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A study on the prevalence of zoonotic important methicillin resistant and vancomycin resistant *Staphylococcus aureus* (MRSA & VRSA) and coagulase negative *Staphylococci* (MR-CNS & VR-CNS) in raw milk samples of Tirupati, Andhra Pradesh

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Abstract
The antimicrobial resistance in food producing animals is a global problem particularly in developing countries and the indiscriminate use of antimicrobial agents is a major concern for the emergence of resistant zoonotic bacterial pathogens. The Coagulase positive (CPS) and Coagulase negative *Staphylococci* (CNS) strains, opportunistic pathogens, which commonly resides on the coverings of humans and animals and cause wide range of infections as well as food poisoning in both humans and animals Hence, the present study was aimed to detect presence of methicillin resistant as well as Vancomycin resistant *Staphylococcus aureus* and CNS in a total of 100 raw milk samples by phenotypic methods i.e. disc agar diffusion tests. Among 57 *Staphylococcus* isolates, 42 and 15 isolates were confirmed as *S. aureus* and CNS. Out of 42 *S. aureus* strains, 10 were identified as methicillin resistant (MRSA) and 5 as Vancomycin resistant (VRSA). Among 15 CNS strains, 5 and 4 were identified as methicillin resistant (MR-CNS) and Vancomycin resistant (VR-CNS). The presence of antibiotic resistance among *S. aureus* and CNS in veterinary may cause hazard for human health via food chain or through direct transmission of resistant pathogens between humans and animals.

Keywords: *staphylococcus* aureus, coagulase positive *staphylococci*, coagulase negative *staphylococci*, methicillin resistant, vancomycin resistant

Introduction
WHO defined Food borne diseases as diseases of infectious or toxic nature caused by, or thought to be caused by the consumption of food or water [52]. Milk is one of the most important and essential component in human diet and it is an excellent growth medium for many microorganisms. Milk borne illnesses are majorly caused by variety of agents specifically pathogenic bacteria [12]. Milk acts as vehicle for transmission of many pathogens including *Staphylococci strains* [58].

Staphylocccus genus, a heterogenous group, consists of 51species and 27 sub species. They are gram positive bacteria which look grape like clusters under microscope**.** Based on the ability to coagulate plasma, *Staphylococci* strains are divided into two groups i.e. Coagulase Positive *Staphylococci* (CPS) and Coagulase Negative *Staphylococci* (CNS or CONS) [44]. In veterinary, they play a vital role in causing wide range of diseases like bovine mastitis (both Coagulase positive and Coagulase negative *Staphylococcal* species), canine pyoderma, exudative epidermitis in pigs, septicaemia in poultry [25]. Among CPS, *Staphylococcus aureus* is most significant pathogen as it is capable of causing food intoxication and many diseases in both humans and animals [41]. The other group, CNS, are responsible for causing variety of opportunistic infections in both humans and animals [56]. *S. aureus* and CNS are normally reside on the healthy skin of teat, vagina, coat, nostrils of healthy animals as well as on the hands of milker; from which they colonize the teat canal and penetrate the secretory tissue and cause infections. Hence, they are often called as opportunistic pathogens.

Coagulase Positive *S. aureus* will cause wide variety of diseases like pyoderma, osteomyelitis, endocarditis, septicaemia, surgical wound complications, pneumonia in humans and animals; it will also cause clinical or subclinical mastitis with severe systemic signs. CNS are associated with nosocomial infections in neonatal intensive care units and also cause food poisoning, but

less virulent than *S. aureus* as they posses smaller array of virulence factors [8]. Coagulase Negative *Staphylococci* species will also causes clinical or subclinical mastitis but with less severe signs. Besides *S. aureus*, CNS causing mastitis rate is increasing substantially in recent years [32]. Previously, the presence of CNS in the clinical specimens is considered as contaminant due to presence of many species within this group [8]. Recently, this perception is changing because of emergence of many new species which are important in causing nosocomial infections particularly in relation to foreign device-related infections and infections in immunocompromised patients [33, 37].

Based on epidemiological studies, it was confirmed that dairy products can be contaminated with CNS by using unpasteurized raw milk and also during manufacturing practices by workers [13] and there was also a case study of food poisoning outbreaks linked CNS strains with unpasteurized milk [23]. The CNS strains isolated from food have the ability to produce several virulence factors such as *Staphylococcal* enterotoxins [23, 10, 40].

Even though, CNS is recognized hygienically a very important bacteria in food production and preservation [48]**;**due to possible promulgation of Antimicrobial Resistance Bacteria (AMRB) and antimicrobial resistant genes (AMRG), CNS presence in food is considered as public health significance [57, 14]. Both animal and human origin CNS are believed to serve as important reservoirs of AMRG [8] which may transfer and integrate in to *S. aureus* genome leading to emergence of new, potential more resistant strains [42, 55].

The tendency of developing antimicrobial resistance in *Staphylococci* group is a great cause for concerning in both human and veterinary medicine [54] and the antimicrobial mechanisms are almost similar in both CNS and *S. aureus* [41]. Due to indiscriminate use of antibiotics in Veterinary resulted in failure treatment of infected livestock which led to drop in food supply and it is due to increase of antimicrobial resistant bacteria which is a globally highlightened issue [41].

In 1994, *Staphylococci* became penicillin resistant by destroying penicillin with penicillinase [15]. Later, methicillin was used to treat penicillin resistant staphylococci in 1959 which resulted in evolution of methicillin resistant *Staphylococci* in 1962 [15]. MRSA was first found in human; later detected in animals also [35]. Now-a-days, methicillin/oxacillin resistant *Staphylococci* strains are increasing which has become a serious clinical and epidemiological problem. In general, MRSA strains only harbour mec A gene and later it was assumed that methicillin resistance encoding gene has evolved in CNS [7]. The methicillin resistance is due to presence of mec A gene which is localized on Staphylococcal Cassette Chromosome (SSC_{mec}). The mec A gene present in *Staphylococci* strains will encodes penicillin-binding protein (PBP_{2a}) and localizes in bacterial cell wall which results low affinity for β lactam antibiotics [24]. Hence, β lactam antibiotics are not effective against mec A gene expressing bacteria [24]. Therefore, the mec A gene is used as a marker for detection of oxacillin/methicillin resistant *Staphylococcal* strains [26]. Normally, MRSA strains only harbour mec A gene and later it was assumed that methicillin resistance encoding gene has evolved in CNS also [7]. According to epidemiological and genetic characteristics, MRSA are divided into 3 groups: Hospital acquired MRSA (HA-MRSA), Community-associated MRSA (CA-MRSA) and livestock associated (LA-MRSA). Based on location and size of SSC_{mec} and the presence of Panton-valentine leukocidin (PVL) gene, these three groups differ in sensitivity to antibiotics [31]. After emergence of MR *staphylococcal* strains, Vancomycin, a glycopeptides, continues to be an important antimicrobial agent to treat MR staphylococcal infections. But in 1996, *S. aureus* strain with Vancomycin was finally emerged in Japan [30]. Later, several reports were recorded on evolution of Vancomycin resistant *Staphylococcal* strains from patients of different places. In contrary to this, only few reports were reported on Vancomycin resistant *Staphylococcal* strains in veterinary medicine as Vancomycin is not regularly used for treating the infected animals. Emerging of such Vancomycin resistant *Staphylococcal* strains from animals may be due to contamination of pasture land or environment with Vancomycin-resistant isolates originated from human patients who are residing very close to animals [19] and also milching of animals by those patients manually will substantially increases possibility of infection or transfer of pathogens from milkman to cows [22].

Though, the PCR is considered as "gold standard" test for detection of mec A gene to identify methicillin resistant *Staphylococcal* strains; but it still remains time consuming, expensive and not available in most of routine laboratories. Therefore, methicillin resistant *Staphylococci* can be detected by phenotypic methods or agar diffusion methods i.e. oxacillin disc agar diffusion tests, oxacillin screen agar test and Cefoxitin disc diffusion agar test [2, 4, 44].

The present study was aimed to isolate and identify methicillin resistant and Vancomycin resistant *S. aureus* (MRSA & VRSA) and Coagulase Negative *Staphylococci* (MR-CNS and VR-CNS) from raw milk samples collected from different places in and around Tirupati region.

Materials and Methods

1. Study site and sample collection:

A total of 100 raw milk samples were collected from different sources like organized and unorganized dairy farms, local milk vendors of different places in the Tirupati region of Andhra Pradesh, India. Samples were collected aseptically in a 25 ml of pre sterilized screw capped bottles and transported to Department of Veterinary Public Health and Epidemiology, College of Veterinary Science, Tirupathi in an ice box maintaining temperature at $4^0C \pm 1^0c$ for further processing and analysis.

2. Isolation and identification of *S. aureus*:

Each 10ml of each sample was inoculated into 90ml of Buffered Peptone Water and incubated at 37^0C for 18-24hrs as per standard protocol. After this pre-enrichment, a loopful of inoculum from each positive sample was streaked on MeReSa agar, Baird Parker agar, Mannitol Salt agar plates and incubated at 37^0C for 18-24 hrs. The positive colonies on the agar plates were further confirmed as *S. aureus* and Coagulase negative Staphylococci strains by grams staining and biochemical tests like haemolysis, Coagulase test, catalase test, methyl red test, Voges-Proskauer test, oxidase test as per standard protocols.

3. Phenotypic detection of Methicillin resistant *S. aureus* (MRSA) and Methicillin resistant-CNS

a. The Oxacillin Screen agar (OSA) test.

A swab was dipped in 0.5 Mc Farland's suspension of the

positive isolate and it is deposited as a spot the Muller-Hinton agar plates containing 4% NaCl and 6µg/ml of oxacillin. These plates were incubated at 37^0C for 24 hrs and observed in transmitted light for any growth after incubation. Any growth after 24hrs of incubation was considered as oxacillin/methicillin resistance *Staphylococcal* strains.

b. Oxacillin disc diffusion agar (ODD) test.

This test is performed by using oxacillin discs on Muller-Hinton agar plates supplemented with 2% NaCl to detect MRSA as per CLSI guidelines [3]. The *S. aureus* colonies and CNS strains were inoculated into nutrient broth media and incubated at 37^0C for 18-24hrs. A sterile swab is dipped into culture and made lawn culture on Muller Hinton agar plates. An oxacillin disc of 1µg was kept at the centre of pate and was pressed down at the centre to get complete contact with agar. Then these plates were incubated at 37^0C for 24 hrs. After incubation, zone of inhibitions were measured in all plates. If it is ≤10mm then the isolates were considered as Methicillin resistant, 11-12mm indicates the isolates as intermediate resistant and a diameter of ≥13mm were considered as Methicillin sensitive [16].

c. The Cefoxitin disc diffusion test.

All the positive isolates of *S. aureus* and CNS were subjected to Cefoxitin disc diffusion test by using 30µg Cefoxitin disc on Muller Hinton agar plates which is almost similar to procedure of ODD test. A lawn culture was done on Muller Hinton agar plates and Cefoxitin discs was placed and pressed at the centre to get contact with surface of agar. These plates were incubated at 37^0C for 18 hrs and zone of inhibitions were measured. If the zone of inhibition ≤21mm was reported then those isolates were considered as methicillin resistant and a diameter of ≥22mm were considered as methicillin sensitive [16].

4. Detection of Vancomycin resistant *S. aureus* (VRSA) and Vancomycin resistant CNS by Vancomycin disc diffusion agar test.

This test was carried out on Muller Hinton agar by using Vancomycin disc. The positive culture which was inoculated and incubated in nutrient broth was swabbed on Muller-Hinton agar plates. Vancomycin (30µg) discs were placed and gently pressed to come in contact with agar surface. These plates were incubated and zone of inhibitions were measured. The diameters with ≥15mm were considered as Vancomycin susceptible and the remaining were as Vancomycin resistant as per CLSI guidelines [15].

Results

A total of 57 (57%) isolates of *Staphylococci* species were isolated from 100 raw milk samples which were collected from various places of Tirupati region, Andhra Pradesh, India. Out of 57 *Staphylococci* species, 42 isolates (73.6%) were confirmed as *S. aureus* as they produced typical bluish green colonies on MeReSa agar, fermented Mannitol on MSA, produced black colour colonies with surrounding halo zone on Baird-Parker agar, shown Coagulase positive, exhibited hemolysis on blood agar, methyl red test and VP test were positive.

The remaining 15 isolates (26.31%) were considered as Coagulase negative *Staphylococci* strains (CNS) by observing Coagulase test, no Mannitol fermentation on MSA (didn't fermented Mannitol), no hemolysis on blood agar, no growth on MeReSa agar (Figure 1).

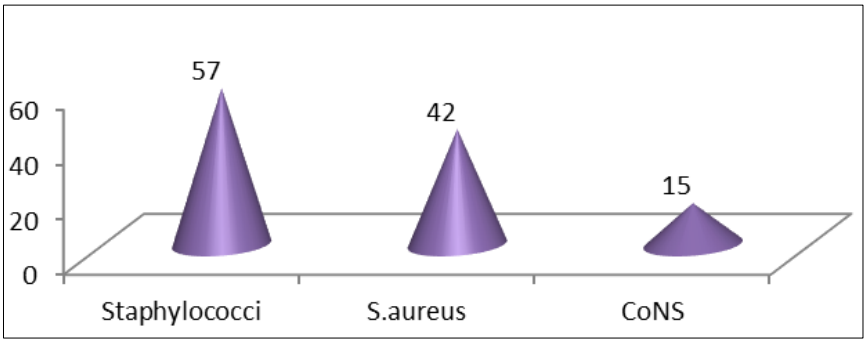

Fig 1. Number of isolates isolated from raw milk

Prevalence of MRSA, VRSA, MR-CNS and VR-CNS in raw milk

MRSA, VRSA, MR-CNS and VR-CNS were detected by using three different phenotypic methods. Out of 42 *S. aureus*, only 10 (23.8%) isolates were confirmed as Methicillin resistant *S. aureus* (MRSA) and the remaining 32 (76.2%) isolates were confirmed as Methicillin Susceptible *S. aureus* (MSSA). Only 5 isolates (11.9%) among 42 isolates of S. aureus were confirmed as Vancomycin Resistant (VRSA) and the remaining 37 isolates (88.1%) were confirmed as Vancomycin susceptible *S. aureus* by Vancomycin Disc Diffusion agar test (Table-1 and Fig. 2).

Among 15 Coagulase negative *S. aureus* isolates, only 5 isolates (33.3%) were shown methicillin resistant (MR-CNS) and the other 10 (66.7%) isolates were methicillin susceptible (MS-CNS). Out of 15 CNS isolates, only 4 isolates (26.7%) and 11 isolates (73.3%) were confirmed as Vancomycin resistant (VR-CNS) and Vancomycin susceptible (VS-CNS) Coagulase negative *Staphylococci* (Table 1 & Fig. 2).

Table 1: Prevalence of methicillin resistance and Vancomycin resistant *S. aureus* and CNS

Type	*Staphylococcus aureus* (42)	Coagulase negative *Staphylococcus* (CNS) (15)
Methicillin resistant	10	5
Methicillin Susceptible	32	10
Vancomycin resistant	5	4
Vancomycin Susceptible	37	11

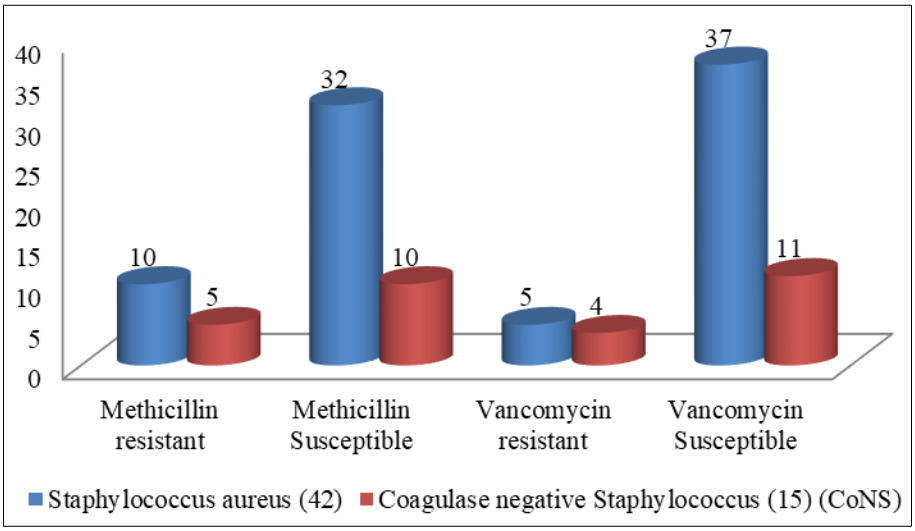

Fig 2. Prevalence of methicillin resistance and Vancomycin resistant *S. aureus* and CNS

Comparison of phenotypic methods used for detection of Methicillin Resistant *Staphylococci* (MRS)

Among the three different phenotypic methods used for detecting Methicillin Resistant *Staphylococci* strains, the number of positives shown in oxacillin screen agar test and Cefoxitin disc diffusion agar test were similar; while oxacillin disc diffusion agar test shown less number of positives than the other two methods (Fig. 3 & Table 2).

Table 2: Comparison of phenotypic methods for detection of methicillin resistant *Staphylococcal* strains

Test	*S. aureus*		CNS	
	Resistant	Susceptible	Resistant	Susceptible
Oxacillin Disc Diffusion agar test (ODD)	8	34	4	11
Cefoxitin Disc Diffusion agar test (CDD)	10	32	5	10
Oxacillin Screen agar test	10	32	5	10

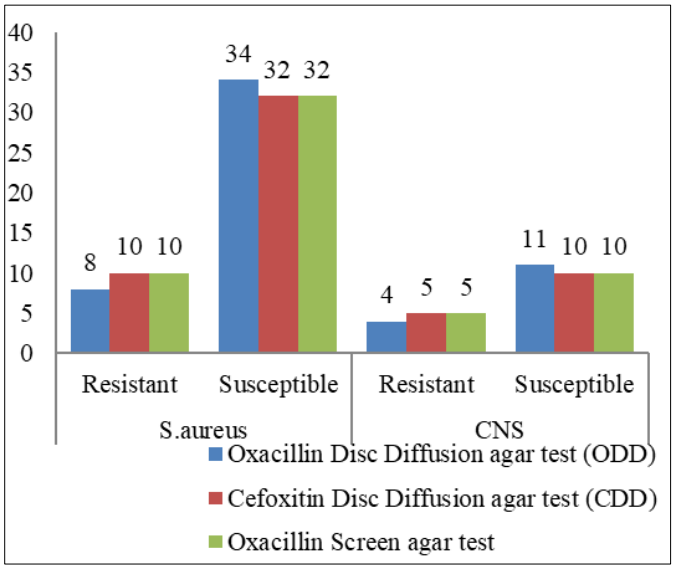

Fig 3: Comparison of phenotypic methods for detection of methicillin resistant *Staphylococcal* strains

Discussion

Staphylococci are more frequently found bacteria on the coverings of humans and animals, the mucosal surfaces of the respiratory, upper alimentary and urogenital tracts of animals and birds [24]. Both Coagulase Positive *Staphylococci* (CPS) and Coagulase Negative *Staphylococci* (CNS) are commensal opportunistic infectious bacteria of animals and humans [11]. Generally, both of them will cause superficial skin infections, mastitis and severe invasive diseases in farm animals and wound infections in humans [8, 21]. They are well known in causing food poisoning via their enterotoxins (Staphylococcal Food Poisoning) and also well known for their ability to acquire AMR [39].

Among the *Staphylococci* strains, *S. aureus* is a well-known commensal and opportunistic pathogen of both bovines and humans and often considered synonymous with CPS [40]. CNS are considered as emerging cause of subclinical mastitis in bovines as they normally present on the bovine skin and commonly carry the gene for methicillin resistance [51].

Manual method of milching of animals may leads to contamination of environment or Pasteur lands which results in transfer of MRSA, VRSA strains or infection from the milkman to cows [19]. Mandal *et al.* (2015) [37] reported that hospital associated discharge/effluent is the convenient vehicle for dissemination of MRSA and VRSA strains. Tattevin *et al.* (2009) [52] recorded that Vancomycin resistance in MRSA strains occurs due to use of other cell wall acting antimicrobials which will change cell wall structure.

The presence of CNS in raw milk may due to bad conditions of hygiene during milking and lack of hygiene practices while handling, transporting and storage. As CNS are a part of normal teat skin flora and mucosa of humans and animals, hence they are common causes of contamination of raw milk and milk products [9].

Out of 100 raw milk samples examined, 57 samples (57%) were shown positive for *Staphylococcus* strains which is lower than the prevalence rate recorded by Ananya and Pranab (2015) [5] (48.2%); whereas Angela *et al.* (2015) [6] reported 62.02% of *Staphylococci* strains from health care workers and medical devices in Rio de Janeiro.

Among 57 samples of *Staphylococci*, 73.7% was the prevalence rate of *S. aureus* recorded in this present study which is almost similar to the studies of Tahoun (2009) [50],

Akindolire *et al.* (2015) [1], Sarkar *et al.* (2014) [46] who reported 78%, 75% and 74.5% of *S. aureus* in raw milk where as de Oliveria *et al*. (2011) [18], Andre *et al.* (2008) [6], Singh *et al.* 2011 [48], Lingathurai and Vellathurai (2011) [37], Debaraj *et al*. (2016) [20], Bharathy *et al.* (2015) [13] reported lower prevalence of *S. aureus* than the present study i.e. 68%, 66.7%, 62.34%, 61.7%, 61.36%, 61% in milk. Angela *et al.* (2015) [7] (2) and Amita *et al.* (2008) [2] reported 71.42% of *S. aureus* from the clinical specimens. While the studies of Bendahou *et al.* (2008) [10], Ananya *et al.* (2015) [5] (7), Kumar *et al.* (2010) [35], Etinosa *et al.* (2016) [25], Sulaj *et al.* (2013) [49], Umaru *et al.* 2014 [55], Thaker *et al.* (2013) [54] reported very low incidence rates than the present study i.e. 46.6%, 33.3%, 26%, 22%, 18%, 12.6%, 6% from raw milk. Osman *et al.* (2016) [43] also reported very low incidence rate of *S. aureus* (21.4%) from poultry meat.

In the present study, only 15 samples (26.3%) of raw milk were positive for CNS while Ananya *et al.* (2015) [5], Bendahou *et al*. (2008) [10] reported high incidence of CNS i.e. 66.6% and 54% in their studies. The studies of Angela *et al.* (2015) [7] (28.57%) and Amita *et al*. (2008) [2] (32.6%) reported almost similar prevalence of CNS from the clinical specimens while Cemil *et al.* (2016) [14] also reported almost similar incidence rate (23.6%) from cheese samples in Turkey. In contrary to the present study, Osman *et al.* (2016) [43] reported 72% of CNS from poultry meat.

In this study, three different phenotypic methods i.e. oxacillin disc diffusion agar test, oxacillin screen agar test, Cefoxitin disc diffusion agar test were used to detect methicillin resistance *Staphylococcal* strains and simultaneously Vancomycin resistance was detected by Vancomycin disc diffusion agar test using the principle of Kirby-Bauer method.

Out of 42 *S. aureus* isolates isolated, only 10 isolates (23.8%) were methicillin resistant (MRSA) which is less than the incidence rates of 40% and 36% reported by Umaru *et al.* 2014 [55] and Sulaj *et al.* (2013) [49] in raw milk. While Neeraj *et al.* (2017) [41] also reported 16.47% prevalence of MRSA in mastitic dairy cattle. Almost similar findings of the present study, Osman *et al.* (2016) [43] recorded 21.4% incidence rate of MRSA in poultry meat. The studies of Amita *et al.* (2008) [2], Anand *et al.* (2009) [4], Anamika *et al.* (2015) [3], Pramodhini *et al.*(2011)[45], Angela *et al.* (2015) [7], Ghias *et al.* (2016) [29] reported 75.26%, 64%, 46%, 36.4%,31.24%, 25.5% from clinical specimens.

Only 5 isolates (11.9%) of *S. aureus* were shown resistance to Vancomycin (VRSA). In contrary to the present study, Sulaj *et al*. (2013) [49] reported higher incidence of VRSA (47%) in milk samples while Umaru *et al.* (2014) [55] from Nigeria, Collins *et al.* (2010) [18] from S.Africa, Debaraj *et al.* (2016) [20] from West Bengal were reported lower prevalence of VRSA i.e. 4%, 3.4%, 0.95% in raw milk. Angela *et al*. (2015) [7], Ghias *et al.* (2016) [29] reported 2.8% and 2.5% prevalence of VRSA from clinical specimens.

Among 15 isolates of CNS isolated in the present study, 33.3% and 26.7% were the prevalence rates of Methicillin resistant CNS (MR-CNS) and Vancomycin resistant CNS (VR-CNS). In contradistinction, Fontes *et al.* (2013) [28] from Brazil and Osman *et al.* (2016) [43] reported very high prevalence of MR-CNS i.e. 81.5% and 52.8% in Minar cheese and poultry meat samples whereas Cemil *et al*. (2016) [14], De Neeling *et al.* (1998) [14] , Guran and Kahya (2015) [30] reported 23.5% from cheese samples in Turkey, 17% in Netherlands and 10% from ground beef and lamb in Turkey. Osman *et al*. (2016) [43] reported almost similar incidence of VR-CONS (27.8%) as in the present study from poultry meat.

Antimicrobial resistance patterns of each pathogen to various drugs used to treat the infections will vary from place to place i.e. between and within the countries [43]. The results of different researchers are incomparable with several variations which may be due to many factors like sampling and culture methods used for isolation and identification of different pathogens from different samples, the temperature of samples maintained while transporting to the laboratory as well as during processing for analysis, contamination of samples with MRSA and VRSA stains while handling and processing, number of pathogens in samples, processing of samples and cross contamination may change pathogen titre, food contamination due to infected food handlers , the last but not least which is very important i.e. indiscriminate and illegal use of different antibiotics to treat animals without correct dose [43].

Along with oxacillin and methicillin, Cefoxitin, other β lactam antibiotic is used to detect methicillin resistance in *Staphylococcal* strains recently. As it is a potent inducer of mec A regulatory system, it is used as accurate marker to detect Methicillin resistant *Staphylococcal* strains in routine susceptibility testing [14].

In the present study, Cefoxitin disc diffusion and Oxacillin screen agar test results shown almost similar positive methicillin resistant *Staphylococcus* strains while Pramodhini *et al.* (2011) [45] reported that Cefoxitin disc diffusion agar test is far superior than the other two phenotypic methods which is nearer to the present study and is accepted for detection of MRS by many reference groups including CLSI. The present investigation also suggested that the phenotypic methods can be used as alternative to PCR which is similar to findings of Anand *et al.* (2009) [4] who reported that Cefoxitin disc diffusion agar test results were in concordance with PCR in identifying MRS strains for mec A gene and it can be used as alternative to technically demanding PCR which is costlier, time consuming and some routine laboratories may not have such facilities for identification of methicillin resistant *Staphylococcal* strains.

Conclusion

The prevalence of CNS in food neither neglected nor its pathogenic potential is considered as insignificant as a food-borne pathogen. Safety measures should take immediately after its occurrence in food either to reduce or to eliminate them in food chain [43]. In conclusion, the present study has given an idea about necessity of maintaining hygiene practices while milking, handling, processing and transporting of raw milk and also emphasized the irrational use of antibiotics either therapeutically or prophylactically in preventing as well as in controlling the spread of drug resistant bacteria which may cause health hazard to humans. Both CPS and CNS were given significance because of their zoonotic importance. Further, there is an urgent need to implement various schemes/policies which are energetic to prevent spread of Methicillin Resistant and Vancomycin Resistant *Staphylococci* (MRS and VRS) through the food chain besides safeguarding the microbiological food safety. There should be continuous education of farmers and milkers about the hygienic measures to practice at the time of milking such as washing their hands before milking; avoiding spitting, coughing, sneezing, chatting during milking; washing the udder with disinfectants; proper sterilization of equipment; wearing apron, head caps, face masks and gloves while

milking; trimming of their nails; avoiding milking while they are suffering from diseases; milk from healthy animals shouldn't mix with milk of diseased animals. Consumers are advised to go for pasteurization of milk before consumption to reduce or remove multidrug *Staphylococci* strains in milk.

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