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RESEARCH ARTICLE

ISOLATION, CHARACTERIZATION AND ANTIBIOTIC RESISTANCE OF *BACILLUS SPECIES* FROM BOVINE MASTITIS MILK

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ABSTRACT

The present investigation was carried out to isolate the *Bacillus species.* From clinical and subclinical Bovine mastitis milk and to determine antimicrobial susceptibility. The samples were collected from mannargudi Taluk, Thiruvarur (Dt) Tamilnadu.from January 2016 to March 2016. A total of 41 Milk samples suffering from mastitis were screened and a total 9 Bacillus species. Were recovered. The isolated were subjected to the antibiotics resistance screening. The antibiotic resistance test showed that the isolated *Bacillus species* were resistant Methicillin (100%) followed by Penicillin G (91.40%), Oxacillin (80.54%), Cefixime (54.75%), Ampicillin (50.67%), Ceftriaxone (35.29) , Streptomycin (28.50%), Erythromycin (20.36%), Amikacin (17.64%), Gentamicin (12.21%), Cefpodoxime (8.59%), (12.21%), Tetracycline (7.69%), Chloramphenicol (6.33%), Azithromycin (5.42%), Ciprofloxacin (4.07%), Ofloxacin (2.26%) and all Bacillus specie were susceptible to vancomycin. The present study demonstrated the presence of alarming level of resistance of frequently and commonly used antimicrobial agents to the isolated bacteria. Therefore , an examination of the antibiotics resistance profiles of the isolated must be done earlier to the use of antibiotics in both to choose appropriate antibiotic for treatment and prevention of Bovine mastitis.

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INTRODUCTION

Bovine mastitis is a common disease entity of dairy cows, accompanied by physical, chemical, pathological and bacteriological changes in milk and grandular tissue (Samad, 2008). It is a harmful disease affecting the dairy industry worldwide and is a matter of great concern for leading milk producing country like India because of the losses incurred due to high morbidity, discarded milk, treatment costs and reduced milk production, thus drawing in more attention towards its treatment and control (Nihar *et al*., 2013). Apart from the economic losses, mastitis can have serious implications on public health. Mastitis which is mostly caused by the interaction of multiple pathogenic agents (primarily bacteria), can expose human beings to various organisms through infected milk, thus serving as a media for transmission of various zoonotic disease like T.B, brucellosis, diphtheria, scarlet fever and Q fever (Mahantesh and Kaliwal, 2011). Mastitis is produced by a variety of pathogenic microorganisms.

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The majority of cases in bovine are infectious and it has been estimated that up to 200 microbial species are potential causative agents (Blowey and Edmondson, 1995). Cows and herds vary in susceptibility and extent, type and duration of infection; although some of mammary pathogens can be isolated from the environment of the cow, manure and bedding, water supplies, soil and inadequately cleaned milking machines (Jain, 1979). In bovine mastitis bacteria isolated with greatest frequency are staphylococcus *aureus, staphylococcus spp., Bacillus spp., corynebacterium spp., Escherichia coli, streptococcus spp., Pseudomonas spp., and klebsiella spp.,*(EI-Khodery *et al.,*2008). Variation in prevalence of mastitis might be due to the different regions, breeds, therapeutic practices, management conditions and presence of microorganisms in environment (Sadashiv and Kaliwal, 2013). Bacteria belonging to the genus *Bacillus* have been associated with bovine, ovine, and porcine abortions worldwide (Agerholm *et al.,* 1995; Kirkbride *et al*., 1993). The success of bovine mastitis basically depends on the understanding of clinical presentation and antimicrobial susceptibility of the etiological agent, among various other factors (Miltenburg *et al*., 1996) and the increased antimicrobial resistance of the organisms in animals treated with antibiotics and their zoonotic transmission

continues to be a matter of great concern globally (Unakal and Kaliwal, 2010). The important reason for therapy failure in the management of mastitis could arise from various factors involving pathological changes in the udder, etiology lower efficacy of antimicrobials, and improper veterinary services (Adesola, 2012). Isolation identification and characterization of mastitis pathogens are a fundamental aspect of milk quality and udder health control programs. There is a need to discuss public health and food safety issues associated with food borne pathogens found in the dairy environment. Because of worries about antimicrobial residues, antimicrobial resistance, milk quality and animal welfare, there is an increasing demand for development and evaluation of the milk culture method and rapid and accurate identification of bacterial species.

Antibiogram studies of mastitis pathogens are important to suggest suitable antibiotic treatment to provide quality milk to be consumers and to prevent antibiotic resistance, potential health risk for humans (Nadeem *et al*.,2013). Identification of mastitis pathogens, and their antimicrobial susceptibility is important when selecting appropriate treatment regimen (Sadashiv and Kaliwal, 2014). Therefore, the present investigation was designed to isolate, characterize the *Bacillus spps*. And their antibiotic resistance, isolated from clinical and subclinical bovine mastitis milk.

MATERIALS AND METHODS

Study Site

The milk samples was collected from different area of mannargudi Taluk such as sundarakottai, mannargudi, peraiyur, koopachikkottai, serumagulam, Neduvakkottai, paravakkottai, kumarapuram, Adichapuram, Nemmeli, Thiruvarur Dt Tamilnadu.

Animal management

All the cows used for this study of were owned by pastoralists and were raised under the traditional management systems. The animals were kept in open yard constructed mainly from local materials. The pens were made from wooden bars and the floor was converted with sand.

The pregnant animals were kept separate yards. The cows were entirely fed on range vegetation. Supplementary food was uncommon. Routine grazing was carried out twice daily (morning and evening) on natural pasture comprising mainly guinea grass (*panicum maximum*).

Milking of the cows is usually carried out manually by the owner before morning grazing into the collected pail. The length of the lactation period ranged between 9 to 10 months.

Sample collection, handling and storages

Milk samples were collected by a standard milk samplings techniques (NMC 1990). To reduce contamination of the teat ends during sample collection, the near teats were sampled first followed by the far once. Approximately 10 ml of milk were collected in to a sterile test tube after discarding the first 3 milking stream. Then samples were placed in racks for ease of handling and transported in an ice to the laboratory and stored at 4^0C for a maximum of 24 hour until inoculated on a standard bacteriological media.

Surf field mastitis test (Muhammad *et al*., 2010)

Surf field mastitis test (SFMT) and increased pH of the milk have been done to confirm the clinical and subclinical mastitis. The samples were subjected to surf field mastitis test (SFMT). The principal of the test is that when detergent is added into milk samples, it causes rupture of somatic cell and release DNA and other cell contents. DNA is acid in nature, while detergent contains alkyl- arysulfonate, which is basic in nature. DNA and detergents unite to form a gel; consistency of gel depends upon the number of somatic cells. More cells more thick gel and vice versa.

Sampling method

Quarter foremilk samples were collected aseptically for bacteriological assay as described by Honkanen-Buzaliski. Before sampling, test ends were disinfected with cotton swaps soaked in 70% ethanol and allowed to dry and the first streams of milk were discarded. Milk samples were colleted in sterile 15ml tubes. The milk samples were transported in a cold container to the laboratory of the P.G, department of studies in microbiology and biotechnology, karnatak University, Dharwad for further analysis.

Identification and Biochemical characterization

A Total of 41 milk samples suffering from mastitis were brought to the laboratory.

Table 1. Prevalence of Clinical and Sub Clinical Mastitis From Mannargudui Taluk

S.No	Areas	Total prevalence			Sub Clinical		Clinical	
		No of animals tested	No of animal affected	Percentage %	Positive	Percentage %	Positive	Percentage %
1	Sundarakottai	3	2	66.6	2	66.6	2	66.6
2	Mannargudi	2	2	100	1	50	1	50
3	Peraiyur	6	4	66.6	2	33.3	2	33.3
4	Koopachikkottai	4	2	50	1	25	1	25
5	Serumagulam	3	2	66.6	1	33.5	1	33.3
6	Neduvakkottai	5	3	60	2	40	1	20
7	Paravakkottai	4	3	75	1	25	1	25
8	Kumarapuram	6	3	3.33	1	16.6	1	16.6
9	Adichapuram	5	2	40	1	20	1	20
10	Nemmeli	3	2	66.6	2	66.6	1	33.3
Total		41	24	6.893	14	3.717	12	3.71

The isolation of *Bacillus* spps. It was carried out using the standard method (Fall, 2011). Briefly, 100ml of aseptically collected milk samples from each sample was spread over a nutrient agar and incubated at 37^oC 24-48 hrs.

Table 2. Biochemical Characteristics of Isolated *Bacillus subtilis*

S.No.	Tests	*Bacillus subtilis*
Morphological characteristics		
1	Gram's staining	Gram positive
2	Shape	Rod
3	Motility	Motile
Biochemical characters		
4	Indole	-
5	Methyl red	-
6	Voges proskauer	+
7	Catalase	+
8	Oxidase	-
9	Citrate	-
10	Urease	-

After incubation, the selected colonies were subjected to Gram nature, morphological character.

Antibacterial Resistance Test

Antibiotic resistance screening was done as per the guidelines of national committee for clinical laboratory standards (NCCLS). Kirby-Bauers 1987. Disc diffusion technique was adapted for antibiogram. The following antibiotics are used for resistence test Amikacin, Ampicillin, Methicillin, Oxacillin, Penicillin G, Cefixime, Cefpodoxime, Ceftriaxone, Ciproflaoxacin, Ofloxacin Gentamycin, Azithromycin, Erythromycin, Streptomycin, Tetracycline and Chloromphenicol.

RESULTS AND DISCUSSION

A total of 41 amimals from mannagudi Taluk in Thiruvarur (Dt) Tamilnadu. Were tested for mastitis. The number of confirmed subclinical and clinical mastitis from showed that total 6.893% animals affected. From which 3.717% and3.71% subclinical and clinical mastitis respectively. (Table1) A total of *9 Bacillus* species were recovered from 10 milk samples based on gram nature and morphological character, (Table 2).

may be due to the environmental factors like soil, water and manure, these are the main sources of bacteria and manure these bacteria infected animals via teat canals (Mohammed *et al.,* 2012). Therefore , the present study suggest that mastitis can be controlled by hygienic conditions cleaning manure, keeping the animals away from the stagnant water washing udder before milking with germicidal solution. In the present study the antibiotic resistance test showed that the isolated *Bacillus species* were resistant Methicillin (100%) followed by Penicillin G (91.40%), Oxacillin (80.54%), Cefixime (54.75%), Ampicillin (50.67%), Ceftriaxone (35.29) , Streptomycin (28.50%), Erythromycin (20.36%), Amikacin (17.64%), Gentamicin (12.21%), Cefpodoxime (8.59%), (12.21%), Tetracycline (7.69%), Chloramphenicol (6.33%), Azithromycin (5.42%), Ciprofloxacin (4.07%), Ofloxacin (2.26%) and all *Bacillus specie*s. Were susceptible to vancomycin (Table3).

In the mastitis the improper or incomplete treatment of animals also contributes significantly to the development of bacterial resistance against them. the usage of antibiotics correlates with the emergence and maintenance of antibiotic resistant traits within pathogenic strains (Shitandi *et al*., 2004). These traits are coded for by particular genes that may be carried on the bacterial chromosome, plasmids (Rychlik *et al*., 2006), hence these are easily transferred among isolates. In the present study, in vitro antimicrobial susceptibility test of twenty one antimicrobial agents was conducted and studied against isolated nine *Bacillus* species. The most commonly used antibiotics on conventional dairies were penicillin, cephalosporin and Tetracycline's for mastitis penicillin Ampicillin and Tetracycline were commonly used (Mohammed *et al.,*2012). The antibiotics resistant of the present study revealed that the isolated *Bacillus* species. showed resistant to multi drugs. These results were in line with the reports of (Mohammed *et al*., 2012), were the resistance of Ampicillin (84%), Cefotaxime (77%), Ceftizoxime (55%), Amikacin and Ofloxacin (25%) and Tetracycline (17%).

Table 3. Antibacterial Resistance Pattern For Isolated Bacillus Sps

Bacterial isolated	Antibiotics Used															
Antibobiotic	AK	AMP	MET	OX	P	CFM	CPD	CTR	CIP	OF	GEN	AZM	E	S	TE	C
Bacillus Spps (m.m)	39	112	00	178	202	121	19	78	09	5	27	12	45	63	17	14
%	17.64	50.67	100	80.54	91.40	54.75	8.59	35.29	4.07	2.26	12.21	5.42	20.36	28.50	7.69	6.33

Notes: AK-Amikacin, OX-Oxacillin, CPD-Cefpodoxim, OF-Ofloxacin, E-Erythromycin, C-Chloramphenicol, Ampicillin, P-Penicillin, TR-Ceftriaxone , GEN-Gentamicin, S-Streptomycin, MET-Methicillin, CFM-Cefixime, CIP-Ciprofloxacin, AZM-Azithromycin, TE-Tetracycline

Similariy (Mohammed *et al*.,2012) also isolated *B. coagulans, B. cereus, B.subtilis, B. licheniformis, B. amyloliquefaciens, B. megaterium,* from bovine mastitis (Nieminen *et al*., 2007) also reported the presence of *B. pumilus B.licheniformis* and *B. cereus.* (Parkinson *et al.,* 1999) reported the presence of Bacillus cereus from the mastitis milk *Bacillus cereus* is recognized as being ubiquitous in the farm environment and the numbers of *Bacillus cereus* spores in soil rises throughout the winter (Davies and Wray, 1996). It is a common contaminant of milk at all stages of processing (Crielly *et al.,* 1994). The organisms is not generally considered to be a primary mastitis pathogen, but cauese mastitis after accidental introduction to the udder (Parkinson *et al*., 1999). The presence of bacillus species. In the study

The reports were higher to the reports of (Firaol *et al*., 2013) to the pencillin G (66.67%), lower to Chloramphenicol (88.89%) and Gentamycin (100%). From to be resistant to previous and established antibiotics compared to the newer developed antibiotics. Appearance of resistance against a particular antibiotic in a specific region may be due to its frequent and long-term use (Moon *et al*., 2007; Kumar *et al*., 2010).

Conclusion

In the present study of *Bacillus species.* Were isolated and characterized from the collected milk samples. Many isolates of *Bacillus species.* were showed multi drug resistant.

It is difficult to treat as many strains are resistant to antibiotics used in mastitis. The development of antibiotic resistance in the bacteria that affects animal health is of growing concern in veterinary medicine. Therefore, the present study suggests the examination of the antibiotic resistance profiles of the isolates must be done earlier to the use of antibiotics in both to choose appropriate antibiotics for treatment and prevention of Bovine mastitis.

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