufacturer's instructions (HiMedia laboratories). The prepareded medium was placed under 15 lb pressure and autoclaved for 15 minutes (121°C) and allowed to cool. The sterility of the prepared plates was examined. Plates that had no growth were carefully packed and kept at 2-8^{0}C until needed.

b. Passage of Gram-negative rods on Klebsiella Selective Agar:

On Klebsiella Selective Agar, a selective medium, Klebsiella sp. was cultivated. After streaking, the plates were incubated for 24 hours at 37°C, bacterial colonies were observed for alterations in color, shape, and other attributes. After use, culture plates were either stored at 2-8^{0}C for further bacterial culture or decontaminated using a liquid cycle in an autoclave.

3.2.5. Identification and antibiogram of bacterial isolates in the BD phoenix M50 instrument

Bacterial identification and antibiogramGram were carried out in Becton Dickinson (BD) phoenix M50 instrument as per its instruction manual. Gram positive PMIC/ID-70 (BD, catalogue no. 448763) combo panels were used to detect Gram +ve bacteria, as well as SMIC/ID 9 (BD, catalogue no. 448858) combo panels specifically for Streptococcus sp., Gram -ve NMIC/ID-55 (BD, catalogue no. 448935) combo panels were used for Gram -ve bacteria.

The reagents, AST indicator, and calibration tubes were brought to room temperature prior to beginning the work of identification of bacterial agents. With the use of a sterile cotton swab (HiMedia, catalogue no. PW005-1X500NO), colonies of an overnight-grown bacterial culture on nutrient agar were taken and suspended in the BD phoenix ID broth (BD, catalogue no. 246001) by proper mixing. The optical density (OD), as determined by the Nephelometer (BD), was adusted to 0.5.

The AST broth (BD, catalogue no 246003) was prepared by mixing of 25 µl BD phoenix ID broth culture with one drop AST indicator.

Based on the Gram staining reaction, ID broth and AST broth contents were transferred to the respective portion of Gram positive or Gram-negative panels. After closing the AST and ID portions with the Phoenix panel closure, the panels were placed in BD transportation rack and inserted into the instrument through login into the system and entering sample details. The results of bacterial identification and antibiogram were obtained after 4 hours and 12 hours of incubation respectively.

3.2.6. Identification and antibiogram of *Str. uberis* isolates:

The isolates were identified based on morphological, cultural and biochemical characteristics (Quinn *et al.*, 2000). Mueller-Hinton Agar was used in the Kirby-Bauer disk diffusion procedure to conduct the antimicrobial susceptibility test. The Clinical and Laboratory Standards Institute's (CLSI) breakpoints were used to evaluate the outcomes (Wayne, 2019). The in-vitro antibiotic sensitivity of the bacterial isolates was carried out with discs coated with five various antibacterial agents: Amoxicillin (AMX), Ceftriaxone (CTR), Oxytetracycline (O), Ampicillin (AMP), and Penicillin-G (P). According to the chart provided by the company, the sensitivity was determined based on the diameter of zone of inhibition surrounding the disc (Hi- Media).

3.2.7. Identification of bacterial isolates by molecular technique:

Take 200µl (each) of active bacterial broth culture & PBS in 1.5 ml tube

↓ Vortex

Add 20 µl protease & 200 µl lysis buffer

↓

Incubate @56°Cfor 10 minutes and centrifuge

↓

Add 200µl, 100 % ethanol in microcentrifuge tube and mixed it

↓

Transfer the mixture in QIAamp mini spin column with 2 ml collection tube

↓ Centrefuged@8000 rpm for a minute

Collect the flow in fresh collection tube

↓

Add 500 µl of AW2 (washing buffer 2) in mini spin column

↓ Centrifuged @14000 rpm for 3 minutes

Through the flow & discard the collection tube

↓

Take QIA amp mini spin column in 1.5 ml microcentrifuge tube

↓

Add 60 µl elution buffer & incubate it for 10 minutes (room temp.)

↓ Centrefuged@8000 rpm for a minute

Take elute for PCR amplification stored in -20°C

Table 1: Details of Thermal profile of species-specific PCR used for identified bacteria

Step	Temp (in ˚C)	Time	Cycles
Initial Denaturation	94˚C	5min	1
Denaturation	94˚C	35sec	30
Annealing	60˚/55 C*	30sec	
Extension	72˚C	45 sec	
Final extension	72˚C	10mins	1
*(varies as per table 4)			

Table 2: Details of Thermal profile of AMR PCR used for identified bacteria

Step	Temp (in ˚C)	Time	Cycles
Initial Denaturation	94˚C	5min	1
Denaturation	94˚C	30 sec	35
Annealing	55˚C*	60 sec	
Extension	72˚C	45 sec	
Final extension	72˚C	10 mins	1
*(varies as per table 5)			

Table 3: PCR reaction mix for PCR used for identified bacteria

Reaction setup (Total volume =20µl)	
Components	**Volume per reaction**

Ampi Taq Gold Master mix(1x)	10µl
Primer (F)10 picomoles	1µl
Primer (R)10 picomoles	1µl
NF Water	3µl
DNA, of respective isolate	5µl

After adding every component of the reaction mix to the PCR tubes, the tubes were placed into the thermal cycler. After configuring every parameter to match the suggested thermal profile for that specific type of bacteria, the thermal cycler was started. The PCR products were run on a 2% agarose gel in the electrophoretic unit once the run was finished. The information on the PCR reaction mix and thermal profile is given in Tables 1, 2, and 3. Table 4 provides information on the primers, product size, and target for PCR particular to species.

Table 4: Details of primers used for identified bacteria and their respective amplicon size

S. No.	Function / Description	Target gene/	Primer Pair (Forward (F)/ Reverse ('R)	Primer Sequence (5'-3')	PCR Product size (in bp)	Annealing Temperature (°C)
	Species specific PCR					
1	*E. coli* (bacterial alkaline phosphatase gene)	phoA	ECPF*	GGTAACGTTTCTACCGCAGAGTTG	468 bp	55.0
			ECPR	CAGGGTTGGTACACTGTCATTACG		
2	*Staph. aureus* (Nuclease gene)	nuc	nuc-F	GCGATTGATGGTGATACGGTT	798	55.0
			nuc-R	AGCCAAGCCTTGACGAACTAAAGC		
3	*Kleb. pneumoniae* (DNA gyrase gene*)*	gyrA	F	CGCGTACTATACGCCATGAACGTA	468	55.0
			R	ACCGTTGATCACTTCGGTCAGG		
4	*Str. agalactiae*	16SrRNA	F	GCTAATACCGCATAAGAGTAATTAAC	317	60.0
			R	GGTAGATTTTCCACTCCTACCAA		
5	*Str. dysgalactiae*	16SrRNA	STDGF	GGGAGTGGAAAATCCACCAT	572	55.0
			STAGR	AAGGGAAAGCCTATCTCTAGACC		
6	*Str. uberis*	*cpn 60*	STUBF	TCGCGGTATTGAAAAAGCAACAT	400	55.0
			STUBR	TGCAATAATGAGAAGGGGACGAC		

3.2.6.1. Screening of antimicrobial resistance genes (ARGs):

The key reason of bacterial resistance to antibiotics is the presence of Antimicrobial resistance Genes (ARGs). Through horizonal gene transfer (HGT), bacteria may spread the resistance among strains within species and even inter species. It may be through plasmid exchange, integrons (In), and transposons (Tn) at the gene level (Bertolla *et al.,* 2000, Dantas *et al.,* 2008).

The selection pressure on bacteria due to wide application of antibiotics results in getting ARGs. Based on the ARGs, different bacteria can develop resistance to different types of antibiotics (Zonghui *et al.,* 2021). I*n his review article,* Zhuang *et al. (2021) has* reported multidrug *(mec),* β-lactamide (*bla),* tetracycline (*tet),* glycopeptides (*van),* sulfonamide *(sul),* aminoglycoside *(aad),* amphenicol (*flo*) and trimethopolyl (*dfr*) as the common ARGs during last 30 years.

During our present study, following AMRs were attempted to screen in mastitis causing bacterial agents from bovine:

Mec (mecA, mecC), beta-lactamases (bla Z, bla TEM, bla SHV, bla CTXM) and tet (tetA, tetO, tetM and tetK) as per availability of standardised PCR in the laboratory.

Those bacteria possess mecA gene, allows resistance to methicillin, penicillin and penicillin like antibiotics (Ubukata *et al.,* 1989). Methicillin-resistant *Staph. aureus* (MRSA) strains most commonly carries mecA gene (Deurenberg and Stobberingh, 2009). mecA encodes penicillin-binding protein 2A (PBP2A) for the formation of bacterial cell walls, which have lower affinity for beta-lactam group of antibiotics. Thus, the formation of bacterial cell walls could not be prevented by beta-lactams (Fogarty *et al., 2015)*

During 2011, MRSA with a divergent mecA gene was reported which was designated as mecC, a highly significant for human and veterinary microbiology and had isolated from 14 different host species (Gavin *et al.,* 2014)

blaZ gene in *Staph. aureus* confer penicillin resistance by production of beta-lactamase which hydrolyse beta-lactam ring (Zhang *et al.,* 2001).

Extended Spectrum β-lactamases (ESBL) can confer resistance to bacteria for cephalosporins (1st to 3rd generation), penicillin and aztreonam (Paterson and Bonomo, 2005).

TEM-1 is the most commonly found beta-lactamase in Gram-ve bacteria. Production of TEM-1 confers up to 90 % ampicillin resistance in *E. coli (*Cooksey *et al*., 1990). More than 150 TEM varieties are known (George, 2006).

More than 88 SHV varieties are known (George, 2006). SHV-1 is similar to TEM-1 with 68 % sharing of its amino acids, commonly found *in Kleb. pneumoniae (*Paterson *et al.,* 2003)

More than 172 CTX-M enzymes are known (Ramadan *et al.,* 2019) and these have greater activity against cefotaxime than ceftazidime. CTX-M are not closely related to TEM or SHV (around 40 % identical) (Tzouvelekis *et al.* 2000)

tet gene governs the resistance to tetracycline, a broad-spectrum antibiotic. There are at least 40 tet genes causing resistance to tetracycline (Roberts, 2005). In Gram -ve bacteria, tetA, tetB, tetD, tetE, tetG (Jones *et al.,* 2006) and in Gram +ve bacteria, tetK, tetL, tetM, tetO and tetS (Roberts, 1996) are found significantly.

Details of primers used for identified bacteria, their respective amplicon size and presence of antimicrobial resistant genes provided in Table 5.

Table 5: Details of primers used for identified bacteria, their respective amplicon size and presence of antimicrobial resistant genes

S. No.	Function/ Description	Target gene/	Primer Pair (Forward (F)/ Reverse ('R)	Primer Sequence (5'-3')	PCR Product size (in bp)	Annealing Temperature (°C)
1	Penicillin Resistance (*Staph. aureus*)	*Bla-Z*	blaZ-F	AAGAGATTTGCCTATGCTTC	850-900	55
			blaZ-R	GCTTGACCACTTTTATCAGC		
2	ESBLs (*Kleb. pneumoniae*)	Bla-TEM	TEM-F	GCGGAACCCCTATTTG	846	55
			TEM-R	ACCAATGCTTAATCAGTGAG		
3		bla-SHV	SHV-ALL-F	TTATCTCCCTGTTAGCCACC	~790- 854	60
			SHV-ALL-R	GATTTGCTGATTTCGCTCGG		
4		bla-CTXm	CTX-M-ALL-F	ATGTGCAGYACCAGTAARGTKATGGC	~593	62
			CTX-M-ALL-R	TGGGTRAARTARGTSACCAGAAYSAGCGG		
5	Methicillin Resistance (*Staph. aureus*)	*mecA*	mecA-F	TCCAGATTACAACTTCACCAGG	162	59
			mecA-R	CCACTTCATATCTTGTAACG		
6	Methicillin Resistance (*Staph. aureus*)	mecC	mecC-F	GAAAAAAAGGCTTAGAACGCCTC	138	59
			mecC-R	GAAGATCTTTTCCGTTTTCAGC		
7	Tetracycline resistance	TeT K	Tet-K-F	GTAGCGACAATAGGTAATAGT	500	55
			Tet-K-R	GTAGTGACAATAAACCTCCTA		
8	Tetracycline resistance	TeT M	Tet-M-F	GTTAAATAGTGTTCTTGGAG	640	55
			Tet-M_R	CTAAGATATGGCTCTAACAA		
9	Tetracycline resistance	TeT O	Tet-O-F	GATGGCATACAGGCACAGAC	500	55
			Tet-O-R	CAATATCACCAGAGCAGGCT		
10	Tetracycline resistance	TeT A	Tet-A-F	CGAGCCATTCGCGAGAGC	750-1000	55
			Tet-A-R	GCCTCCTGCGCGATCTGG		

3.3. Study the efficacy of EVHP in the management of bovine mastitis:

3.3.1. Sub-clinical Mastitis field study

50 CMT positive HF cattle were selected for the field study with application of ethnoveterinary herbal medicines in Sabarkantha Milk Union. The EVHP formulation used were as per the protocol mentioned in the NDDB EVHP booklet. The milk production before application of the EVHP and after completion of EVHP were also recorded.

3.3.2. Bacterial isolation and identification for pre and post EVHP Mastitis cases

In order to investigate the effectiveness of EVHP in treating mastitis, milk samples from 41 animals (total 82 milk samples i.e. pre and post-intervention) were gathered for bacteriological isolation and identification along with Post-EVHP status on mastitis recovery.

Of these, in 10 animals (5 sub-clinical and clinical mastitis infected quarter each) more thorough research was done with parameters like disease history, milk colour and consistency, milk quantity, milk constituents, pH of milk, california mastitis test (CMT), somatic cell count (SCC), bacteriological isolation, identification and antimicrobial resistance pattern etc. Total Bacterial Count (TBC) was conducted in 5 mastitic milk samples.

EVHP treatment protocol was followed as per guidance of Prof. N. Punniamurthy (NDDB EVHP booklet), detailed procedure enclosed in Annex. 10.

3.3.2.1. Bacterial count of pre and post EVHP mastitis milk by Total Bacterial Count method (TBC):

Total bacterial count (TBC) was carried out by the method of Griffin *et al*. (1977) on 5% bovine blood agar plates. The detailed procedure is given in Annex. 6.

3.3.2.2. California Mastitis Test (CMT) test

As per the instruction manual of supplier (DeLavel), CMT was conducted for detection of sub-clinical mastitis. After discarding few strips of foremilk, approximately 3 ml of quarter milk was taken in CMT paddle. Equal volume of CMT reagent was added to it and paddle was rotated clock wise direction. Manufactures instructions was followed for CMT interpretation recording the reading within 10-15 seconds. The detailed interpretation is given in Annex. 7.

3.3.2.3. Determination of the pH:

To check the alkalinity of milk samples, pH readings were recorded using pH meter (Make- Oakton, Model- PC 2700) following the manufacturer's instructions.

3.3.2.4. Determination of milk composition:

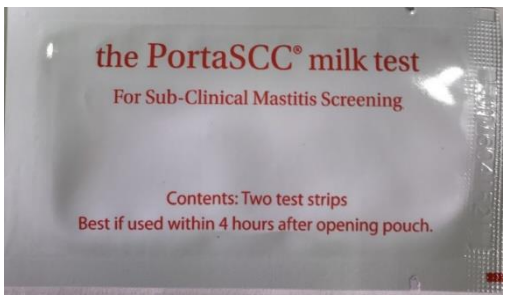
To check milk protein, lactose, fat and SNF, Milk analyser (Lactoscan SL-30) was used following the manufacturer's instructions.

3.3.2.5. Determination of SCC:

To check milk somatic cell count, Porta SCC field test kit instrument (Figure 2) was used following the manufacturer's instructions (details in Annex. 8)

The reader with the control strip

The reader with water droplet during celebration of the PortaSCC reader



Test strips

Figure 3: Porta SCC milk test kits

3.4. Case studies on the recovery of bovine ailments by using EVHP under field condition:

3.4.1. Empirical data on management of bovine ailments through EVHP:

5,60,969 nos. empirical data on management of bovine ailments through EVHP in different milk unions and producer companies for 26 common conditions including FMD were documented. The data documented were captured through an in-house web-based software. The EVHP formulations used in the project areas were as per the protocol mentioned in the NDDB EVHP booklet, EVHP was provided in recommended ratio of herbs or spices as detailed in Annex. 9.

3.4.2. Field study on management of mastitis, diarrhoea and pyrexia cases with use of EVHP at Sabarkantha Milk Union:

In order to determine the effectiveness of EVHP on non-specific pyrexia, non-specific diarrhea, and mastitis, a field level study was conducted at Sabarkantha Milk Union. During study, any animal with history of persistent diarrhea or fever and symptoms associated with hemo-protozoan infection were excluded. The diagnosis was based on clinical observations. The animals were provided with EVHP in recommended ratio of herbs or spices as detailed in Annex. 9.

Following cases were included in the study:

3203 cattle and 500 buffalos with clinical mastitis, 1661 cattle and 579 buffalos with non-specific diarrhoea, 1228 cattle and 399 buffalos with non-specific pyrexia.

The animal included were classified into 4 age groups:

Group I: Below 3 years,

Group II: 3-5 years,

Group III: 6-9 years and

Group IV: above 9 years.

3.4.3. Case reports on management of LSD like conditions with ethno-veterinary practices:

14 LSD suspected cattle (age 1-7 years) in the states from Assam and Maharashtra were included in the study to manage by EVHP. Animals were included as per the clinical signs exhibited by these cattle. The EVHP for LSD preparations having both oral administrations and topical (external) application, in cases of wound formation. The animals were provided with EVHP in recommended ratio of herbs or spices as detailed in Annex. 10.

3.4.4. Management of repeat breeding in bovine by herbal combination:

Thirty five nos. were cows and 73 buffaloes having history of repeat breeding at Kolhapur, Maharashtra were included in the study. 20-day protocol for feeding were provided to all these animals. Artificial Insemination (AI) was performed during the next estrus after completion of EVHP. After 3 months of AI, pregnancy diagnosis was performed by per rectal examination. Follow-up was continued unto calving for all pregnant animals. The animals were provided with EVHP in recommended ratio of herbs or spices as detailed in Annex. 9.

3.5. Empirical data recorded in NDDB portal by veterinarians from different states for various bovine ailments treated by EVHP

A unique approach was initiated in documentation, validation and dissemination of EVHP by TANUVAS, Tamil Nadu and ICAR with co-operation from community-based organizations like the FRLHT, traditional-healers, farmers and field veterinarians. The NDDB, in collaboration with TDU and TANUVAS, has been popularizing the use of EVHP since 2016. This was initiated at Sabarkantha District Co-operative *Milk* Producers' *Union* Limited (Sabar Dairy), Himatnagar, Gujarat. Initially, a considerable no of bovine mastitis cases of chronic nature, unresponsive to commonly used antibiotics by the dairy veterinarians were successfully managed by a team of TAVUVAS and TDU with the use of EVHP. With the encouraging outcomes for mastitis management through EVHP, veterinarians from milk unions/producer companies across India were trained at TDU, Bangalore. In the current study, common bovine ailments viz. foot and mouth disease, diarrhoea, retention of placenta, bloat, indigestion, repeat breeding, fever, lumpy skin disease etc. were managed through the use of EVHP. The EVHP formulations used in the project areas were as per the protocol mentioned in the EVHP booklet (Annex. 10).

3.6. Cost benefit estimation for bovine mastitis, diarrhoea and pyrexia managed by EVHP in place of modern medicine

The total cases recorded in MCPP portal during years 2017-18, 2018-19, 2019-20 and 2021-22 were considered for the estimation. The calculation was carried out with help of cost estimated by Nair *et al.* (2022) for EVHP and modern medicine which is provided in Annex. 9.

3.7. Study the effect of using EVHP in bovine diseases on antibiotic residues in milk:

Representative bulk milk samples from 10 village milk cooperative was taken for the study during 2018 and 2019. The bulk milk samples from DCS bulk milk coolers were taken quarterly for determination of presence or absence of antibiotic residues in milk by field antibiotic residue test kit (Figure 3, Unisensor kit). Milk samples were taken from the bulk milk tanks after proper mixing and transported to the union laboratory at 4°C. Testing were performed immediately as per the manufacturer's instruction (Unisensor, KIT072).

Figure 4: 4sensor kits (Unisensor KIT072 with micropipettes)

3.7.1. Using "Heatsensor Duo" and readsensor:

Two milk samples can be tested at a time in "Heatsensor Duo". After switch on the instrument, allowed it to reach 40^0C (when ready, a beep sound occurs and display appear as "OK"). Microwell (s) from the kit were kept in the testing block which shows 40^0C and milk

samples were put into it using the fixed volume micropipette provided which transfers 200 microlitres. Milk in the microwell were gently mixed at least 5-6 times using the micropipette. There should be no air bubbles in the well and every milk sample should be taken using a new tip. Then, start button was pressed and 3 minutes incubation was commenced displaying on the screen. The dipstick(s) were inserted into each of the dipstick holder in the incubator before the countdown ends. After 3 minutes, the dipstick(s) were automatically fall into the microwell(s) for another 3 minutes round of incubation. After that, reading of the strips were taken in 'Readsensor' machine. The BSTQ strip can detect 4 groups of antibiotics i.e. Betelactam, Sulphadimidine, Tetracycline and Quinolone groups. The detectable limits (ng/ml – ppb) provided by the manufacturers for *4SENSOR BSTQ Milk Assay*) are provided in Annex. 5.

3.8. SCC in bulk milk in study villages for 1.5 years (Sept 2018-Apr'2020)

Bulk milk samples from 15 DCSs were collected at quarterly interval for 1.5 years to assess the Somatic Cell Count (SCC) in the samples. The samples were tested with Porta SCC Kits (details in Annex. 7).

3.9. Safety of Mastitis EVHP formulation

In Central Animal Facility, 10 healthy young adult female Rattus norvegicus (Wistar rats) between 8 and 12 weeks age, of SASTRA Deemed University, were selected for the dermal toxicity study on mastitis. Herbal formulation was comprised of aloevera, turmeric and lime as use for bovine mastitis cases. A dose of 2000 mg/Kg Body weight was applied dermally.

Identification of Animals

Tags having animal number, group number and dose level were attached to respective cages. Each animal was identified by unique identification number by ear tagging (Table 6)

Table 6: Animal identification details for dermal safety study

Animal ID Normal Control	Sex	Animal ID Test substance Treated	Sex
12874	Female	12879	Female
12875	Female	12880	Female

12876	Female	12881	Female
12877	Female	12882	Female
12878	Female	12883	Female

Details regarding animal housing, identifation sanitation, animal welfare and regulatory compliance, diet and water etc. provided in Annex.11.

Preparation of Test Substance

The test substance at the dose 2000 mg/Kg b. wt was mixed in distilled water and applied uniformly over the skin, held in contact with porous gauze dressing and non-irritating.

Treatment

Healthy Wistar rats (10 female) were selected and allowed for an acclimation period of one week. The fur was removed from the dorsal area of trunk of the test animals by trimming using electric trimmer before 24 hours of experiment. The test substance was applied uniformly over the skin and wrapped with a porous gauze dressing and non-irritating tape throughout a 24- hour exposure period. The animals were sent back to their cages immediately after topical application. After the exposure period, any remaining test substance was washed away with water.

Observation Period: 14 days

Mortality: All animals were observed twice everyday for mortality for 14 days.

Body Weight: Each animal body weight was recorded just prior to the test substance treatment (Day 0), Day 7 and 14 using electronic animal weighing balance (Sartorius AG, Germany).

Feed Intake: Feed intake for individual animals was recorded daily for the entire study period.

Toxicity Signs: Examination for alopecia, catalepsy, chromodacryorrhea, clonic, coma, convulsion, diarrhea, dullness, excessive grooming, change in gait, hyperactivity, lacrimation, nasal discharge, nasal irritation, piloerection, polyuria, prostration, repetitive circling, respiratory distress, salivation, scaling, tonic, and tremor were done individually in each animal after treatment with the test substance throughout the entire observation period. In addition, dermal related toxicity signs such as edema, erythema, eschar formation, hyperkeratosis, hyperplasia, scaling, and wound formation were observed.

Gross Pathology: All animals were subjected to necropsy at the end of 14^{th} day observation period for gross pathological examination.

4. RESULTS AND DISCUSSION

4.1. Survey on awareness on bovine mastitis and use of ethnoveterinary herbal preparation (EVHP)

Surveys were conducted among the farmers on the awareness for bovine mastitis and on use of ethnoveterinary herbal preparation (EVHP) in various Milk Unions (MUs) and Milk Producer Companies (MPCs) in India.

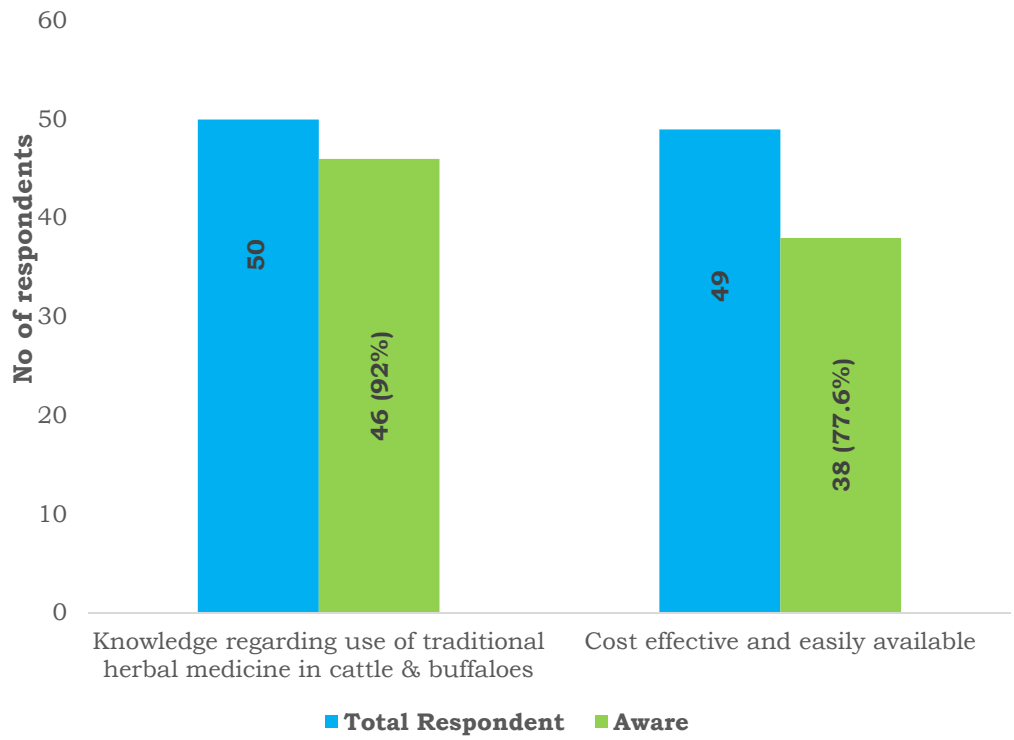
4.1.1. Survey on awareness on bovine mastitis, use of EVHP at Sabarkantha District Co-operative Milk Producers' Union Limited

A survey was performed to assess the awareness level on bovine mastitis and other common ailments in dairy animals and exclusive use of ethnoveterinary herbal preparation (EVHP) for the management of these ailments (Annex.1). With this objective, fifty dairy farmers were randomly chosen from 16 villages of Bayad, Himmatnagar and Idar tehsil of Himmatnagar district of the Gujarat state covered under Sabarkantha District Co-operative Milk Producers' Union Limited (Sabar Dairy). A total of 542 animals used to be reared by these farmers and on an average 4714 liters of surplus milk used to be sold per day. Their understanding towards animal diseases and management could provide us some light on milk quality producing by these farmers. The details of the survey and its findings are given in table 7.

Table 7: Survey on awareness for bovine mastitis and EVHP at Sabar dairy

Name of Tehsil	No of respondents included	Total animals of the respondents	Total surplus milk sold by the respondents (lts/day)
BAYAD	5	62	704
HIMMATNAGAR	35	382	3205
IDAR	10	98	805
TOTAL	50	542	4714

Out of 50 respondents, 92.0 % were aware about use of EVHP in cattle and buffaloes. In addition, 77.6 % of 49 respondents consider that EVHP are cost effective and easily available (Figure 5).



Figures in parenthesis showing %

Figure 5: Level of awareness among farmers regarding use of EVHP in dairy animals

Awareness regarding availability of EVHP for bovine ailments like mastitis, diarrhoea, fever, repeat breeding and FMD (Foot and Mouth Disease) is being depicted in Figure 6. The percentage of awareness were in between 22.0-78.0 % regarding various ailments.

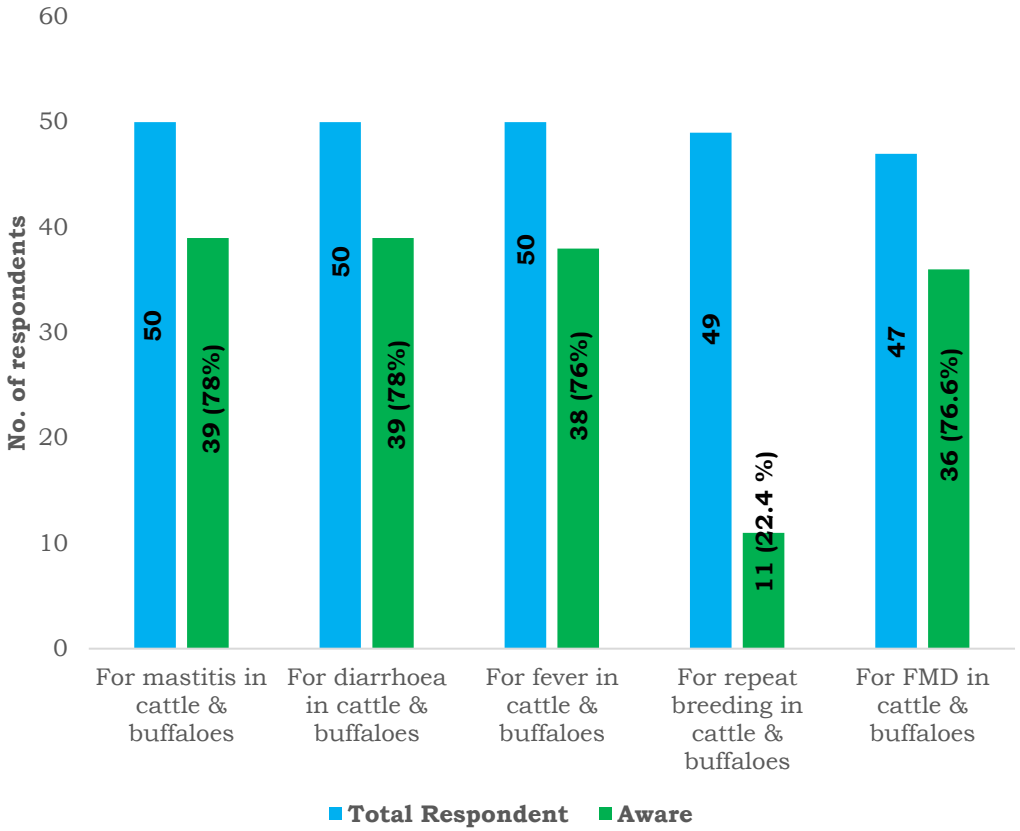
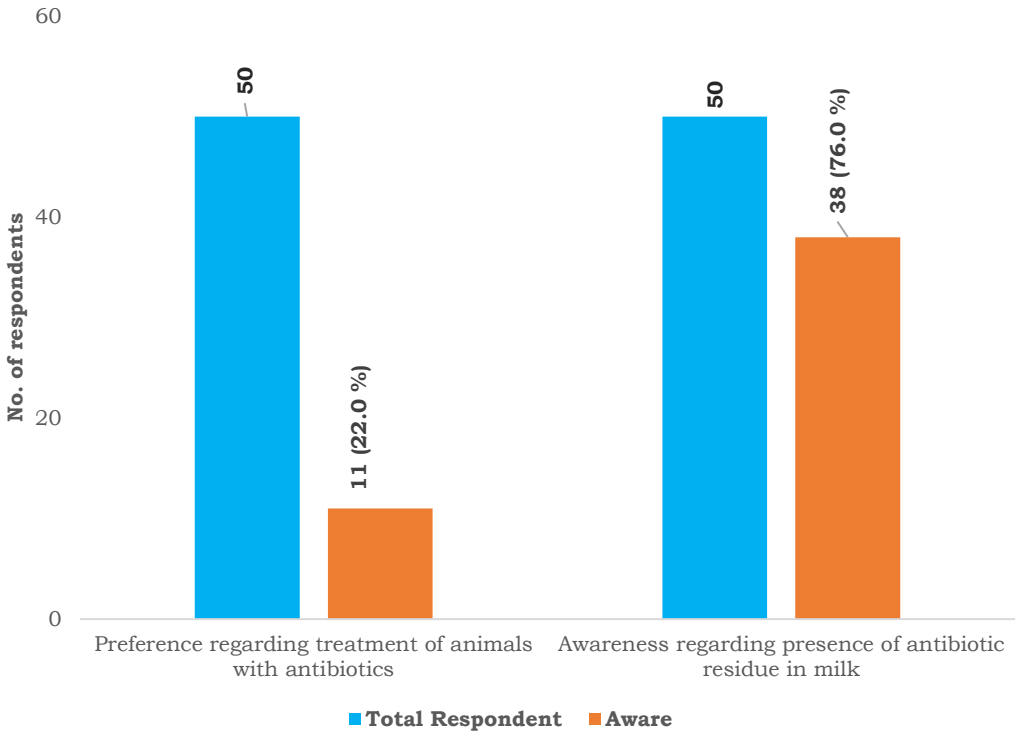


Figure 6. Level of awareness regarding availability of EVHP for various bovine ailments in Sabar Dairy

The preference to use antibiotics in treatment of bovine diseases and awareness regarding presence of its residues in milk have presented in Figure 7. In Sabarkantha milk union, 76.0 % respondents were aware regarding possibility of presence of residues in milk after use of antibiotic in animals.



Figures in parenthesis showing %

Figure 7. Awareness regarding antibiotic residue in milk

4.1.2. Survey on awareness for bovine mastitis and EVHP in other milk unions/producer companies

It was observed, in the current study conducted during 2018-19 that dairy farmers in the Sabar dairy had higher level of awareness regarding use of EVHP for management of animal diseases especially mastitis, and presence of antibiotic residues in milk (Figure 5-7). This high level of awareness was probably due to implementation of Mastitis Control Project (MCP) in Sabarkantha Milk Union since 2014-15 in collaboration with the NDDB.

In view of the above, it was decided to conduct another study in some other areas where Mastitis Control Project (MCP) was at the initial stage and 3 project areas were identified during this study

i. Banni Milk Producer Company Limited, Punjab i.e. Baani MPC

ii. Saahaj Milk Producer Company Limited, Utter Pradesh i.e. Saahaj MPC and

iii. West Assam Milk Producers' Co-operative Union Limited, Assam (WAMUL)

To understand actual field status, a baseline survey was carried out among the randomly identified farmers in the above MU/MPCs at the beginning of MCP in those areas. Subsequently another survey was done about a year after initiation of MCP.

4.1.2.1. Baseline survey on awareness:

A total of 318 dairy farmers were chosen at rendom from these MU/MPCs for the baseline survey. The survey was performed to assess the awareness level on bovine mastitis and other common ailments in dairy animal and exclusive use of ethnoveterinary herbal preparation (EVHP) for management of these against these ailments (Annex. 2a). A total of 1649 animals used to be reared by these farmers and average 4492 liters of surplus milk used to be sold per day. The details of the baseline survey and the findings are depicted in Table 8.

Table 8: Baseline survey on awareness for bovine mastitis and EVHP in different MU/MPCs

Name of MU/MPC	Baseline survey (3 states, 3 MU/MPCs, 10 Districts, 43 villages)		
	No of respondents	Total animal holdings by the respondents	Total amount of surplus milk sold (lts/day)
Baani Milk Producer Company Limited	111	806	2225
Saahaj Milk Producer Company Limited	100	434	1463
West Assam Milk Producers' Co-operative Union Limited	107	409	804

Total respondents	318	1649	4492

4.1.2.2. Post-intervention survey on awareness:

After taking the baseline survey, awareness campaigns were conducted in the study areas through village extension camps in DCS (Dairy Cooperative Societies)/MPP (Milk pooling Points) through a network of dairy veterinarians/Field Level Workers (FLWs). Extension materials like awareness videos, posters, booklets in regional languages (Baani: Punjabi; Saahaj: Hindi and WAMUL: Assamese) were displayed/distributed. When requested, softcopies of the extension materials were also provided to the farmers.

For post-intervention survey, a total of 215 dairy farmers were randomly chosen from these MU/MPCs. The survey was performed to assess the awareness level on bovine mastitis and other common ailments in dairy animal and exclusive use of ethnoveterinary herbal preparation (EVHP) for management of these against common ailments (Annex. 2b). A total of 1407 animals used to be reared by these farmers and average 4871 liters of surplus milk used to be sold per day. The details of the post-intervention survey are given in Table 9.

Table 9: Post-intervention survey on bovine mastitis and EVHP in different MU/MPCs

Name of MU/MPC	Post-intervention survey (3 states, 3 MU/MPCs, 10 Districts, 38 villages)		
	No of respondents	Total animals of the respondents	Total surplus milk sold (lts/day)
Baani Producer Company Limited	66	626	2640
Saahaj Producer Company Limited	100	551	1784

West Assam Milk Producers' Co-operative Union Limited	49	230	447
Total respondents	215	1407	4871

4.1.3. Salient impacts of the awareness programme:

The level of awareness regading mastitis (sub-clinical) in cattle and buffaloes in the milk shed areas of WAMUL, Baani and Saahaj MPC is being depicted in Figure 8. The overall awareness in respondents increased from 9.1 per cent to 85.6 per cent.

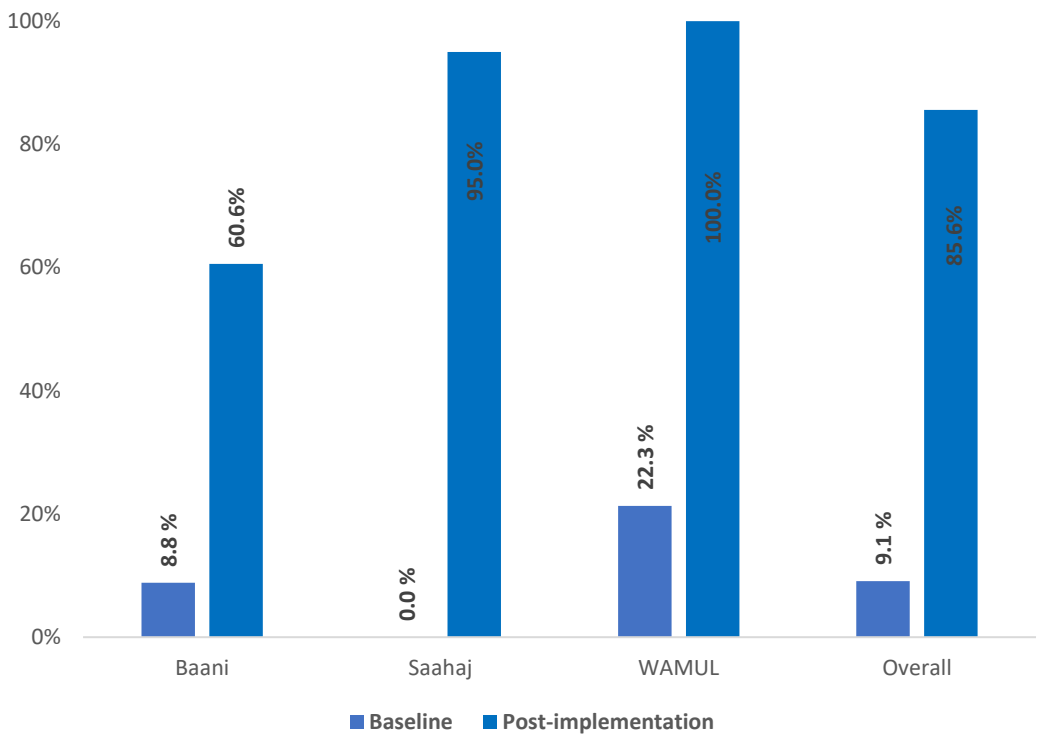


Figure 8: Level of awareness on Mastitis (Sub-clinical)

The increased awareness on use of ethnoveterinary medicines on mastitis in cattle and buffaloes in the milk shed areas of WAMUL, Banni and Saahaj MPC is depicted in Figure 9. The overall farmer awareness on use of EVHP increased from 12.3 % to 45.2 %.

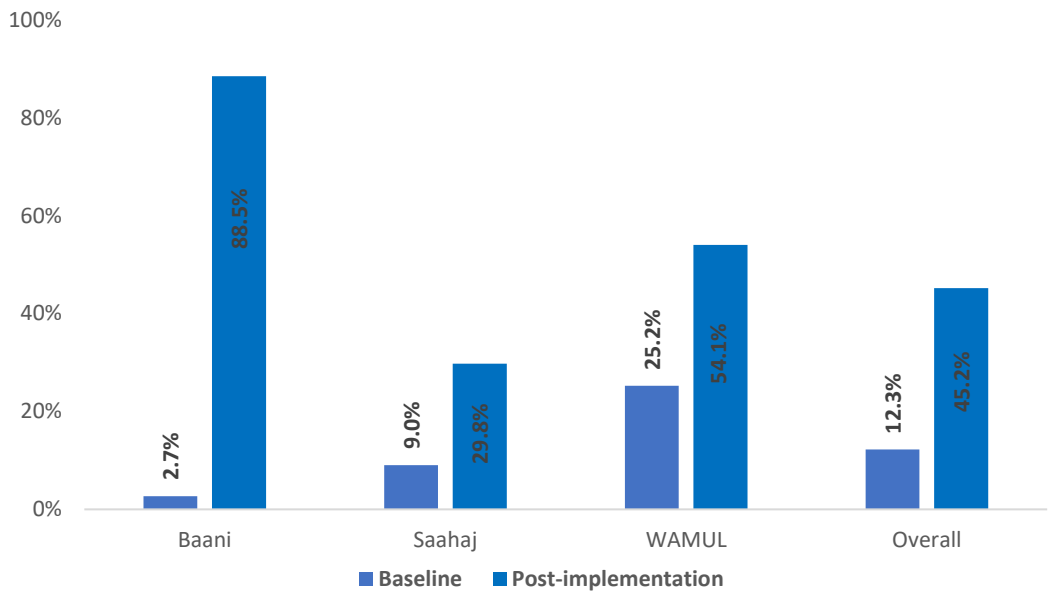


Figure 9: Level of awareness on use of EVHP

The increased in awareness regarding presence of antibiotic residue in milk in the milk shed areas of WAMUL, Banni MPC and Saahaj MPC is depicted in Figure 10. It was observed that the overall farmer awareness increased from 19.2 % to 74.6 % on antibiotic residue significance.

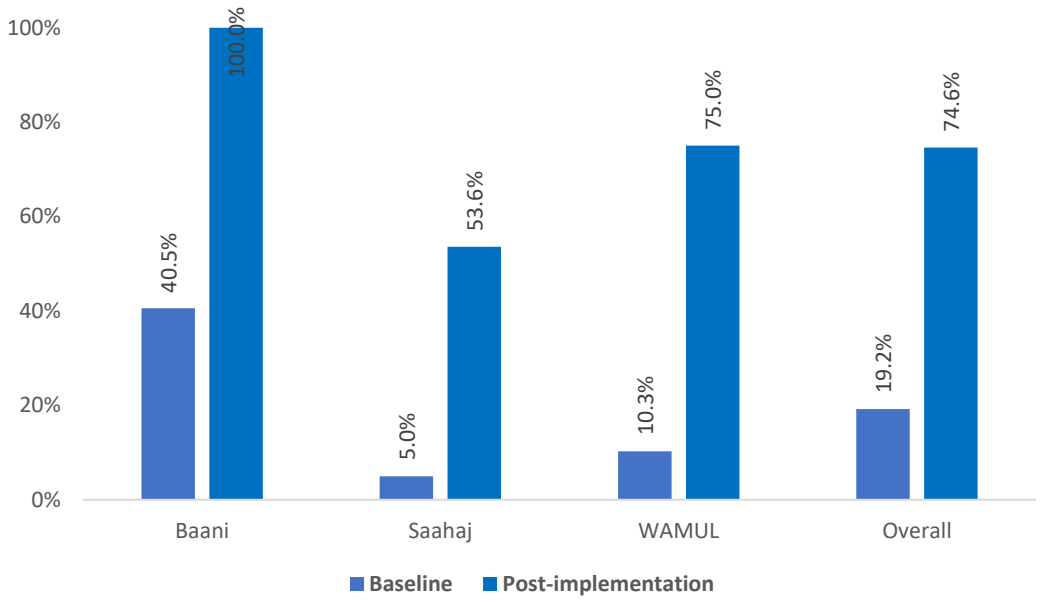


Figure 10: Awareness regarding antibiotic residue in milk

From the survey conducted, it was noticed that in various MU/MPCs that awareness campaign had a great impact in increasing awareness among the dairy farmers on the implication of mastitis, use of cost effective, efficacious EVHP and presence of antibiotic residues in milk due to indiscreminate use of antibiotics.

In the pre and post intervention awareness survey on occurrence of subclinical mastitis (SCM) in cattle and buffaloes was found to be drastically improved from initial 9.12 per cent to 85.6 per cent. Very less awareness on SCM diagnostic technologies among the farmers have been reported (Chelkeba *et al*., 2016, Rathod *et al*., 2017, Nimbalkar *et al., 2020*). Rathod *et al*. (2017) reported that better awareness on bovine mastitis results in better milk quality. Chelkeba *et al.* (2016) also highlighted the significance of extensive extention in rural areas through training, pamphlets, experience sharing in enhancing their awareness on SCM. Kaaya *et al*. (2005) and Dehinenet *et al.* (2014) also emphasized on requirement of knowledge upgradation to increase farmer proclivity towards technology adaptation.

As mastitis control programme was in proress since the year 2014-15 in the milk sheds of Sabar Dairy, a very high parcentage (76%) of farmers were aware regarding presence of antibiotic residues in milk and only a few farmers (22%) were in favour of using antibiotic for the treatment of their animals. In a fresh survey coverining three different milk sheds, pre and post intervention awareness on the significance antibiotic

residue were found to be increased from 19.2 % to 74.6 % indicating the usefulness of the awareness campaign.

In India, around 70 million farmers depand on dairying (Chauhan *et al.,* 2018). Several reports are available on the non-judicious use of antibiotics in milch animals as well as lack of adequate focus on milk quality in small scale dairy farming (Chauhan *et al*., 2018, Mutua *et al*., 2020, Sharma *et al*., 2020). Kumar *et al.* (2021) has documented very little awareness (2%) among farmers regarding milk withdrawal period after the use antibiotics in the lactating animals.

The Food Safety and Standards (Contaminants, Toxins, and Residues) Regulations by the FSSAI published guidelines on the permissible limits of antibiotics (FSSAI, 2011 and 2018) in milk and milk products. Although, there is need to conduct extensive awareness programmes among farmers in simple languages to understand and follow these guidelines by them.

During survey in Sabar dairy, it was observered that 92 % respondents were aware regarding use of traditional herbal medicines in cattle and buffaloes whereas 77.55 % considered traditional herbal medicines as cost effective and easily available. Awareness regarding availability of traditional herbal medicines for management of bovine ailments like mastitis, diarrhoea, fever, repeat breeding and FMD (Foot and Mouth Disease) were in between 22-78 %. In the next survey, the overall farmer awareness on use of EVHP was increased from 12.3 % to 45.22 %.

Bhatt *et al.* (2019) investigated on ethnoveterinary practices at Junagarh, Gujarat among farmers revealed that they use different medicinal plants for 13 different animal ailments like gastrointestinal issues, skin-related disorders, respiratory problems, wound healing etc.

Hence, continual pursuance of awareness drive among the dairy farmers on the harmful effect of mastitis, rationalization on the use of antibiotics and usefulness of EVHP would be of immence advantage on milk production and its enhanced quality.

4.2. Isolation, identification and antibiogram of the bacterial agents from mastitic milk

The milk samples were taken from various MU/MPCs i.e. Aurangabad Dist. Cooperative Milk Producer Union Limited; Baani Milk Producer Company Limited, Bengaluru Urban, Rural and Ramanagara District Cooperative Milk Producers Societies Union Limited; Baramati Taluka Sahakari Doodh Utpadak Sangh; Dakshina Kannada Cooperative Milk Producers' Union Limited; Erode District Cooperative Milk Producers' Union Limited; Kishan Cooperative Milk Producer's Union Limited; Kolhapur Zilla Sahakari Dudh Utpadak Sangh Limited; Krishna District Cooperative Milk Producers' Mutually Aided Cooperative Union Limited; Ludhiana District Cooperative Milk Producers' Union Limited; Malabar Regional Cooperative Milk Producers' Union; Mysore Dist. Cooperative Milk Producers' Societies' Union Limited; Pune Zilha Sahakari Dudh Utpadak Sangh; Rajarambapu Patil Sah.Dudh Sangh Limited; Saahaj Milk Producer Company Limited; Sabarkantha District Cooperative Milk Producers' Union Limited; Salem District Cooperative Milk Producers Union Limited; Shreeja Mahila Milk Producer Company Limited; Tumkur Cooperative Milk Producers Societies Union Limited and West Assam Milk Producers' Co-operative Union Limited. Although, six samples were processed from individual dairy farmers from Telangana and Madhya Pradesh, not affiliated to any MU/MPCs.

The details of the samples processed and results recorded as depicted in Table 10.

Table 10: No. of milk samples processed for bacteriological isolation and identification from different milk unions and Producer Companies

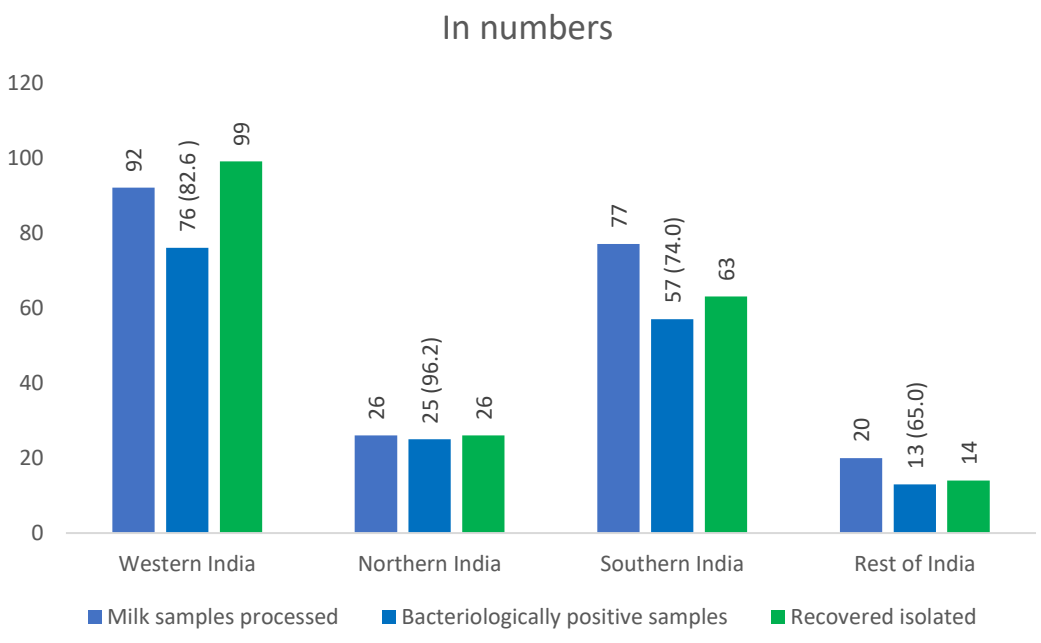
Mastitis Type	No of milk samples subjected for bacteriological isolation	Culture and identification method	No of samples positive for bacteriological culture	No of bacterial isolates obtained from culture positive samples
Clinical Mastitis (CM)	146	BD phoenix	109	120
	10	BD phoenix	8	9*
	19	Conventional	16	22*

Sub-clinical Mastitis (SCM)	23	BD phoenix	21	24
	7	BD phoenix	7	11*
	10	Conventional	10	16*
Total	215		171 (79.5%)	202

* Post EVHP sample were also collected

A total of 215 milk samples were subjected for bacteriological isolation from the above areas, out of which 171 samples were recorded positive for bacterial isolation (79.5%). This finding agrees with Suheyla *et al.* (2010) who recorded 83.2% of milk samples yielding bacterial growth. Although, Makovec and Ruegg (2003) documented only 49.7% culture positive samples from mastitic milk which is not accordance to our present study.

The distribution of milk samples received and bacterial isolates recovered from various Zones in India is shown in Figure 11.



(* figures shown in paranthesis is the per cent culture positive samples)

Figure 11: Distribution of milk samples received and bacterial isolates recovered from various Zones in India

The reasons for obtaining culture negative milk samples may be due to low bacterial concentration in the sample (Sears *et al.*, 1990; Hogan *et al.* (1999), influence of pre-culture incubation, freezing etc (Schukken *et al*., 1989; Dinsmore *et al.*, 1992). Another reason may be due to prior treatment of affected animals with antibiotic before taking sample. Rinaldia *et al*. (2010) stated that microbial species of at least 137 nos. including bacteria, yeast, fungi and algae, could be responsible for bovine mastitis. Therefore, the mastitis affected animal samples with bacterial culture negative results could be attributed due to other etiological agents.

4.2.1. Isolation of bacteria:

For primary isolation of bacteria from the mastitis milk samples, Blood Agar was selected and thereafter special agar such as EMB, MLA, MSA, etc. were used for isolation of specific organism (Figure 12-15). Subsequently, organisms from simple colony subjected to Gram staining for morphological examination (Figure 16-18).

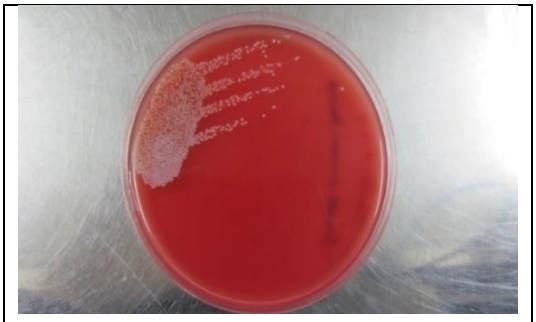


Figure 12: Primary isolation of bacteria on Blood Agar

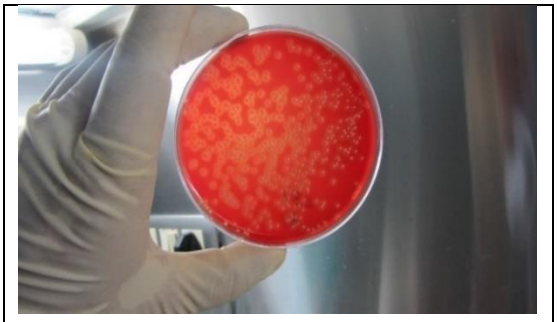


Figure 13: Haemolytic colonies recorded on blood agar

Figure 14: Isolation of *E coli* and Klebsiella in McKonkey Agar

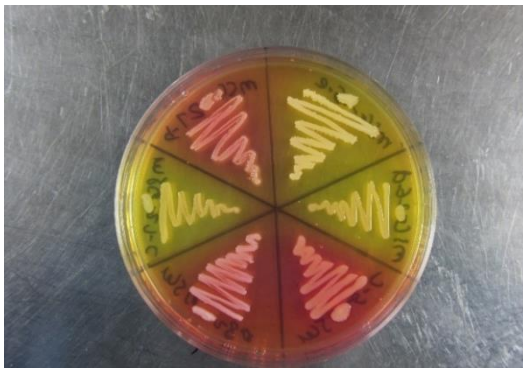


Figure 15: Coagulase positive (yellow) organism on Mannitol salt agar

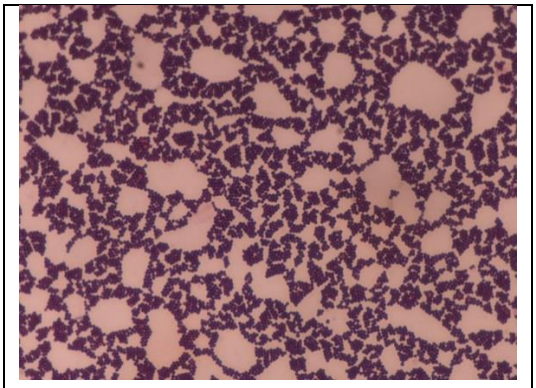


Figure 16: Gram +ve organism (cocci) in clusters

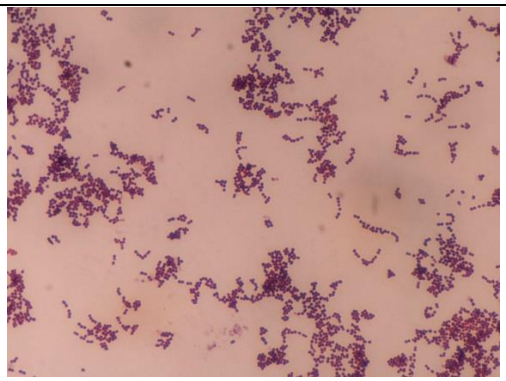


Figure 17: Gram +ve organism (cocci) in chain

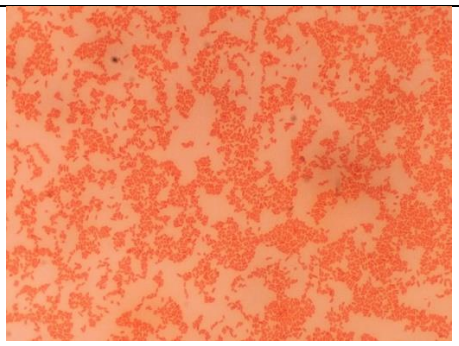


Figure 18: Gram -ve organism (bacilli)

4.2.2. Identification of bacterial isolates

A total of 202 bacterial isolates were recovered, of which 164 isolates were identified using the BD phoenix M50 instrument (manufacturer manual) and remaining 38 were identified through conventional bacterial identification techniques.

4.2.2.1 Identification of bacterial isolates by BD Phoenix M50 detection system

Bacterial agents recovered from mastitic milk were initially screened for Gram +ve and Gram –ve categories. Based on these results, 164 isolates were processed using the BD Phoenix detection system for further cheracterisation, out of which 97 isolates were processed through the Gram-positive combo panel (PMIC/ID-70), 40 isolates through SMIC /ID 9 combo panel for streptococcus and the remaining 27 isolates were screened using the Gram-negative panel (NMIC/ID-55).

Kleb. pneumoniae and E. coli were the main Gram -ve pathogens identified in the present study using the NMIC/ID-55 combo panel. Table 11 provides details on these Gram +Ve isolates.

Table 11: Details of identification of bacterial isolates by Gram -ve combo panel (NMIC/ID-55) of BD Phoenix M50 detection system

Sr. No	Identified bacteria in BD phoenix M50	No of isolates recorded
1	*Kleb. Pneumoniae*	9
2	*Kleb. Aerogens*	1
3	*E. coli*	8
4	*Pseudomonas oryzihabitans*	2
5	*Pseudomonas pseudoalcaligenes*	2
6	*Serratia plymuthica*	2
7	*Moraxella spp*	1
8	*Pseudomonas aeruginosa*	1
9	*Pseudomonas putida*	1
Total		27

Staphylococcus, Kocuria, and Enterococcus spp. were recorded as the predominant Gram +ve bacterial agents (apart from Streptococcus spp.), identified employing the PMIC/ID-70 combo panel and *Staph. aureus* was emerging as the most prevalent organism (Table 12).

Table 12: Details of identification of bacterial agents identified by Gram +ve combo panel (PMIC/ID-70) through BD Phoenix M50 detection system

Sr. No	Identified bacteria in BD phoenix M50	Number of isolates recorded
1	*Staph. Aureus*	39
2	*Other Staphylococcus spp. (38 nos.)*	
A	*Staph. Haemolyticus*	10
B	*Staph. Chromogenes*	5
C	*Staph. Hominis*	4
D	*Staph. Intermedius*	4
E	*Staph. Lentus*	4
F	*Staph. Schleiferi*	3
G	*Staph. Sciuri*	3
H	*Staph. Caprae*	1
I	*Staph. Epidermidis*	1
J	*Staph. Equorum*	1
K	*Staph. Gallinarum*	1
L	*Staph. Vitulinus*	1
3	*Kocuria varians*	9
4	*Kocuria kristinae*	4
5	*Enterococcus faecalis*	4
6	*Aerococcus viridans*	1
7	*Bacillus cereus*	1
8	*Enterococcus hirae*	1
Total		97

Str. uberis, *Str. agalactiae (Strep. group B) and Str. dysgalactiae* were the major agents identified through SMIC/ID 9 combo panel. Details of *Streptococcus* spp isolated are depicted in Table 13.

Table 13: Details of identification of Streptococcus isolates through SMIC/ID 9 Combo panel of BD Phoenix M50 detection system

Sr. No.	Identified bacteria in BD phoenix M50	Number of isolates recorded
1	*Str. Uberis*	17
2	*Str. agalactiae (Strep. group B)*	10
3	*Str. Dysgalactiae*	10
4	*Str. Mitis*	1
5	*Str. group C/G (large colony)*	1
6	*Str. gallolyticus ssp pasteurianus/infantarius*	1
Total		40

Thus, major Gram -ve pathogens recovered during the present study through NMIC/ID-55 combo panel were *Kleb. pneumoniae* and *Escherichia coli.* Major Gram +ve pathogens (except *Streptococcus* spp.) recovered during the present study through PMIC/ID-70 combo panel were *Staphylococcus, Kocuria and Enterococcus spp of which Staph. aureus* was found to be predominant. *Str. uberis, Str. agalactiae (Strep. group B) and Str. dysgalactiae* were the major pathogens isolated through SMIC/ID 9 combo panel.

species-wise, *Staphyloccus spp*. was the most commonly isolated species (51%), followed by *Streptoccus spp*. (21%), *E. coli* (17.8%), *Klebsiella spp.* (6%) and various other species (14%). Thorberg *et al*., 2009; Hegde *et al.* 2013 and Preethirani *et al.* 2015 had also recorded similar pattern of bacterial isolation for *Staphylococcus spp., Streptococcus spp. and E. coli.*

Details of species-wise isolates recovered in the present study being depicted in Figure 19.

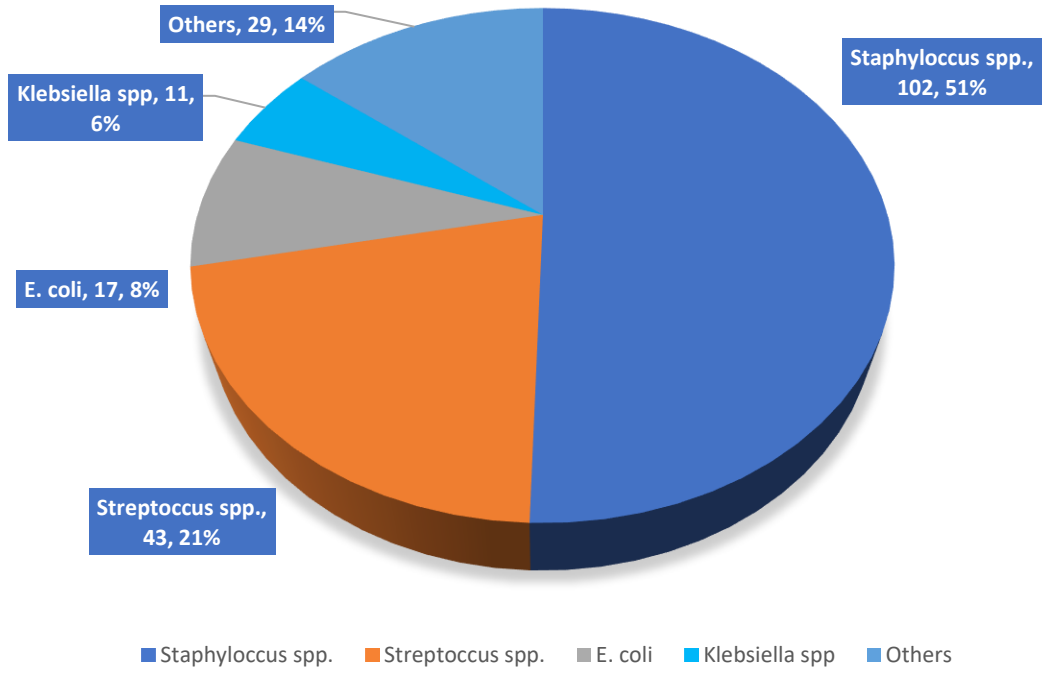


Figure 19: Distribution of bacterial agents those were identified from the cases of bovine mastitis (n=202)

In line with reports, Staphylococcus spp. are the primary etiological agents of mastitis in cattle and buffaloes in India (NAAS, 2013).

In the present study, incidence of *Staph. aureus* (identified by BD system) was recorded to be 27 % (39 nos. of 145 culture positive samples) in respect to overall distribution bacterial agent causining bovine mastitis. Zone wise, the distribution of *Staph. aureus* was found to be maximum in Northern India (36 %), followed by Southern India (28 %) and Western India (22 %). Its average incidence in remaining part of India was 23 % (Figure 20). However, according to a number of studies (*Hazari et al.*, 2018; *Mubarack et al.*, 2012; *Singh et al.*, 2017; *Thakur et al.*, 2016), incidence *Staph aureus* incidence remained generally higher

in cases of mastitis in the states of Chhattisgarh (54.66%), Tamil Nadu (70.30%), Gujarat (12.6%), and Haryana (35.29%).

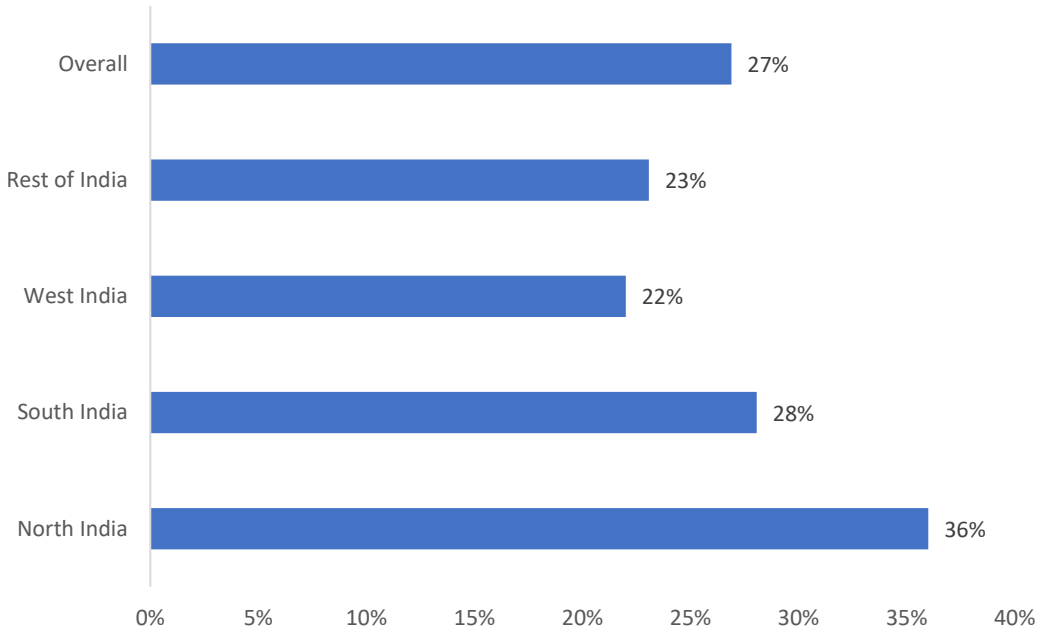


Figure 20: Zone-wise distribution of *Staph aureus* causing bovine mastitis

In a comparable study, *Str. agalactiae, E. coli, Str. dysgalactiae, Staph. aureus* and *Klebsiella spp.* were also found to be the most commonly isolated bacterial agents from clinical caes of bovine mastistis in Gujarat by Dutta *et al.* (2018). According to Rana *et al.* (2019), clinical and sub-clinical mastitis have been caused by *Staph. aureus, Str. dysgalactiae, Srt. agalactiae, Str. uberis, Klebsiella spp., E. coli, Staph. xylosus*, including non-*Staph. aureus, Aerococcus viridians, and Staph. Epidermidis*. Contagious pathogens like *Staph. aureus, Str. agalactiae* and environmental pathogens like *E. coli, Kleb. pneumoniae* are common mastitis pathogens found predominantly. *Streptococcus dysgalactiae* seems to be both environmental and contagious (Radostits and Arundel, 2000; Hogan *et al.* 1999; Pieterse *et al.* 2010).

Isolation of *Staphylococcus haemolyticus, Staphylococcus chromogenes, Staphylococcus hominis, Staphylococcus intermedius, Staphylococcus lentus, Staphylococcus sciuri, Staphylococcus caprae, Staphylococcus epidermidis, Staphylococcus equorum, Staphylococcus gallinarum, Staphylococcus vitulinus, Kocuria varians, Kocuria kristinae, Enterococcus faecalis, Aerococcus viridans, Bacillus cereus. Enterococcus hirae, Streptococcus mitis, Streptococcus gallolyticus ssp pasteurianus/infantarius, Moraxella spp, Pseudomonas aeruginosa and*

Pseudomonas putida from milk have also been reported by several authors (Krishnamoorthy *et al.* 2016; Dabele *et al.*, 2021; Moroni *et al.*, 2005; Traversari *et al.*, 2019; Larissa *et al.*, 2016; Kenar *et al.*, 2019; Bag *et al.*, 2022; Sun *et al.*, 2017; Abraha *et al.*, 2020; Kaya and Turkyılmaz., 2019; Ahmed *et al.*, 2020; Audrey *et al.*, 2009; Abeer *et al.*, 2016; Hsieh *et al.*, 2019).

4.2.2.2. Identification of bacterial isolates by conventional bacterial identification method

Thirty-eight isolates were identified through conventional identification technique due to unavailability of BD system during that period (Table 14). The identification was carried out up-to genus level except *E. coli.* The isolates were identified as per standard method (Quinn *et al.*, 2000).

Table 14: Details of identification of bacterial isolates through conventional method

S No	Identified bacteria	No of isolates	% recovery
1	*Staphylococcus spp.*	25	65.79 %
2	*E. coli*	9	23.68 %
3	*Streptococcus spp.*	3	7.9 %
4	*Klebsiellae spp.*	1	2.63 %
Total		38	

Major isolated bacterial spp. Identified through conventional method were *Staphylococcus spp. (65.79 %) followed by E. coli (23.68 %), Streptococcus* spp. 7.9 % and *Klebsiellae spp.* 2.6 %. Bhalerao *et al*., (2000) had noted that major pathogenic organisms in mastitis were *Staphylococcus. aureus* (54.55%) followed by the *Strepococci spp* (36.36%), *E. coli* (4.55%) and *Klebsiella* spp. (2.27%). In our present study, in the bacteria isolated through conventional method, the recovery percentage of Streptococcus is lesser than findings of Bhalerao *et al*., (2000).

4.2.2.3. Confirmation of bacterial isolates by Polymerase Chain Reaction (PCR)

A total of 60 bacterial isolates were subjected to species specific PCR available for 6 different bacterial species i.e. *E. coli, Kleb. pneumoniae, Staph. aureus, Str. agalactiae, Str. dysgalactiae* and *Str. uberis.* Details of the PCR results for species identification are depicted in Table no 15.

Table 15: Bacterial isolates identified by Species specific Polymerase Chain Reaction (PCR)

S. No.	Bacteria identified by BD system	No. of isolates	Bacteria identified by species specific PCR
1	*E. coli*	6	*E. coli*
2	*Kleb. pneumoniae*	8	*Kleb. pneumoniae*
3	*Staph. aureus*	32	*Staph. aureus*
4	*Str. agalactiae (Strep. group B)*	6	*Str. agalactiae*
5	*Str. dysgalactiae*	3	*Str. dysgalactiae*
6	*Str. Uberis*	5	*Str. Uberis*
	Grand Total	60	

A 100 % matching in species identification through BD system and PCR results was recorded for all the isolates. Species-Specific PCR image in agarose gel electrophoresis of confirmed isolates of *Kleb. pneumoniae is* depicted in Figure 21.

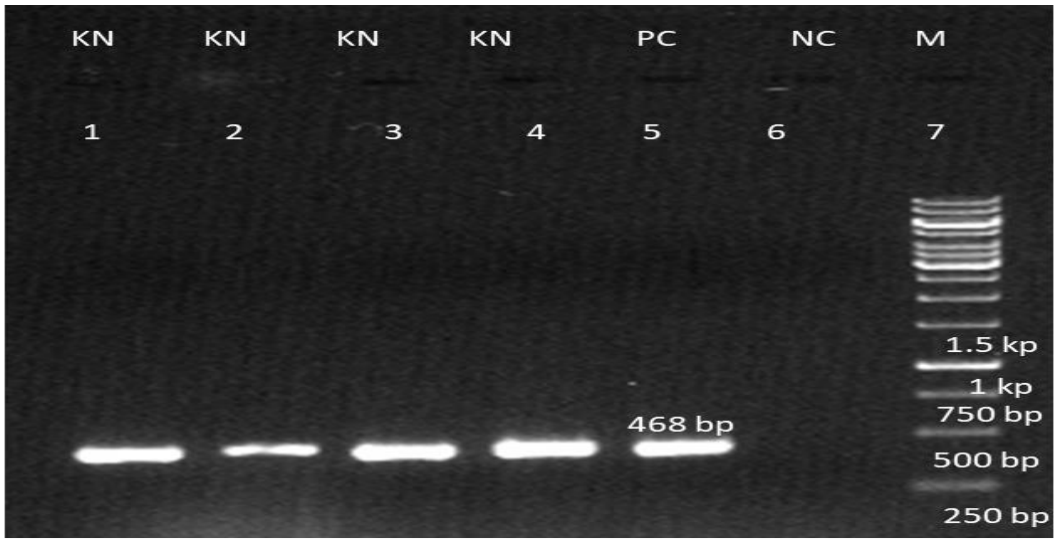


Figure 21: *A*garose gel electrophoresis image depicting the presence of gyrA gene in *Kleb. pneumoniae* (kleb primer: 468bp) Lane 1, 2, 3 and 4 : *Kleb. pneumoniae (KN), Lane 5:* Positive control (PC), Lane 6: Negative Control (NC), M: 1 kb Marker

Species-Specific PCR image of agarose gel electrophoresis of confirmed isolates of *Str. uberis, Str. agalactiae, Str. dysgalactiae in Figure 22.*

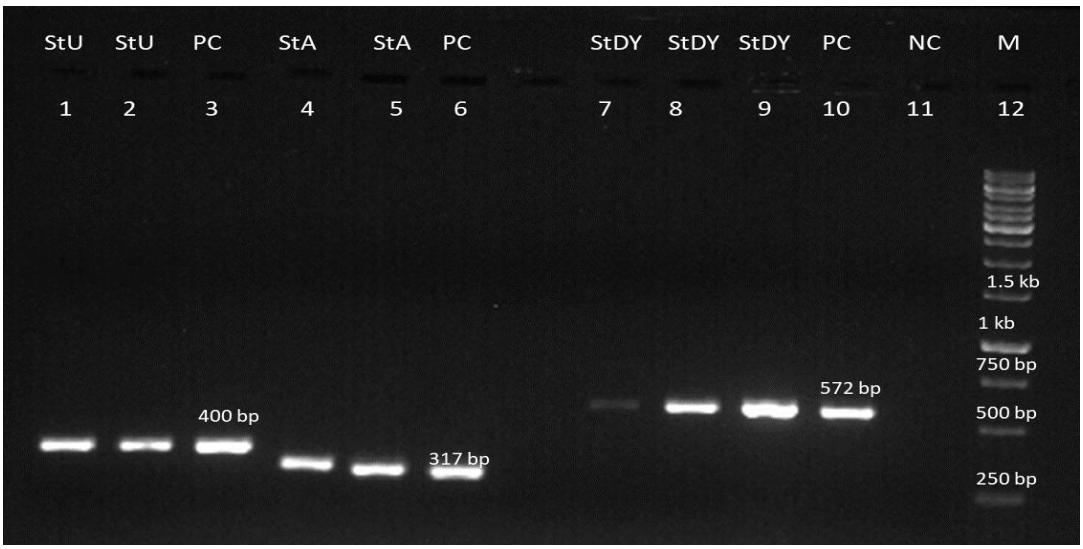


Figure 22: *A*garose gel electrophoresis image depicting the presence of cpn 60, 16Sr RNA and 16Sr RNA gene in *Str. uberis, Str. agalactiae, Str. dysgalactiae respectively* (400bp, 317bp, 572bp) Lane 1, 2 : *Strep uberis (StU), Lane 3:* Positive control (PC), Lane 4,5: *Strep agalactiae* (StA), *Lane 6:* Positive control (PC), Lane 7,8, 9: *Strep. dysgalactiae* (StDy), *Lane 9:* Positive control (PC), Lane 11: Negative Control (NC), Lane 12: 1 kb Marker (M)

Image of Species-Specific PCR agarose gel electrophoresis of confirmed isolates of *Staph. aureus* and *E coli in Figure 23*

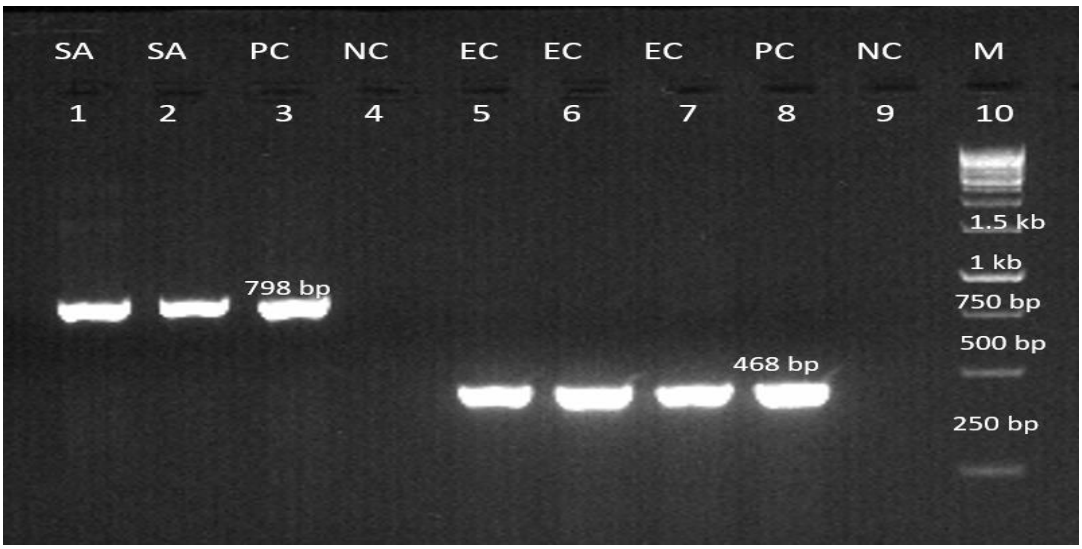


***Figure 23: A*garose gel electrophoresis image depicting the presence of nuc and phoA gene in *Staph. aureus* and *E coli* respectively (798bp, 468bp resp.)** Lane 1, 2 : *Staph. aureus (SA), Lane 3:* Positive control (PC), Lane 4: Negative Control (NC), Lane ,5, 6, 7: *E. coli* (EC), *Lane 8:* Positive control (PC), Lane 9: Negative Control (NC), Lane 10: 1 kb Marker (M)

4.2.3. Antibiogram of bacterial isolates

4.2.3.1. Antibiogram of bacterial isolates through BD phoenix M50 instrument:

The BD Phoenix M50 equipment was used to perform an antibiogram on 123 isolates. Details of bacterial isolates processed for antibiogram through BD phoenix are illustrated in table no 16. Seventy-seven bacterial strains could not be processed for an antibiogram using BD Phoenix system due to lack of instructions in the BD system.

Table 16: No. of bacterial isolates processed for antibiogram

S. No.	Species of bacteria	Nos. subjected for phenotypic antibiotic sensitivity
1	*Staph. Aureus*	39
2	*Kleb. Pneumoniae*	9
3	*Kleb. Aerogens*	1
4	*Staph. Haemolyticus*	10
5	*Str. agalactiae*	10
6	*Str. dysgalactiae*	10
7	*E. coli*	8
8	*Staph. Chromogenes*	5
9	*Staph. Intermedius*	4
10	*Enterococcus faecalis*	4
11	*Staph. Hominis*	3
12	*Staph. Lentus*	4
13	*Staph. Schleiferi*	3
14	*Staph. Sciuri*	3
15	*Pseudomonas pseudoalcaligenes*	2
16	*Staph. Epidermidis*	2
17	*Enterococcus hirae*	1
18	*Serratia plymuthica*	1
19	*Staph. Caprae*	1
20	*Pseudomonas putida*	1
21	*Staph. gallinarum*	1
22	*Staph. equorum*	1
		123

4.2.3.2. Results of antibiogram in BD Phoenix M50 detection system/ Disc diffusion method

The selected isolates' antibiogram was performed in accordance with their classification into three groups: 'Gram -ve bacteria', 'Gram +ve bacteria except for *Streptococcus spp', and 'Streptococcus spp'*. Susceptibility tests were performed on six kinds of Gram -ve microorganisms to 18 different antibiotics and antibiotic combinations, which are shown in Table 17.

The Gram +ve microorganisms were tested for susceptibility to 15 various antibiotics and antibiotic combinations, with the exception of Streptococcus species. The study's inclusion of 14 bacterial species, which include *Staphyloccus* and *Enterococcus* species, are shown in table no. 18.

The Streptococcus species were subjected to sensitivity towards 12 antibiotics/antibiotic combinations depicted in table no 19. The bacteria included were *Str. agalactiae* and *Str. dysgalactiae.*

The disc diffusion method was adopted to assess the antibiotic sensitivity of *Strep. uberis* strains using discs coated with five different antibacterial agents: Amoxicillin (AMX), Ceftriaxone (CTR), Oxytetracycline (O), Ampicillin (AMP), and Penicillin-G (P) (Figure 20 and Table 22)

Table 17: Antibiogram pattern of Gram -ve bacteria through BD Phoenix M50 detection system

S. No.	Identified bacteria in BD phoenix M50	Sensitivity status	Antibiotic name*																
			Amikacin	**Amoxicillin-Clavulanate**	**Ampicillin**	**Aztreonam**	**Cefepime**	**Cefotaxime**	**Cefoxitin**	**Ceftazidime**	**Chloramphenicol**	**Ciprofloxacin**	**Gentamicin**	**Imipenem**	**Levofloxacin**	**Meropenem**	**Piperacillin**	**Piperacillin- Tazobactam**	**Tetracycline**	**Trimethoprim- Sulfamethoxazole**
1	E. coli	*Sensitive*	8	6	4	6	6	**6**	6	6	8	7	7	8	7	8	6	8	5	5
		Intermediate						1												
		Reisistant	0	2	4	2	2	1	2	2	0	1	1	0	1	0	2	0	3	3
		Total	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
		% resistance	0.0	25.0	50.0	25.0	25.0	12.5	25.0	25.0	0.0	12.5	12.5	0.0	12.5	0.0	25.0	0.0	37.5	37.5
2	*Kleb. pneumoniae*	*Sensitive*	9	9	0	8	8	8	8	8	9	9	9	9	9	9	9	9	9	8
		Intermediate						1												
		Resistant	0	0	9	1	1	0	1	1	0	0	0	0	0	0	0	0	0	1
		Total	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
		% resistance	0.0	0.0	100.0	11.11	11.11	0.00	11.11	11.11	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	11.11
3	Pseudomonas pseudoalcaligenes	*Sensitive*	2	0	0	0	2	1	0	1	1	2	2	2	2	2	2	2	2	2
		Intermediate						1			1									
		Resistant	0	2	2	2	0	0	2	1	0	0	0	0	0	0	0	0	0	0
		Total	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2

		% resistance	0.0	100.0	100.0	100.0	0.0	0.0	100.0	50.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
4	*Kleb. aerogenes*	*Single isolate*	S	R	R	R	R	I	R	R	S	S	S	S	S	S	S	S	S	S
5	*Pseudomonas putida*	*Single isolate*	S	R	R	R	S	I	R	S	R	S	S	S	S	S	S	S	S	S
6	*Serratia plymuthica*	*Single isolate*	R	R	R	R	S	S	S	S	S	S	I	S	S	S	S	S	R	S
*No of isolates																				

The strains of *E coli* isolated from mastitic milk showed highest resistance to Ampicillin (50 %) followed by Tetracycline, Trimethoprim- Sulfamethoxazole (37.5 % each); Amoxicillin-Clavulanate, Cefepime, Aztreonam, Cefoxitin, Ceftazidime, Ceftazidime, Piperacillin (25 % each) and Cefotaxime, Ciprofloxacin, Gentamicin, Levofloxacin (12.5 % each). No resistance was observered for Amikacin, Chloramphenicol, Imipenem and Piperacillin-Tazobactam. Sadashiv and Kaliwal (2015) found resistant *E. coli* strains for Cefpodoxime (62.88%), Ampicillin (49.48%), Cefaclor and Ceftriaxone (42.26%), Cefixime (38.14%), Amoxyclav and Gentamicin (31.95%), Amikacin (23.71%), Chloramphenicol (9.27%), Tetracycline (7.21%), Ciprofloxacin (4.12%). Although, in present study, for Amikacin and Chloramphenicol, no resistance was observed.

The strains of *Kleb. pneumoniae* isolated from mastitic milk showed highest resistance to Ampicillin (100 %) followed by Aztreonam, Cefepime, Cefoxitin, Ceftazidime, Trimethoprim- Sulfamethoxazole (11.11 % each). No resistance was observered for Amikacin, Amoxicillin-Clavulanate, Chloramphenicol, Cefotaxime, Ciprofloxacin, Gentamicin, Levofloxacin Imipenem, Piperacillin, Piperacillin-Tazobactam and Tetracycline. Yadav *et al.* (2021) observed resistance of *Kleb. pneumoniae isolates* against penicillin group of antibiotics, streptomycin and aminoglycosides such as amikacin, neomycin and kanamycin. They recorded succeptablity of the isolates to chloramphenicol, oxytetracycline, gentamicin and levofloxacin which are accoredence to our finding.

Klebsiella aerogenes isolate having resistance for Amoxicillin-Clavulanate, Ampicillin, Aztreonam, Cefepime, Cefoxitin and Ceftazidime. *Pseudomonas putida* isolate having resistance for Amoxicillin- Clavulanate, Ampicillin, Aztreonam, Cefoxitin and Chloramphenicol. *Serratia plymuthica* isolate having resistance for Amikacin, Amoxicillin- Clavulanate, Ampicillin, Aztreonam, colistin and Tetracycline.

The strains of Pseudomonas pseudoalcaligenes isolated from mastitic milk showed highest resistance to Ampicillin, Amoxicillin-Clavulanate, Aztreonam, Cefoxitin, (100 %) followed by Ceftazidime (50 %). No resistance was observered for Amikacin, Chloramphenicol, Cefotaxime, Cefepime, Trimethoprim-Sulfamethoxazole, Ciprofloxacin, Gentamicin, Levofloxacin Imipenem, Piperacillin, Piperacillin-Tazobactam and Tetracycline.

Table 18: Antibiogram pattern of Gram +ve bacteria excluding Streptoccus spp. through BD Phoenix M50 detection system

S. No.	Identified bacteria in BD phoenix M50	Sensitivity status	Antibiotic name*													
			Cefazolin	Clindamycin	Daptomycin	Erythromycin	Gentamicin	Linezolid	Nitrofurantoin	Norfloxacin	Oxacillin	Quinupristin-dalfopristin	Rifampin	Teicoplanin	Tetracycline	Trimethoprim-Sulfamethoxazole	Vancomycin
1	*Staph. aureus*	*Sensitive*	32	37	39	34	34	38	39	31	32	37	38	38	37	35	38
		Intermediate								1							
		Resistant	7	2	0	5	5	1	0	7	7	2	1	1	2	4	1
		Total	39	39	39	39	39	39	39	39	39	39	39	39	39	39	39
		% Resistant	17.9	5.1	0.0	12.8	12.8	2.6	0.0	17.9	17.9	5.1	2.6	2.6	5.1	10.3	2.6
2	*Enterococcus faecalis*	*Sensitive*	0	0	4	1	0	4	3	3		0		4	1	0	1
		Intermediate				3			1	1							
		Resistant	4	4	0	0	4	0	0	0		4		0	3	1	0
		Total	4	4	4	4	4	4	4	4		4		4	4	1	1
		% Resistant	100.0	100.0	0.0	0.0	100.0	0.0	0.0	0.0		100.0		0.0	75.0	100.0	0.0
3	*Staph. chromogenes*	*Sensitive*	5	5	5	5	5	5	5	5	5		5	5	5	5	5
		Resistant	0	0	0	0	0	0	0	0	0		0	0	0	0	0
		Total	5	5	5	5	5	5	5	5	5		5	5	5	5	5
		% Resistant	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	0.0	0.0	0.0	0.0

S. No.	Identified bacteria in BD phoenix M50	Sensitivity status	Antibiotic name*													
			Cefazolin	Clindamycin	Daptomycin	Erythromycin	Gentamicin	Linezolid	Nitrofurantoin	Norfloxacin	Oxacillin	Quinupristin-dalfopristin	Rifampin	Teicoplanin	Tetracycline	Trimethoprim-Sulfamethoxazole	Vancomycin
4	*Staph. haemolyticus*	*Sensitive*	8	9	9	6	10	9	10	10	8	0	9	9	8	10	9
		Resistant	2	1	0	4	0	1	0	0	2	1	1	1	1	0	1
		Total	10	10	9	10	10	10	10	10	10	1	10	10	9	10	10
		% Resistant	20.0	10.0	0.0	40.0	0.0	10.0	0.0	0.0	20.0	100.0	10.0	10.0	11.1	0.0	10.0
5	*Staph. hominis*	*Sensitive*	2	2	3	1	3	2	3	3	2		2	2	3	3	2
		Resistant	1	1	0	2	0	1	0	0	1		1	1	0	0	1
		Total	3	3	3	3	3	3	3	3	3		3	3	3	3	3
		% Resistant	33.3	33.3	0.0	66.7	0.0	33.3	0.0	0.0	33.3		33.3	33.3	0.0	0.0	33.3
6	*Staph. epidermidis*	*Sensitive*	0	2	2	1	1	2	2	2	0		2	2	1	0	2
		Intermediate				1											
		Resistant	2	0	0	0	1	0	0	0	2		0	0	1	2	0
		Total	2	2	2	2	2	2	2	2	2		2	2	2	2	2
		% Resistant	100.0	0.0	0.0	0.0	50.0	0.0	0.0	0.0	100.0		0.0	0.0	50.0	100.0	0.0
7	*Staph. sciuri*	*Sensitive*	0	0	3	3	2	3	2	3	0		3	2		3	2
		Intermediate		2													
		Resistant	3	0	0	0	0	0	0	0	3		0	0		0	0

S. No.	Identified bacteria in BD phoenix M50	Sensitivity status	Antibiotic name*													
			Cefazolin	**Clindamycin**	**Daptomycin**	**Erythromycin**	**Gentamicin**	**Linezolid**	**Nitrofurantoin**	**Norfloxacin**	**Oxacillin**	**Quinupristin-dalfopristin**	**Rifampin**	**Teicoplanin**	**Tetracycline**	**Trimethoprim-Sulfamethoxazole**	**Vancomycin**
		Total	3	2	3	3	2	3	2	3	3		3	2		3	2
		% Resistant	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0		0.0	0.0		0.0	0.00
8	*Staph. schleiferi*	*Sensitive*	3	3	3	3	3	3	3	3	3		3	3	3	3	3
		Resistant	0	0	0	0	0	0	0	0	0		0	0	0	0	0
		Total	3	3	3	3	3	3	3	3	3		3	3	3	3	3
		% Resistant	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	0.0	0.0	0.0	0.0
9	*Staph. lentus*	*Sensitive*	0	2	4	4	4	4	4	4	0		3	4	4	4	4
		Intermediate		2													
		Resistant	4	0	0	0	0	0	0	0	4		1	0	0	0	0
		Total	4	4	4	4	4	4	4	4	4		4	4	4	4	4
		% Resistant	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0		25.0	0.0	0.0	0.0	0.0
10	*Staph. intermedius*	*Sensitive*	4	4	4	4	4	4	4	4	4		4	4	4	4	4
		Resistant	0	0	0	0	0	0	0	0	0		0	0	0	0	0
		Total	4	4	4	4	4	4	4	4	4		4	4	4	4	4
		% Resistant	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	0.0	0.0	0.0	0.0
11	*Enterococcus hirae*	*Single isolate*	R	R	S	I	R	S	R	S	R		-	S	S	R	S

S. No.	Identified bacteria in BD phoenix M50	Sensitivity status	Antibiotic name*													
			Cefazolin	**Clindamycin**	**Daptomycin**	**Erythromycin**	**Gentamicin**	**Linezolid**	**Nitrofurantoin**	**Norfloxacin**	**Oxacillin**	**Quinupristin-dalfopristin**	**Rifampin**	**Teicoplanin**	**Tetracycline**	**Trimethoprim-Sulfamethoxazole**	**Vancomycin**
12	*Staph. caprae*	*Single isolate*	S	S	S	S	S	S	S	S	S		S	S	S	S	S
13	*Staph. equorum*	*Single isolate*	S	S	S	I	S	S	S	S	S		S	S	S	S	S
14	*Staph. gallinarum*	*Single isolate*	R	S	S	S	S	S	S	S	R		S	S	S	S	S
*No. of Isolates																	

Strains of *Staph. aureus* isolated from mastitic milk showed highest resistance to Cefazolin, Norfloxacin (18.4 %) followed by Oxacillin (17.9 %), Erythromycin, Gentamicin (12.8 %), Trimethoprim- Sulfamethoxazole (10.3 %), Clindamycin, Quinupristin-dalfopristin, Tetracycline (5.1 %), Linezolid, Rifampin, Teicoplanin and Vancomycin (2.6 %). No resistance was observered for Daptomycin and Nitrofurantoin. Neelam *et al. (2022) has recorded* maximum resistance of *Staph. aureus* isolates from milk sample to oxytetracycline, penicillin, oxacillin, gentamicin, enrofloxacin, lincomycin and minimum resistance to methicillin and cefuroxime antimicrobials.

Strains of *Staph. haemolyticus* isolated from mastitic milk showed highest resistance to Erythromycin (40 %) followed by Cefazolin, Oxacillin (20 %), Tetracycline (11.1 %), Clindamycin, Linezolid, Quinupristin-dalfopristin, Rifampin, Teicoplanin, Vancomycin (10 %). No resistance was observered for Daptomycin, Gentamicin, Norfloxacin, Nitrofurantoin and Trimethoprim- Sulfamethoxazole. Strains of *Staphylococcus hominis* isolated from mastitic milk showed highest resistance to Erythromycin (66.7 %) followed by Cefazolin, Clindamycin, Linezolid, Oxacillin Rifampin, Teicoplanin, Vancomycin (33.3 %), No resistance was observered for Daptomycin, Gentamicin, Norfloxacin, Nitrofurantoin, Tetracycline and Trimethoprim- Sulfamethoxazole. Strains of *Staph. epidermidis* isolated from mastitic milk showed highest resistance to Cefazolin, Oxacillin, Trimethoprim-Sulfamethoxazole (100 %), followed by Gentamicin, Tetracycline (50 %), No resistance was observered for Clindamycin, Daptomycin, Erythromycin, Linezolid, Norfloxacin, Nitrofurantoin, Rifampin, Teicoplanin and Vancomycin. Strains of *Staphylococcus sciuri* isolated from mastitic milk showed highest resistance to Cefazolin, Oxacillin (100 %). No resistance was observered for Clindamycin, Erythromycin, Daptomycin, Linezolid, Gentamicin, Norfloxacin, Nitrofurantoin, Trimethoprim- Sulfamethoxazole, Rifampin, Teicoplanin and Vancomycin. Strains of *Staphylococcus lentus* isolated from mastitic milk showed highest resistance to Cefazolin, Oxacillin (100 %) followed by Rifampin (25 %). No resistance was observered for Clindamycin, Erythromycin, Daptomycin, Linezolid, Gentamicin, Nitrofurantoin, Norfloxacin, Trimethoprim- Sulfamethoxazole, Teicoplanin and Vancomycin. Strain of *Staph. caprae* and *Staph. equorum* isolated from mastitic milk showed no resistance to Cefazolin, Clindamycin**,** Gentamicin, Nitrofurantoin Oxacillin and Trimethoprim-Sulfamethoxazole, Daptomycin, Erythromycin, Linezolid, Norfloxacin, Rifampin, Tetracycline, Teicoplanin and Vancomycin. Strain of *Staph.*

gallinarum isolated from mastitic milk having resistance for Cefazolin and Oxacillin only. No resistance was shown to Clindamycin**,** Gentamicin, Nitrofurantoin, Trimethoprim-Sulfamethoxazole, Daptomycin, Erythromycin, Linezolid, Norfloxacin, Rifampin, Tetracycline, Teicoplanin and Vancomycin.

Ibrahim *et el.* (2022) recorded maximum resistance of coagulase-negative staphylococci isolates, predominantly *Staph. haemolyticus* from milk sample to oxacillin, clindamycin, erythromycin and ciprofloxacin which is accordance to our present findings. Dabele *et al.* (2021) reported presence of multiple drug resistance for *Staph. sciuri*, and *Staph. lentus* isolates from milk samples. Waller *et al*. (2011) documented prevalence of $-lactamase producing isolates in Staph. epidermidis and Staph. haemolyticus.

No resistance was observered in our present study for Cefazolin, Clindamycin**,** Erythromycin, Daptomycin, Linezolid, Gentamicin, Nitrofurantoin, Norfloxacin, Oxacillin, Rifampin, Trimethoprim-Sulfamethoxazole, Tetracycline, Teicoplanin and Vancomycin for the strains of *Staph. chromogenes, Staph. schleiferi and Staph. intermedius* isolated from mastitic milk.

Strains of *Enterococcus faecalis* isolated from mastitic milk showed highest resistance to Cefazolin, Clindamycin**,** Gentamicin Quinupristin-dalfopristin, Trimethoprim- Sulfamethoxazole (100 %) followed by Tetracycline (75 %). No resistance was observered for Oxacillin, Norfloxacin Daptomycin and Nitrofurantoin Erythromycin, Linezolid, Rifampin, Teicoplanin and Vancomycin. Although, as the number of isolates is quite less, the resistance percentage is showing towards higher side. Bag *et al.* (2022) observed maximum resistance of *Staph. faecalis* isolates from milk sample to tetracycline and sensitive to vancomycin in accordance to our findings. But gentamicin was found sensive by them which is in contrast to our outcome.

Strain of *Enterococcus hirae* isolated from mastitic milk having resistance for Cefazolin, Clindamycin**,** Gentamicin, Nitrofurantoin Oxacillin and Trimethoprim-Sulfamethoxazole. No resistance was shown to Daptomycin, Erythromycin, Linezolid, Norfloxacin, Tetracycline, Teicoplanin and Vancomycin. Kaya and Turkyılmaz (2019) found tetracycline, erythromycin and gentamycin resistant to *Enterococcus hirae* isolated from mastitic milk. Although, in our study, tetracycline and erythromycin were found sensitive to the isolated *Enterococcus hirae strain.*

Table 19: Antibiogram pattern of Streptococcus spp. through BD Phoenix M50 detection system

S. No.	Identified bacteria in BD phoenix M50	Sensitivity status	Antibiotic Name										
			Amoxicillin	**Cefepime**	**Cefotaxime**	**Chloramphenicol**	**Clindamycin**	**Erythromycin**	**Levofloxacin**	**Linezolid**	**Meropenem**	**Penicillin G**	**Tetracycline**	**Vancomycin**
1	*Str. agalactiae*	*Sensitive*	10	10	10	10	10	10	10	10	10	10	0	10
		Resistant	0	0	0	0	0	0	0	0	0	0	10	0
		Total	10	10	10	10	10	10	10	10	10	10	10	10
		% Resistant	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0
2	*Str. dysgalactiae*	*Sensitive*	10	10	10	10	8	8	10	10	10	10	0	10
		Intermediate											3	
		Resistant	0	0	0	0	2	2	0	0	0	0	7	0
		Total	10	10	10	10	10	10	10	10	10	10	10	10
		% Resistant	0.0	0.0	0.0	0.0	20.0	20.0	0.0	0.0	0.0	0.0	70.0	0.0

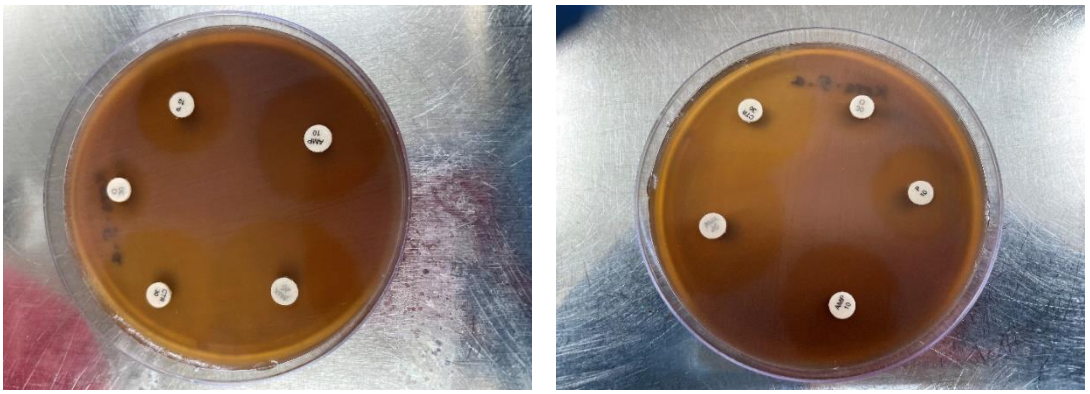


Figure 24.: Antibiogram by disc diffusion for *Str. uberis* identified isolates

Table 20: Antibiogram pattern of *Str. uberis* through Disc Diffusion Method

No of isolates	**Amoxicillin (AMX)**	**Ampicillin (AMP)**	**Penicillin -G (P)**	**Ceftriaxone (CTR)**	**Oxytetracycline (O)**
Total nos.	17	17	17	17	17
Nos. resistant	1	1	2	2	6
% resistant	5.9%	5.9%	11.8%	11.8%	35.3%

Str. uberis strains showed the highest resistance towards Oxytetracycline (35.3 %) followed by Penicillin-G and Ceftriaxone (11.8 % each). Amoxicillin and Ampicillin were least resistance (5.9 %) for *Str. uberis* strains (Table 21 And Figure 24)

Strains of *Str. agalactiae* isolated from mastitic milk in the present study showed highest resistance to Tetracycline (100 %). No resistance was observered for Amoxicillin, Cefepime, Cefotaxime, Chloramphenicol, Clindamycin, Erythromycin, Levofloxacin, Linezolid, Meropenem, Penicillin G and Vancomycin. Singh *et al.* (2023) observed maximum resistance of *Str. agalactiae* isolates from milk sample to tetracycline. They also found high sensitivity towards to meropenem, vancomycin, and erythromycin which are accordance to our present study finding.

Strains of *Str. dysgalactiae* isolated from mastitic milk showed highest resistance to Tetracycline (100 %) followed by Clindamycin and Erythromycin (20 % each). No resistance was observered for Amoxicillin, Cefepime, Cefotaxime, Chloramphenicol, Levofloxacin, Linezolid, Meropenem, Penicillin G and Vancomycin. Earlier researcher also recorded higher sensitivity twowards Chloramphenicol, penicillin-G, amoxicillin (Charaya *et al.* 2014).

Different researchers found ampicillin having least sensitivity to different isolates such as *E coli, Staph aureus* etc. (Moon *et al.*, 2007; Sumathi *et al.* 2008, Kaur *et al.* 2015) which is accordance to present study.

Resistance to antimicrobial agents in bacteria may occur due to a spontaneous mutation or it may be acquired via plasmids, transposons and integrons from resistant bacteria. (Woodford and Ellington, 2007)

Many factors could be responsible for this, like indiscriminate use of antibiotics without prescription, lack of proper veterinary consultation, not performing an antibiogram when the situation warrants, improper treatment regimens, etc. Giving antimicrobials to domestic livestock for preventing and treating diseases, as well as promoting growth, may act as an important factor in the emergence of antibiotic-resistant bacteria. These may subsequently transfer to humans through the food chain.

4.2.3.3. Screening of Antimicrobial Resistant (AMR) genes by PCR

All the 60 bacterial isolates belonging to 6 species were genotyped for the presence of various ARGs. Screening was carried out for bla_Z, bla_tem_all, bla_SHV, bla_CTX_M, mecA, mecC, TeT_K, TeT_M, TeT_O and TeT_A ARGs, however presence of bla_Z, bla_SHV, bla_CTX_M, mecA, TeT_K and TeT_M could be detected (Table 21).

Table 21: Details of ARGs present in bacterial isolates confirmed by PCR

S. No.	Bacteria identified by PCR	No. of isolates processed	Presence of ARGs in number of isolates									
			Mec A	**Mec C**	**bla_Z**	**bla_TEM-all**	**bla-SHV**	**bla-CTX-M**	**Tet A**	**Tet K**	**Tet O**	
1	*E. coli*	6	NA	NA	0	0	0	2	0	0	0	
2	*Kleb. Pneumoniae*	8	NA	NA	0	0	6	0	0	0	0	
3	*Staph. Aureus*	32	4	0	4	NA	0	0	0	5	0	
4	*Str. Agalactiae*	6	0	0	0	0	0	0	0	0	0	
5	*Str. Dysgalactiae*	3	0	0	0	0	0	0	0	0	0	
6	*Str. Uberis*	5	0	0	0	0	0	0	0	0	0	
	Total	60										

Presence of ARGs i.e. mecA (in *Staph. aureus, 4 isolates),* bla_Z (in *Staph. aureus, 4 isolates),* bla-SHV (in *Kleb. pneumoniae, 6 isolates),* bla CTX-M (in *Escherichia coli, 2 isolates),* tet K (*Staph. aureus, 5 isolates)* and tetM (*Str. agalactiae,* 1 isolate; *Str. uberis, 5 isolates)* were obtained.

Image of *A*garose gel electrophoresis depicting the presence of ARGs mecA and bla-Z in confirmed isolates of *Staph. Aureus isbeing presented in Figure 25.*

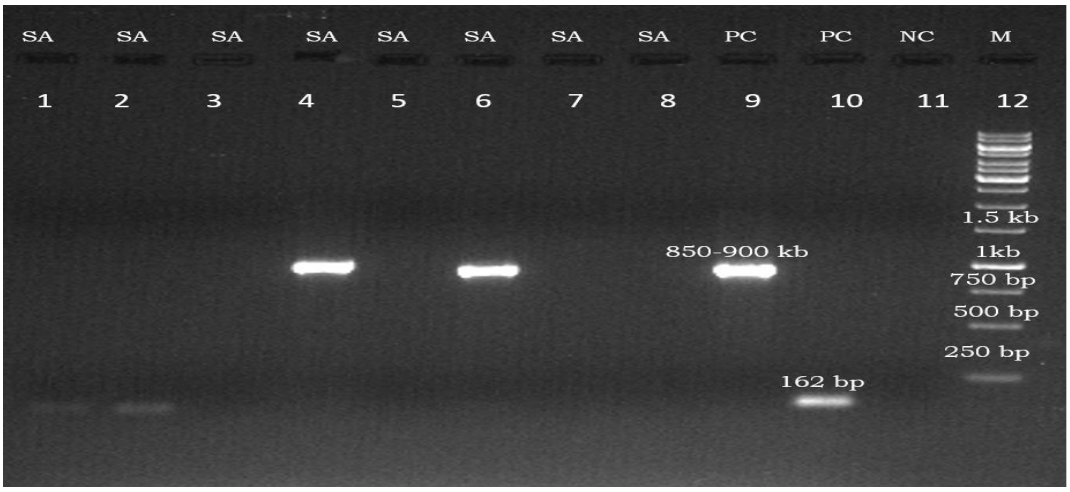


Figure 25: *A*garose gel electrophoresis image depicting the presence of ARGs mecA and bla-Z in confirmed isolates of *Staph. aureus (162 and 850-900 bp)* Lane 1-8 : *Staph. aureus (SA),* Lane 9, 10: Positive control (PC), Lane 11: Negative Control (NC), Lane 12: 1 kb Marker (M)

Image of *A*garose gel electrophoresis depicting the presence of ARGs SHV and CTXM in confirmed isolates of *Kleb. pneumoniae* and *E coli being shown in Figure 26.*

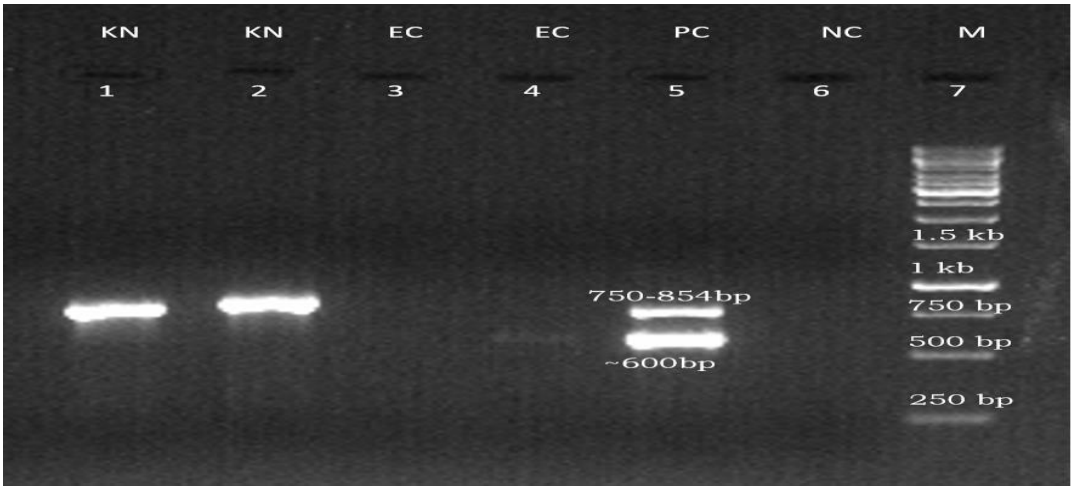


Figure 26: *A*garose gel electrophoresis image depicting the presence of ARGs SHV and CTXM in confirmed isolates of *Staph. aureus, Kleb. pneumoniae* and *E coli* (750-854bp, ~600bp) Lane 1, 2 : *Kleb. pneumoniae (KN),* Lane 3, 4: *E. coli* (EC), *Lane 5:* Positive control (PC), Lane 6: Negative Control (NC), Lane 7: 1 kb Marker (M)

Image of *A*garose gel electrophoresis depicting the presence of ARGs TET K in confirmed isolate of *Staph. aureus shown in Figure 27.*

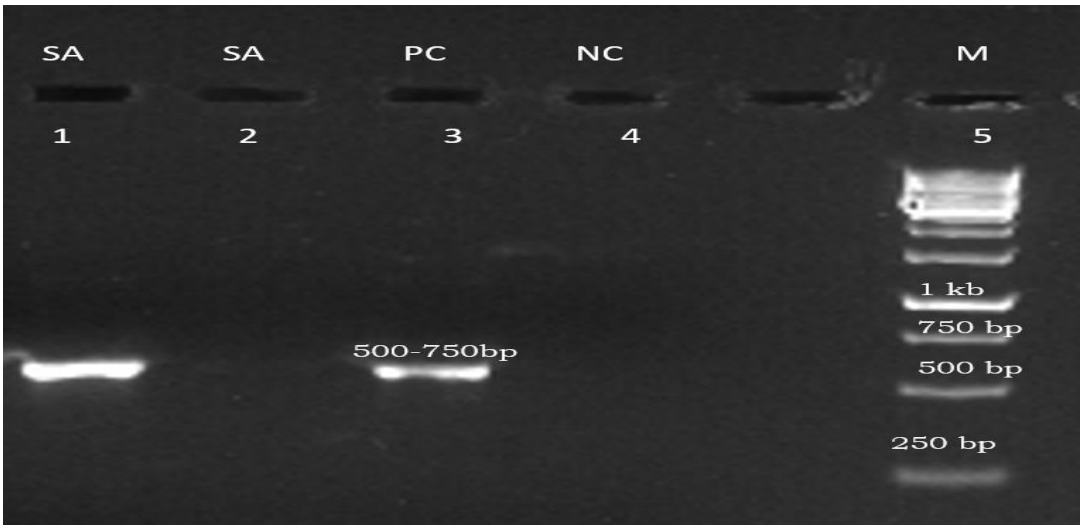


Figure 27: *A*garose gel electrophoresis image depicting the presence of ARGs TET K in confirmed isolates of *Staph. aureus* (500-750bp) Lane 1, 2: *Staph. aureus (SA), Lane 3:* Positive control (PC), Lane 4: Negative Control (NC), Lane 5: 1 kb Marker (M)

Image of *A*garose gel electrophoresis depicting the presence of ARGs tetM in confirmed isolate of *Strep. Agalactiaeis shown in Figure 28.*

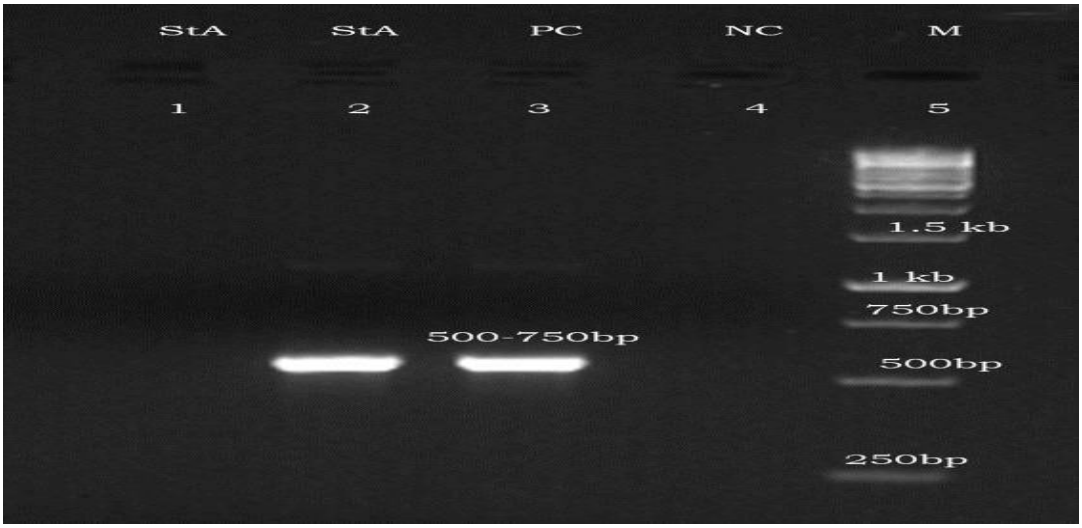


Figure 28: *A*garose gel electrophoresis image depicting the presence of ARGs TET M in confirmed isolate of *Strep. agalactiae* (500-750bp) Lane 1, 2 : *Strep. agalactiae (StA), Lane 3:* Positive control (PC), Lane 4: Negative Control (NC), Lane 5: 1 kb Marker (M)

4.2.4. Comparative analysis of phenotypic and genotypic antibiogram data

Bacteria wise, in *Escherichia coli, presence of bla-CTX-M, gene was recorded. In Kleb. pneumoniae islates,* bla-SHV gene was detected. In *Staph. aureus, resistant genes for* mecA, bla_Z, and tet K was found. In *Str. agalactiae,* tet M was found. In *Str. uberis isolates,* tet M gene was detected. Although, in isolates of *Str. dysgalactiae,* no antimicrobial resistant genes were detected.

4.2.4.1. *Staph. aureus* isolates

Multiple drug resistance was observed in phenotyic test where mecA and blaZ ARGs are present (Table 22). Cefazolin and Oxacillin resistance by phenotypic test was found for all the isolates where mecA and blaZ ARGs are present (100 %). Olugbenga *et al*. (2013) recorded 70 % sensitivity for oxacillin when compared with mecA ARG as gold standard. Isolates where tetK was detected, not showing resistance pattern for tetracycline in disc diffusion. It may be due to presence of other tet genes not covered in the present study. The minor deviations observed for each antibiotic may be attributed to the either other intrinsic factors that aid in resistance or non-functional ARG. In isolate no. ‘pdma146’, phenotypic antimicrobial resistance was found against 5 antimicrobial agents, but no ARG was detected in the present study. Further study with other ARGs and/or whole genomic study is required to know the ARGs present.

Table 22: Comparative analysis of phenotypic and genotypic antibiogram data for Staph. aureus isolates

Isolate ID	Identified bacteria in BD phoenix M50	Cefazolin	Clindamycin	Daptomycin	Erythromycin	Gentamicin	Linezolid	Nitrofurantoin	Norfloxacin	Oxacillin	Quinupristin-dalfopristin	Rifampin	Teicoplanin	Tetracycline	Trimethoprim-Sulfamethoxazole	Vancomycin	ARGs present
pdma1	*Staph. Aureus*	S'-	S	S	S	S	S	S	S	S	S	S	S	S	S	S	
pdma11	*Staph. Aureus*	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	
pdma15	*Staph. Aureus*	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	
pdma18	*Staph. Aureus*	R	S	S	R	R	S	S	R	R	S	S	S	S	R	S	mecA, bla_Z
pdma21	*Staph. Aureus*	S	S	S	S	S	S	S	R	S	S	S	S	S	R	S	
pdma23	*Staph. Aureus*	S	S	S	S	S	S	S	R	S	S	S	S	S	R	S	
pdma25	*Staph. Aureus*	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	
pdma29	*Staph. Aureus*	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	
pdma31	*Staph. Aureus*	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	
pdma36	*Staph. Aureus*	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	
pdma39	*Staph. Aureus*	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	tet-K
pdma50	*Staph. Aureus*	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	
pdma51	*Staph. Aureus*	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	
Pdma76	*Staph. Aureus*	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	
pdma80	*Staph. Aureus*	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	

Isolate ID	Identified bacteria in BD phoenix M50	Cefazolin	Clindamycin	Daptomycin	Erythromycin	Gentamicin	Linezolid	Nitrofurantoin	Norfloxacin	Oxacillin	Quinupristin-dalfopristin	Rifampin	Teicoplanin	Tetracycline	Trimethoprim-Sulfamethoxazole	Vancomycin	ARGs present
pdma86	*Staph. Aureus*	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	
pdma93	*Staph. Aureus*	R	S	S	S	R	S	S	S	R	S	S	S	S	S	S	mecA, bla_Z
pdma96	*Staph. Aureus*	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	
pdma105	*Staph. Aureus*	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	
pdma106	*Staph. Aureus*	R	S	S	S	R	S	S	S	R	S	S	S	R	S	S	mecA, bla_Z, tet-K
pdma109	*Staph. Aureus*	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	tet-K
pdma111	*Staph. Aureus*	R	S	S	R	R	S	S	R	R	S	S	S	S	S	S	mecA, bla_Z
pdma115	*Staph. Aureus*	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	
pdma120	*Staph. Aureus*	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	
pdma122	*Staph. Aureus*	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	tet-K
pdma125	*Staph. Aureus*	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	
pdma136	*Staph. Aureus*	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	
pdma145	*Staph. Aureus*	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	
pdma146	*Staph. Aureus*	S	R	S	R	S	S	S	I	S	R	S	S	R	R	S	
pdma151	*Staph. Aureus*	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	
pdma152	*Staph. Aureus*	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	
pdma157	*Staph. Aureus*	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	

4.2.4.2. *E. coli* and *Kleb. pneumoniae* isolates

Table 23: Comparative analysis of phenotypic and genotypic antibiogram data for *E. coli* and *Kleb. pneumoniae* isolates

Isolate ID	Identified bacteria in BD phoenix M50	Amikacin	Amoxicillin-Clavulanate	Ampicillin	Aztreonam	Cefepime	Cefotaxime	Cefoxitin	Ceftazidime	Chloramphenicol	Ciprofloxacin	Gentamicin	Imipenem	Levofloxacin	Meropenem	Piperacillin	Piperacillin-Tazobactam	Tetracycline	Trimethoprim-Sulfamethoxazole	ARGs present
pdma8	*E. coli*	S	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	R	R	
pdma32	*E. coli*	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	
pdma34	*E. coli*	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	
pdma44	*E. coli*	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	bla-CTX-M
pdma57	*Kleb. Pneumoniae*	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	bla-SHV
pdma59	*Kleb. Pneumoniae*	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	bla-SHV
pdma61	*Kleb. pneumoniae*	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	bla-SHV
pdma62	*Kleb. pneumoniae*	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	bla-SHV

pdma65	*Kleb. pneumoniae*	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	bla-SHV
pdma79	*Kleb. pneumoniae*	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	
pdma99	*E. coli*	S	R	R	R	R	R	S	R	S	R	R	S	R	S	R	S	R	R	
pdma112	*Kleb. pneumoniae*	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	bla-SHV
pdma153	*Kleb. pneumoniae*	S	S	R	R	R	I	R	R	S	S	S	S	S	S	S	S	S	R	
pdma154	*E. coli*	S	S	R	R	R	I	R	R	S	S	S	S	S	S	S	S	S	R	bla-CTX-M

Multiple drug resistance was observed in phenotypic test in one isolate where bla-CTX-M ARG is present (Table 23). Ampicillin resistance by phenotypic test was found for all the isolates where bla-SHV ARG are present (100 %). Bla (SHV) is a key beta-lactamase gene and thought to be resistance to ampicillin intrinsically (Yingmei *et al.*, 2007). In isolate no 'pdma99', 'pdma153' and 'pdma8' phenotypic AMR was found against 12, 6 and 5 antimicrobial agents respectively, but no ARG was detected in the present study. Further study with other ARGs and/or whole genomic study is required to know the ARGs present in those isolates. The minor deviations observed for each antibiotic may be attributed to the either other intrinsic factors that aid in resistance or non-functional ARG.

4.2.4.3. Streptococcus isolates

Table 24: Comparative analysis of phenotypic and genotypic antibiogram data for *Streptococcus* isolates

		Antibiogram by BD												Disc diffusion					
Isolate ID	**Identified bacteria in BD phoenix M50**	**Amoxicillin**	**Cefepime**	**Cefotaxime**	**Chloramphenicol**	**Clindamycin**	**Erythromycin**	**Levofloxacin**	**Linezolid**	**Meropenem**	**Penicillin G**	**Tetracycline**	**Vancomycin**	**Amoxicillin (AMX)**	**Ampicillin (AMP)**	**Penicillin -G (P)**	**Ceftriaxone (CTR)**	**Oxytetracycline (O)**	**ARGs present**
pdma2	*Str. dysgalactiae*	S	S	S	S	S	S	S	S	S	S	R	S						
pdma26	*Str. agalactiae*	S	S	S	S	S	S	S	S	S	S	R	S						*tet-M*
pdma35	*Str. agalactiae*	S	S	S	S	S	S	S	S	S	S	R	S						
pdma78	*Str. agalactiae*	S	S	S	S	S	S	S	S	S	S	R	S						
Pdma82	*Str. Dysgalactiae*	S	S	S	S	S	S	S	S	S	S	I	S						
pdma110	*Str. agalactiae*	S	S	S	S	S	S	S	S	S	S	R	S						
pdma124	*Str. Dysgalactiae*	S	S	S	S	S	S	S	S	S	S	R	S						
pdma144	*Str. agalactiae*	S	S	S	S	S	S	S	S	S	S	R	S						
pdma156	*Str. agalactiae*	S	S	S	S	S	S	S	S	S	S	R	S						
pdma12	*Str. uberis*													S	S	S	S	R	*tet-M*
pdma123	*Str. uberis*													S	S	S	S	R	*tet-M*
pdma128	*Str. uberis*													S	S	S	S	R	*tet-M*
pdma129	*Str. uberis*													S	S	S	S	R	*tet-M*
pdma131	*Str. uberis*													S	S	S	S	R	*tet-M*

Isolates where tetM was detected, showing resistance pattern for tetracycline/oxytetracycline in phenotypic test. Although, in few isolates where phenotypic test shows resistance to tetracycline, but no resistance gene was detected. It may be due to presence of other tet genes not covered in the present study. The minor deviations observed for each antibiotic may be attributed to the either other intrinsic factors that aid in resistance or non-functional ARG.

4.2.5. Isolation and identification of Bacterial agents from milk samples collected before and after EVHP:

Thirty-nine animals were treated with EVHP for mastitis with a recovery rate of 79.5 %. From these animals, 41 quarter milk samples were taken before EVHP application for bacteriological examination. The results of their thorough bacteriological examination have already been explained in "bacterial isolation and identification" portion through Tables-10-14.

Out of these 39 animals, 22 had clinical mastitis and remaining 17 were positive for SCM. Subsequent to use of EVHP, 77.2% and 82.4% animals were recovered from mastitis (Table 25)

Table 25: Details of bacterial agents isolated from milk collected before and after EVHP

Particulars	Nos. of animals treated with EVHP	Animals recovered clinically	% recovery	Total samples positive for bacteriology		Total isolates recovered	
				Before EVHP	After EVHP	Before EVHP	After EVHP
Total CM animals	22	17	77.2 %	24	24	31	29
Total SCM animals	17	14	82.4%	17	17	27	24
All total	39	31	79.5%	41	41	58	53

During pre and post-EVHP sampling for bacteriology, same bacterial species were recovered irrespective of clinical recovery status except in five quarter milk samples. The bacteria isolated from pre and post EVHP samples are listed in table no 26.

Table 26: List of bacterial agents isolated from pre and post EVHP mastitis milk

Bacteria recovered from pre-EVHP samples	**Bacteria recovered from post-EVHP samples**
Staph. aureus	*Staph. aureus*
Str. dysgalactiae;	*Str. dysgalactiae*
E. coli	*E. coli*
Klebsciella spp.	*Klebsciella spp.*
Str. uberis	*Str. uberis*
Staph. hominis	*Staph. hominis*
Staph. lentus;	*Staph. lentus;*
Staphylococcus spp.	*Staphylococcus spp.*
Streptococcus spp.	*Streptococcus spp.*
Kocuria kristinae	*Kocuria kristinae*
Kocuria varians	.
Moraxella spp.	
Pseudomonas oryzihabitans	

Although, the bacterial count and SCC in milk from the infected quarters were observed to be reduced (Table 29) and overall clinical recovery recorded was 79.5%.

This might be due to improved udder immunity as a result of EVHP application. Further studies need to be conducted to follow-up the bacteria sheding staus from the infected quarters.

The bacterial isolates from pre-EVHP milk samples of 8 animals were screened for exsistance of AMR genes i.e. mecA, mecC, bla_Z, bla_TEM-all, bla-SHV, bla-CTX-M, Tet A, Tet K, Tet O and Tet M. Eleven bacterial isolates were recovered from quarter milk samples of these eight animals. Presence of Tet M gene was recorded in the pre-EVHP milk samples of four of these animals. Details of EVHP treated individual animals' recovery status from mastitis and presence of ARGs in pre-EVHP milk has shown in Table 27.

Table 27: Details of EVHP treated individual animals' recovery status from mastitis and presence of ARGs in the isolates.

Isolate ID	Status of mastitis	Mastitis recovery status by EVHP	PCR STATUS	mecA	mecC	TEM_Z	bla_TEM-all	bla-SHV	bla-CTX-M	Tet -A/K/O/M
pdma1	SCM	*Recovered*	*Staphylococcus aureus*	N	N	N	N	N	N	N
pdma2			*Streptococcus dysgalactiae*	N	N	N	N	N	N	N
pdma8	SCM	*Recovered*	*Escherichia coli*	NA*	NA*	N	N	N	N	N
pdma11	SCM	*Recovered*	*Staphylococcus aureus*	N	N	N	N	N	N	N
pdma12	SCM	*Not recovered*	*Streptococcus uberis*	N	N	N	N	N	N	tet-M
pdma123	CM	*Recovered*	*Streptococcus uberis*	N	N	N	N	N	N	tet-M
pdma124	CM	*Not recovered*	*Streptococcus dysgalactiae*	N	N	N	N	N	N	N
pdma125			*Staphylococcus aureus*	N	N	N	N	N	N	N
pdma128	CM	*Recovered*	*Streptococcus uberis*	N	N	N	N	N	N	tet-M
pdma129			*Streptococcus uberis*	N	N	N	N	N	N	tet-M

pdma131	CM	*Recovered*	*Streptococcus uberis*	N	N	N	N	N	N	tet-M
NA: Not applicable; N Negative, tet-M: Positive for tet M										

Out of two animals which could not be recovered with EVHP, one animal milk sample carried tetM gene containing bacteria. While, rest six animals were recovered with the use of EVHP. Out of these six animals, three animal's bacterial isolates have herbour ARG tet-M. Therefore, it is obvious that recovery of mastitis with EVHP is not influenced by presence of resistance genes in isolates recovered from mastitic milk. However, a largescale study is required for better observation as well as to get a firm conclusion.

Our present finding is in accordance to Hegde *et al.* (2021) where they analysed that average abundance of *Staphylococcus, Streptococcus, Pseudomonas, Klebsiella and Enterobacteriaceae family* were reduced by use of this herbal combination.

Punniamurthy *et al.* (2017a) investigated that the fresh herbal formulation for mastitis has antibacterial properties against *E. coli* and *S. aureus*. During analysing the mechanism of action of active ingredients of aloevera, Termaric and lime by molecular docking studies using the *in-silico* approach, Punniamurthy *et al.*, 2017b found the combination effective in mastitis treatment. The pharmacodynamic study using the online server PASS revealed that the compounds in the herbal preparation having anti-inflammatory, anti-healing and anti-bacterial properties.

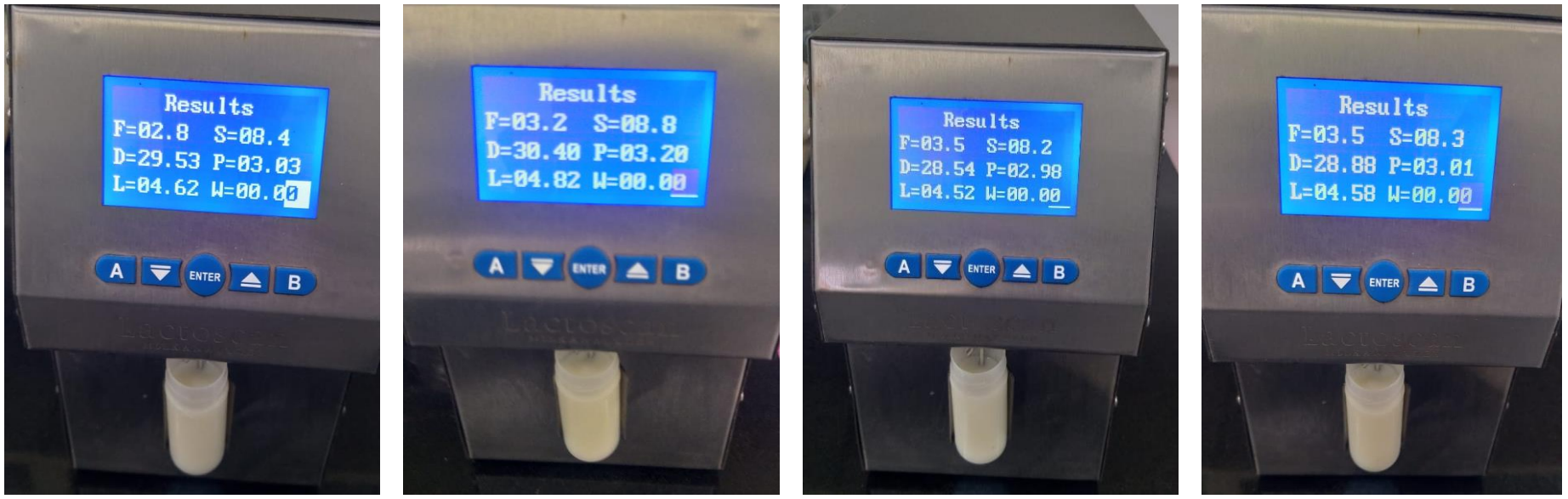
4.2.6. a. Milk composition and other parameters in mastitis treated with EVHP:

Out of 39 animals treated with EVHP, 7 sub-clinically infected animals (within 4 months of calving) were followed for changes in pH, CMT, SCC, TPC and milk composition i.e. fat, SNF, lactose and milk protein before and after EVHP treatment. The details of the same depicted in table 28.

Table 28: Change in physical parameters and milk composition in SCM animals treated by EVHP

Particulars	Milk yield all quarters (in Lit)		Quarter Milk fat		Quarter Milk SNF		Quarter Milk TS		Quarter Milk protein		Quarter Milk lactose		Quarter SCC (per ml) in lakh		Quarter Milk pH		Quarter TPC (per ml) in lakh	
	Before EVHP	On 15th day	Before treatment	After EVHP	Before treatment	After EVHP	Before treatment	After EVHP	Before treatment	After EVHP	Before treatment	After EVHP	Before EVHP	On 30th day	Before treatment	After EVHP	Before EVHP	On 30th day
Mean of 6 quarters	7.63	8.13	3.73	3.93	8.68	8.87	12.42	12.80	3.31	3.44	4.49	4.62	4.42	1.83	6.90	6.72	3.72	1.11
Standard Deviation	1.22	1.30	0.65	0.58	0.33	0.31	0.88	0.78	0.32	0.33	0.28	0.27	0.74	0.10	0.12	0.06	0.88	0.40
p-value	0.01		0.05		0.01		0.02		0.01		0.00		0.00		0.00		0.00	
Reading of 7th quarter	10.1	9.8	3.6	3.6	8.4	8.5	12	12.1	3.1	3.1	4.3	4.2	4.6	4.2	6.9	7.1	4.67	4.00

Out of 7 sub-clinically affected udder quarters, 6 quarter become CMT -ve, SCC, pH and TPC comes down. In one quarter, pH remains high (7.1) and CMT positivity remains (+++). Rest of quarter milk samples become CMT negative. Representative image of milk composition captured is showed in Figure 29.



Before EVHP **After EVHP** **Before EVHP** **After EVHP**

Figure 29: Image of Milk analysis report for fat, SNF, protein and lactose (before and after EVHP)

Out of 39 animals treated with EVHP, 6 clinical mastitis affected animals were followed up for change in milk colour and consistency before and after EVHP treatment. pH, CMT, SCC and milk composition after clinical recovery were also recorded as shown in table no 29.

Table 29: Physical appearence and milk quality data in CM cases treated with EVHP (from cattle)

Sample No	Milk yield (all quarters)		Quarter Milk pH		Quarter Milk fat		Quarter Milk SNF		Quarter Milk protein		Quarter Milk lactose		Quarter milk SCC (per ml) in lakh	
	During mastitis	After EVM	Before EVM	After EVM	After 7time application	After completion of EVM	After 7time application	After completion of EVM	After 7time application	After completion of EVM	After 7time application	After completion of EVM	After 7 ime application	After 30 days of EVM
Mean	7.28	12.4	6.89	6.66	3.13	4.14	7.90	8.87	3.43	3.60	4.71	4.81	7.52	1.80
SD	5.0	4.6	0.1	0.0	0.3	0.4	0.2	0.2	0.2	0.2	0.2	0.2	2.5	0.1
P value	0.00		0.00		0.00		0.00		0.00		0.01		0.00	

Out of 7 Clinically affected swellen quarters, in 3 quarters milk was having flakes and 2 each having watery and cardled milk. With five days EVHP treatment, 6 quarters milk become apperantly normal. But, in one affected quarter, flakes remain in milk and could not be cured. Due to presence of flakes in milk, milk composition also could not be measured. pH remains high (7) and CMT positivity remains (+++). Rest of quarter milk samples become CMT negative.

It was observed during the present study that animals having mastitis were treated successfully with EVHP has shown increased milk fat, SNF, total solid, milk protein and lactose along with improvement in milk production. Decreasing trend of milk pH, SCC and TPC were recorded. These findings are in accordance to Swami *et al.*, 2017, Nair *et al*. 2017 and Abase & Kurtu, 2018.

4.2.7. b. Clinical Mastitis Case studies recorded along with udder photographs:

In another study, clinical mastitis infected udder photographs of six cows were recorded to compare before and after application of mastitis EVHP for 5 days (Table no 30-35). From these studies, it was observed that clinical symptoms of mastitis disappear within 5 days of EVHP application. The ‘milk yield before mastitis’ column in the below case studies were taken as per the farmer’s observation during milk recording in NDDB’s PT (Progeny testing) programme. The milk production of these cows was observed to be back near normalcy or even higher. The higher yield might be due to clearance of subclinical stage beore converted to clinical one or might be due to overall enhancement in udder immunity. Further study is required to observe the milk yield status after EVHP completion for further duration.

Table 30: Clinical recovery of Mastitis with use of EVHP: Case study 1

Clinical mastitis case details

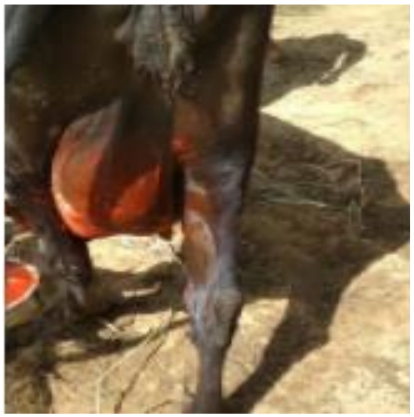
No. of days EVHP applied:5 days; application/day: 5 times; EVHP used: oil based

Case no. 1: Date: 16.03.2021

INAPH ID: [redacted] Owner name: [redacted] Village: Bhuval; District: Sabarkantha; State: Gujarat

Species: Cattle Age: 7 years Quarter: LF, LH, LF

Disease History:



Symptom	Before EVHP	After EVHP	Symptom	Before EVHP	After EVHP
Fever	N	N	Pain in udder	Y	N
Redness in udder	Y	N	Watery milk	Y	N
Swelling in udder	Y	N	Difficulty in milk	Y	N
Blood in milk	N	N	Flakes in milk	Y	N
Repeated clinical mastitis case					N

On 1st day of EVHP application Y: Yes; N: No **On 5th day of EVHP application**

Milk yield (liters/day):

Milk yield before mastitis detection	On Day of mastitis detection	After completion of EVHP application
5.22	4.57	6.14

Conclusion: Animal recovered from clinical mastitis with use of EVHP for 5 days and milk production also restored to near normalcy

Table 31: Clinical recovery of Mastitis with use of EVHP: Case study 2

Clinical mastitis case details

No. of days EVHP applied:5 days; application/day: 5 times; EVHP used: oil based

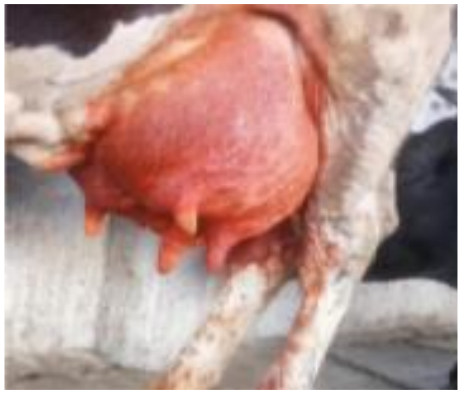
Case no. 2: Date: 13.03.2021

INAPH ID: [redacted] Owner name: [redacted] Village: Aminpur; District: Sabarkantha; State: Gujarat

Species: Cattle Age: 8 years Quarter: RH, LH

Disease History:

Symptom	Before EVHP	After EVHP	Symptom	Before EVHP	After EVHP
Fever	N	N	Pain in udder	N	N
Redness in udder	Y	N	Watery milk	Y	N



Swelling in udder	Y	N	Difficulty in milk	N	N
Blood in milk	N	N	Flakes in milk	Y	N
Repeated clinical mastitis case					N

On 1st day of EVHP application Y: Yes; N: No **On 5th day of EVHP application**

Milk yield (liters/day):

Milk yield before mastitis detection	On Day of mastitis detection	After completion of EVHP application
4.22	2.54	4.40

Conclusion: Animal recovered from clinical mastitis with use of EVHP for 5 days and milk production also restored to near normalcy

Table 33: Clinical recovery of Mastitis with use of EVHP: Case study 3

Clinical mastitis case details

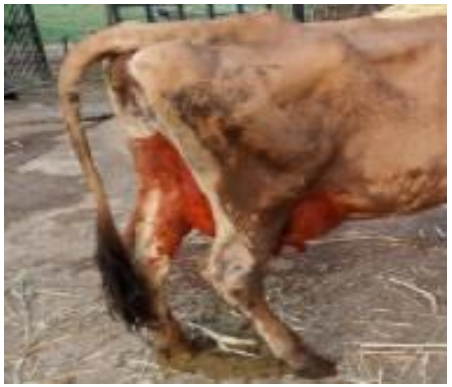
No. of days EVHP applied:5 days; application/day: 5 times; EVHP used: oil based

Case no. 3: Date: 16.03.2021

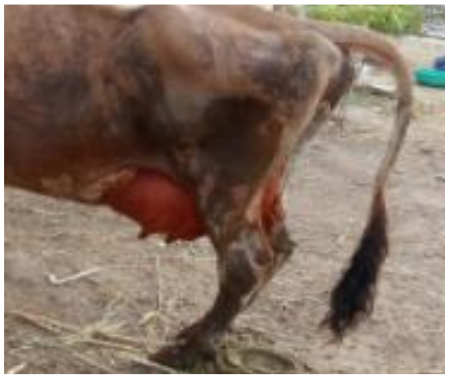
INAPH ID: [redacted] Owner name: [redacted] Village: Bhuval; District: Sabarkantha; State: Gujarat

Species: Cattle Age: 5 years Quarter: RH; LF, LH

Disease History:



Symptom	Before EVHP	After EVHP	Symptom	Before EVHP	After EVHP
Fever	N	N	Pain in udder	Y	N
Redness in udder	Y	N	Watery milk	N	N
Swelling in udder	Y	N	Difficulty in milk	Y	N
Blood in milk	N	N	Flakes in milk	Y	N
Repeated clinical mastitis case					N



On 1st day of EVHP application Y: Yes; N: No **On 5th day of EVHP application**

Milk yield (liters/day):

Milk yield before mastitis detection On Day of mastitis detection After completion of EVHP application

5.74	4.15	7.39

Conclusion: Animal recovered from clinical mastitis with use of EVHP for 5 days and milk production also restored to near normalcy

Table 34: Clinical recovery of Mastitis with use of EVHP: Case study 4

Clinical mastitis case details

No. of days EVHP applied:5 days; application/day: 5 times; EVHP used: oil based

Case no. 4: Date: 16.03.2021

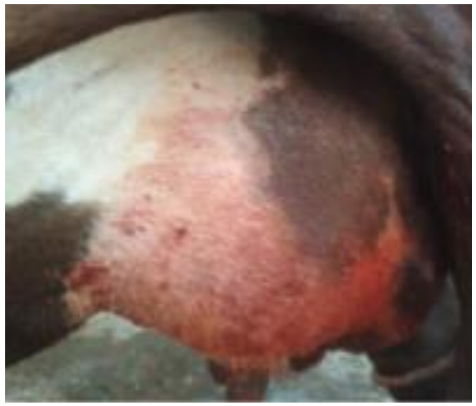
INAPH ID: [redacted] Owner name: [redacted] Village: Bhuval; District: Sabarkantha; State: Gujarat

Species: Cattle Age: 4 years Quarter: RF, RH, LF

Disease History:

Symptom	Before EVHP	After EVHP	Symptom	Before EVHP	After EVHP
Fever	N	N	Pain in udder	Y	N
Redness in udder	Y	N	Watery milk	N	N
Swelling in udder	Y	N	Difficulty in milk	Y	N

Blood in milk	N	N	Flakes in milk	Y	N
Repeated clinical mastitis case					N



On 1st day of EVHP application Y: Yes; N: No **On 5th day of EVHP application**

Milk yield (liters/day):

Milk yield before mastitis detection	On Day of mastitis detection	After completion of EVHP application
6.93	5.10	7.10

Conclusion: Animal recovered from clinical mastitis with use of EVHP for 5 days and milk production also restored to near normalcy

Table 35: Clinical recovery of Mastitis with use of EVHP: Case study 5

Clinical mastitis case details

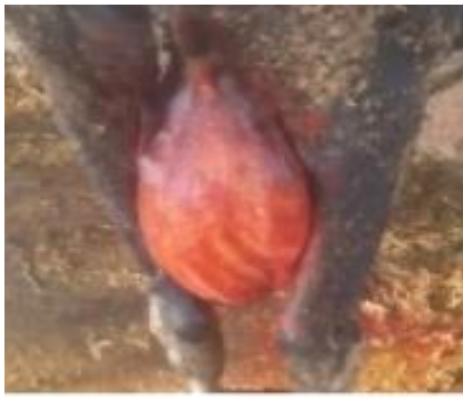
No. of days EVHP applied:5 days; application/day: 5 times; EVHP used: oil based

Case no. 5: Date: 16.03.2021

INAPH ID: [redacted] Owner name: [redacted] Village: Bhuval; District: Sabarkantha; State: Gujarat

Species: Cattle Age: 7 years Quarter: RH, LF, LH

Disease History:



Symptom	Before EVHP	After EVHP	Symptom	Before EVHP	After EVHP
Fever	N	N	Pain in udder	Y	N
Redness in udder	Y	N	Watery milk	N	N
Swelling in udder	Y	N	Difficulty in milk	Y	N
Blood in milk	N	N	Flakes in milk	Y	N
Repeated clinical mastitis case					Y

On 1st day of EVHP application Y: Yes; N: No **On 5th day of EVHP application**

Milk yield (liters/day):

Milk yield before mastitis detection	On Day of mastitis detection	After completion of EVHP application
6.35	4.58	8.35

Conclusion: Animal recovered from clinical mastitis with use of EVHP for 5 days and milk production also restored to near normalcy

Table 36: Clinical recovery of Mastitis with use of EVHP: Case study 6

Clinical mastitis case details

No. of days EVHP applied:5 days; application/day: 5 times; EVHP used: oil based

Case no. 6: Date: 12.03.2021

INAPH ID: [redacted] Owner name: [redacted] Village: Akodra; District: Sabarkantha; State: Gujarat

Species: Cattle Age: 6 years Quarter: RF, RH

Disease History:

Symptom	Before EVHP	After EVHP	Symptom	Before EVHP	After EVHP
Fever	N	N	Pain in udder	Y	N
Redness in udder	N	N	Watery milk	N	N

Swelling in udder	Y	N	Difficulty in milk	Y	N
Blood in milk	N	N	Flakes in milk	Y	N
Repeated clinical mastitis case					Y

On 1st day of EVHP application Y: Yes; N: No **On 5th day of EVHP application**

Milk yield (liters/day):

Milk yield before mastitis detection	On Day of mastitis detection	After completion of EVHP application
5.48	3.45	5.68

Conclusion: Animal recovered from clinical mastitis with use of EVHP for 5 days and milk production also restored to near normalcy

4.3. Case studies on EVHP applications in different MU/MPCs

4.3.1. EVHP application in cows having sub-clinical mastitis

A total of 50 Holstein Friesian (HF) crossbred cows at Sabarkantha Milk Union with SCM were treated with EVHP. The cows were in various parity [2nd lactation (n=5), 3rd lactation (n=24), 4th lactation (n=15) and 5th lactation (n=6)] and were positive for SCM by California Mastitis Test (CMT) before application of EVHP. Every animal underwent CMT testing once more, after the EVHP treatment was completed. 44 (88%) out of 50 SCM positive cows turned CMT negative after completion of EVHP application and an average increase of 605 ml (7.3 %) of daily milk production was recorded in recovered animals.

4.3.2. Animals treated for clinical mastitis, pyrexia and diarrhoea with EVHP

Total nos. of animals covered for EVHP treatment for clinical mastitis, pyrexia and diarrhoea were 7570, out of which 3703 nos. were of clinical mastitis cases followed by 2240 nos. of cases of diarrhoea and 1627 cases were of non-specific pyrexia (table 36). Representative photograph of EVHP application for mastitis is showing in Figure 30.

Figure 30: Application of EVHP on udder of a mastitis affected cow

Table 36: Age wise split-up of cases in cattle and buffalo

Disease condition	Animals below 3 years	Animals in age group 3-5 years	Animals in age group 6-9 years	Animals above 9 years
No of cattle treated with EVHP				
Clinical mastitis	74	439	1405	1170
Non-specific pyrexia	91	231	561	335
Diarrhoea	184	273	694	467
No of buffaloes treated with EVHP				
Clinical mastitis	Nil	58	206	236
Non-specific pyrexia	11	63	163	162
Diarrhoea	67	92	205	215

More than 92% of 3703 cattle and buffaloes treated with EVHP for clinical mastitis, clinically recovered after a 5-day treatment protocol. The recovery rate recorded in cattle and buffaloes were 92.6% and 89% respectively.

A total of 2240 cattle and buffaloes with non-specific acute diarrhoea were treated with EVHP and 97.4% clinical recovery was recorded. Comparable rate of recovery was noted in cattle (96.99%) and buffaloes (98.62%).

A total of 1627 cattle and buffaloes were included in the study and the overall recovery rate for pyrexia through EVHP application was found to be 97.79%. The recovery rate in cattle and buffaloes were 97.72% and 97.99 % respectively. Age wise distribution of clinical recovery rate is tabulated in Table 37.

Table 37: No. of animals recovered from clinical mastitis, pyrexia and diarrhoea with use of EVHP (Figure in parenthesis indicates percentage)

Disease condition	Animals below 3 years	Animals in age group 3-5 years	Animals in age group 6-9 years	Animals above 9 years
No of cattle recovered				
Clinical mastitis	73 (98.65)	423 (96.36)	1299 (92.46)	1285 (91.05)
Non-specific pyrexia	89 (97.80)	229 (99.13)	547 (97.50)	345 (97.10)
Diarrhoea	182 (98.91)	268 (98.17)	706 (98.30)	498 (93.78)
No of buffaloes recovered				
Clinical mastitis	Nil	55 (94.83)	184 (89.32)	206 (87.29)
Non-specific pyrexia	11 (100.00)	62 (98.41)	161 (98.77)	157 (96.91)
Diarrhoea	67 (100.00)	91 (98.91)	202 (98.54)	211(98.14)

In 50 SCM cases, 88% recovery rate with EVHP along with an average increase of 605 ml (7.3 %) of daily milk production recorded.

More than 92% of 3703 cattle and buffaloes treated with EVHP for clinical mastitis were clinically recovered after a 5-day treatment protocol. The recovery rate recorded in cattle and buffaloes were 92.6% and 89% respectively.

In case of non-specific acute diarrhoea, 97.4% clinical recovery was recorded with EVHP. Comparable rate of recovery was noted in cattle (96.99%) and buffaloes (98.62%).

In case of bovine pyrexia, 97.79% clinical recovery was recorded with EVHP. The recovery rate in cattle and buffaloes were 97.72% and 97.99 % respectively.

A slight lower recovery rate with an increase in age was observed both in cattle and buffaloes treated by EVHP for mastitis. In respect to cattle, recovery rate in Group I (n=74) recorded the highest cure rate of 98.65% followed by Group II (n=439) at 96.36%, Group III (n=1405) at 92.46% and Group IV at 91.05%. In buffaloes, a similar pattern was also observed with Group II (n=58) recording the highest cure rate of 94.83% followed by Group III (n=206) at 89.32% and group IV (n=236) at 87.29%.

There were no animals in Group I in lactating buffaloes due to their delayed maturity. On the contrary, no such age dependent trends were observed in cattle and buffaloes treated by EVHP for non-specific pyrexia and diarrhoea.

Earlier reseachers like Bhatt *et al*., 2019; Nair and Punniamurthy, 2017; Aruna *et al*. 2019 were also stated ethno-veterinary medicines effective against bovine mastitis cases. Verma (2014) documented the use of ethno-veterinary medicinal plants by rural farmers and traditional herbal healers of villages at Bundelkhand region for different animal ailments including diarrhoea and fever.

The results of EVHP intervention without the use of synthetic drugs were very encouraging in terms of clinical remission for SCM, clinical mastitis, diarrhoea and pyrexia, both in cattle and buffaloes. In developing countries like India, in which the resources with the stakeholders are limited, EVHP has a great potential as a cost-effective and efficacious alternative bovine disease management option. This also minimises the drug residues in milk and milk products and thereby helps to stall the emergence of AMR.

4.3.3. Lumpy Skin Disease like conditions managed in animals with EVHP

Ethnoveterinary (EVHP) preparations were used for management of 14 Lumpy Skin Disease (LSD) suspected cattle in field condition, aged 1-7 years, in the states of Assam and Maharashtra. Clinical signs exhibited by these cattle were suggestive of LSD. However, because of the COVID-19 scenario during the treatment time, it was not able to confirm the individual samples of infected animals in the laboratory.

All the 14 animals were provided with herbal preparations both for oral and external use as outlined in Materials and Methods. The table 38 displays the details of the animals included in the study. Animal nos. 1-6 and 7-14 were from were from Assam and Maharashtra respectively. The range of initial rectal temperature were in between 101-104°F.

Table 38: Details of the LSD affected Animals

Animal No	Age in year	Sex	Days required for apparent cure	Symptoms (Y: yes; N:no)									
				Depression	Anorexia	Lameness	Swelling in joint	Oedema in limbs and brisket	Swelling of Lymph node	Abortion	Nasal discharge	Lacrimal discharge/ salivation/corneal opacity	Lung infection
1	5	Female	10	Y	Y	N	N	N	Y	N	N	N	N
2	7	Female	8	Y	Y	N	N	Y	Y	N	N	N	N
3	4	Female	10	Y	Y	N	N	Y	Y	N	N	N	N
4	3	Female	8	Y	Y	N	N	Y	Y	N	N	N	N
5	7	Female	9	Y	Y	N	N	N	Y	N	N	N	N
6	4	Female	15	Y	Y	Y	N	N	Y	N	Y	N	Y
7	6.5	Female	8	Y	Y	N	N	Y	Y	Y	Y	N	N
8	7.1	Female	8	N	Y	N	Y	N	N	N	N	N	N
9	1	Male	8	Y	Y	N	N	N	N	-	N	N	N
10	5	Female	8	N	Y	N	N	N	N	N	N	N	N
11	1.5	Male	10	Y	Y	N	N	N	N	-	Y	Y	N
12	4.5	Male	10	Y	Y	Y	Y	Y	Y	-	N	N	N
13	4	Male	10	N	Y	N	Y	Y	N	-	N	N	N
14	7	Female	7	N	N	N	N	N	N	N	N	N	N
*				**71.4**	**92.9**	**14.3**	**21.4**	**42.9**	**57.1**	**10**	**21.4**	**7.1**	**7.1**

** % animals exhibited the symptom*

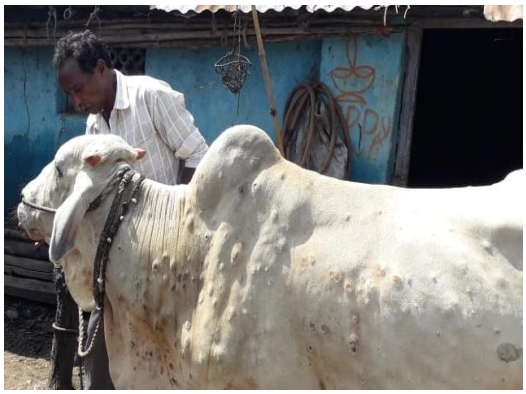
All the animals had variable degrees of skin lesions (nodules) on several parts of the body as detailed in Table 39.

Table 39: *Presence of skin nodules* in the LSD affected animals

Animal No	Location of skin nodule						
	Head	**Neck**	**Udder**	**Trunk**	**Perineum**	**Near Eye**	**Buccal mucosa**
1	Yes	Yes	No	Yes	No	Yes	No
2	Yes	Yes	No	Yes	No	Yes	No
3	Yes	Yes	No	Yes	No	Yes	No
4	Yes	Yes	Yes	Yes	No	Yes	No
5	Yes	Yes	No	Yes	No	Yes	No
6	Yes	Yes	No	Yes	No	No	No
7	No	Yes	No	No	Yes	No	No
8	No	Yes	No	No	No	No	No
9	No	Yes	-	No	No	No	No
10	Yes	No	No	No	No	No	No
11	Yes	Yes	-	Yes	Yes	No	No
12	Yes	Yes	-	Yes	Yes	No	Yes
13	No	Yes	-	No	No	No	No
14	No	Yes	No	Yes	No	No	No
**	64.3%	92.9%	10 %	64.3%	21.4%	35.7%	7.1%

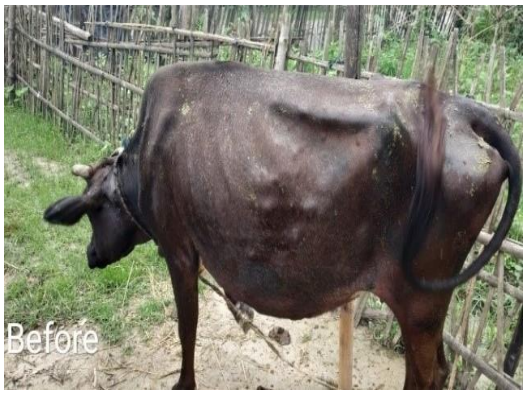
** % animals exhibited the symptom

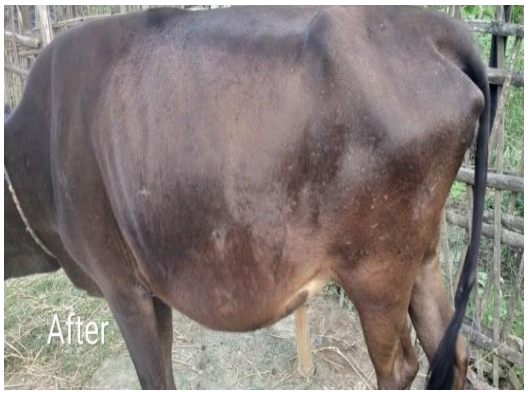
Animals photograph before and after application of EVHP for LSD:



Before EVHP **On 7th day of EVHP**

Figure 31: In a Gaolao cattle breed, Kharangana village, Arvi tehsil, Wardha District, state Maharashtra





Before EVHP After EVHP

Figure 32: In a HF crossbred, Panigaon village, Pachim Nalbari tehsil, Nalbari District, state Assam

Within 7-10 days after initiation of the EVHP intervention, symptomatic recovery of the animals was recorded. Although, one animal required 15 days' time to recover as there was involvement of lower respiratory tract. Once the clinical signs subsided, *viz.,* rectal temperature, disappearance of skin nodules and other symptoms indicated in the table 41 and 42, animals were considered apparently cured from LSD.

Symptomatic recovery of LSD affected animals treated with EVHP were recorded within 7-10 days after initiation of the EVHP intervention, except in one animal which took 15 days' time to recover due to involvement of lower respiratory tract. As per FAO (2020), LSD affected animals may become debilitated for up to six

months. Feyisa (2018) also recorded three months recovery time in a bull affected with LSD.

4.3.4. Management of repeat breeding in bovine by herbal combination

Out of 108 bovines, 84 (77.7 %) conceived after providing the EVHP preparation for the management of repeat breeding. Species wise, 26 cows i.e. 74.3 % and 56 buffaloes i.e. 79.5 % resulted in pregnancy. All the 84 pregnant cattle and buffaloes had completed the full-term pregnancy and delivered calves successfully. No abortion or still birth was reported. The details of animals with variable repeat breeding status and the conception rate after EVHP intervention have been depicted in Table-40.

Table 40: Conception rates following EVHP intervention in repeat breeding cases

S. No.	Repeat breeding history (post-partum AI service attempts)	Cattle		Buffalo	
		Total	Conceived	Total	Conceived
1	Animals did not conceive even after 3-6 AI services.	19	13 (68.4%)	25	20 (80.0%)
2	Animals did not conceive even after 7 AI services.	16	13 (81.3%)	49	38 (77.6%)
Total		35	26 (74.3%)	73	58 (79.5%)

Out of 108 bovines, 77.7 % were conceived after providing the EVHP preparation for the management of repeat breeding. Species wise, 26 cows i.e. 74.3 % and 56 buffaloes i.e. 79.5 % were resulted in pregnancy. All the 84 pregnant cattle and buffaloes had completed the full-term pregnancy and delivered calves successfully. No abortion or still birth was reported.

Although reports on the effect of these EVM preparations on repeat breeding cases are scanty, however Satheshkumar *et al.* (2021) reported an overall conception rate of 57.1% among the post-partum anoestus cows (n=14) treated with the similar EVM preparations mentioned in the current study. Aloevera (*A. vera)* and white radish (*R. sativus)* are reported to have anti-microbial and anti-inflammatory effacts (Surjushe

et al., 2008, Bettaieb *et al.,* 2011). Elamaran *et al.* (2018) reported that the combination of Moringa (*M. oleifera),* Hadjod stem (*C. quadrangularisis)* and Curry leaves (*M. koenigii)* can improve the follicular maturation and ovulating capacity whereas *A. vera* and *R. sativus* help in cleansing the uterine environment. *M. oleifera (Moringa)* leaves rich in minerals and vitamins and also a source of insulin like proteins (Gopalakrishnan *et al.,* 2016). *C. quadrangularisis* phytoestrogen rich fraction have the capacity to increase the serum oestrogen, blood calcium level and vitamin D3 (Seema, 2015). In light of the foregoing, a combination of the aforementioned herbs and spices may have improved general health status and helped the reproductive system facilitate conception. Dey *et al.* (2020) documented various ethno-veterinary practices (EVPs) for retention of placenta, anoestrus and repeat breeding in dairy animals of rural Punjab which were found to be very effective as per farmers' observation.

For profitable dairy husbandry practices, a calf is expected every year from each cow. Enormous loss is caused to the farmers in such failure of conception. The animals of present study were not conceived even after performing several AI services and with conventional treatment in many of such cases. The EVM intervention in the present study has resulted in the successful management of a large population of the repeat breeding cases. However, further studies may be required on a large population to draw a firm conclusion.

4.4. Empirical data recorded in NDDB portal by veterinarians from different states for various bovine ailments treated by EVHP

EVHP therapy being carried out in the field across the country in the project areas have been captured online in which a total of 7,59,792 empirical cases of various ailments with overall 80 % cure rates have been recorded between 2017-18 and 2022-2023 (upto October 2022). The details depicted in Table 41. A representative photograph of demonstrating mastitis EVHP application to the veterinarians showed in Figure 33.

Figure 33: Demonstraion of EVHP application for mastitis to veterinarians and dairy farmers

Table 41: Empirical data on cure rates of various bovine ailments treated by EVHP between 2017-18 and 2022-23

S. No.	Ailment/ Symptoms	No of cases		% recovery
		Treated cases with EVHP	Clinically recovered	
1	Mastitis	255475	199785	78.2%
2	Fever	161015	132415	82.2%
3	Diarrhoea	149869	126444	84.4%
4	Indigestion	32158	26830	83.4%
5	Anoestrus	20300	14695	72.4%
6	Blood in milk	19133	16062	83.9%
7	Repeat breeder	15953	10567	66.2%
8	Worm infestation	14849	13409	90.3%
9	FMD mouth lesion	13947	12046	86.4%
10	Udder oedema	11547	9633	83.4%
11	FMD foot lesion	9703	7995	82.4%
12	Wound	9289	7531	81.1%
13	Retention of placenta	7761	5602	72.2%
14	Bloat	6493	4939	76.1%
15	Ticks	4999	4003	80.1%
16	Teat obstruction	5144	3451	67.1%
17	Endometritis	4247	3365	79.2%
18	Agalactia	3370	2466	73.2%
19	Downer syndrome	3095	2038	65.8%
20	Wart	3287	2326	70.8%
21	Lumpy Skin Disease (LSD)	3270	2165	66.2%
22	Swelling of Joints	2343	1711	73.0%
23	Prolapse	1807	1260	69.7%
24	Poisoning of unknown origin	738	509	69.0%
Total		7,59,792	6,11,247	80.4%

No of cases treated by the veterinarians with EVM for mastitis, fever and diarrhoea were above 1 lakh for each ailment; recovery rate details depicted in Figure 34.

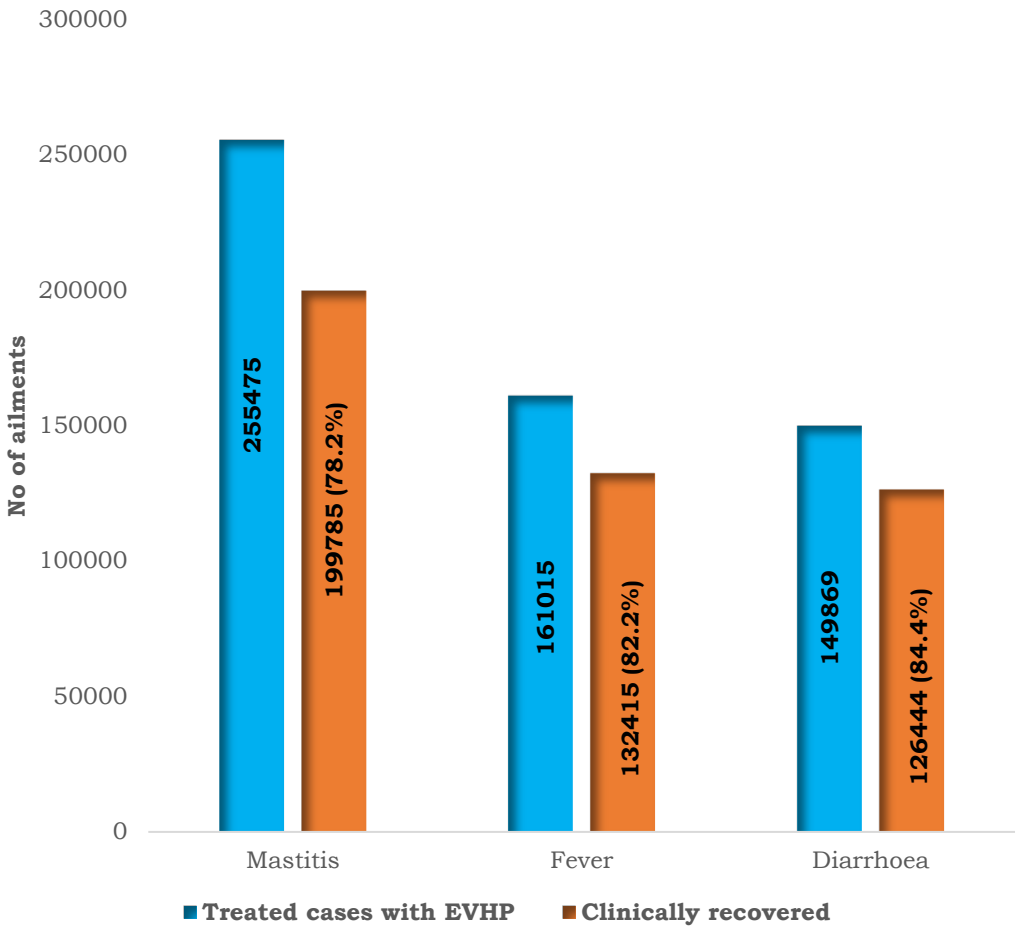


Figure shows in parenthesis is the per cent recovery rate

Figure 34: No of bovine ailments managed by EVHP with >100,000 empirical data

No of cases treated by the veterinarians with EVM for Indigestion, Anoestrus, Blood in Milk, Repeat Breeder, Worm Infestation, FMD mouth lesion and Udder Edema were between 10,000-1,00,000. The details of cases and their recovery rate are depicted in Figure 35.

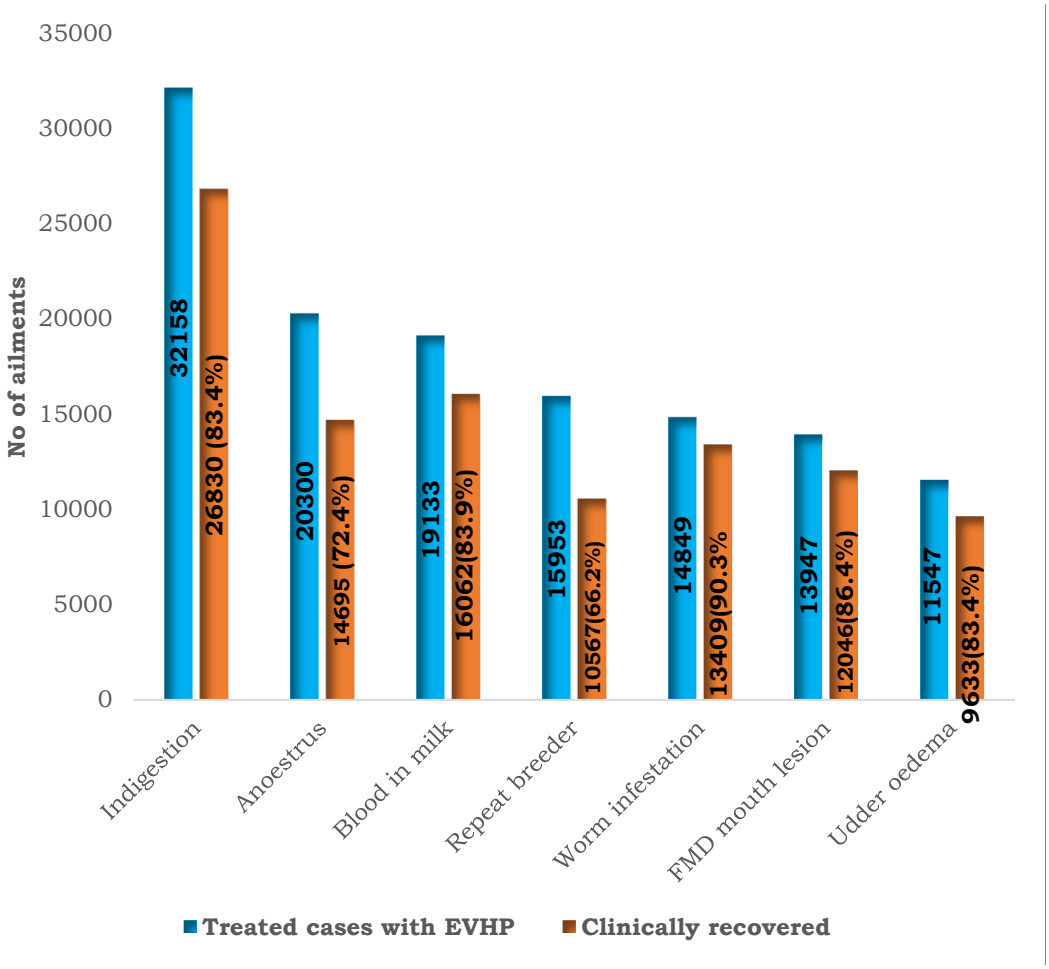


Figure shows in parenthesis is the per cent recovery rate

Figure 35: No of bovine ailments managed by EVHP with >10000 to <100,000 empirical data

No of cases treated by the veterinarians with EVM for FMD foot Lesion, Wound, Retension of Placenta, Bloat, Ticks and Teat Obstruction were between 5,000-10,000. The details of cases and their recovery rate are depicted in Figure 36.

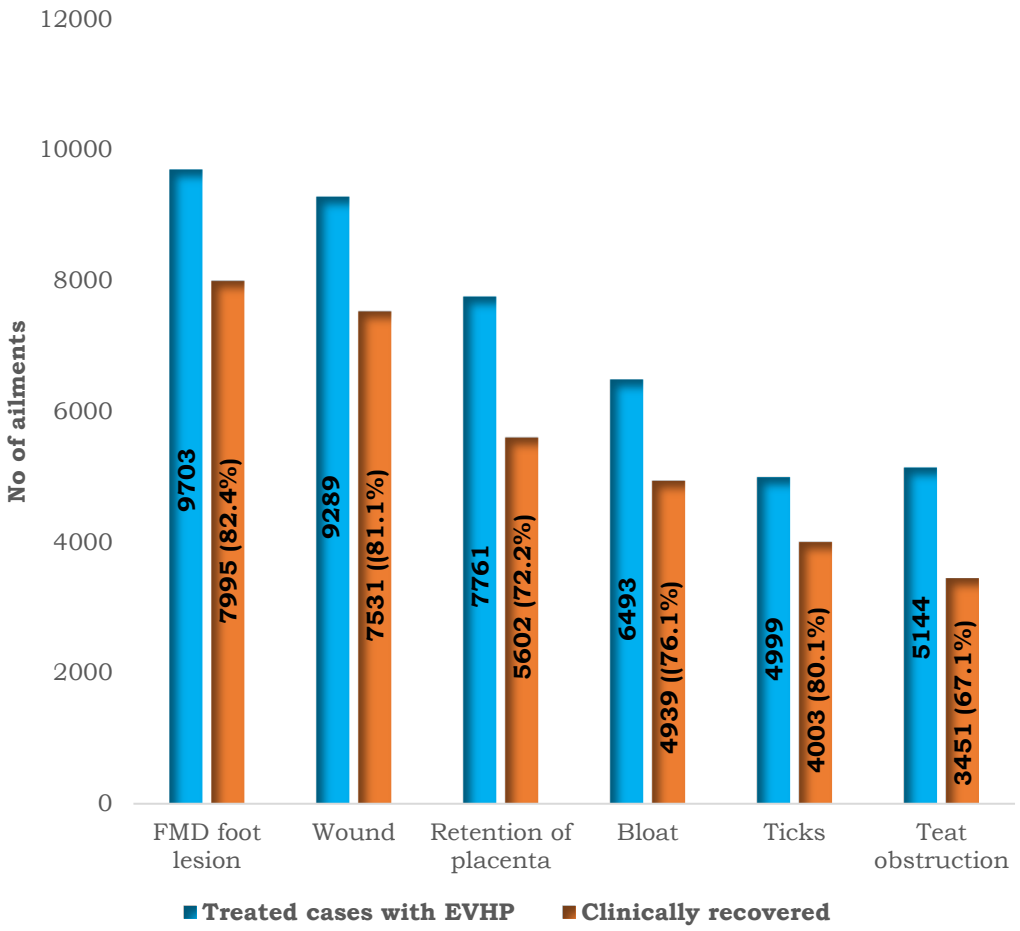


Figure shows in parenthesis is the per cent recovery rate

Figure 36: No of bovine ailments managed by EVHP with >5000 to <10,000 empirical data

No. of cases treated by the veterinarians with EVM for Endometritis, Agalactia, Downer syndrome, wart, Lumpy skin disease, Swelling of Joints, Prolapse and Poisoining of unknown origin were below 5000. The details of cases and their recovery rate are depicted in Figure 37.

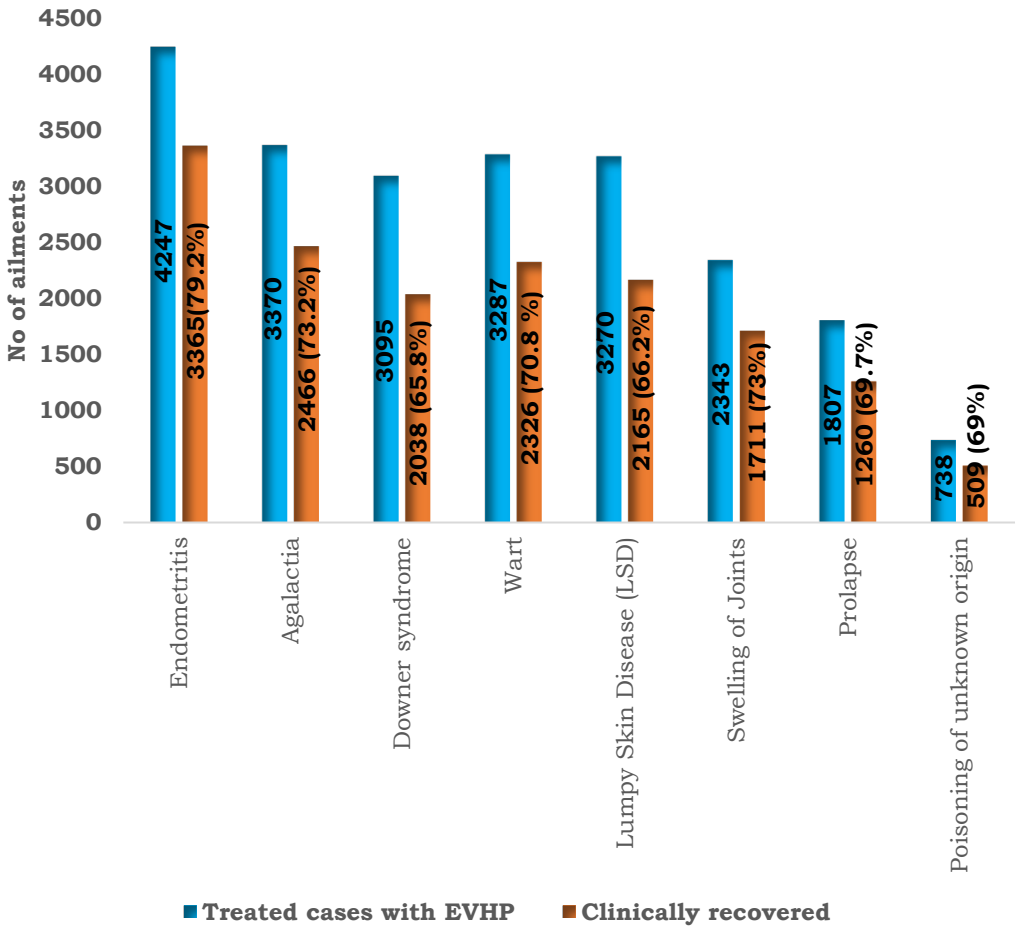


Figure shows in parenthesis is the per cent recovery rate

Figure 37: No of bovine ailments managed by EVHP with <5,000 empirical data

EVHP therapy being carried out in the field across the country in the project areas have been captured online in which a total of 7,59,792 empirical cases of various ailments with overall 80 % cure rates have been recorded between 2017-18 and 2022-2023 (upto October 2022).

In the present study, on an average 80 % clinical recovery rate was observed for different ailments/ symptoms managed with EVM which clearly indicates that EVM may play a great role in the management of bovine ailments in India. Similar observations were also recorded by Nair *et al.* (2017) indicating that a combination of Aloe vera 250 g, Curcuma longa 50 g and calcium hydroxide 15 g (NDDB Dairy Knowledge Portal, EVM booklet) is effective in managing bovine mastitis. Satheshkumar *et al.* (2021) recorded an overall 57.1% conception rate among the post-partum anoestus cows treated with EVM (NDDB Dairy Knowledge Portal, EVM booklet).

Currently, EVHP has been extensively used in the milk sheds of various MU/PCs. In one such MU, a 20 % reduction in the number of veterinarian's visit for attending the cases at farmer's doorstep has been recorded during 2020-21 in comparison to 2017-18, which might be attributed to more farmers adopting EVM to manage common ailments.

More than 1000 veterinarians and, around 7000 milk society personnel have been trained on EVM under the DCAM (Disease Control through Alternate Method) project. NDDB has prepared extension material like brochure, posters and videos in 12 languages (Hindi, Malayalam, Tamil, Kannada, Telugu, Odia, Marathi, Gujarati, Punjabi, Bengali, Assamese and English) on the EVM formulations for various ailments so as to reach the last mile farmer. The *e-Gopala* application has been developed and is available on Google Play Store as well as on the Web which provides the option of viewing both the document and the video for any specific ailment in question for which the formulation is available. A focused and diligent effort need to be made for popularization of EVM among farmers, paraveterinarians and veterinarians through demonstrations and vocational trainings. Regional compilation of EVM practices should be encouraged and authenticated. Good practices for herbal cultivation, harvest, processing and storage must be documented.

4.4.1. Preliminary Cost Benefit Analysis of EVHP use for the management of bovine mastitis

The estimated cost savings in mastitis management by use of EVHP in place of modern medicine during year 2017-18 to 2021-22 were revealed as Rs 847.96 million. The zone covered for the estimation was DCAM project areas. MCPP portal as well as milk union data was considered for this analysis. Details being tabulated in Table 42 and depicted in Figure 38.

Table 42: Preliminary Cost Benefit Analysis of EVHP use for the management of bovine mastitis

For EVHP intervention (mastitis)	17-18	18-19	19-20	20-21	21-22
Estimated cost for conventional treatment* (Rs in million)	23.18	94.92	223.86	295.36	245.98
Treatment cost with EVHP* (Rs in million)	0.93	3.80	8.95	11.81	9.84
Difference (cost saved) (Rs in million)	22.25	91.12	214.90	283.54	236.14
Total estimated cost saved during 2017-18 to 2021-22	Rs 847.96 million				

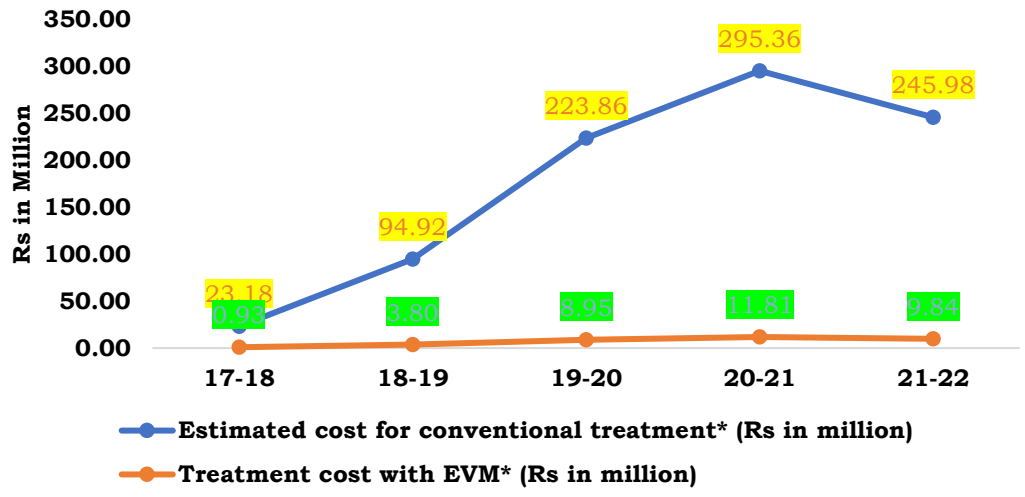


Figure 38: Cost saved by EVHP intervention for bovine mastitis (Cases = 294 thousands)

The estimated cost savings in treatment of bovine Indigestion cases by use of EVHP in place of modern medicine during year 2017-18 to 2021-22 were revealed as Rs 15.23 million. The zone covered for the estimation was DCAM project areas. Details being tabulated in Table 43 and depicted in Figure 39.

Table 43: Preliminary Cost Benefit Analysis of EVHP use for the management for Indigestion cases

Cost saved by EVHP intervention for Indigestion (Cases 30.74 thousand)	18-19	19-20	20-21	21-22
Estimated cost for conventional treatment* (Rs in million)	5.80	6.92	5.28	4.11
Treatment cost with EVHP* (Rs in million)	1.81	2.15	1.64	1.28
Difference (cost saved) (Rs in million)	4.00	4.76	3.64	2.83
Total estimated cost saved during 2017-18 to 2021-22	Rs 15.23 million			

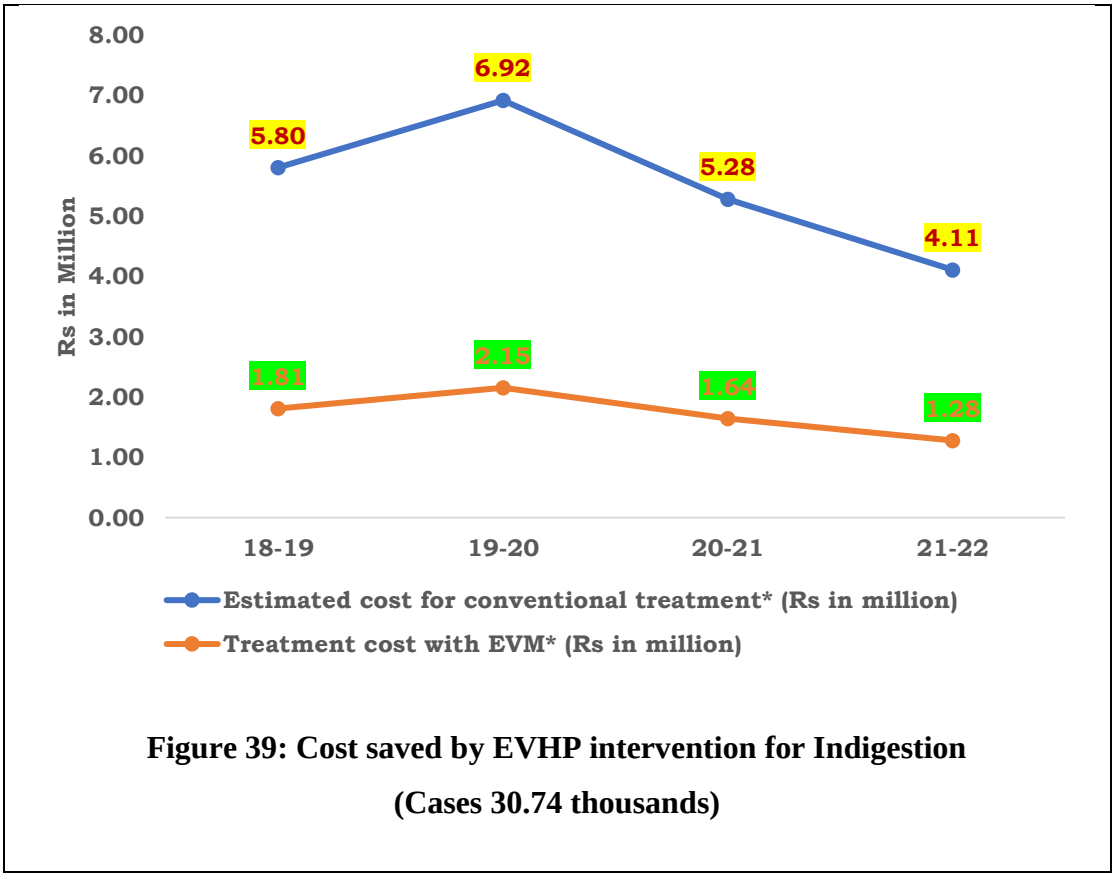


Figure 39: Cost saved by EVHP intervention for Indigestion (Cases 30.74 thousands)

The estimated cost savings in treatment of non-specific diarrhoea cases in bovine by use of EVHP in place of modern medicine during year 2017-18 to 2021-22 were revealed as Rs 61.36 million. The zone covered for the estimation was DCAM project areas. Details being tabulated in Table 44 and depicted in Figure 40.

Table 44: Preliminary Cost Benefit Analysis of EVHP use for the management for diarrhoea cases

Cost saved by EVHP intervention for Diarrhoea (Cases 183.71 thousand)	17-18	18-19	19-20	20-21	21-22
Estimated cost for conventional treatment* (Rs in million)	0.02	12.33	37.81	24.39	17.30
Treatment cost with EVHP* (Rs in million)	0.01	4.09	12.55	8.10	5.74
Difference (cost saved) (Rs in million)	0.01	8.24	25.26	16.29	11.56
Total estimated cost saved during 2017-18 to 2021-22	Rs 61.36 million				

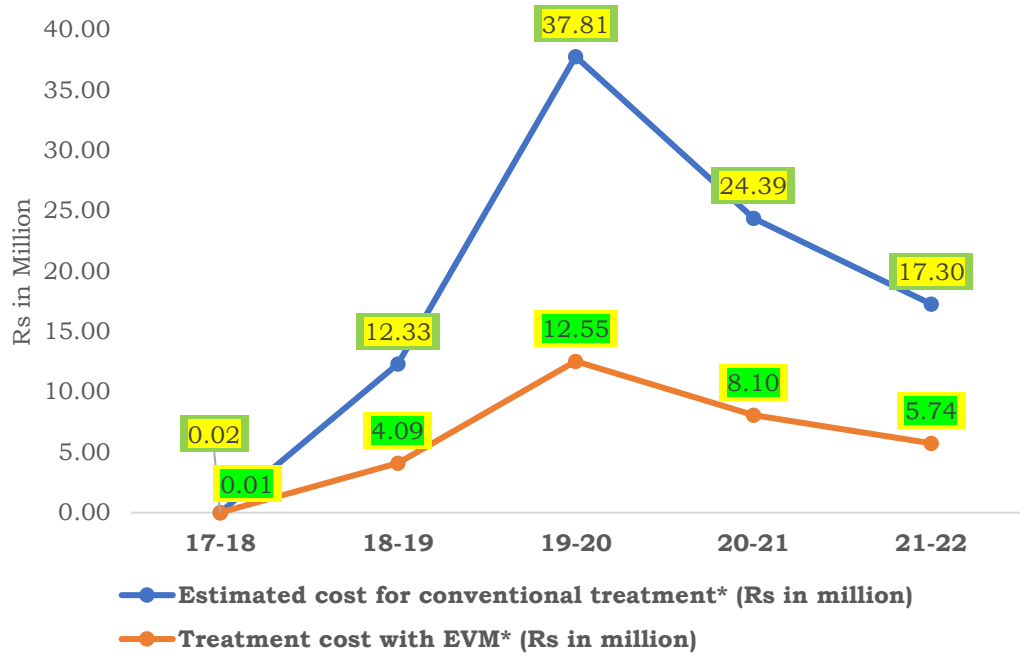


Figure 40: Cost saved by EVHP intervention for Diarrhoea (Cases 183.71 thousands)

4.5. Effect of using EVHP in bovine diseases on antibiotic residues in bulk milk in the study villages

Screening for presence of antibiotic residues in milk by BSTQ field kits was carried out quarterly in bulk milk samples from 10 villages. The sample wise result is shown in Table 45.

Table 45: Details on presence of antibiotic residues in milk samples tested by BSTQ kits (Quarterly tested in bulk milk)

Villag code	BSTQ_MK_MRL_V5 results 2019 (betalactam group)				BSTQ_MK_MRL_V5 results 2019 (quinoline group)				BSTQ_MK_MRL_V5 results 2019 (Sulfadimidine group)				BSTQ_MK_MRL_V5 results 2019 (Tetracycline group)			
	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4
VD1	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
VD2	POS	NEG	NEG	NEG	POS	NEG	NEG	NEG	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG
VD3	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
VD4	POS	LPOS	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
VD5	POS	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
VD6	POS	NEG	NEG	NEG	POS	NEG	NEG	NEG	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG
VD7	POS	NEG	NEG	POS	POS	NEG	NEG	NEG	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG
VD8	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
VD9	NEG	NEG	NEG	NEG	NEG	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
VD10	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
Q: Quarter																

4.5.1. Betalactam gr by BSTQ_MK_MRL_V5 field kits (2019-2020)

Out of bulk milk samples collected from 10 DCSs from one Milk Union, 70 % milk samples were found having presence of residues of betalactam group of antibiotics during 2019 1st quarter. By use of extensive EVHP in these villages, during 3rd quarterly bulk milk sampling, it was reduced to zero presence by the third quarter. The details are given in figure 41.

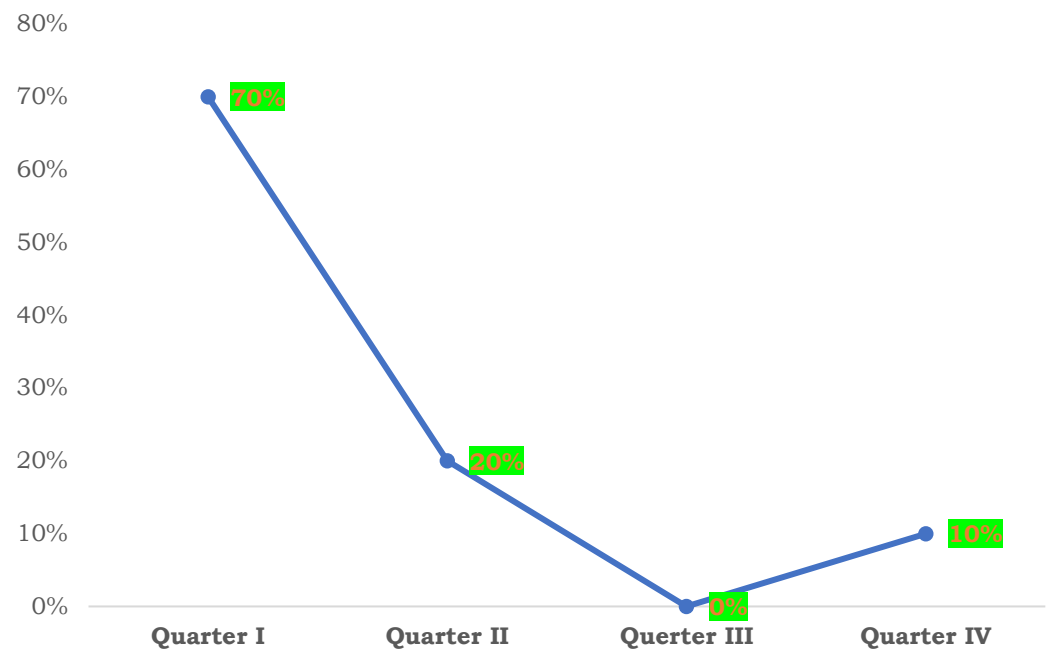


Figure 41: % positivity for Betalactam gr residue in bulk milk samples (N=10)

4.5.2. Quinolones gr by BSTQ_MK_MRL_V5 field kits (2019-2020)

Out of bulk milk samples collected from 10 DCSs from one Milk Union, 30 % milk samples were found having presence of residues of Quinolone group of antibiotics during 2019 1st quarter. By use of extensive EVHP in these villages, during 3rd quarterly bulk milk sampling, it was reduced to zero presence in the 3rd and 4th quarters. The details are given in figure 42.

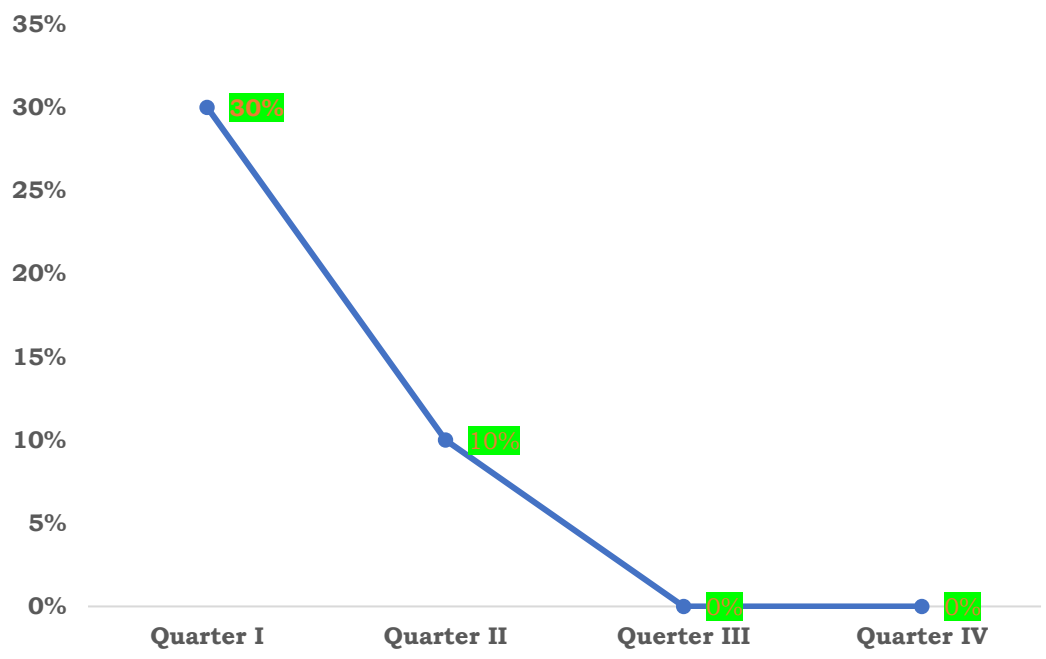


Figure 42: % positivity for Quinolones gr residue in bulk milk samples (N=10)

4.5.3. Sulphadimidine gr by BSTQ_MK_MRL_V5 field kits (2019-2020)

Out of bulk milk samples collected from 10 DCSs from one Milk Union, 30 % milk samples were found having presence of residues of Quinolone group of antibiotics during 2019 1st quarter. By use of extensive EVHP in these villages, during quarterly bulk milk sampling, it was reduced to zero presence by 2nd, 3rd and 4th quarters. The details are given in figure 43.

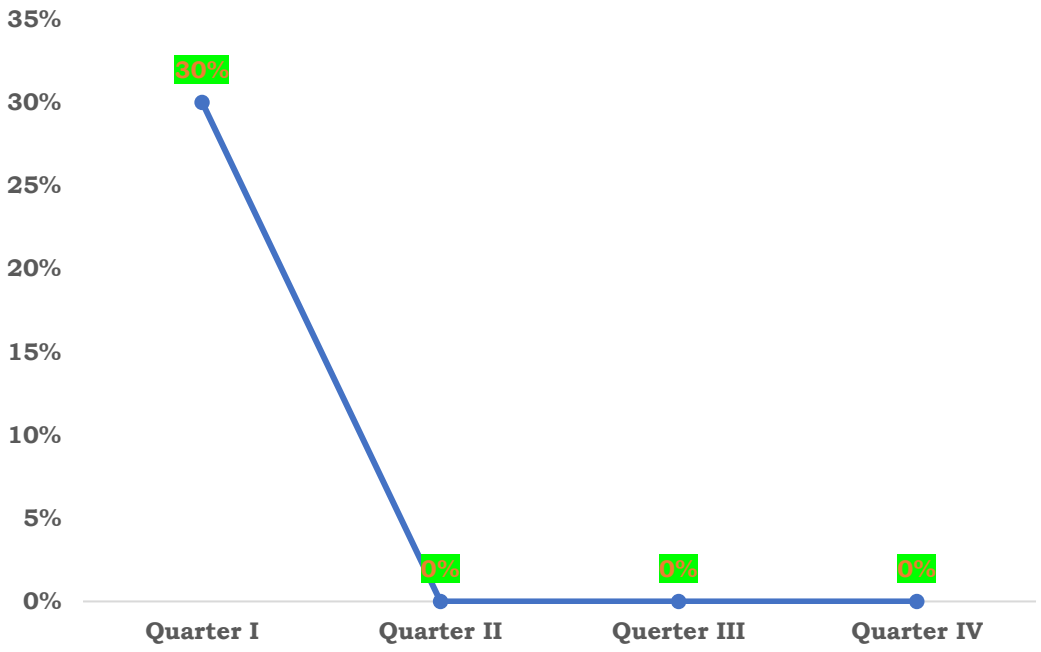


Figure 43: % positivity for Sulphadimidine gr residue in bulk milk samples (N=10)

4.5.4. Tetracycline gr by BSTQ_MK_MRL_V5 field kits (2019-2020)

Out of bulk milk samples collected from 10 DCSs from one Milk Union, no milk samples were found having presence of residues of tetracycline group of antibiotics during 2019 1st quarter and was continued no presence of this group antibiotic residues in milk up to 4th quarter.

Globally, the use of antimicrobial in animals is doubled than use in human (Aarestrup *et al,* 2012). out of these, 30%–70% being released unaltered into the environment (Kummerer, 2001). In our present study, before EVHP awareness and subsequent use in treatment of bovine diseases, the antibiotic residues in the test DCSs were found in around 70 % samples. Within 12 months of study period, antibiotic residues were found to be negative in the bulk milk samples of these DCSs.

The main reason of getting antibiotic residues in milk is injudicious usage of antibiotics in animals for treating infectious diseases (Zhang *et al.*, 2009). In analysing 224 articles across globe, Sachi *et al*., (2019) found that residues of β-lactam group have been detected mostly followed by the other antibioitics viz., tetracyclines, fluoroquinolones, sulfonamides and aminoglycosides which is a serious public health concern. Antibiotic residues in milk i.e. for antibiotics like penicillin, tetracycline, oxytetracycline, beta lactam group, enrofloxacin, neomycin, streptomycin, sulphonamides, chloramphenicol, gentamicin has reported by several authors in India (Kalla *et al*., 2015, Kumarswamy *et al.*, 2018, Nirala *et al.*, 2017, Lunden, 2015, Lejaniya *et al.*, 2017, Gaurav *et al.*, 2014). Antibiotic residues in milk is suspected to have potential effects on public health. It may cause antibiotic resistance allergic reactions, carcinogenicity, mutagenicity, teratogenicity and disturbances in the normal intestinal environment (Beyene, 2016).

4.6. SCC in bulk milk in study villages for 1.5 years (Sept' 2018-Apr'2020)

The average SCC of the milk samples from 15 villages were recorded as 7.36 lakh/ml during the initial study at August 2018. By March 2020, the bulk milk average SCC for these villages were coming down to 2.97 lakh/ml. The details are shown in Table 46 and Figure 44.

Table 46: SCC of bulk milk samples at study villages

Sr No	Village code	DCS bulk milk SCC per ml (in lakh)					
		Aug'18	Dec'18	Apr-19	Sep-19	Dec-19	Mar-20
1	VD1	5.15	2.54	16.05	4.55	4.2	3.45
2	VD2	4.97	1.39	2.57	2.78	2.4	2.22
3	VD3	4.64	4.8	10.21	6.71	5.31	2.42
4	VD6	6.45	3.07	5.19	4.01	3.01	3.8
5	VD8	7.96	19.63	1.37	4.72	4.22	2.35
6	VD11	7.6	9.49	3.83	2.91	2.24	1.56
7	VD12	9.15	3.16	10.18	4.4	4.14	5.98
8	VD13	9.41	5.22	14.56	3.69	2.72	1.37
9	VD14	9.41	11.8	7.26	6.52	5.54	4.19
10	VD15	9.09	8.49	7.27	5.23	4.87	3.39
11	VD16	8.1	3.65	6.11	5.4	4.87	3.18
12	VD17	6.91	5.19	9.29	1.65	2.65	2.93
13	VD18	6.58	7.47	6.03	2.59	3.2	3.52
14	VD19	6.74	5.16	7.27	1.87	2.66	1.52
15	VD20	8.18	1.04	6.19	1.39	2.54	2.73
Mean		7.36	6.14	7.56	3.89	3.64	2.97

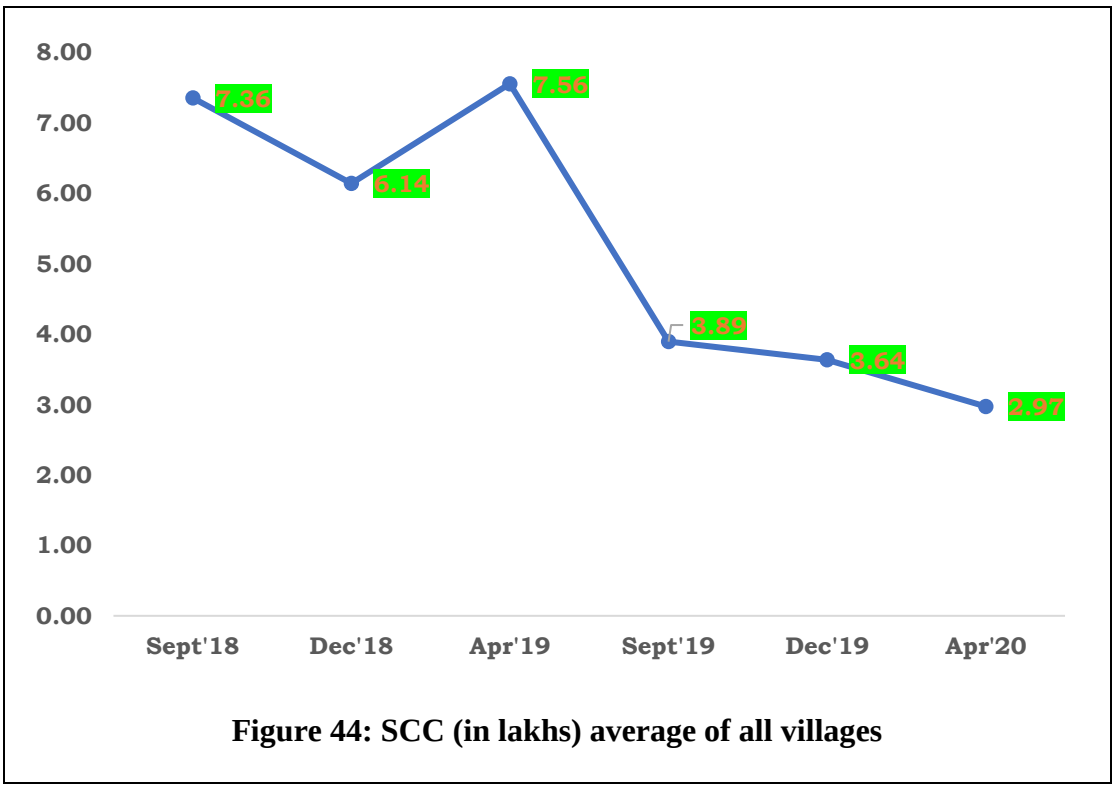


Figure 44: SCC (in lakhs) average of all villages

Nair *et al.* (2017) recorded external application of mixture of *Aloe vera* leaves, *Curcuma longa* rhizome and calcium hydroxide on udder also reduces SCC in mastitis

cows. In our present study, the initial record of 7.36 lakh per ml bulk milk SCC were coming down to 2.97 lakh per ml with the EVHP application in 15 DCSs. Mukherjee *et al*. (2005, 2010) also observed that use of EVHP can reduce SCC in sub-clinically mastitis infected cows.

4.7. Safety study of EVHP Mastitis formulation

Mortality: No mortality was found in treated group of rats throughout the observation period (**Table 47**).

Body Weight: The animals treated with test substance did not show a significant change in body weight hike on day 7 and 14 when compared with Day 0 **(Table 48a & b)**.

Feed Intake: The daily feed consumption of rats remained unaffected throughout the experimental period **(Table 49a & b).**

Toxicity Signs: No observed signs of toxicity such as Edema, Erythema, Eschar formation, Hyperkeratosis, Hyperplasia, Scaling, Wound formation, changes in circulation, respiration, central and autonomic nervous system, behavioral pattern were observed during the entire observation period **(Table 50a & b and 51a & b)**.

Gross Pathology:

1. No test compound linked findings were observed during necropsy. Gross findings were agonal in nature and had no relation to the treatment with the test substance **(Table 52)**.

2. All animals survived until the end of study.

Single topical administration of test substance at the dose 2000 mg/kg b wt caused no adverse toxic effects on the body weight / body weight changes, feed and the gross anatomy of treated female Wistar rats.

Table 47: Mortality Data of female rats

Details of the Group	Total no. of rats treated	Dose (mg/kg b.w)	Percent mortality (upto 14 days)
Distilled water (Vehicle)	5 Female	NA	0
Test substance Treated	5 Female	2000	0

B. w. - Body Weight

Table 48a: Weekly Mean Body Weight Changes in vehicle treated rats

Animal ID	Sex	Body Weight(g)		
		Day 0	Day 7	Day 14
2283161	Female	196.59	207.86	205.84
2283162	Female	195.83	194.32	197.02
2283163	Female	197.56	194.74	197.74
2283164	Female	201.77	204.71	209.89
2283165	Female	191.08	191.24	189.65
Mean		**196.57**	**198.57**	**200.03**
Standard Deviation		**3.83**	**7.25**	**7.95**

Table 48b: Weekly Mean Body Weight Changes in Test substance treated Rats

Animal ID	Sex	Body Weight(g)		
		Day 0	Day 7	Day 14
12879	Female	185.02	187.07	188.28
12880	Female	193.30	195.75	192.88
12881	Female	206.39	198.84	200.50
12882	Female	207.34	204.90	206.79
12883	Female	210.05	208.93	214.68
Mean		**200.64**	**199.10**	**200.63**
Standard Deviation		**10.74**	**8.46**	**10.58**

Table 49a: Daily Feed Intake by Rats Treated with Distilled Water

Animal ID	Sex	Day													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
12874	**F**	13.32	12.66	15.23	14.30	11.25	13.65	12.97	16.92	10.91	11.41	13.33	12.33	11.79	14.21
12875	**F**	15.94	10.74	13.46	13.00	10.84	12.89	9.96	11.71	13.15	11.31	11.49	10.97	15.10	13.26
12876	**F**	11.83	11.09	8.19	11.54	13.02	9.97	10.48	11.26	11.91	10.19	11.36	11.07	12.54	9.16
12877	**F**	12.24	11.67	14.94	13.34	14.69	12.50	13.72	19.91	13.78	9.63	15.16	12.19	13.89	14.04
12878	**F**	13.21	15.94	14.35	11.16	12.73	14.32	11.88	11.57	12.86	11.69	13.31	10.47	11.27	14.04
Mean		13.31	12.42	13.23	12.67	12.51	12.67	11.80	14.27	12.52	10.85	12.93	11.41	12.92	12.94
SD		1.60	2.10	2.90	1.30	1.54	1.66	1.60	3.93	1.13	0.89	1.57	0.81	1.57	2.15

M-Male; F-Female; SD- Standard deviation

Table 49b: Daily Feed Intake by Rats Treated with Test Substance

Animal ID	Sex	Day													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
12879	**F**	11.20	10.97	14.27	11.18	12.89	10.06	14.88	11.59	13.02	12.70	12.96	12.85	11.95	11.95
12880	**F**	11.27	14.13	13.82	16.90	11.90	13.36	9.77	12.69	12.27	12.03	13.51	10.31	12.16	12.45
12881	**F**	11.17	10.43	15.90	13.23	12.80	11.36	12.45	11.26	13.44	10.11	11.90	12.58	11.63	11.92
12882	**F**	11.27	11.78	15.98	17.22	12.36	14.13	11.82	15.08	8.56	12.57	14.03	13.14	11.82	11.88

12883	**F**	10.87	11.59	17.39	13.58	15.51	12.01	14.29	13.88	13.60	9.44	13.48	13.99	16.08	12.88
Mean		11.16	11.78	15.47	14.42	13.09	12.18	12.64	12.90	12.18	11.37	13.18	12.57	12.73	12.22
SD		0.17	1.42	1.44	2.58	1.41	1.61	2.04	1.59	2.09	1.50	0.81	1.37	1.88	0.44

F-Female; SD- Standard deviation

Table 50a: Toxicity signs observed in Female Rats Treated with Vehicle

Observation*	Day														
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Appeared Normal	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
Found death	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Catalepsy	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Chromodacryorrhea	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Clonic	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Coma	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Convulsion	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Diarrhea	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Dullness	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Excessive grooming	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Change in Gait	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Hyperactivity	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Lacrimation	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Nasaldischarge	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Nasal irritation	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Piloerection	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5

Polyuria	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Prostration	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Repetitive circling	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Respiratory distress	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Salivation	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Tonic	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Tremor	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Uro-genital staining	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5

*****No. of animals showing the clinical sign/No. of animals per group

Table 50b: Toxicity signs observed in Female Rats Treated with Test Substance

Observation*	**Day**														
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
Appeared Normal	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
Found death	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Catalepsy	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Chromodacryorrhea	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Clonic	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Coma	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5

Convulsion	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Diarrhea	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Dullness	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Excessive grooming	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Change in Gait	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Hyperactivity	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Lacrimation	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Nasaldischarge	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Nasal irritation	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Piloerection	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Polyuria	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Prostration	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Repetitive circling	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Respiratory distress	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Salivation	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Tonic	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Tremor	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Uro-genital staining	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5

*****No. of animals showing the clinical sign/No. of animals per group

Table 51a: Dermal Toxicity signs observed in Female Rats Treated with vehicle

Observation*	Day														
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Edema	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Erythema	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Eschar formation	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Hyperkeratosis	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Hyperplasia	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Scaling	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Wound formation	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5

*****No. of animals showing the clinical sign/No. of animals per group

Table 51b: Dermal Toxicity signs observed in Female Rats Treated with Test Substances

Observation*	Day														
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Edema	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Erythema	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Eschar formation	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Hyperkeratosis	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5

Hyperplasia	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Scaling	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Wound	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5

*****No. of animals showing the clinical sign/No. of animals per group

Table 52: Gross Pathology of female rats

Sr. No.	Sex	Animal No.	Status of the animal at the time of receipt at necropsy	Lesions observed at necropsy
1	F	12874	Live	NAD
2	F	12875	Live	NAD
3	F	12876	Live	NAD
4	F	12877	Live	NAD
5	F	12878	Live	NAD
6	F	12879	Live	NAD
7	F	12880	Live	NAD
8	F	12881	Live	NAD
9	F	12882	Live	NAD
10	F	12883	Live	NAD

F-Female; NAD- No Abnormalities Detected

Young adult Wistar female rats in good health who had EVHP treatment for mastitis on skin were found to be non-toxic and no mortality was observed. No outward manifestations of toxicity such as Edema, Erythema, Eschar formation, Hyperkeratosis, Hyperplasia, Scaling, Wound formation, changes in circulation, respiration, central and autonomic nervous system, behavioral pattern were observed throughout the whole observation period. Gross observations were agonal in nature and had no relation to the treatment with the test substance. Thus, it can be concluded that single topical administration of test substance at the dose 2000 mg/kg b wt caused no adverse toxic effects on the body weight/body weight changes, feed and the gross anatomy of treated female Wistar rats. The entire study was conducted in a NABL accredited laboratory. Details provide in Annex. 12.

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7. ANNEXTURES

7.1. Annex. 1 Survey form for Sabarkantha Milk Union

QUESTIONNAIRE FOR FARMER/PRODUCER

Society Name: ……

Veterinary Centre name: ……

Name of Farmer: ……

Village: ……

Education: ……

(Illiterate=1, Up to 5th class=2, Up to 12th Class=3, Graduate=4, PG & above=5)

Social group: ……

(General=1, Scheduled Caste=2, Scheduled Tribe=3, Other Backward Class=4)

Mobile ……

Land (Acres) ……

Annual Income: …… thousand

Occupation: …… (Dairying=1, Others=2)

No of cows (indigenous): In milk …..... Dry…….

Milk production/day…… liters,

No of Buffaloes: In milk …… Dry……...

Milk production/day…… liters,

No of cows (crossbred): In milk ….. Dry…….

Milk production/day…… liters,

1. Have you heard about traditional herbal Medicine use in cattle & Buffalo? (Yes=a, No=b) ☐

2. If yes, from where you come to know about herbal medicines for cattle & Buffalo? ☐
   a. Television
   b. Newspaper
   c. Milk Union Veterinarians
   d. State Veterinarians
   e. Other, specify……..

3. Have you ever use or seen use of any traditional herbal application for treatment of mastitis? ☐
   a. Yes
   b. No
   c. I have no idea

4. If yes, what is your experience regarding it? ☐
   a. Useful
   b. Not useful
   c. Sometimes beneficial

5. If yes (no 3), what was the approximate cost involved in the entire days treatment with the herbs? ☐
   a. Less than Rs 200
   b. In between Rs 201-500
   c. In between Rs 501-999
   d. Above Rs 1000

6. Are you aware of use of herbal medicine for Diarrhoea in cattle & Buffaloes? (Yes=a, No=b) ☐

7. If yes, what is your experience regarding it? ☐
   a. Useful
   b. Not useful
   c. Sometimes beneficial

8. Are you aware of use of herbal medicine for fever in cattle & Buffalo?
(Yes=a, No=b) ☐

9. If yes, what is your experience regarding it?
   a. Useful ☐
   b. Not useful
   c. Sometimes beneficial

10. Are you aware of use of herbal medicine for repeat breeding in cattle & Buffalo?
(Yes=a, No=b) ☐

11. If yes, what is your experience regarding it?
   a. Useful ☐
   b. Not useful
   c. Sometimes beneficial

12. Are you aware of use of herbal medicine for FMD in cattle & Buffalo?
(Yes=a, No=b) ☐

13. If yes, what is your experience regarding it?
   a. Useful ☐
   b. Not useful
   c. Sometimes beneficial

14. Are the herbal medicines are cost effective and easily available?
(Yes=a, No=b) ☐

15. Do you prefer to treat your animals with antibiotics for recovery from mastitis?
   a. Yes ☐
   b. No
   c. I have no idea

16. Are you aware that after antibiotic treatment, the treated antibiotic may pass through the milk of that animal?
   a. Yes ☐
   b. No
   c. I have no idea

7.2. Annex. 2a: Baseline survey form for various Milk union/Milk Producer Companies

Mastitis Control Popularization Project

Baseline survey format

QUESTIONNAIRE FOR FARMER/PRODUCER

Society Name..... Veterinary Centre name .....

Name of Farmer..... Village.....

Education (Illiterate = 1 Up to 5th Class = 2 , Up to 12th Class= 3 , Graduate= 4 PG & above = 5) ☐

Social group (General=1, Scheduled Caste=2, Scheduled Tribe = 3 , Other Backward Class= 4) ☐

Mobile ..... Land "Acres" ..... Annual Income: .....

Occupation: (Dairying=1, Others=2) ☐

No of cows (indigenous): In milk ... .. Dry..... ; Milk production/day..... liters,

No of Buffaloes: In milk ..... Dry..... ; milk production/day..... liters,

No of cows (crossbred): In milk ... Dry.....; milk production/day..... liters,

1. Have you heard about mastitis? (Yes = 1, No=2) ☐
2. What are the symptoms of mastitis? ☐
   (Multiple responses possible, tick applicable)
   1) Swelling and redness of Udder
   2) Pain in Udder
   3) Changes in milk colour
   4) Others.....
3. Are you aware of Sub-Clinical Mastitis (SCM)? (Yes = 1, No=2) ☐
4. If yes (no 3), how did you aware about SCM? ☐
   (Multiple responses possible, tick applicable)
   1) From Veterinarian
   2) Some training programme
   3) Extension literature
   4) Others.....
5. If yes (no 3), according to you, what are the main losses due to SCM? ☐
   (Multiple responses possible, tick applicable)
   1) Loss of milk
   2) Animal to animal contamination
   3) I don't know
   4) Others.....
6. Do you have SCM positive cattle/buffalo? ☐
   1) Yes
   2) No
   3) Don't know

Mastitis Control Popularization Project

Baseline survey format

7. If yes, how did you know that your animal has SCM? ☐
   (Multiple responses possible, tick applicable)
   1) From veterinarian
   2) By using paper strip
   3) CMT testing
   4) Other.....

8. Do you know about any testing to detect SCM? (Yes = 1, No=2) ☐

9. If yes, how frequently do you test your animal? ☐
   1) Six monthly
   2) Monthly
   3) When having doubt for mastitis
   4) Never testing
   5) Specify.....

10. When animal suffers from mastitis, what do you do? ☐
    1) Call a Vet
    2) Call a technician
    3) Treat by self
    4) Others.....

11. Do you prefer to treat your animals with antibiotics for recovery from mastitis? ☐
    1) Yes
    2) No
    3) I have no idea

12. Are you aware that after antibiotic treatment, the treated antibiotic may pass through the milk of that animal? ☐
    1) Yes
    2) No
    3) I have no idea

13. Have you ever use or seen use of Trisodium citrate (TSC) for treatment of subclinical mastitis? (Yes = 1, No=2) ☐

14. Have you ever use or seen use of any traditional herbal application for treatment of mastitis? ☐
    1) Yes
    2) No
    3) I have no idea

15. If yes, what is your experience regarding it? ☐
    1) Useful
    2) Not useful
    3) Sometimes beneficial
    4) Beneficial but time consuming

.....

7.3. Annex. 2b: Post-implementation survey form for vasrious Milk union/Milk Producer Companies

Mastitis Control Popularization Project
Cost benefit analysis questionnaire (Awareness creation)

Society Name :		Village:	
Name of Farmer:		Farmer No:	
Mobile:	Land (Acres) :	Annual Income:	
Details of milch animals owned and milk production			
	Cows (Indigenous)	Cows (Crossbred)	Buffaloes
No.of in-milk animals			
No.of dry animals			
Milk production/day (ltrs)			
Tick (√) whichever is applicable			
Education:	Illiterate = 1; Up to 5th Class = 2; Up to 12th Class= 3; Graduate= 4; PG & above = 5		
Social group :	General=1; Scheduled Caste=2; Scheduled Tribe = 3; Other Backward Class= 4		
Occupation:	Dairying=1; Others=2		

i	Are you aware of the Mastitis Control Project in your society? (Yes = 1; No=2)		
ii	Are you aware of Sub-Clinical Mastitis (SCM)? (Yes = 1; No=2)		
iii	If yes, according to you, what are the main losses due to SCM?		
	(1) Loss of milk	(3) Do not know	
	(2) Animal to animal contamination	(4) Others…	
iv	If yes, how did you come to know about SCM? (Multiple responses possible)		
	(1) Training programme by Union	(3) Extension posters	(5) Others……
	(2) From other milk producers	(4) Through media	
v	How often is your milk tested at DCS for SCM?		
	(1) Monthly	(3) Every 3-4 months	(5) Never
	(2) Every 1-2 months	(4) Every 6-7 months	(6) Do not know
vi	Have any of your animals tested positive from SCM? (Yes = 1; No=2)		
vii	If yes, how did you know that your animal had SCM?		
	(1) From Veterinarian	(3) CMT testing	
	(2) By using paper strip	(4) Others…	
viii	If yes to the above, what was the treatment given for the SCM positive animal?		
	(1) Oral TSC therapy	(3) EVM therapy	(5) EVM+antibiotic
	(2) Antibiotic therapy	(4) TSC+ Antibiotic	(6) No treatment
ix	Was there improvement in milk production after treatment for SCM?		
	(1) Yes	(2) No	(3) Don't know
x	Do you test/get your animal tested for SCM if a reduction in milk production is seen?		
	(1) Yes	(3) Sometimes	(5) Others……
	(2) No	(4) Would like to but do not know how	

Mastitis Control Popularization Project
Cost benefit analysis questionnaire (Awareness creation)

xi	Have you been made aware of ethno-veterinary medicine (EVM) for mastitis? (Yes = 1; No=2)		
xii	If yes to the above, how did you come to know about it? (Multiple responses possible)		
	(1) Awareness by Union	(3) From other producers	(5) Others.....
	(2) Extension posters	(4) From media (print, TV etc)	
xiii	Have you treated any of your animals by EVM for clinical mastitis? (Yes = 1; No=2)		
xiv	If yes, what is your experience regarding it?		
	(1) Very effective	(2) Somewhat effective	(3) Not effective
xv	Why would you want to adopt EVM for clinical mastitis? (Multiple responses possible)		
	(1) Very effective	(3) Easy to prepare and apply	(5) Can do it by self
	(2) Very cheap	(4) Reduces the use of drugs	(6) Others.....
xvi	Would you advocate EVM for clinical mastitis and other ailments to others?		
	(1) Yes	(2) No	(3) Don't know
xvii	Are you aware that after antibiotic treatment, the treated antibiotic may pass through the milk of that animal? (Yes = 1; No=2)		
xviii	How would you prefer to treat clinical mastitis? (Antibiotic = 1; EVM=2)		
xix	In what way has the mastitis control programme helped you? (Multiple responses possible)		
	(1) Improved production	(3) Reduced dependency on antibiotics	(5) Provided knowledge on EVM
	(2) Reduced costs	(4)Helped manage other ailments also	(6) Others.....
xx	How would you rate the mastitis control project in your DCS? (covering SCM control and EVM)		
	(1) Excellent	(3) Average	
	(2) Good	(4) Not satisfactory	

7.4. Annex. 3: List of Consumables, culture media etc used for Isolation, Identification, antibiogram of bacterial agents from mastitic milk.

Consumables:

- Triangular Metal spreader (HiMedia, Catalogue no: LA876-1X10NO)
- Cotton swabs (HiMedia, Catalogue no: PW005-1X500NO)
- Petri plates 90 mm in diameter (Himedia, Catalogue no: PW 1132)
- Gloves (Himedia, Catalogue no:LA915-10X20NO)
- Iso-propyl alcohol 99% (Loba Chemie Pvt.Limited, Product code: 0027005000)
- PhoenixSpec Calibrator kit (BD, REF: 440911)
- PID panel (BD, REF: 448008)
- PMIC-84 panel (BD, REF: 448420)
- NID panel (BD, REF: 448007)
- NMIC-404 panel (BD, REF: 448788)
- PMIC/ID Combo panel (BD, REF: 448763)
- NMIC/ID Combo panel (BD, REF: 448935)
- SMIC/ID Combo panel (BD, REF: 448858)
- Centrifuge (Eppendorf, 5810 R)
- BSTQ strips
- PortaSCC strips

Equipment:

- For continuing the present study, following equipment were used from NDDB's R & D laboratory, Hyderabad
- Laminar air flow (Laminar),
- Vortex Mixer (Thermo scientific 88880018),
- Table top centrifuge (Eppendorf, 5810R),
- BD Phoenix M50 detection system (BD, M50),
- Nephelometer (BD, Phoenixspec)
- Light Microscope (Olympus BX50),
- electrophoresis assembly (BioRad Power Pac HV),
- PCR machine (Veriti, 96 well Thermal cycle, Applied Biosystems),
- Gel Doc system (Bio-Rad XR+) and
- Freeze (New Brunswick Scientific, U725 INNOVA),

Culture media

- Blood agar base, modified (Himedia, Catalogue no: M1989-500G)
- EMB Agar (Himedia, Catalogue no: M317-500G)
- MacConkey agar Medium 8 (Himedia, Catalogue no: MM081-500G)
- Mannitol salt agar base (Himedia, Catalogue no: M118-500G)
- Nutrient agar No. 2 (Himedia, Catalogue no: M1269-500G)
- Nutrient broth (Himedia, Catalogue no: M002-500G)
- Baird parker agar medium (Himedia, Catalogue no: MU043-500G)
- HiCrome Klebsiella Selective Agar Base (Himedia, Catalogue no: M1573-500G)

Reagents

- BD Phoenix ID broth (BD, REF: 246001)
- BD Phoenix AST broth (BD, REF: 246003)
- BD Phoenix AST-S broth (BD, REF: 246007)
- BD Phoenix AST indicator (BD, REF: 246004)
- BD Phoenix AST-S indicator (BD, REF: 246009)
- Phosphate buffered saline (Dulbecco A) (Himedia, Catalogue no: RM7385)
- Gram's staining kit (Himedia Catalogue no: K001L-1KT)
- AmpliTaq Gold Master mix (Applied Biosystems, REF: 4398886)
- GelPilot 100 bp Ladder (Qiagen, Catalogue no: 239035)
- GelPilot 50 bp Ladder (Qiagen, Catalogue no: 239025)
- Primers (Eurofins Genomics)
- Nuclease free water (Integrated DNA technologies)

7.5. Annex. 4. Identification of different types of Bacteria on the basis of Colony characteristics on Blood Agar

Sr. No	Name of bacteria	Pigmentation	Size	Texture	Elevation	Edges	Haemolysis
1	*E. coli*	Creamy	Big	Shiny	Convex	Regular/ Circular	Beta haemolysis
2	*Klebsiella pneumonia*	Creamy	Big	Mucoid	Convex	Regular/ Circular	Non-haemolytic
3	*Str. agalactiae*	Semi-transparent	Small	Shiny	Convex	Regular/ Circular	Non-haemolytic
4	*Str. dysgalactiae*	Semi-transparent	Small	Shiny	Convex	Regular/ Circular	Non-haemolytic
5	*Str. uberis*	Semi-transparent	Small	Shiny	Convex	Regular/ Circular	Alpha prime Haemolysis
6	*Staph. aureus*	Yellow-White	Big	Glistening	Convex	Regular/ Circular	Beta haemolysis
7	*Staph. epidermidis*	White	Small	Glistening	Convex	Regular/ Circular	Non-haemolytic

7.6. Annex 5: Table on detection limits of various antibiotics in milk by field antibiotic residue detection kit (BSTQ, Unisensor)

Beta Lactam	Limit of Detection (ppb)
Penicillins	
Penicillin G	2 to 3
Ampicillin	3-4
Amoxicillin	3-4
Oxacillin	12-18
Cloxacillin	6-8
Dicloxacillin	6-8
Nafcillin	30-40
Cefalosporines	
Ceftiofur	10 to 15
Cefquinome	30-35
Cefazolin	18-22
Cefapirine	6-8
Cefacetrile	30-40
Cefoperazone	3-4
Cefalexin	1000-1200
Cefalonium	3-5
Sulfonamides	
Sulfadiazine	8-10
Sulfapyridine	0.5-1
Sulfathiazole	7.5-8.5
Sulfamethoxazole	320-360
Sulfamethazine	1-2
Sulfamethoxypyridazine	2-3

Beta Lactam	Limit of Detection (ppb)
Sulfadimethoxine (Sdm)	10-15
Sulfacetamide	300-600
Sulfamerazine	2-3
Sulfamonomethioxine	8-12
Sulfaquinoxaline	14-18
Sulfachloropyridazine	5-10
Sulfaguanidine	15-25
Sulfamethizole	220-260
Sulfasalazine	250-350
Tetracycline	
Tetracycline	80-100
Oxytetracycline	60-70
Chlortetracycline	50-60
Doxycycline	20-30
(Fluoro) Quinolone	
Norfloxacin	15-20
Enrofloxacin	10 to 15
Danofloxacin	15-20
Difloxacin	15-20
Marbofloxacin	10 to 15
Sarafloxacin	15-25
Ciprofloxacin	15-20
Flumequine	15-30
Oxolinic acid	50-75
Enoxacin	15-20
Lomefloxacin	20-30

7.7. Annex 6: Bacterial count of pre and post EVHP mastitis milk by Total Bacterial Count method (TBC):

- Milk samples were taken from the refregerator and keep at room temperature for one hour and afterwards, vortexed the milk samples to disperse bacteria uniformly from milk fat.
- Addition of 0.5 ml of milk sample was carried out to the 4.5 ml of nutrient broth in 15 ml falcon tube which was further vortex properly for uniform mixing.
- Serial dilution of milk was done in nutrient broth up to three dilutions. 0.1 ml of diluted milk samples from each dilution poured on the 5-7% blood agar plates.
- Spreading the samples on blood agar was done uniformly with the L-shaped spreader.
- The blood agar plates were kept at 37 ^{0}C for 24 hours of incubation in the incubator.
- The colonies on blood agar were identified after incubation of 24 hours on the basis of colony morphology.
- Blood agar plates with no growth of microbes were further incubated at 37 ^{0}C within incubator for further 48-72 hours.

7.8. Annex. 7: Table on interpretation of CMT gel formation

Observation	Grading	Score	Interpretation
No thickening of the mixture	Negative (-)	N	No infections
Slight thickening of the mixture. disappeared quickly	Trace (-)	T	Possible infections
Distinct thickening of the mixture. No gel formation tendency	Weak Positive (+)	1	Infected (If CMT paddle is rotated more than 20 seconds, thickening may disappear)
Immediate thickening of the mixture, with a slight gel formation. As mixture is swirled, it moves toward the center of the cup, exposing the bottom of the outer edge. When motion stops, mixture levels out and covers bottom of the cup.	Distinct Positive (++)	2	Infected
Gel is formed and surface of the mixture becomes elevated (like a fried egg). Central peak remains projected even after the CMT paddle rotation is stopped	Strong Positive (+++)	3	Infected

7.9. Annex. 8: Use of Porta SCC (Field Somatic Cell Count) kits for detection of SCC in milk

Field Somatic Cell Count (SCC) testing SOP:

1. Ensure proper mixing of milk before sampling.
2. Add 1 drop of sample to the test strip sample well by using the specific pipet.
3. Allow the milk to be absorbed into the well.
4. Add 3 drops of '*Activator Solution'* to the sample well.
5. Wait for 45 minutes after which the test strip is ready for the reader.

Calibrating the reader:

1. Turn on the reader by pressing the blue button on the left.
2. Code 543 should appear on screen.
3. Insert the control strip into the reader (face downward and forward).
4. Remove control strip when a strip and drop symbol appears.
5. Reader is ready for test strips when the drop symbol disappears.

Reading:

1. Insert the prepared test strip into the reader, face down and forward
2. Enter the result as shown in the reader (including LO or HI) in the locked excel sheet provided against the respective unique DCS codes.
3. Repeat as necessary for additional test strips.
4. Re-calibrate the reader after reading a maximum of 5 test strips.

Precautions:

1. Use only freshly collected bulk milk with no preservatives.
2. Perform the test within 8 hours of collection.
3. Milk samples should be brought to room temperature before testing.
4. Test strips should be stored at 2-25°C. Refrigerate if possible.
5. Do not run the test under direct sunlight.
6. Use only the pipette provided in the kit.
7. Do not touch the pipette tip during testing
8. Do not press and hold the down arrow of the reader for more than 5 seconds.

   It will change the calibration of the reader and '543' code will not appear.

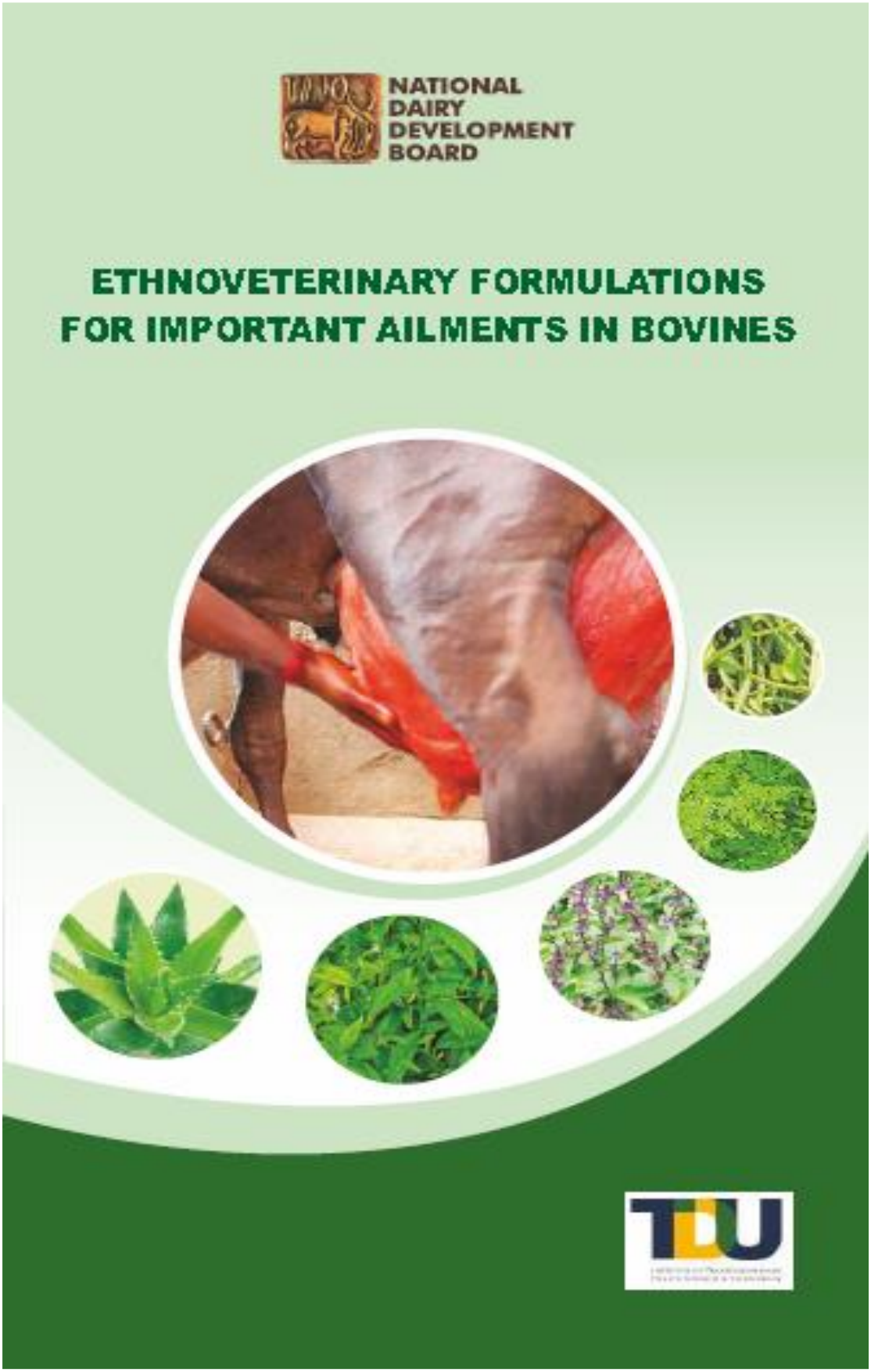
Interpretation of messages displayed on the reader:

1. LO: If cell count is less than 50,000 cells/ml
2. HI: If cell count is greater than 30,00,000 cells/ml
3. E2: Test strip was removed before a reading could be taken. Turn it back on, blank the reader and reread the test strip.
4. No display: The reader shuts itself off automatically after 2 minutes of inactivity. Turn it back on, blank it, and continue to read test strips.

7.10. Annex 9: Estimated cost for EVHP and modern medicine by Nair *et al.* (2022)

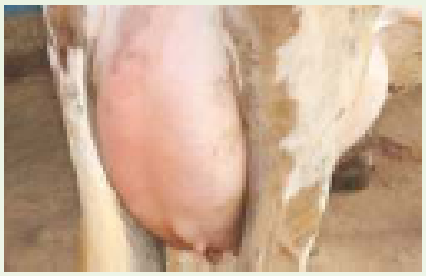
S. No.	Ailment	In Indian Rupee		
		Modern Drug Treatment cost	**EVHP treatment cost**	**Amount saved per case**
1	Bovine mastitis	3000.00	120.00	2880.00
2	Indigestion	719.40	224.00	495.40
3	Diarrhoea	500.00	166.00	334.00

7.11. Annex 10: EVM treatment booklet (NDDB)



Mastitis (all types)

Aloe vera



Turmeric Powder

Lemon

Lime

Water based Preparation

Ingredients: For one day

a) Aloe vera – 250 g; b) Turmeric powder – 50 g; c) Calcium Hydroxide (lime) – 15 g; d) Lemon – 6 nos.

Preparation:

(i) Cut Aloe vera whole leaf into small pieces (after removing the thorns). (ii) Blend along with turmeric powder and lime to form a reddish paste.

Application:

(i) Wash, clean and completely milk out all quarters (including unaffected). (ii) Take a handful of the paste and add 200ml of water to make it thin. (iii) Apply the paste diluted in water ten times a day for 5 days each time after following the step (i) above. (iv) Last application of the day should be oil based preparation. (v) Feed two lemons at a time orally (cut into halves) thrice a day for 3 days.

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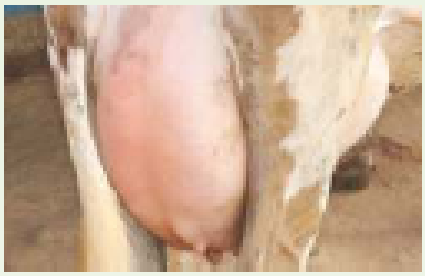
Mastitis (all types)

Aloe vera



Mustard or Gingelly oil

Lemon

Lime

Turmeric Powder

Oil based Preparation

Ingredients : For one day

Aloe vera (whole leaf) - 250 g; Turmeric powder - 50 g; Calcium hydroxide (lime) - 15 g; Lemon - 6 no.s ; Mustard or Gingelly oil - 600 ml.

Preparation:

(i) Cut Aloe vera whole leaf into small pieces (after removing the thorns).(ii) Blend along with turmeric powder and lime to form a reddish paste.

Application:

(i) Wash, clean and completely milk out all quarters (including unaffected) and dry the udder.(ii) Take a handful of the paste and add 200ml of mustard or gingelly oil to make it thin.(iii) Apply the paste diluted in oil three times a day for 5 days each time after following the step (i) above. (iv) Feed two lemons orally at a time (cut into halves) thrice a day for 3 days.

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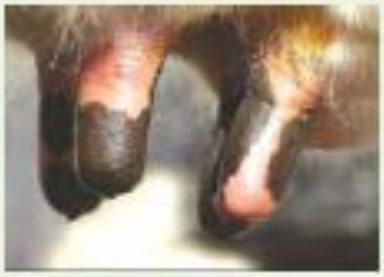
Teat obstruction

Neem leafstalk



Turmeric Powder

Butter

or

Ghee

Ingredients:

Freshly plucked & clean neem leafstalk-1; Turmeric powder; Butter or Ghee

Preparation:

(i) Nip the neem leafstalk from the top at the required length based on teat length, leaving the base intact. (ii) Coat the turmeric powder & butter/ghee mixture thoroughly on the neem leafstalk. (iii) Clean the affected teat opening thoroughly.

Application:

(I) Insert the coated neem leafstalk with cut end up, base down into the affected teat in an anti-clockwise direction. (ii) Replace with fresh neemstalk after each milking.

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Udder Oedema

Sesame/
Mustard oil

Turmeric
Powder

Garlic

Ingredients: For one time

Sesame or mustard oil - 200 ml; Turmeric powder- 1 handful; Garlic-2 pearls.

Preparation:

(i) Heat oil, add turmeric powder and sliced garlic. (ii) Mix well and remove from flame just as the flavour develops (no need to boil). (iii) Allow to cool.

Application:

(i) Apply in a circular manner with force over the entire oedematous region and udder. (ii) Apply 4 times a day for 3 days.

Note: Rule out mastitis before using the formulation.

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Retention of Placenta

Radish

Lady's finger

Jaggery

Salt

Ingredients:

White radish -1 full tuber; Lady's finger - 1.5 kg; Jaggery - as required; Salt - as required

Preparation:

(i) Cut each lady's finger into 2 pieces.

Application:

(i) Feed one full tuber radish within two hours of calving. (ii) Feed 1.5 Kg of fresh lady's finger with jaggery and salt if ROP persists after 8 hours of calving. (iii) In case ROP persists even after 12 hours of calving, tie a knot very close to the base and cut 2 inches below the knot and leave it. The knot will go in. (iv) Do not try to remove the retained placenta by hand. (v) Feed one full tuber of radish once a week for four weeks.

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Repeat breeding


Radish


Aloe vera


Cissus


Curry leaves


Salt


Moringa


Jaggery


Turmeric Powder

Application:

(i) Start treatment on 1st or 2nd day of heat. (ii) Feed orally in fresh form in the following order once a day along with jaggery and salt:

(a)1 white radish daily for 5 days (b) 1 Aloe vera leaf daily for 4 days. (c) 4 handfuls of moringa leaves for 4 days. (d) 4 handfuls of cissus stem for 4 days. (e) 4 handfuls of curry leaves with 5 gram turmeric powder for 4 days. (f) Repeat the treatment once again if the animal has not conceived.

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Prolapse

Aloe vera gel

Turmeric Powder

Mimosa leaves

Ingredients:

Aloe vera gel - from one full leaf; Turmeric powder - one pinch; Mimosa pudica leaves - 2 handfuls.

Preparation:

(i) Remove the gel from a whole leaf. (ii) Wash it multiple times till the sliminess is reduced. (iii) Make volume upto 1 litre by adding water. (iv) Add a pinch of turmeric powder and boil to half the original volume and allow to cool (v) Prepare a paste of M. pudica leaves separately.

Application:

(i) Clean the prolapsed mass (ii) Sprinkle the gel on the prolapsed mass. (iii) Apply M.pudica paste after the gel dries. (iv) Repeat the process as frequently as possible till the condition improves.

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FMD mouth lesions

Ingredients: For one dose

Cumin seeds - 10 g; Fenugreek seeds - 10 g; Black pepper - 10 g; Turmeric powder - 10 g; Garlic - 4 pearls; Coconut - 1; Jaggery - 120 g.

Preparation:

(i) Soak cumin, fenugreek and black pepper seeds in water for 20-30 mts. (ii) Blend all ingredients to a fine paste. (iii) Add 1 full grated coconut to the paste and mix by hand only. (iv) Prepare dose freshly for each application.

Application:

(i) Apply gently inside the mouth, tongue and palate. (ii) Give the preparation thrice a day for 3 to 5 days.

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FMD foot lesions/wound


Acalypha


Neem


Garlic


Coconut oil

or


Sesame oil


Mehndi


Turmeric Powder


Tulsi


Anona

Ingredients:

Acalypha indica leaves - 1 handful; Garlic-10 pearls; Neem leaves - 1 handful; Coconut or Sesame oil - 500 ml; Turmeric powder - 20 g; Mehndi leaves - 1 handful; Tulsi leaves - 1 handful.

Preparation:

(i) Blend all the ingredients thoroughly. (ii) Mix with 500 ml coconut or sesame oil and boil and bring to cool.

Application:

(i) Clean the wound and apply directly or bandage with a medicated cloth. (ii) Apply Anona leaf paste or camphorated coconut oil for the first day only if maggots are present.

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Fever

Ingredients: For one day

Garlic - 2 pearls; Coriander- 10 g; Cumin -10 g; Tulsi - 1 handful; Dry cinnamon leaves - 10 g; Black pepper - 10 g; Betel leaves - 5 no.s; Shallots/Onion - 2 bulbs; Turmeric powder - 10 g; Chirata leaf powder - 20 g; Sweet basil - 1 handful; Neem leaves - 1 handful; Jaggery - 100 g.

Preparation:

(i) Soak cumin, pepper and coriander seeds in water for 15 mts. (ii) Blend and mix all ingredients to form a paste.

Application:

(i) Administer orally in small portions in the morning and evening.

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Diarrhoea

Fenu greek

Pepper

Onion

Poppy

Cumin

Turmeric Powder

Garlic

Curry leaves

Jaggery

Asafoetida

Ingredients: For one day

Fenugreek seeds - 10 g; Onion - 1 no.; Garlic - 1 pearl; Cumin seeds - 10 g; Turmeric Powder - 10 g; Curry leaves - 1 handful; Poppy seeds - 5 g; Pepper- 10 g; Jaggery - 100 g; Asafoetida - 5 g.

Preparation:

(i) Dry fry cumin seeds, asafoetida, poppy seeds and fenugreek seeds till smoke emanates.(ii) Cool and powder the fried seeds.(iii) Blend it with rest of the ingredients to form a paste.

Application:

(i) Roll the paste into small balls. (ii) Administer orally in small portions once daily for 1-3 days till condition cures.

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Bloat and Indigestion


Onion


Pepper


Garlic


Betel leaves


Chilly


Turmeric Powder


Jaggery


Ginger


Cumin


Salt

Ingredients: For one day

Onion - 100 g; Garlic-10 pearls; Dry Chilly - 2; Cumin seeds - 10 g; Turmeric Powder - 10 g; Jaggery - 100 g; Pepper - 10 g; Betel leaves - 10 no.s; Ginger - 100 g

Preparation:

(i) Soak pepper and cumin seeds for 30 mts. (ii) Blend along with other ingredients to form a paste.

Application:

(i) Roll the paste into small balls. (ii) Administer orally in small portions with salt 3-4 times a day for 3 days.

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Worms

Onion


Pepper

Garlic


Turmeric Powder


Mustard


Neem


Cumin


Jaggery


Banana stem


Common leucas


Bitter gourd

Ingredients: For one day

Onion- 1 no; Garlic-5 pearls; Mustard seeds - 10 g; Neem leaves - 1 handful; Cumin - 10 g; Bitter gourd - 50 g; Turmeric Powder - 5 g; Pepper- 5 g; Banana stem - 100 g; Common leucas -1 handful; Jaggery - 100 g.

Preparation:

(i) Soak pepper , cumin and mustard seeds for 30 mts. (ii) Blend along with other ingredients to form a paste.

Application:

(i) Roll the paste into small balls. (ii) Administer in small portions with salt once daily for 3 days.

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Tick/Ectoparasites

Ingredients:

Garlic - 10 pearls; Neem leaves - 1 handful; Neem fruit - 1handful; Acorus rhizome - 10 g; Turmeric powder - 20 g; Lantana leaves - 1 handful; Tulsi leaves - 1 handful.

Preparation:

(i) Blend all the ingredients. (ii) Add one litre of clean water. (iii) Strain with a fine sieve or muslin cloth.(iv) Transfer to a bottle attached to a sprayer.

Application:

(i) Spray on the entire body of the animal. (ii) Also spray on any cracks and crevices in the cattle shed.(iii) Application can also be done using a cloth dipped in the solution.(iv) Repeat once a week till the condition resolves. (v) Do the application only during sunny part of the day.

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Pox/wart/cracks


Garlic


Butter


Turmeric Powder


Neem


Cumin


Sweet basil

Ingredients:

Garlic-5 pearls; Turmeric powder - 10 g; Cumin seeds - 15 g; Sweet basil - 1 handful; Neem leaves - 1 handful; Butter(preferred) or ghee - 50 g.

Preparation:

(i) Soak cumin seeds in water for 15 mts. (ii) Blend all ingredients to a fine paste. (iii) Add butter and mix well.

Application:

(i) Apply on affected part as many times as possible till condition resolves. (ii) Apply after drying the skin surface.

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Allergy/Poisoning/Venomous sting/bite

Ingredients: For one dose

(Three Kings: as per Tamil traditional Siddha lore)

Betel leaves -10 no.s; Black pepper - 10 g; Salt - 10 g; Jaggery - as required.

Preparation:

(i) Blend the ingredients to form a paste. (ii) Mix with jaggery.

Application:

(i) Feed the dose in small portions (ii) Administer 3 doses daily for 2 weeks.

Note : Alternatively 2-3 drops may be instilled in eye every one hour in critical conditions (without jaggery).

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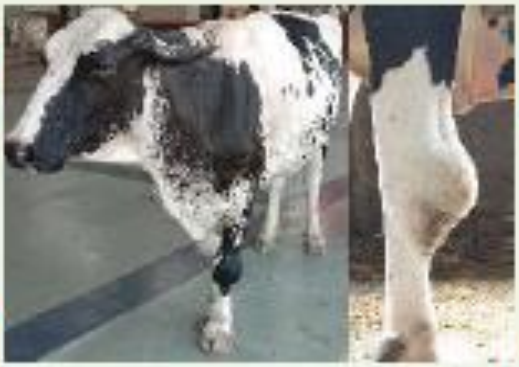
Hygroma (swelling of joints)

Aloe vera



Garlic

Lime

Cissus

Gingelly oil

Turmeric Powder

Ingredients:

Aloe vera - 100 g; Lime (Calcium hydroxide) - 10 g; Cissus quadrangularis stem - 100g; Turmeric Powder - 15 g; Garlic - 5 cloves; Gingelly oil - 1 litre.

Preparation:

(i) Blend all the ingredients to a paste (ii) Boil in 1 litre gingelly oil and allow to cool.

Application:

(i) Apply four or five times a day on affected part.
(ii) Give hot water fomentation twice daily.

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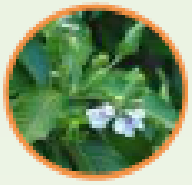
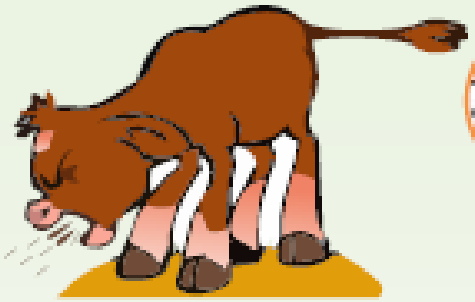
Cough



Adhathoda (Adusa)



Pepper

Tulsi

Garlic

Turmeric Powder

Jaggery

Ingredients: For one day

Adhathoda (Adusa) - 1 leaf; Tulsi - 1 handful; Garlic - 5 cloves; Turmeric Powder - 10 g; Pepper - 10 g; Jaggery - as required.

Preparation:

(i) Soak pepper for 15-20 minutes and grind separately (ii) Blend all the ingredients together to form a paste with jaggery.

Application:

Feed orally 2-3 times daily till the condition resolves.

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Downer (not able to get up)

Desi chicken eggs

Cissus

Moringa

Jaggery

Ingredients: For one dose

Desi chicken eggs - 2; Moringa leaves - 4 handfuls; Cissus quadrangularis - 4 handfuls; Jaggery - as required.

Preparation:

(I) Take fresh unboiled eggs (ii) Make a paste of Moringa and Cissus stem separately with jaggery.

Application:

(i) Feed 2 eggs (including shell) at a time, three times a day (make a small hole in the shell before feeding the egg). (ii) Feed moringa and cissus stem paste alternatively every 2 hours (four handfuls at a time) (iii) Do not attempt to lift the animal till the 4th day.

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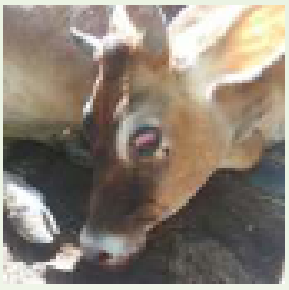
Toxicity (Pesticide/HCN/Mycotoxin)

Betel



Moringa

Pepper

salt

Jaggery

Tamarind

Ingredients:

Three kings : For one dose

Betel leaves - 10 nos; Black pepper - 10 g; Salt - 10 g; Jaggery - as required.

Other preparation: For one day

Tamarind - 1 Kg; Water - 1 Litre; Moringa extract from 1 Kg leaves.

Preparation:

Three kings preparation

(i) Blend Betel leaves, Black pepper and salt to form a paste (ii) Mix with jaggery.

Other ingredients preparation

(i) Soak tamarind pulp for 15 minutes. (ii) Extract the juice from the pulp (iii) Add water, moringa leaf extract and jaggery (iv) Mix it thoroughly.

Application:

(i) Give the first dose of three kings (ii) Feed 200 ml of the tamarind-moringa-jaggery as a thick slimy mixture every 2 hours (iii) Feed doses of three kings mixture in between.

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Blood in Milk

Curry leaves

Jaggery

Moringa

Lemon

Ingredients: For one day

Curry leaves - 2 handfuls; Moringa leaves - 2 handfuls; Jaggery - 100 g; Lemon - 6 no.s

Preparation:

Blend curry and moringa leaves to a paste along with jaggery. Cut the lemon in two halves.

Application:

(i) Feed the paste twice daily till the condition resolves.

(ii) Feed two lemons at a time orally (cut in two halves) thrice a day for 3 days.

Note: Carry out EVM treatment for mastitis also.

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Anoestrus

Application:

Feed orally in fresh form in the following order along with jaggery and salt: (i) One white radish twice a day for 5 days (ii) One Aloe vera whole leaf twice a day for 4 days.(iii) Four handfuls of moringa leaves twice a day for 4 days. (iv) Four handfuls of cissus stem twice a day for 4 days. (v) Four handfuls of curry leaves with 5 gram turmeric Powder twice a day for 4 days.

Note: Carry out deworming 15 days prior to start of treatment.

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Prepared with technical inputs from Prof. N. Punniamurthy (profpunniya@gmail.com)
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Website: www.nddb.coop
facebook.com/NationalDairyDevelopmentBoard/

July 2021

7.12. Annex. 11: Dermal toxicity study of Mastitis EVHP on mice

TOXICOLOGY REPORT

Report Date	: 27.06.2022
SR No.	: CAF/S/264
Sample ID No.	: 345
Sample Name	: Herbal Formulation for Mastitis
Identification and condition of the test item	: Samples received in good condition
Test item received on	: 19.05.2022
Sample description	: Coarse Powder
Report prepared on	: 26.06.2022
Name & address of the customer	: Dr Panbaj Dutta, Manager, Animal Health, National Dairy Development Board, Anand.

Name & Address of the Testing Laboratory
Central Animal Facility, SASTRA Deemed University, Thanjavur - 613 401.
Email : panchapakesan@sastra.edu
Phone : +914362-264101-108 Extn. 3680
Fax : +914362-264120

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Methods

1. Standard procedure for Euthanasia & Necropsy as per CARISM/SOP/CAF/01
2. Standard procedure for Receipt, Handling and Preparation of test Substance as per CARISM/SOP/CAF/05
3. Standard procedure for Route of Administration in animals as per CARISM/SOP/CAF/06
4. Standard procedure for Animal Handling as per CARISM/SOP/CAF/07
5. Standard procedure for Animal Identification as per CARISM/SOP/CAF/08
6. OECD Guidelines for the Testing of Chemicals Acute Oral Toxicity – Up-and-Down-Procedure (UDP) Adopted: 3 October 2008

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TEST SYSTEM

Test Species : *Rattus norvegicus*

Strain : Wistar rats

Age : Healthy young adult animals between 8 and 12 weeks

Source : Central Animal Facility, SASTRA Deemed University

Number of Animals : Ten

Dose : 2000 mg/Kg. B wt.

Route of administration : Dermal

Identification of Animals

Tags marked with animal number, group number and dose level were attached to the respective cages. Each animal was identified by unique identification number by ear tagging.

Animal ID Normal control	Sex	Animal ID Test substance treated	Sex
12874	Female	12879	Female
12875	Female	12880	Female
12876	Female	12881	Female
12877	Female	12882	Female
12878	Female	12883	Female

Acclimatization

Seven days prior to the experiment.

ANIMAL HUSBANDRY

Animal House Condition

Temperature of the test room was maintained between 22±3°C and relative humidity between 50 to 70 % during the experimental period. The experimental room was provided with a 12h light and 12h dark lighting condition using an automatic timer.

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Housing

Standard polypropylene rat cages with stainless steel top grill was used to house the animals. The cages were autoclaved. Sieved and sterilized paddy husk was used as the bedding material. Animals were housed individually.

Sanitation

Bedding material, cages, grills and water bottles were changed weekly twice.

Animal Welfare and Regulatory Compliance

The experiment was conducted at the Central Animal Facility (CPCSEA Registration No. 817/PO/ReRcBiBt/S/04/CPCSEA dated 06-08-2004) for Breeding and Experiments of Animals by the Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Forest and Environment, Govt. of India.

The study was conducted after the approval by the Institutional Animal Ethical Committee, SASTRA Deemed University (IAEC Approval Number: 701/SASTRA/IAEC/RPP).

Diet and Water

Standard rodent pellet feed supplied by M/s. ATNT Laboratories, Mumbai, India and Reverse Osmosis (RO) water were provided to the animals *ad libitum*.

METHODS

Preparation of Test Substance

The test substance (345) at the dose 2000 mg/Kg b. wt was mixed in distilled water and applied uniformly over the skin, held in contact with porous gauze dressing and non-irritating.

Treatment

Healthy Wistar rats (10 female) were selected and allowed for an acclimation period of 7 days. The fur was removed from the dorsal area of the trunk of the test animals by clipping using electric trimmer before 24 hours of experiment. The test substance (345) was applied uniformly over the skin and wrapped with a porous gauze dressing and non-irritating tape throughout a 24-hour exposure period. The animals were returned to their cages immediately after topical application. At the end of the exposure period, residual test substance was removed using water.

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OBSERVATIONS

Observation Period: 14 days

Mortality

All animals were observed twice every day for mortality for 14 days.

Body Weight

Body weight of each animal was recorded just prior to the test substance treatment (Day 0), Day 7 and 14 using electronic animal weighing balance (Sartorius AG, Germany).

Feed Intake

Feed intake for individual animals was recorded daily for the entire study period.

Toxicity Signs

All the animals were observed individually after the treatment of the test substance during the entire observation period for the presence of any signs of toxicity including alopecia, catalepsy, chromodacryorrhea, clonic, coma, convulsion, diarrhea, dullness, excessive grooming, change in gait, hyperactivity, lacrimation, nasal discharge, nasal irritation, piloerection, polyuria, prostration, repetitive circling, respiratory distress, salivation, scaling, tonic, tremor and uro-genital staining. In addition, dermal related toxicity signs such as edema, erythema, eschar formation, hyperkeratosis, hyperplasia, scaling, and wound formation were observed.

Gross Pathology

All animals were subjected to necropsy at the end of 14 day observation period for gross pathological examination.

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RESULTS

Mortality

No mortality was observed in treated group of rats throughout the observation period (**Table 1**).

Body Weight

The animals treated with test substance (345) did not show a significant change in body weight gain on day 7 and 14 when compared with Day 0 **(Table 2a & b)**.

Feed Intake

The daily feed intake of rats remained unaffected throughout the experimental period **(Table 3a & b)**.

Toxicity Signs

No visible signs of toxicity such as Edema, Erythema, Eschar formation, Hyperkeratosis, Hyperplasia, Scaling, Wound formation, changes in respiration, circulation, autonomic and central nervous system, behavioral pattern were observed during the entire observation period **(Table 4a & b and 5a & b)**.

Gross Pathology

1. No test compound related findings were observed at necropsy. All gross observations were agonal in nature and bore no relation to treatment with the test substance **(Table 6)**.
2. All animals survived until the end of study.

CONCLUSION

Single topical administration of test substance (345) at the dose 2000 mg/kg b wt caused no adverse toxic effects on the body weight / body weight changes, feed and the gross anatomy of treated female Wistar rats.

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Table 1: Mortality Data

Details of the Group	Total no. of rats treated	Dose (mg/kg b.w.)	Percent mortality (upto 14 days)
Distilled water (Vehicle)	5 Female	NA	0
Test substance (345) treated	5 Female	2000	0

b.w. - Body Weight.

Table 2a: Weekly Mean Body Weight Changes in vehicle treated rats

Animal ID	Sex	Body Weight (g)		
		Day 0	Day 7	Day 14
2283141	Female	196.59	207.86	205.84
2283142	Female	195.83	194.32	197.02
2283143	Female	197.56	194.74	197.74
2283144	Female	201.77	204.71	209.89
2283145	Female	191.08	191.24	189.65
Mean		**196.57**	**198.57**	**200.03**
Standard Deviation		**3.83**	**7.25**	**7.95**

Table 2b: Weekly Mean Body Weight Changes in Test substance (345) treated Rats

Animal ID	Sex	Body Weight (g)		
		Day 0	Day 7	Day 14
12879	Female	185.02	187.07	188.28
12880	Female	193.50	195.75	192.88
12881	Female	206.39	198.84	200.50
12882	Female	207.34	204.90	206.79
12883	Female	210.05	208.93	214.68
Mean		**200.46**	**199.10**	**200.63**
Standard Deviation		**10.74**	**8.46**	**10.58**

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Table 3a: Daily Feed Intake by Rats Treated with Distilled Water

Animal ID	Sex	Day													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
12874	F	13.32	12.66	15.23	14.30	11.25	13.65	12.97	16.92	10.91	11.41	13.33	12.33	11.79	14.21
12875	F	15.94	10.74	13.46	13.00	10.84	12.89	9.96	11.71	13.15	11.31	11.49	10.97	15.10	13.26
12876	F	11.83	11.09	8.19	11.54	13.02	9.97	10.48	11.26	11.91	10.19	11.36	11.07	12.54	9.16
12877	F	12.24	11.67	14.94	13.34	14.69	12.50	13.72	19.91	13.78	9.63	15.16	12.19	13.89	14.04
12878	F	13.21	15.94	14.35	11.16	12.73	14.32	11.88	11.57	12.86	11.69	13.31	10.47	11.27	14.04
Mean		13.31	12.42	13.23	12.67	12.51	12.67	11.80	14.27	12.52	10.85	12.93	11.41	12.92	12.94
SD		1.60	2.10	2.90	1.30	1.54	1.66	1.60	3.93	1.13	0.89	1.57	0.81	1.57	2.15

M - Male; F- Female; SD – Standard deviation;

Table 3b: Daily Feed Intake by Rats Treated with Test Substance (345)

Animal ID	Sex	Day													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
12879	F	11.20	10.97	14.27	11.18	12.89	10.06	14.88	11.59	13.02	12.70	12.96	12.85	11.95	11.95
12880	F	11.27	14.13	13.82	16.90	11.90	13.36	9.77	12.69	12.27	12.03	13.51	10.31	12.16	12.45
12881	F	11.17	10.43	15.90	13.23	12.80	11.36	12.45	11.26	13.44	10.11	11.90	12.58	11.63	11.92
12882	F	11.27	11.78	15.98	17.22	12.36	14.13	11.82	15.08	8.56	12.57	14.03	13.14	11.82	11.88
12883	F	10.87	11.59	17.39	13.58	15.51	12.01	14.29	13.88	13.60	9.44	13.48	13.99	16.08	12.88
Mean		11.16	11.78	15.47	14.42	13.09	12.18	12.64	12.90	12.18	11.37	13.18	12.57	12.73	12.22
SD		0.17	1.42	1.44	2.58	1.41	1.61	2.04	1.59	2.09	1.50	0.81	1.37	1.88	0.44

F - Female; SD – Standard deviation

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Table 4a: Toxicity signs observed in Female Rats Treated with Vehicle

Observation *	Day														
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Appeared Normal	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
Found death	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Catalepsy	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Chromodacryorrhea	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Clonic	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Coma	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Convulsion	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Diarrhea	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Dullness	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Excessive grooming	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Change in Gait	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Hyperactivity	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Lacrimation	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Nasal discharge	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Nasal irritation	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Piloerection	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Polyuria	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Prostration	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Repetitive circling	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Respiratory distress	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Salivation	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Tonic	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Tremor	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Uro-genital staining	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5

* No. of animals showing the clinical sign / No. of animals per group

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Deputy Technical Manager-Toxicology

Authorised by

Technical Manager

Table 4b: Toxicity signs observed in Female Rats Treated with Test Substance (345)

Observation *	Day														
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Appeared Normal	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
Found death	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Catalepsy	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Chromodacryorrhea	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Clonic	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Coma	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Convulsion	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Diarrhea	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Dullness	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Excessive grooming	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Change in Gait	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Hyperactivity	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Lacrimation	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Nasal discharge	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Nasal irritation	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Piloerection	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Polyuria	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Prostration	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Repetitive circling	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Respiratory distress	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Salivation	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Tonic	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Tremor	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Uro-genital staining	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5

* No. of animals showing the clinical sign / No. of animals per group

Analysed by

Deputy Technical Manager-Toxicology

Authorised by

Technical Manager

Table 5a: Dermal Toxicity signs observed in Female Rats Treated with vehicle

Observation *	Day														
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Edema	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Erythema	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Eschar formation	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Hyperkeratosis	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Hyperplasia	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Scaling	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Wound formation	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5

* No. of animals showing the clinical sign / No. of animals per group

Table 5b: Dermal Toxicity signs observed in Female Rats Treated with Test Substance (345)

Observation *	Day														
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Edema	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Erythema	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Eschar	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Hyperkeratosis	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Hyperplasia	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Scaling	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Wound	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5

* No. of animals showing the clinical sign / No. of animals per group

Table 6 : Gross Pathology

S. No.	Sex	Animal No.	Status of the animal at the time of receipt at necropsy	Lesions observed at necropsy
1	F	12874	Live	NAD
2	F	12875	Live	NAD
3	F	12876	Live	NAD
4	F	12877	Live	NAD
5	F	12878	Live	NAD
6	F	12879	Live	NAD
7	F	12880	Live	NAD
8	F	12881	Live	NAD
9	F	12882	Live	NAD
10	F	12883	Live	NAD

F –Female; NAD- No Abnormalities Detected

Analysed by

Deputy Technical Manager-Toxicology

Authorised by

Technical Manager