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Research Article

ANTIBIOGRAM OF *KLEBSIELLA PNEUMONIAE* ISOLATED FROM MASTITIC MILK SAMPLES OF CATTLE AND BUFFALO

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ABSTRACT

Klebsiella pneumoniae is an opportunistic pathogen that can cause bovine mastitis in various forms depending upon severity. It can deeply invade into the udder tissue and can damage the secretory capacity of the gland resulting into long-term decreased milk production. The present study was aimed to isolate *K. pneumonae* from mastitic milk samples of cattle and buffalo. The overall prevalence of *K. pneumoniae* in mastitic milk samples of cattle and buffalo was recorded as 2.18% (18/823). Of these isolates, 13 were from buffalo origin and five isolates were recovered from cattle. All the 18 isolates were susceptible to chloramphenicol, followed by enrofloxacin, oxytetracycline, gentamicin and levofloxacin. All the isolates were resistant to penicillin group of antibiotics followed by streptomycin (77.08%), moxifloxacin (61.10%) and aminoglycosides such as amikacin, neomycin and kanamycin except gentamicin. All the isolates were multi drug resistant having multiple antibiotic resistance index value more than 0.2.

Keywords: Antibiogram, Buffalo, Cattle, *Klebsiella pneumoniae*, Mastitis

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Bovine mastitis is a serious concern in the dairy industry worldwide and invariably requires treatment with antimicrobials (Gupta *et al.*, 2020). Opportunistic pathogens of environmental origin or commensals such as *Staphylococcus* species, *Streptococcus* species, *E. coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* are the major agents of mastitis (Podder *et al.*, 2014; Mittal *et al.*, 2018). *K. pneumoniae* is an opportunistic human pathogen mostly prevalent in organic matter, such as animal house bedding of moist soils and manure (Munoz *et al.*, 2007). Mastitis caused by *K. pneumoniae* may be mild, moderate, and severe depending upon the symptoms. The bacterium deeply invades the udder tissue and damages the glandular tissue. This type of infection becomes chronic and reduces the milk production of the affected animal (Pinzon-Sanchez *et al.*, 2011). *K. pneumoniae* are Gram-negative rods having pink-colored mucoid colonies on the MacConkey agar plate. The hyper mucoidness is a typical characteristic of virulence. The hyper-virulent and β-lactam drug-resistant strains of *K. pneumoniae* are capable of causing fatal, life-threatening infections both in animals and humans (Shon *et al.*, 2013). The drug-resistant strains can rapidly spread and transfer the resistance phenotype (Tzouvelekis *et al.*, 2012) through horizontal gene transfer by certain mobile genetic elements, such as transposons, bacteriophages, and plasmids (Ahmed *et al.*, 2010). The present study was conducted to determine incidence of antibiotic resistant *K. pneumoniae* isolates in mastitic milk of cattle and buffalo.

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MATERIAL AND METHODS

Isolation and Identification

Milk samples from cattle and buffalo were received from livestock's owners in sterile containers at College Central Laboratory situated in College of Veterinary and Animal Science, Lala Lajpat Rai University of Veterinary and Animal Science (LUVAS), Hisar, Haryana, India for routine diagnosis of mastitis and antibiotic susceptibility assay of isolates. Briefly, 0.01 ml of milk sample was inoculated on Nutrient agar and MacConkey agar plate following aseptic procedures and then plates were incubated at 37 ºC overnight in incubator. Bacterial isolates were identified by their colony characteristics and Gram's staining as described by Quinn *et al.* (2002).

Amplification of 16S rRNA gene sequence

Presumptive isolates were confirmed by PCR (polymerase chain reaction) amplification of 130bp amplicon of species-specific 16S rRNA nucleotide sequence primers (Osman *et al.*, 2014). The DNA extraction was carried out as per protocol suggested by DNeasy Blood and Tissue Kit (Qiagen). The forward primer sequence is 52-ATTTGAAGAGGTTGCAAACGAT-32 and reverse primer sequence is 52 -TTCACTCTGAATTTTCTTG TGTTC-32 . The reaction mixture (total volume 25µl) was prepared by mixing 12.5µl GoTaq® Green Master Mix (Promega), 1µl Primer-1 and 2 each (10 pM/µl), 3µl Template DNA (25ng/µl) and 7.5µl nuclease-free water (up to final volume 25µl). PCR Amplification was carried

out in 'Thermal cycler gradient (BR Gradient Thermal Cycler)' as follows: initial cycle of denaturation at 95 ºC for 5 min, 30 cycles (denaturation at 95 ºC for 1 min, primer annealing at 55 ºC for 1 min and primer extension at 72 ºC for 1 min), and a final extension at 72 ºC for 5 min. The PCR products were analysed by electrophoresis on 1-1.5% agarose gel with ethidium bromide (0.5µg/ml) in 1X TAE buffer for 60 min at 100 V. The gel was then visualized by the Azure C-15 UVP gel documentation system.

Antibiotic susceptibility testing

An antibiotic susceptibility test was conducted as per the guidelines described by Bauer (1966) against sixteen antibiotics. Briefly, pure culture colonies of *K. pneumoniae* isolates from Nutrient agar and MacConkey agar plates were inoculated in Nutrient broth and incubated for 6-8 hrs in incubator. The turbidity of broth should be as equivalent to 0.5 McFarl and standards after incubation. Sterilized swab socked with inoculum was uniformly spread on the Muller Hinton agar (MHA) surface and allowed to dry for 10 min (Quinn *et al.*, 1994). Antibiotic discs were carefully placed on the surface with enough space around each disc for the diffusion of the antibiotic and further incubated at 37 ºC overnight. The zone of inhibition of growth of the organism around each disc was measured in millimetres. Clinical and Laboratory Standards Institute (CLSI) guidelines (2011) were followed for the zone of inhibition interpretation of antimicrobial resistance or susceptibility.

Determination of Multi antibiotic resistance (MAR) value

All Multidrug-resistant (MDR) isolates were evaluated for their multiple antibiotic resistances (MAR) index. In an effort for risk assessment of MDR isolates, this index was calculated as per method given by Krumperman (1983).

MAR Index of single isolate = a/b, (where a -represents the number of antibiotics to which the isolate was resistant and b-represents the number of antibiotics to which the isolate was exposed.)

RESULTS AND DISCUSSION

A total of 32 *Klebsiella* spp. isolates were presumptively identified from the received mastitic milk samples (823 quarter milk samples) at College Central Laboratory, LUVAS, Hisar. All of them had typical pink colored mucoid colonies on MacConkey agar plates. Organisms were seen as pink-colored Gram-negative rods on Gram's staining as method described by Paulin-Curlee *et al.* (2007). After presumptive identification, isolates were directly processed for DNA isolation and molecular confirmation by PCR amplification of *K. pneumoniae* species-specific rRNA nucleotide sequence without any other biochemical identification. All the 18 phenotypically identified isolates amplified 130bp amplicon of 16S rRNA *K. pneumoniae* species-specific nucleotide sequence (Fig. 1). Of the 18 isolates, 13 were isolated from buffalo and five were isolated from cattle. Over all prevalence of *K. pneumoniae* isolates was detected as 2.18 % (18/823).

Antimicrobial susceptibility revealed 100% susceptibility to chloramphenicol followed by enrofloxacin (83.30%), oxytetracycline (72.20%), gentamicin (55.60%) and levofloxacin (38.9%). All isolates were resistant to the penicillin group (penicillin, cloxacillin, ampicillin, and amoxycillin) of antibiotics as this group act on the bacterial cell wall (not found in gram-negative

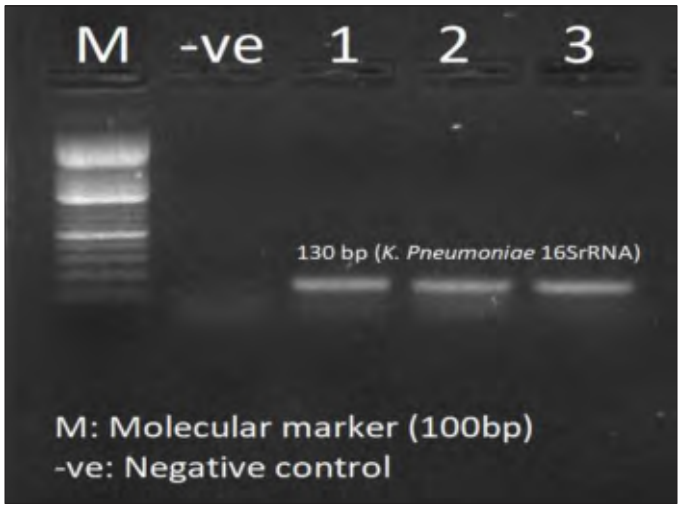

Fig. 1. Amplification of 16S rRNA *K. pneumoiae* species-specific nucleotide sequence (130 bp)

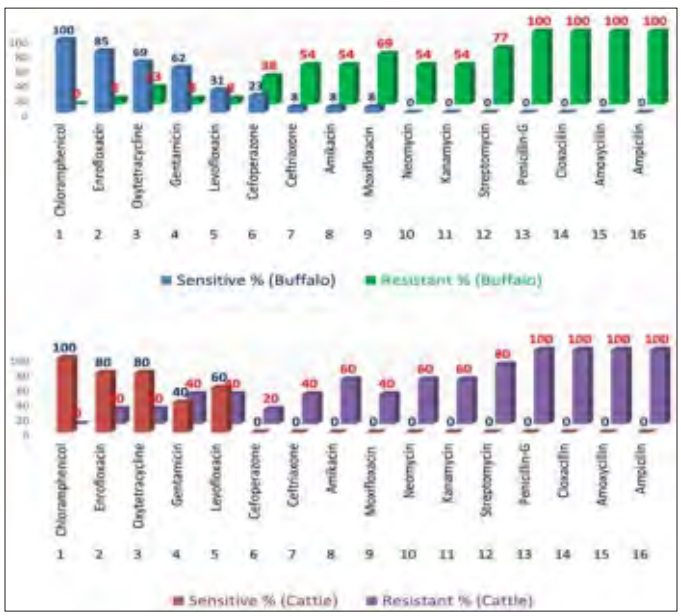

Fig. 2. Antibiogram (%) of *K. pneumoiae* isolates from cattle and buffalo

organism). However, the result of penicillin group was expected but still these antibiotics were included in study to cross check any contamination of any Gram's positive organisms in pure culture. Overall 77.80% and 61.10% of the isolates were resistant to streptomycin and moxifloxacin, respectively. Following that, 55.60% of the isolates from both species were resistant to the aminoglycoside group of antibiotics (amikacin, neomycin, and kanamycin) except gentamicin (Fig. 2). Isolates have highest MAR index value of 0.88 and lowest MAR index value 0.25 with average group MAR index value of 0.53. The MAR index value of more than 0.20 was considered as multi drug resistance (MDR) value (Krumperman, 1983). It signifies that all the *K. pneumoniae* isolates of present study were resistant to atleast two or more than two antibiotics. Previously, many researchers observed similar findings with present study that *K. pneumoniae* from mastitic milk origin were found susceptible to gentamicin, chloramphenicol, enrofloxacin and ciprofloxacin whereas resistant to carbenicillin, piperacillin, ampicillin, co-trimoxazole, cefotaxime (Mittal *et al.*, 2018; Singh *et al.*, 2018; Karimi and Momtaz, 2019; Arya *et al.*, 2020; Masse *et al.*, 2020). Similar to our study, Bhanot *et al.* (2012) also found that enrofloxacin is the most sensitive drug in 86.62% cattle milk samples and 87.96% in buffalo milk samples.

The present study data provide a good assessment about therapeutic management of mastitis caused by opportunistic pathogens such as *K. pneumoniae* in field conditions. The antibiotic susceptibility testing plays important role in the judicial use of antibiotics in dairy livestock. However, lack of antibiotic susceptibility testing facilities in remote areas is big a hurdle to achieve the goal. Inappropriate doses and indiscriminate use of antibiotics may lead to high antibiotic resistance (Yadav *et al.*, 2015; Sharma *et al.*, 2015; Mittal *et al.*, 2018). The multidrug resistant bacterium may transfer resistance genes to sensitive population of other bacteria through chromosomal changes, exchange of genetic material via plasmids and transposons (Sharma *et al.*, 2015).

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