

**Antimicrobial susceptibility of coagulase negative *Staphylococci* isolates from suspected cases of bovine subclinical mastitis in parts of Bundelkhand region**Balendra Singh<sup>1\*</sup>, Ramesh Kumar<sup>2</sup><sup>1\*</sup>Department of Biotechnology, Bundelkhand University, Jhansi, India<sup>2</sup>Department of Biochemistry, Bundelkhand University, Jhansi, India

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**Abstract**

**Background:** Increasing importance of Coagulase negative *Staphylococci* as etiological agent in health concerns of domestic animals is recognized in various studies. A significant concern of CoNS isolates from bovine subclinical mastitis is development of resistance towards antimicrobials routinely used in management diseases like mastitis. Isolates from study area had not been worked out properly in previous reports. **Methodology:** 125 *Staphylococci* sp. isolates identified in previous study by the authors were screened for resistance against 9 antimicrobials of common use using the Kirby Bauer disk diffusion method. Minimal inhibitory concentrations of 8 antibiotics were measured by using Ezy™ Strips supplied by Himedia. Multiple drug resistance was also determined by analyzing results with individual antimicrobial agent. **Results:** Significant resistance towards  $\beta$ -lactam group of antibiotics penicillin and oxacillin was observed in isolates with 85.6 and 41.40 % respectively. Trimethoprim was second group of antibiotics for which isolates were found resistant with 81.60%. 20.8, 12, 10.40, 8.80, and 3.20% isolates were found resistant to erythromycin, tetracyclin, gentamycin, ciprofloxacin and ofloxacin respectively. Thus highest susceptibility was observed to fluoroquinolones. Data analysis also revealed multiple drug resistance in isolates as 44% of isolates was also found resistant to 3 or more antibiotics. On regional basis highest number of antibiotic resistant CoNS isolates was reported from Jhansi followed by Lalitpur and Jalaun. **Conclusion:** The study revealed the incidence of multiple antibiotic resistances in Coagulase negative *Staphylococci* isolates from possible cases of bovine subclinical mastitis and if not managed properly may be responsible for horizontal transfer of resistance from animal to man through food born infections.

**Key Words:** CoNS, Antibiotic resistance, Staphylococci, Mastitis, Bovines.**Introduction**

Bovine mastitis is considered as major economic problem in dairy sector, worldwide, with involvement of different pathogens. Among the gram positive bacteria, species of *Staphylococci* and *Streptococci* are of serious concern in occurrence of mastitis and frequently isolated from clinical mastitis cases.

In contrast Coagulase negative *Staphylococci* sp. (CoNS) are mostly associated with subclinical infection in udder inflammation and generally correlated with increased somatic cell count in milk samples followed by decrease in milk samples [1,17,38,41]. Coagulase negative *Staphylococci* have become a frequently isolated causative agent in some infections. It was not considered as contaminant, initially and was mostly found to occur in hospitalized patients, nosocomial infections and other cases [16] and considered as important blood stream pathogen resistant to multiple antibiotics by Larissa *et al.* 2014. Now frequent isolation of CoNS from many cases of subclinical mastitis in different times and locations from various countries have established it as major causative agent in this disease [42,43,35] However, in recent studies CoNS species is reported in both subclinical and clinical mastitis [41]. Levels of antibiotics resistance is continually increasing in last

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few decades due to a deadly combination of bacterial character sticks, evolution and selective pressure because of very much use of antimicrobials[10]. One of the important reason behind failure of treatment is given as indiscriminate use of antibiotics without prior , In Vitro evaluation of its susceptibility, this practice at one side increase the cost of treatment, mean to economic loss and at other side results in higher resistance to routinely used common available antibiotics [26].These bacteria have gain resistance to many routinely used antimicrobials, which is reported recently in many studies followed by significant increase in resistance against methicillin [23,46]. CoNS reported to be evolve more resistant to antibiotics as compared to *Staphylococcus aureus* and develop multiresistance[36,8,9] Even CoNS species is discovered as less virulent as compared to *S. aureus* but these exhibit higher resistances against commonly used antibiotics [34]. Antimicrobial agents are routinely used to treat and manage the bacterial infections in bovines to avoid the production loss in animals. Resistance to more than one antimicrobial was reported between 7-9% respectively in subclinical and clinical CoNS isolates by Waller *et al.* 2011[45]. Person *et al.* 2011 in his studies reported 35% of isolates resistant to penicillin. In a study in Dharwad District of Karnataka by Kaliwal *et al.* 2011, lowest susceptibility (Only 23.23%) was reported to penicillin[20,30].

In India many studies have been conducted and reported development of resistance in CoNS isolates against multiple antibiotics [37], specially, resistance to beta lactam antibiotics is of serious concern[33]. Bansal *et al.* 2015 reported development of resistance in CoNS isolates to Chloramphenicol, Gentamycin & streptomycin, while higher resistance was reported to commonly use penicillin group. In backward areas like Bundelkhand in India having animal husbandry as major source of income to the rural farming sector, it is important to manage mastitis in any form either it is clinical or subclinical immediately to reduce unwanted sufferings. Thus, in vitro antimicrobial susceptibility studies can guide the field veterinarians to use potent antibiotics in prophylactic and therapeutics interventions [21]. Which is also important to reduce the irrational use of antibiotics so development of resistance and failure of therapy can be checked as stated in several studies[32,43].

No previous studies on antibiotic resistance in coagulase negative *Staphylococci* isolates with specific reference to this area had been seen in literature surveyed, and it remains scanty. In context, the present study was conducted to investigate the susceptibility of CoNS isolates from milk samples of presumptive

subclinical mastitis infected bovines, against the antibiotics used in common. Antibiotic study profiles reported in this study will be helpful in recommendation of therapy to manage subclinical mastitis in this region.

### Methodology

#### CoNS isolates used in the study

Different CoNS strains isolated during 2015-16 and characterized by the authors in previous study were used[39]. Isolates were characterized up to species level but here in this study isolates used as single group as Coagulase negative *Staphylococci* to study antimicrobial resistance pattern.

#### Antibiotic susceptibility testing

Kirby Bauer disc diffusion method was used to determine antibiotic sensitivity of CoNS isolates [7]. A discrete colony was picked with sterile wire loop from 18 hour old culture of test isolate on mannitol salt agar. The culture was transferred to sterile brain heart infusion broth followed by incubation for 4 hours at 37°C. Standardization of inoculums was achieved with 0.5 McFarland turbidity standards, corresponding to 1.5 x 10<sup>8</sup> cfu/ml of bacterial cells. A sterile cotton swab was dipped in inoculum solution, and excess of inoculum was removed by pressing swab with wall of the tube. Mueller Hinton agar plates were prepared as per the manufacturer guidelines (Himedia Laboratories). Swab was streaked over entire surface of Mueller Hinton agar to inoculate the culture. The even distribution of inoculums was confirmed by repeating procedure two additional times by rotating about 60° each time. The antibiotic discs were place on the inoculated agar with help of applicator provided. Plates were left for 1 hour followed by incubation for 18 hours at 35°C. After 16 to 18 hours plates were considered to examine and diameter of zone of inhibition was noted down with the help of ruler. All the antibiotic discs were purchased from Himedia laboratories limited, Mumbai, India. Results were classified into three categories as Susceptible, Intermediate and resistant in accordance to guidelines established by Clinical and Laboratory Standards Institute.

#### Broth micro dilution method to determine minimal Inhibitory concentration

Minimal inhibitory concentration was determined for oxacillin, erythromycin, tetracycline, cefotaxime, gentamycin, ciprofloxacin and trimethoprim with commercial MIC determination paper strips i.e. Ezy MIC strips purchased from Himedia laboratories, Mumbai, India. These discs were provided with impregnated antimicrobial compound on a paper strip in concentration gradient manner. MICs on these strips were in range of 0.01 µg/ml to 240 µg/ml to or 0.016

µg/ml to 256 µg/ml, on testing against the test organism. Plates were prepared by the same method as described in previous section, followed by transfer of inoculum by rubbing sterile but dipped with inoculum, cotton swab on the medium surface. Ezy MIC strips, stored at 2-80C were first normalized at room temperature followed by placing at inoculated medium surface with the help of sterile forceps. Plates were incubated at 370C for 18 hours; an elliptical zone of inhibition was measured with scale. MIC values recorded were compared with interpretation chart provided by manufacturer in accordance to standards prescribed by Clinical Laboratory Standards Institute. (2010).

### Results

A total of 125 *Staphylococcus* isolates screened Coagulase negative, were tested for susceptibility to 9 antimicrobials of general use in veterinary practices including methicillin. Result of susceptibility test is presented in Table-1 as percentage of resistant isolates against each of the antimicrobial used in the study. Highest percentage of resistance was recorded against the penicillin (85.6%) followed by trimethoprim

(81.60%) and oxacillin (41.60%). While isolates in general were found most susceptible to cefotaxime (97.60%) followed by ofloxacin (96.80%), ciprofloxacin (91.20%) gentamycin (89.60) and tetracyclin (88.0%). No resistant isolate was reported from samples from district Jalaun against Cefotaxime and similar results were found against Ofloxacin for samples from district Lalitpur. Isolates from Jhansi district were reported to be most resistant against penicillin, trimethoprim and oxacillin as compare to Jalaun and Lalitpur, but for Ciprofloxacin, ofloxacin and gentamycin, isolates from Jalaun district were reported to be most resistant. Susceptibility to cefotaxime was highest in all samples considered in this study without any relation to source type or any other parameter. Among two antimicrobials from group fluoroquinolones, CoNS were found more resistant to ciprofloxacin with percentage value of 8.80 followed by ofloxacin with 3.2% resistance. Even no resistant isolate was found for ofloxacin in Lalitpur during the study. On holistic basis higher number and percentage of resistant isolates were reported from Jhansi.

**Table: 1:Percentage antimicrobial resistance in Cogulase –Ve *Staphylococci* sp. isolates along with distribution pattern**

Antibiotics	% resistance in CoNS in general, isolates from milk samples			
	Total	Jhansi	Jalaun	Lalitpur
	125	n=65	n=35	n=25
<b>Pencillin G</b>	107 (85.6%)	61 (93.85%)	26 (74.29%)	20 (80.00%)
<b>Trimethoprim</b>	102 (81.60%)	59 (90.77%)	25 (71.43%)	18 (72.00%)
<b>Oxacillin</b>	52 (41.60%)	30 (46.15%)	12 (34.29%)	10 (40.00%)
<b>Ciprofloxacin</b>	11 (8.80%)	5 (7.7%)	4 (11.43%)	2 (8.00%)
<b>Erythromycin</b>	26 (20.80%)	16(24.62%)	5 (14.29%)	4 (16.00%)
<b>Tetracycline</b>	15 (12.00%)	8 (12.31%)	4 (11.43%)	3 (12.00%)
<b>Gentamycin</b>	13 (10.40%)	7 (10.77%)	4 (11.43%)	2 (8.00%)
<b>Ofloxacin</b>	04 (3.20%)	2 (3.10%)	2 (5.71%)	0 (0.00%)
<b>Cefotaxime</b>	03 (2.40%)	1 (1.50%)	0 (0.00%)	2 (8.00%)

All the isolates except one that were found resistant to penicillin were also observed resistant to one or another antibiotics used. This was found resistant to trimethoprim only. Data on phenotypic resistance pattern of CoNS isolates to different classes of antibiotics is presented in table 2. Isolates were found resistant to all 5 classes of antimicrobials used in the study. Out of 125 isolates all but 18 isolates were found resistant to one or other or combination of antibiotics in

individual capacity. Highest number (19) Isolates were reported resistant to both PE combination followed by PT. Isolates resistant to 3 different classes of antimicrobials were 13 against PET. Orderly, 14, 8,8,5,4 numbers of isolates were reported resistant towards 4 different classes of antimicrobials PCTE, PETT, PETG, POTEC and PCTEO respectively. Only 3 isolates were found resistant towards all 5 different classes of antibiotics.

Table: 2- Phenotypic antibiotic resistance pattern in CoNS isolates

Resistance Phenotypes	No. of strains	No. of antibiotic classes
None	18	0
Pencillin,	17	1
Trimethoprim	01	1
Pencillin, Erythromycin	19	2
Pencillin, Trimethoprim	15	2
Pencillin, Erythromycin, Trimethoprim	13	3
Pencillin, Cefotaxime, Trimethoprim, Erythromycin	14	4
Pencillin, Erythromycin, Trimethoprim, Tetracycline	8	4
Pencillin, Erythromycin, Trimethoprim, Gentamycin	8	4
Pencillin, Oxacillin, Trimethoprim, Erythromycin, Ciprofloxacin	5	4
Pencillin, Ciprofloxacin, Trimethoprim, Erythromycin, Ofloxacin	04	4
Pencillin, Ciprofloxacin, Trimethoprim, Erythromycin, Cefotaxime	03	5

Table-3 revealed the data on Minimal inhibitory concentrations of 8 different antibiotics against

Coagulase negative Staphylococci isolates. Breakpoints for different antibiotics are considered as described by manufacturer in accordance with CLSI guidelines.

Table: 3- MIC (mcg/ml) of 8 antimicrobials used in management practices of mastitis for CoNS isolates

Antimicrobial agent	Break point	MIC90	Range
Oxacillin	4	32	0.016-256
Erythromycin	4	8	0.016-256
Tetracycline	8	30	0.01-240
Gentamycin	16	24	0.064-1024
Trimethoprim	16	128	0.001-240
Ciprofloxacin	4	5	0.01-240
ofloxacin	4	8	0.001-64
cefotaxime	32	30	0.01-240

Note: Breakpoints were considered as per CLSI guidelines and Supplier's instructions.

### Discussion

Prevalence of antibiotic resistance in CoNS isolates from suspected cases of subclinical mastitis on random basis is in agreement with previous studies done by Villari *et al.* 2000. This is the most comprehensive study on antibiotic resistance in Coagulase negative Staphylococci isolates in Bundelkhand region. Rationale behind this study was deprivation of status of knowledge regarding these isolates and its impact on animal husbandry sector in parts of Bundelkhand. A total of 9 routine use antibiotics (table-1) were selected

in view of running managerial practices[49]. A total of 125 isolates characterized in previous study were used to further investigate their antimicrobial profile due to their potential of being clinically significant, on the basis amount of organism and source or on the basis of combination of both. About 40% isolates among these were other than *S. epidermidis*.

Curing rate of mastitis less than 25% is considered as poor while response rate attains 75% or above is favorable [22]. Lowest susceptibility towards penicillin (15%) in this study is lower than the values found by

Basappa et al, 2011 (235) in the study conducted in Dharwad region India, but in agreement with Fowoyo & Ogunbanwo, 2017 (14%). Findings are in correlation with frequent production of penicillinases by *Staphylococcus* sp. specially by CoNS species. A large percentage of isolates were found to resistant against Erythromycin, Gentamycin, Ciprofloxacin and ofloxacin is also in agreement with Fowoyo and Ogunwanwo, 2017[16]. Ozkan *et al.* 2014 also reported higher percentage resistance against Erythromycin, Pencillin, Vancomycin and Tetracycline in similar type of study. In previous studies various authors have revealed the significant variation in resistance towards tetracycline ranged from 10 -100% [8,9,11,24]. Finding in this studies lies within the range. Higher resistance with 81% isolates were observed as resistant to trimethoprim, these results are in agreement with Habib *et al.* 2015, who reported 100% resistance to this antibiotic in bovine isolates. In developing countries, sulfonamides are commonly used antimicrobials due to nonjudicial uses of antibiotics, this results in development of resistance in bacterial pathogens of domestic animals(Then, 1989). In present investigation, lowest resistance were found to cefotaxime followed by ofloxacin and ciprofloxacin, which proved as drug of choice in the representative area supported by the similar study carried out by Bansal et al. 2015. It might be due to lowest exposure of infected animals to these antimicrobials. Large variations in susceptibility studies were reported by various authors in similar studies [49,18-20]. Cefotaxime a good antimicrobial agent against *S.aureus* is not used frequently in the representative area. It was also not studied in most of the antimicrobial studies of Coagulase negative *Staphylococci* but in this study CNS was found highest susceptibility and least resistance to this antibiotic with percentage (only 2.4% resistant isolates) followed by the similar studies of Santosh *et al.* 2018 who reported 4.3% resistance.

Data revealed that isolates from Jhansi were found more resistant to antimicrobials considered in this study. Possible reason could be the long term exposure due to more reachability of healthcare facilities but uncontrolled use of antimicrobials is evidenced here. Resistance to multiple antibiotics becomes a serious issue now a day's specially in nosocomial infections[4]. Higher percentage of isolates were found resistant against multiple antibiotics it may be due to selective pressure caused by abusive use of antibiotics in disease management[14]. Multidrug resistant Coagulase negative *Staphylococci* have been reported by different authors in dairy products[40,13,15,19,21]. Shubhra *et al* 2008 revealed the 76% of CoNS isolates

of particular species resistant towards oxacillin (76%), gentamycin (76%), erythromycin (80%) in human infections from Lucknow but in our study resistance to these antibiotics was found significantly lower in isolates from bovines which was 41.6, 20.8, 10.40% respectively, which show the spatial and host specific difference in resistance pattern. Overall 55 (44%) isolates were found to resistance for 3 or more antibiotics, significantly higher than 29% reported by Antonio *et al.* 1999[3]. Spread of such strains in this region is of serious concern in management of animal husbandry and related issued directly impacting the agrarian economy.

### Conclusion

Increased incidence of antibiotic resistance in Coagulase negative *Staphylococci* from healthy animals and possible cases of subclinical mastitis shows that the milk may act as reservoir for horizontal transfer of antibiotic resistance in human infections through food borne disease. It may be of serious concern to public health. Results also show the unregulated use of antibiotics leading to multiple drug resistance in CoNS species in interior and semiurban areas of Bundelkhand region. It also suggests the search and use of newer antimicrobials as resistance to routinely used antibiotics is continually increasing. Variability in resistance pattern with other studies from different areas shows the disease triangle basis of infectious diseases may change the pattern of resistance in different antibiotics based on host, pathogen and environmental interaction. Further on the basis of this study we recommend the continuous work on surveillance of antibiotic resistance in study area.

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