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Research Article

Mastitis Prevalence in Suburban Villages of Bhubaneswar City, Associated Risks Factors, and Antimicrobial Susceptibility of the Isolated Pathogens from Milk Samples of Dairy Cows

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Abstract

Mastitis is characterized by increase of somatic cell counts (SCC) in secreted milk, local inflammation associated with swelling, heat, pain, redness of the udder, and abnormal looking milk. The problem continues to remain highly prevalent in high yielders and causes significant economic losses to the farmers. The present study was carried out for one year in small holder dairy farms in the suburban villages of Bhubaneswar city. A total of 1024 cows, each with yield record of minimum seven litres milk per day, belonging to 398 farmers, were recruited for the study. A total of 4094 quarter milk samples from 1024 lactating cows were screened for mastitis. The overall prevalence of mastitis, subclinical and clinical mastitis in current study was 41.1%, 32.8% and 8.3%, respectively. The prevalence of subclinical and clinical mastitis in Holstein Friesian cross was 38.8% and 11.9%, respectively. The highest prevalence was recorded in cows aged more than 7years. The parity number was an important risk factor as mastitis was more common in the animals with increasing parity number. The incidence of mastitis increased with higher hygiene score. A total of 422 pooled milk samples showed bacterial growth and 417 (98.63%) bacterial isolates could be identified, either as single (94.50%) or mixed (1.37%) infection. The most common bacterial cause of infectious SCM was Staphylococcus species constituting 55.4% of infections. The highest degree of antimicrobial sensitivity was recorded for marbofloxacin (98.88%). The outcome of the present study may facilitate the mastitis control by developing specific strategies to reduce cost of treatment and antibiotics overuse, and to enhance food safety and public health.

Keywords: Mastitis; Hygiene, Teat Dipping; Prevalence; Quarter, Sensitivity

Introduction

Mastitis is a multi-etiological disease, associated with inflammation of parenchyma of mammary glands. It continues to remain a highly prevalent disease despite innovations in therapeutic regimens and improved management practices for its prevention. The disease is characterized by an increase of SCC in secreted milk or in the mammary tissue of the udder. The disease causes local inflammation of the udder with swelling, heat, pain, redness, and abnormal-looking milk production such as discoloration or clots, called Clinical mastitis (CM), or an increased SCC with normal appearance of the milk, termed as SCM [1]. Thus, in contrast to visible changes in the clinical form of mastitis, gross abnormalities in the milk or udder are absent in subclinical mastitis. The average incidence of clinical mastitis ranges from 10 to 20 % in most of the herds and the prevalence of mammary infection stands around 50 % of quarters [2]. The SCM is 3-4 times more prevalent than clinical mastitis [3]. However, SCM is 15 to 40 times more prevalent than CM

as per available data retrieved from four electronic databases [4]. The CM in dairy cows can be immediately intervened by veterinary services because of visible changes. Thus, the incidence of CM has decreased considerably worldwide as a result of comprehensive control measures. Dairy cows with SCM might be overlooked by farm owners asthe infected cows do not present any clinical signs [5]. Therefore, milk production losses are more in SCM than CM [6]. Bovine mastitis, particularly SCM, is one of the most devastating diseases in dairy cows [7,8]. The incidence of mastitis, especially SCM, has increased continuously in most dairy farms due to lack of comprehensive strategy to prevent and control this disease. Thus, an overall assessment of SCM prevalence is necessary to facilitate its prevention and control. The prevalence also differs considerably among studies, possibly due to variation in the location, the number of dairy cows examined, and the stage of lactation and parity in cows. Therefore, regular monitoring of region-wise SCM prevalence is a prerequisite to implement prevention and control strategies of subclinical mastitis in dairy cattle [5].

Mastitis induced erythrocytic oxidative stress, and incorporation of ascorbic acid in conventional therapy had therapeutic benefits, particularly when used concurrently with antibiotics by modulating udder immunity, preventing noxious insult to mammary tissue, and potentiating antimicrobial activity of antibacterial drugs [9]. Therefore, a comprehensive assessment of mastitis prevalence in the regional and national level and associated risk factors in dairy cattle management are essential to control the disease and improve economic returns.

Materials and Methods

Area of study: The study was carried out during April to May in small holder dairy farms in 19 Panchayats covering 125 revenue villages in suburban Bhubaneswar city, the state capital of Odisha. The city is located at the latitude of **20.296059,** and the longitude of **85.824539, and it has** a tropical savannas climate. The ambient temperature of the city ranges from 11 to 44°C (52 to 111°F). The study area experiences four primary seasons: winter (December to February), when temperatures drop to 11°C (52°F); summer (March to May), when temperatures can reach 44°C (111°F) or higher; monsoon (June to October); and post-monsoon (November). The annual mean temperature is 27.4°C (81.3°F); monthly mean temperatures are 22-32°C (72-90°F). Summers are hot and humid, with temperatures as low as 30°C. The maximum temperatures during dry spells often exceed 40°C (104°F) in May. These villages were distributed into 4 different regions such as north-east, north-west, south-east and south-west regions as per geographical locations. Eight different villages were randomly selected from each region, 8-16 households were selected from each village, and a total of 398 households were visited for the prevalence study (Table 1). As per Livestock Census 2011, the population of cows in the suburban villages and in urban areas of Bhubaneswar city were 1,38,353 and 8, 53,363, respectively.

A total of 1024 cows yielding a minimum of seven litres milk per day with the history of gradual reduction in milk yield without any change of dietary management were randomly screened for detection of mastitis. The udder was examined for teat lesions and signs of clinical mastitis, such as hard, warm, painful or swollen udder. The udder of the selected cows was first examined visually and then palpated to detect fibrosis, inflammatory swelling or atrophy of the tissue. The size and consistency of the mammary quarter was inspected for the presence of any abnormalities such as disproportional symmetry, swelling, firmness and blindness of the teat canal.

The cows recruited for the study belonged to three different breeds such as Indigenous zebu, Jersey cross and Holstein Friesian cross. The study animals were examined visually and information on potential risk factors such as age, parity, and stage of lactation, frequency of milking and hygiene of the farm were collected by a questionnaire survey. The deviation in milk appearance, with or without signs of inflammation of the udder, is classified as CM. A positive MCMT test without overt signs of mastitis was categorized as SCM. The animals were categorized into three groups with respect to different stages of lactation such as early (<30 days), mid (30-120 days) and late (>120 days) lactation. The prevalence of mastitis was also analyzed on the basis of the parity number like 1st, 2nd, 3rdand 4th, 5thlactation or above5thlactation.A total of 4094 quarter milk samples from 1024 lactating cows were screened for mastitis. The milk was examined for the presence of clots, flakes, blood or changes in colour. Two streaks of milk from each quarter in a strip cup was inspected by visual inspection for presence of any flakes, clots, pus, watery appearance, blood and colour change. The MCMT was performed for qualitative assessment of SCC and changes in pH. The test was based on the increased leukocyte counts in milk and increased alkalinity [10]. The milk somatic cells were counted following the general principle of Prescott and Breed [11]. The milk samples were thoroughly mixed to obtain a uniform distribution of cells and were allowed to stand for 2-5 minutes to settle air bubbles and for disappearance of the foams. A clean, grease-free microscopic slide was placed over the template

to outline four one square cm areas. Ten µl of milk was placed exactly in the centre of the one square cm template and was spread evenly to cover all the area delineated by the template. Two films were prepared for each sample using successive areas of the slide. The films were dried at room temperature and then stained by Newman-Lampert staining technique. The slides with milk smears were placed on the slide rack and flooded with modified Newman-Lampert stain (HiMedia) for 2 min. The excess stain was drained off by keeping the slides in standing position on absorbent paper and then air dried. The slides were rinsed and air-dried. Counting of cells in stained films was performed under oil immersion objective and the cells in 30 different fields were counted. The average number of cells per field was multiplied by the microscopic factor.

The degree of dirtiness was assessed by a hygiene scoring system from 1 to 5, with one being cleanest [12]. The hygiene score was based on a combined assessment of the hygiene of tail head, upper rear limb, ventral abdomen, udder and lower rear limb. The prevalence of mastitis was evaluated based on the floor type, presence of drainage system, pre-milking cleaning of the udder and usage of post-milking teat disinfection.

- **Bacteriological culture:** The milk samples were collected following the standard procedure [11,13]. Udders as well as teats were cleaned before collection of milk sample. Each teat end was scrubbed with cotton gauze moistened with 70% ethyl alcohol in a sequential manner (the teats on the far side of the udder first, then those on the near side). Approximately, five millilitre of milk sample was collected in a sterile vial from each quarter after discarding first few streams of milk and brought to the laboratory for further study.
- Preparation of media and reagents: Nutrient broth, Nutrient agar, Mannitol salt agar, Maconkey's agar, Edward's medium, EMB agar, Mueller Hinton agar and Gram's staining kit were procured from M/S Hi Media Laboratories Limited, Mumbai and working media/ stain were prepared as recommended by the manufacturer. Plating of the agar and tubing of the agar were carried out following the standard procedures.
- **Inoculation of milk sample:** The milk samples collected in sterile vials were shaken well for proper mixing. Two to three loops of milk was inoculated into sterile test tubes containing sterile nutrient broth and incubated at 37ºC. Streaking was done with a loop of the nutrient broth culture 24 hours after incubation into five solid media like Mannitol salt agar, Mac Conkey agar, Edward's medium, Eosin Methylene Blue agar and Nutrient agar and incubated aerobically at 37ºC for 24 hours [14]. Smears from respective colonies grown on the agar plates were stained by Gram's staining method to study morphological and staining characteristics. Isolated single colony was picked up from the agar plates, inoculated into fresh nutrient broth tubes and incubated at 37ºC aerobically for 24hours. A loop of the inoculums from each broth tube was streaked onto the nutrient agar plates and incubated aerobically at 37 ºC for 24 hours. Subcultures were further carried out until the isolates were considered as pure [14].
- **Identification of the bacteria:** Gram's staining was done for all the pure cultures for primary identification with respect to Gram positive or negative, shape, size and arrangements. Identification and differentiation of the Gram positive bacterial isolates were done using the HiStaph TM Identification Kits and HiStrep TM Identification Kits whereas the Gram negative bacteria were identified using Hi Assorted TM Identification Kits and Hi *E.coli* TM Identification Kits.
- **Identification of Staphylococcus species:** The HiStaph TM identification Kit (HiMedia Laboratories Pvt. Ltd.) was used for identification and differentiation of the Staphylococcal isolates as per the procedure mentioned by the manufacturer. The suspected isolates were grown on nutrient agar plates at 37ºC for 24 hour. Single colony was picked up and inoculated in 5 ml Brain Heart Infusion broth and incubated at 37 ºC for 4-6 hours until the inoculums turbidity was 0.1 OD at 620 nm or 0.5 McFarland standards. Each well of the kit was inoculated with 50 µl of the above inoculums by surface inoculation method. The samples were incubated at 35-37ºC for 18-24 hours.
- **Voges-Proskauer's (VP) Test:** Well No.1: Baritt reagent A and B were provided with the kit. After 18 -24 hours of incubation, 2-3 drops of Baritt reagent A and 1 drop of Baritt reagent B was added to Well No.1. Development of pinkish red colour in 5-10 minutes indicated a positive reaction whereas no colour change or development of copper colour indicated a negative reaction.
- **Alkaline phosphatase test:** Well No. 2: After 18-24 hours of incubation, 1-2 drop of 40% NaOH was added to well no. 2. Positive test was indicated by development of bright pink colour within few seconds. The reagent remained colourless when the test was negative.
- **Interpretation of results:** Results were interpreted as per the standards given in the result interpretation chart provided by the manufacturer.
- **Identification of Streptococcus species:** The Streptococcal isolates were subjected to biochemical characterization using HiStrep TM Identification Kit (HiMedia Laboratories Pvt. Ltd)

as per the standard procedure mentioned by the manufacturer. The preparation of the inoculums, inoculation of the kit and incubation was done as per the procedures mentioned for *Staphylococcus* spp.

- **Voges-Proskauer's (VP) Test:** Well No.1: Baritt A and Baritt B reagents were provided along with the kit. After 18-24 hour incubation period 1-2 drops of both the reagents were added to Well No. 1 of the kit. Development of pinkish red colour within 5-10 minutes indicated positive reaction. No change in colour or a slight change in colour denoted negative reaction.
- **PYR Test:** Well No.3: PYR reagent was provided along with the kit. After 18-24hour incubation of the kit, 1-2 drops of the reagent was added to Well No. 3. Positive reaction was indicated by development and retention of cherry red colour. Development of pink, orange or yellow colour indicated negative reaction. The results were interpreted as per the chart provided by the manufacturer.
- **Identification of Gram negative rods:** The Hi Assorted TM Biochemical Test Kit (HiMedia Laboratories Pvt. Ltd) was used for the identification of gram- negative rods as per the standard procedure mentioned by the manufacturer. The preparation of the inoculums, inoculation of the kit and incubation was done as per the procedures mentioned above for *Staphylococcus* spp.
- **Phenylalanine Deamination Test:** Well No.5: TDA reagent was provided with the kit and 2-3 drops of the reagent was added to Well No.5 after 18-24 hours of incubation. Development of dark green colour within one minute indicated positive reaction whereas no change in colour denoted negative reaction.
- **Nitrate Reduction Test:** Well No.6: Sulphanilic acid and N, N-Dimethyl-1Napthylamine reagent was provided along with the kit. After 18–24-hour incubation of the kit, 1-2 drops of sulphanilic acid and 1-2 drops of N, N-Dimethyl-1-Napthylamine reagent was added to Well No. 6. Positive reaction was indicated by immediate development of pinkish red colour whereas no change in colour denoted negative reaction.
- **Identification of *E. coli*:** Identification and differentiation of *Escherichia coli* isolated from milk samples were done using Hi *E.coli* TM Identification Kit (HiMedia Laboratories Pvt. Ltd) as per the procedure mentioned by the manufacturer.
- **Methyl Red Test: Well No.1:** Methyl red reagent was provided with the kit and 1-2 drops of the reagent was added to Well No.1 after 18-24 hours of incubation. The reagent remained red in colour in positive cases where as the reagent decolourises and becomes yellow in negative cases.
- **Voges-Proskauer's (VP) Test:** Well No.2: Baritt A and Baritt B reagents were provided along with the kit. After 18-24 hour incubation period 2-3 drops of Baritt reagent A and 1-2 drop of Baritt reagent B were added to Well No. 2 of the kit. Development of pinkish red colour within 5-10 minutes indicated positive reaction. No change in colour or a slight change in colour denoted negative reaction.
- **Indole Test: Well No. 4:** One to two drops of Kovac's reagent provided with the kit was added to Well No. 4 after 18-24 hour incubation. Development of reddish pink colour within 10 seconds indicated positive reaction where-as the reagent remained pale coloured in negative cases.

In vitro Antibiotic Sensitivity Test

The *in vitro* antibiotic sensitivity test was performed by disc diffusion methodusing 20 antimicrobial discs [15]. Mueller Hinton agar media (M/S HiMedia Laboratories Pvt. Ltd, Mumbai) was used for the antimicrobial sensitivity test. An amount of sterile Mueller Hinton agar medium cooled to 50ºC was poured carefully into sterilized petriplates kept on a levelled surface, sufficient to get a thickness of approximately 4 mm and allowed to solidify. The solidified agar plates were then dried for 30 minutes in the laminar air flow to remove excess moisture from the plates. The Petriplates containing the medium were incubated at 37ºC for 24 hours to check for any contamination.

One isolated colony of the test strain grown overnight on Nutrient agar plate was picked up and inoculated into nutrient broth. The inoculated tube was incubated at 37 ºC for 18-24 hours. A sterile swab stick was dipped into the inoculated broth and rotated on, pressing against the inner wall of the tubes and then swabbed on to the Mueller Hinton agar plates in three directions turning the plates at 60ºC between each applications. The antimicrobial discs were picked up from their respective vials and were placed on the plates with the help of a disc dispenser keeping sufficient space (30 mm) from each other to avoid overlapping of the zones of inhibition. The plates were incubated aerobically at 37 ºC for 24 hours. The zones of inhibition were measured up to the nearest mm (including the disc) and interpreted from the standard chart provided by the manufacturer. On the basis of the diameter of the zones of inhibition a strain was interpreted as sensitive (S), resistant (R) or intermediately resistant (I). The antibiotic discs selected for the *in vitro* test were Marbofloxacin, Gentamicin, Enrofloxacin, Tetracycline, Cloxacillin, Colistin, Cefoperazone, Ceftriaxone plus Sulbactam, and Amikacin. The pattern of antibiotic sensitivity of the bacterial isolates was assessed following the standard *in vi-*

tro procedure. The antibiotic discs were included for the said test based on the availability of intramammary preparations in the local market for treatment purpose. Besides, some antibiotic discs were used based on their availability as parenteral preparations.

Results

The overall prevalence of mastitis in current study was 41.1%. 32.8% cows suffered from subclinical and 8.3% from clinical mastitis. The cow-wise prevalence of sub clinical mastitis in 4 different regions, such as north-east, north-west, south-west and south-east was 30.4%, 33.3%, 30.9%, 35.8% respectively. Similarly, the cow-wise prevalence of clinical mastitis in 4 different regions was 7.9%, 9.6%, 7.6%, 8.3% respectively (Table 1). The highest overall prevalence of mastitis was revealed in Holstein Friesian cross (50.7%), followed by Jersey cross (47.7%) while the lowest was recorded in Indigenous zebu (20.1%). The prevalence of subclinical and clinical mastitis in Holstein Friesian cross was 38.8% and 11.9%, respectively and that of Jersey cross was 38.1% and 9.6% respectively (Table 2). The prevalence of subclinical mastitis was recorded as 8.1%, 13.4%, 37.8%, 38.0%, 38.2%, 40.8% in the age group of <3years, 3-4 years, 4-5years, 5-6years, 6-7years and >7 years respectively. The prevalence of clinical mastitis was recorded as 2.0%, 3.3%, 9.1%, 10.3%, 9.9%, 10.2% in the age group of <3years, 3-4 years, 4-5years, 5-6years, 6-7years and >7 years respectively (Table 2) suggesting that the prevalence of mastitis increases with increasing age and the highest prevalence was noticed in >7years cows followed by 5-6years, and 6-7years.

Sl. No.	Suburban regions	Villages	Farmers consulted	Cows studied	No of cows affected with mastitis		
					Subclinical	Clinical	Total
1	North-east	Basudeipur	10	28	9	2	11
		Singada	12	32	10	3	13
		Brajamohanpur	8	29	7	1	8
		Ostapada	11	28	8	2	10
		Khairapada	12	35	7	2	9
		Nandankanan	14	30	11	3	14
		Barimunda	13	36	12	4	16
		Bhimpur	12	35	13	3	16
	Total		92	253	77(30.4%)	20(7.9%)	97(38.3%)
2	North-west	Andharua	13	35	13	4	17
		Dasapur	12	34	11	2	13
		Bhola	15	39	13	5	18
		Dalua	16	38	15	4	19
		Kujimahala	14	30	8	3	11
		Malipada	10	26	10	2	12
		Kantabad	11	30	9	2	11
		Gangapatna	13	38	12	4	16
	Total		104	270	91(33.3%)	26(9.6%)	117(43.3%)
3	South-west	Tamando	14	27	8	3	11
		Madanpur	9	30	9	2	11
		Retanga	12	25	8	2	10
		Parbatipur	11	23	7	1	8
		Bhagabanpur	10	29	6	1	7
		Jhinkarada	15	29	11	2	13
		Kantilo	13	36	11	3	14
		Subudhipur	14	37	13	4	17
	Total		98	236	73(30.9%)	18(7.6%)	91(38.5%)

4	South-east	Hirapaur	14	32	13	3	16
		Benupur	16	38	15	3	18
		Sarakana	13	36	10	2	12
		Satyabhamapur	11	35	14	3	17
		Dhauli	14	30	11	2	13
		Samantarapur	15	33	12	4	16
		Jashuapur	9	27	8	2	10
		Lenkudi	12	34	12	3	15
	Total		104	265	95(35.8%)	22(8.3%)	117(44.1%)
Grand Total			398	1024	336(32.8%)	86(8.3%)	422(41.1%)

Table 1: Suburban regions and villages recruited for mastitis prevalence study.

The highest prevalence of SCM was recorded in early lactation (56.7%) followed by mid (42.9%) and late lactation (20.2%). The prevalence of CM was the highest in early lactation (12.5%) followed by mid (7.6%) and late (5.5%) lactation (Table 2). Parity number was an important risk factor as the animals become more prone to mastitis with increasing parity number (Table 2) and the highest prevalence was highly seen in 5th lactation (37.97%) and the least in 1stlactation (19.5%). A total 1487 out of 4094 quarters (36.2%) examined, were positive, among which 1202 (29.3%) quarters were positive for subclinical mastitis and 285 (6.9%) for clinical mastitis (Table 2). The prevalence of both the clinical and subclinical mastitis was the highest in right hind quarters (7.5%, 30.6%) and the least in the left fore quarters (19.35%).

Risk factors	group	Cows screened	No of cows affected with Mastitis		
			Clinical	Subclinical	Total
Breed	Indigenous breed	258	9 (3.5%)	43 (16.6%)	52 (20.1%)
	HF cross	134	16 (11.9%)	52 (38.8%)	68 (50.7%)
	Jersey cross	632	61 (9.6%)	241 (38.1%)	302 (47.7%)
	Total	1024	86	336	422
Age (Years)	<3	98	2 (2.0%)	8 (8.1%)	10 (10.1%)
	3-4	122	4 (3.3%)	17 (13.4%)	21 (16.7%)
	4-5	164	15 (9.1%)	62 (37.8%)	77 (46.9%)
	5-6	242	25 (10.3%)	92 (38.0%)	117 (48.3%)
	6-7	212	21 (9.9%)	81 (38.2%)	102 (48.1%)
	>7	186	19 (10.2%)	76 (40.8%)	95 (51%)
Lactation stage	<30 days	312	39 (12.5%)	138 (44.2%)	177 (56.7%)
	31-120 days	368	28 (7.6%)	130 (35.3%)	158 (42.9%)
	>120 days	344	19 (5.5%)	68 (14.7%)	87 (20.2%)
Parity no	1	148	6 (4.0%)	23 (15.5%)	29 (19.5%)
	2	212	14 (6.6%)	51 (24.0%)	65 (30.6%)
	3	234	19 (8.1%)	76 (32.4%)	95 (40.5%)
	4	198	22 (11.1%)	85 (41.4%)	107 (52.5%)
	5	166	19 (11.4%)	77 (46.3%)	96 (57.7%)
	>5	66	6 (7.5%)	24 (36.3%)	30 (43.8%)

Quarter	RF	1024	77 (7.5%)	314 (30.6%)	391 (38.1%)
	RH	1023	74 (7.2%)	303 (29.6%)	377 (36.8%)
	LF	1024	69 (6.7%)	298 (29.1%)	367 (35.8%)
	LH	1023	65 (6.3%)	287 (28.0%)	352 (34.3%)
	Total	4094	285 (6.9%)	1202 (29.3%)	1487 (36.2%)
Hygiene score	1	47	0 (0 %)	2 (4.2%)	2 (4.2%)
	2	89	2 (2.2%)	7 (7.8%)	9 (10%)
	3	248	18 (7.2%)	60 (24.1%)	78 (31.3%)
	4	317	30 (9.4%)	118 (37.2%)	148 (46.6%)
	5	323	36 (11.1%)	149 (46.1%)	185 (57.2%)

Table 2: Prevalence of mastitis with respect to breed, age, lactation stage, parity and hygiene score.

The incidence of mastitis increased with higher hygiene score. The incidence of CM and SCM were the highest in hygiene score of 5 (46.1%,11.1%) and lowest in hygiene score-1 (4.2%, 0%). Of the three different floor types, concrete flooring was associated with a significantly (P = 0.002) lower prevalence of subclinical mastitis, compared to earthen and brick floor. The prevalence was more where post-milking disinfection was not adopted (45.5%) than when it was used sometimes (36.8%). Similarly, the prevalence was more (52.3%) when pre-milking cleaning of the udder was not practised than when it (36.5%) was used sometimes (Table 3).

risk factors	Types	No of cows screened	No of cows affected with Mastitis		
			Clinical	Subclinical	Total
Floor type	Concrete	96	2 (2.1%)	7 (7.3%)	9 (9.4%)
	Earthen	584	55 (9.4%)	231 (39.5%)	286 (48.9%)
	Brick	344	29 (8.4%)	98(28.4%)	127 (36.8%)
Drainage system	Yes	828	64 (7.7%)	246(29.7%)	310 (37.4%)
	No	196	22 (11.2%)	90(45.4%)	112 (56.6%)
Pre milking cleaning of udder	Never	447	47 (10.5%)	187(41.8%)	234 (52.3%)
	Sometimes	369	29(7.8%)	106(28.7%)	135 (36.5%)
	Always	208	10(4.8%)	43(20.6%)	53 (25.4%)
Use of post milking teat disinfectant	Never	673	61(9.1%)	245(36.4%)	306 (45.5%)
	Sometimes	312	25(8.0%)	90(28.8%)	115 (36.8%)
	Always	39	0(0.0%)	1(2.5%)	1 (2.5%)

Table 3: Management practices as risk factor for Prevalence of mastitis.

A total of 422 pooled milk samples were found positive for bacterial growth and 417 (98.63%) bacterial isolates could be identified, either as single (94.50%) or mixed (1.37%) infection. The most common infectious bacterial cause of SCM was *Staphylococcus* spp constituting 55.4% of infections (Table 4). Out of which, 104 (30.40%) isolates were coagulase negative *Staphylococcus* spp. (CNS) which included *Staphylococcus epidermidis*, *Staphylococcus haemolyticus, Staphylococcus simulans*, *Staphylococcus warneri*, *Staphylococcus xylosus, Staphylococcus sciuri*, *Staphylococcus lugdunensis*, *Staphylococcus chromogenes,* and *Staphylococcus kloosii*,and the rest 84 (25.0%) isolates are *Staphylococcus aureus*. Besides, *Staphylococcus* spp,other Gram positive bacterial isolates were *Streptococcus uberis* (13.05%) and *S. agalactiae* (8.4%). Theidentified gram negative bacterial isolates in the milk samples were *E.coli* (8.0%), *Pseudomonas aerogenosa* (2.7%) *Proteus* spp (1.80%), *Klebsiella* (1.5%) and*Enterococcus* spp (0.90%). The bacteria isolated from milk sample of CM were most commonly *Staphylococcus aureus* (37.2%), coagulase negative *Staphylococcus* spp (25.6%), *E. coli* (10.4%), *Streptococcus uberis* (3.5%), and *S. agalactiae* (2.3%). The association of Gram positive bacteria (79.55%) in causing SCM and CM was more than Gram negative bacteria (14.95%).

Pathogen isolated from milk sample positive for mastitis	No. of positive isolates and percentage of total			
	Sub-clinical		Clinical	
	Number	Percent	Number	Percent
Coagulase negative *Staphyloccus* spp.	104	30.4%	22	25.6%
Staphylococcus aureus	84	25.0%	32	37.2%
Streptococcus uberis	47	13.5%	3	3.5%
Streptococcus agalctiae	30	8.4%	2	2.3%
E. coli	27	8.0%	9	10.4%
Pseudomonas aeroginosa	9	2.7%	1	1.1%
Proteus spp.	6	1.8%	0	0.0%
Klebsiella spp.	5	1.5%	2	2.3%
Enterococcus spp.	3	0.9%	0	0.0%
Mixed	17	5.1%	14	16.2%
Staphylococcus aureus + *S. agalctiae*	10	2.5%	7	8.1%
Staphylococcus aureus + *E. Coli*	6	1.8%	6	6.4%
E. coli + *Pseudomonas aeroginosa*	1	0.2%	1	1.1%
Unidentified	4	1.1%	1	1.1%
Total	336		86	

Table 4: Bacterial isolates from milk samples positive for subclinical and clinical mastitis.

Antibiotic sensitivity pattern of Gram +ve isolates: *In vitro* sensitivity pattern of all the isolated bacteriato various antimicrobials is shown in the table 5. The highest degree of antimicrobial sensitivity was recorded for marbofloxacin (98.88%), followed by gentamicin (95.28%), cefoperazone (92.93%), ceftriaxone plus sulbactam (92.39%), enrofloxacin (90.38%). However, colistin (89.96%), tetracycline (93.67%), cloxacillin (90.66%), amikacin (87.66%) were comparatively more resistant in majority of the milk samples. The coagulase negative *Staphylococcus* isolates were highly sensitive to marbofloxacin (98.41%) followed by gentamicin (98.62%), cefoperazone (96.03%), ceftriaxone plus sulbactam (94.44%), enrofloxacin (91.26%). The spectrum of resistance was seen with colistin (88.10%), tetracycline (92.07%), cloxacillin (88.60%), amikacin (83.58%). The degree of sensitivity of *S. aureus* isolates was encouraging for marbofloxacin (99.13%) followed by gentamicin (96.55%), cefoperazone (93.10%), ceftriaxone plus sulbactam (91.37%), enrofloxacin (89.65%).The majority of bacterial isolates were resistant to colistin (93.11%), tetracycline (94.83%), cloxacillin (91.38%) and amikacin(89.56%).The Gram negative organisms were sensitive to marbofloxacin (98.47%), gentamicin (96.29%), ceftriaxone plus sulbactam (95.35%), cefoperazone (94.78%), and enrofloxacin (92.78%). More number of isolates was resistant to colistin (89.79%), tetracycline (90.14%), cloxacillin (91.33%) and amikacin (81.66%). The isolated *E.coli* was sensitive to marbofloxacin (99.18%), gentamicin (97.24%), ceftriaxone plus sulbactam (94.32%), cefoperazone (93.28%), enrofloxacin (91.76%). The *E. coli* isolates were resistant to colistin (90.53%), tetracycline (91.56%), cloxacillin (91.44%) and amikacin (80.52%).

Discussion

Mastitis is a highly prevalent in dairy cows worldwide resulting in serious economic losses to the dairy farmers, especially due to subclinical mastitis [16,17]. It is the second most challenging disease in high-yielding dairy cows inflicting economic losses to the tune of $35 billion worldwide, and nearly Rs. 7165.51 crore (~ $961.23 million) in India per annum [18]. Mastitis is one of the most common and costly diseases affecting dairy cows, despite scope of intensive prevention and control strategies, and socio-ecological consideration for mastitis control in suburban areas help reducing the cost of mastitis control and for clean milk production [19]. India is the number one milk producing country in the world, with diverse agro-climatic conditions. Therefore, it becomes imperative to know the prevalence of mastitis in a particular region for planning of proper therapeutic, preventive and control measures [20].

Antimicrobials	Percentage of sensitivity to bacterial isolates				
	CNS (n = 126)	*S. aureus* (n = 116)	*S. uberis*(n = 50)	*S. agalactiae* (n = 32)	Average
Gentamicin	98.62	96.55	94.0	93.75	95.28
Tetracycline	7.93	5.17	6.0	6.25	6.33
Cloxacillin	11.40	8.62	8.0	9.37	9.34
Colistin	11.90	6.89	12.0	9.37	10.04
Cefoperazone	96.03	93.10	92.0	90.62	92.93
Marbofloxacin	98.41	99.13	98.0	100	98.88
Amoxicillin	39.68	38.79	38.0	37.5	38.49
Ceftriaxone	94.44	91.37	90.0	93.75	92.39
Amikacin	16.42	10.44	10.0	12.5	12.34
Enrofloxacin	91.26	89.65	90.0	90.62	90.38

Table 5: *In vitro* antibiotic susceptibility pattern of gram-positive bacteria isolated from milk.

CNS: Coagulase Negative *Staphylococcus* spp.

The prevalence of clinical (CM) and subclinical mastitis (SCM) varies from region to region. However, there are meagre reports on the overall prevalence of mastitis, especially SCM, in dairy cattle that possibly compromised the implementation of specific strategies to prevent and control the disease. In the present study, we reported prevalence of 8.3% and 32.8% for clinical and subclinical mastitis, approaching the SCM prevalence in other countries or regions. The prevalence of 40.1% SCM has been reported in Ethiopia [21], 59.2% in South Ethiopia [22], 48.8% in Tanzania [23], and 46.35% in India [24], and 36.7% in Poland [25]. The distribution of samples in our current study included four suburban regions of Bhubaneswar City. In this meta-analysis, parity was strongly associated ($P < 0.001$) with the prevalence of SCM during the selected periods. Similarly, the parity had significant associations with the occurrence of SCM in Ethiopia [21]. We found some differences among potential risk factors as compared with other studies. The pooled estimates and statistical analysis demonstrated that the prevalence rate of SCM in dairy cows declined with progressive stages of lactation that ranged from 14.7% to 44.2% (Table 2). However, the highest prevalence of SCM was reported in the early lactation stage, followed by late stages, with the lowest prevalence in mid-stage [26]. On the contrary, the positive rate of SCM ($P = 0.0004$) was highest in late stage lactation, followed by early stage, and the lowest in mid-stage lactation [27]. Age is a determinant factor in the distribution of various diseases. In the present study, it was taken into consideration and the prevalence of mastitis was measured for different age groups of lactating cows. The highest percentage of cows diagnosed with subclinical (40.8%) and clinical mastitis (10.2%) falls under age group of above 7 years. The age of dairy cows was also a potential risk factor for SCM, though statistical analysis demonstrated nonsignificant ($P = 0.146$) relationship. Similarly, age had significant associations with the occurrence of SCM [21]. On the contrary, age had no significant ($P=0.48$) association with prevalence of SCM [27]. Breed difference can play a vital role in the prevalence of different animal diseases. Apart from seasonality, age, parity, lactation stage, the difference in dairy cow breed may create a significant variation in SCM prevalence. Holstein Friesian dairy cattle are the predominant breed in most dairy farms in China, besides Jersey and Jersey × Holstein crossbred cows, and the overwhelming majority of clinical mastitis was diagnosed in Holstein cows [20]. In the present investigation, prevalence of subclinical and clinical mastitis was more common in Holstein Friesian crosses (38.8%, 11.9%) followed by Jersey crosses (38.1%, 9.6%).

The affected quarter did not seem to be associated with the prevalence of SCM in this study. The positive rate of SCM in the right rear quarter was higher than other quarters. However, there were no significant variations among the quarters, affected with SCM (Table 2). The epidemiological surveys relating to the quarter and SCM risk are scarce. However, affection of single quarter and the fore left quarter with SCM ($P < 0.05$) was more common [28]. Therefore, the variation in the SCM occurrence related to the quarters in dairy cows remains unexplained.

The variations in hygiene and sanitization procedures may account for potential risk for SCM in dairy cattle. Subclinical mastitis occurs due to a variety of causes, including pathogenic microor-

ganisms such as *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Streptococcus dysgalactiae*, poor environmental hygiene, faulty milking procedures, and unscientific feeding management [26,29,30]. Therefore, the difference in SCM prevalence in various management systems could be related to factors such as environmental conditions, hygienic practices, and milking conditions that determine the proliferation and transmission of pathogens [31]. The prevalence of SCM among cows in semi-intensive management system is higher than intensive management system. Dairy cattle were fed under semi-intensive management, thus leaving lesser chance for the occurrence of SCM [32,33]. The favourable surviving conditions in summer may lead to the growth of pathogenic bacteria, especially *Streptococcus* species, leading to a higher incidence of SCM [7]. The risk factors such as location, bedding and season should be considered while considering mastitis prevention and control plans. In addition, regular national SCM incidence studies are essential in order to develop appropriate mastitis prevention programs [34]. The actual SCM prevalence in some regions may be overestimated or underestimated owing to a small number of clinical samples, indicating that a large variation in the SCM prevalence among dairy cattle may exist. This systematic analysis demonstrated that pooled estimates of the overall positive rate of SCM and CM in dairy herds in suburban Bhubaneswar regions reached 32.8%and 8.3%during 2012-2021, and there was a large variation in the occurrence of SCM by region or province.

Conclusion

The most commonly identified mastitis-causing pathogens in dairy cattle are *Staphylococcus aureus*, non-aureus Staphylococci, *Escherichia coli*, *Corynobacterium bovis*, *Streptococcus uberis*, *Streptococcus dysgalactiae*, *Mycoplasma* spp., and *Enterococcus* spp. [35,36]. In the present investigation, coagulase negative *Staphyloccus* spp was the most predominant (30.4%) pathogen in SCM and *Staphylococcus aureus* (37.2%) in clinical mastitis. The most prevalent pathogens showed highest sensitivity to marbofloxacin followed bygentamycin. It is concluded that management practices such as use of post milking teat disinfectant and pre milking cleaning of udder were important risk factors for subclinical and clinical mastitis, and the prevalence of mastitis was influenced by animal factors such as breed, age, lactation stage, and parity. The isolated pathogens from milk samples were most sensitive to marbofloxacin. It is concluded that the prevalence of subclinical and clinical mastitis in suburban villages of Bhubaneswar was 38.8% and 11.9%, respectively. The mastitis was a common problem in dairy cows above 7years of age, and marbofloxacin was the drug of choice for the treatment of mastitis with 98.88% antimicrobial sensitivity.

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