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Isolation, identification and antimicrobial resistance in *Klebsiella* spp. isolated from diarrheic cattle calves in Mhow

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Abstract

Livestock is an essential component of the Indian agricultural production system and is crucial to the advancement of the country. In dairy, calf diarrhoea is a complex disease that can have major financial and animal welfare ramifications. High risks of calf illness and mortality result in significant financial losses for dairy industries. The present study was undertaken to isolate *Klebsiella* from diarrheic cattle calves to assess the antimicrobial resistance pattern of these isolates by *in vitro* method. A total of 137 rectal swabs were collected from diarrheic calves directly from the rectum. BHI broth, MacConkey agar, EMB agar and Nutrient agar were used to isolate the *Klebsiella*. From 137 diarrheic samples of cattle calves, 13 (9.48%) *Klebsiella* isolates were isolated and identified on the basis of cultural, morphological, and biochemical characteristics. These *Klebsiella* isolates on subjecting to PCR using genus specific primers revealed the presence of an expected amplicon of 441 bp size (Plate 3) and, hence confirmed genotypically as *Klebsiella*. The *in vitro* AST revealed that all isolates of *Klebsiella* were 100% resistant to enrofloxacin followed by ampicillin (92.31%), and aztreonam, colistin and nitrofurantoin (84.62% each), ceftazidime plus clavulanic (76.92%), ceftriaxone and sulphadiazine (46.15% each), azithromycin (38.46%), and co-trimoxazole and tetracycline (30.77% each), and cefotaxime, ciprofloxacin, gentamycin and streptomycin (23.08% each). All the isolates were found to be multidrug resistant as each of the isolate was resistant to minimum one agent of at least three antimicrobial groups.

Keywords: *Klebsiella*, diarrhea, calves, antimicrobial resistance

Introduction

Livestock is an essential component of the Indian agricultural production system and is crucial to the advancement of the country. Young animals die more frequently than older animals, which has a significant negative impact on the cattle industry's finances. (Radostits *et al.*, 2010) [13]. In dairy, calf diarrhoea is a complex disease that can have major financial and animal welfare ramifications. According to estimates, pre-weaning acute diarrhoea accounts for 75% of early calf mortality in dairy herds. (Svensson *et al.*, 2006) [14]. One of the clinical symptoms that veterinarians see the most commonly in agricultural animals is diarrhoea, particularly in young calves. The economic losses result from both mortality and morbidity, as well as from the later chronic ill-thrift nature of calf diarrhoea and the resulting drop-in growth rate (Bazeley, 2003) [15].

According to Wudu *et al.*, (2008) [17], diarrhoea is the most common cause of calf-hood disease in livestock and is frequently accompanied with pneumonia, joint pain, and septicaemia (Razzaque *et al.*, 2009) [16]. Overfeeding, overpopulation, cold weather, poor cleanliness, artificial feeding, and lack of colostrum are all predisposing variables that can have a big impact on the disease's complex aetiology. High risks of calf illness and mortality result in significant financial losses for dairy industries (Svensson *et al.*, 2006) [14].

The *Enterobacteriaceae* family member *Klebsiella* is common in the environment and benignly colonises both human and animal gastrointestinal tracts. It is an opportunistic pathogen that can infect people and several animal species with a variety of diseases (Davis and Price 2016) [18]. *Klebsiella* have been identified from both clinically ill and seemingly healthy animals. As a member of the ESKAPE bacterial group, *Klebsiella* poses a serious challenge to clinicians worldwide because of the advent of several isolates of bacteria that are resistant to numerous drugs (Chaudhary *et al.*, 2020) [5].

Antimicrobial medications are frequently used to treat calf diarrhoea. The development of antimicrobial tolerance to many antimicrobial drugs has nonetheless become a well-known phenomenon due to their broad spectrum of activity, which is cause for serious concern (Hajipour *et al.*, 2013) [19]. Many people are becoming more aware of the common resistance of many bacteria to most antimicrobial treatments. The World Health Organization (WHO) made a statement regarding the serious threat that antibiotic-resistant bacteria pose to both human and livestock health (Raffi *et al.*, 2010) [20].

Animals make up a sizable portion of the several niches from which *Klebsiella* species might acquire genes for antimicrobial resistance (AMR). Thus, animal-derived *Klebsiella* species may play a significant role in the trafficking of drug resistance genes. The present study was undertaken to isolate *Klebsiella* from diarrheic cattle calves to assess the antimicrobial resistance pattern of these isolates by *in vitro* method.

Material and Methods

Sample collection

The animals used in this investigation were calves under 4 months of age with clinical diarrhoea, showing symptoms of systemic sickness (such as impaired suckling reflex, poor appetite, fever and dehydration), and pasty-watery feces. A total of 137 rectal swabs were collected from diarrheic calves directly from the rectum and kept in sterile test tubes, and transferred immediately in ice box to the laboratory of department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, Mhow, for processing.

Isolation of *Klebsiella*

The sample was inoculated in BHI broth and incubated aerobically at 37 °C for 24 hours, followed by streaking on MacConkey agar plate and incubated aerobically at 37 °C for 24 hours to obtain isolated single bacterial colonies. Single, mucoid, isolated, lactose fermenting pink colony was inoculated on EMB agar to rule out the presence of *E. coli*. The gross morphological characteristics of the colonies were observed and Gram staining of bacterial growth was carried out. A Single, mucoid, well-defined colonies from the MacConkey agar plate suggestive of *Klebsiella* were streaked on nutrient agar plate and incubated at 37 °C for 24 hours. Single colony was then picked up and inoculated on nutrient agar slant. Following this a series of various biochemical tests like Catalase, Oxidase, Indole, Methyl Red, Vogues Proskauer, Citrate utilization, Urease production test was applied for identification of the *Klebsiella* species.

Genotypic confirmation of *Klebsiella*

The phenotypically identified *Klebsiella* species were confirmed genotypically by PCR using genus specific primers. For this purpose, whole bacteria DNA of each bacterial isolates was extracted by using DNA isolation kit (GenElute, Sigma) as per the manufacturer's instructions.

The *Klebsiella* genus species specific primers having sequence of (5'-3') F: CGCGTACTATACGCCATGAACGTA, R: ACCGTTGATCACTTCGGTCAGG (Younis *et al.*, 2017) [21]. PCR reaction mixture was prepared that consisted of 12.5 µl mastermix (ReadyMix™ Sigma-Aldrich), 1 µl of 20 pmol of each forward and reverse primers, 2 µl of template DNA

and finally the reaction volume was made up to 25 µl using Nuclease free water (Sigma). PCR was performed on a thermocycler (Applied biosystem, USA) with the following conditions: an initial denaturation at 94 °C for 5 minutes followed by 35 cycles each of denaturation at 94 °C for 30 seconds, annealing at 60 °C for 45 seconds and extension at 72 °C for 45 seconds. This was followed by a final extension at 72 °C for 10 minutes. The PCR product was run on a 1.5% agarose gel at 80 V for 50 minutes. After the gel electrophoresis, DNA bands were visualized and the images were captured by using Gel Documentation System.

Antimicrobial susceptibility testing

The *in vitro* Antibiotic sensitivity test (AST) of all *Klebsiella* isolates was performed according to disc diffusion method of Bauer *et al.*, (1966) [22]. Fifteen antibiotic discs *viz.*, ampicillin (AMS) 10 µg, azithromycin (AZM) 15 µg, aztreonam (AT) 30 µg, cefotaxime (CTX) 30 µg, ceftazidime plus clavulanic (CAC) 30 µg, ceftriaxone (Ci) 10 µg, ciprofloxacin (CIP) 5 µg, colistin (CL) 10 µg, co-trimoxazole (COT) 25 µg, enrofloxacin (Ex) 10 µg, gentamycin (G) 10 µg, nitrofurantoin (NF) 100 µg, streptomycin (S) 10 µg, sulphadiazine (SZ) 100 µg, tetracycline (TE) 10 µg were used in the study.

Results

From 137 diarrheic samples of cattle calves, 13 (9.48%) *Klebsiella* isolates were isolated and identified on the basis of cultural, morphological, and biochemical characteristics (Plate 1, 2 and 3). These *Klebsiella* isolates on subjecting to PCR using genus specific primers revealed the presence of an expected amplicon of 441 bp size (Plate 3) and, hence confirmed genotypically as *Klebsiella*. The incidence of *Klebsiella* in diarrheic fecal samples of was 9.48%.

The *in vitro* AST revealed that all isolates of *Klebsiella* were 100% resistant to enrofloxacin followed by ampicillin (92.31%), and aztreonam, colistin and nitrofurantoin (84.62% each), ceftazidime plus clavulanic (76.92%), ceftriaxone and sulphadiazine (46.15% each), azithromycin (38.46%), and co-trimoxazole and tetracycline (30.77% each), and cefotaxime, ciprofloxacin, gentamycin and streptomycin (23.08% each) (Plate 4, Figure 1). All the isolates were found to be multidrug resistant as each of the isolate was resistant to minimum one agent of at least three antimicrobial groups.

Discussion

In present research 9.48% (13/137) *Klebsiella* isolated from diarrheic fecal cattle calves. Many researchers reported *Klebsiella* from calves' diarrhea and other sources with varying degree. Okela *et al.*, 2010 [29] reported only 2.6% *Klebsiella* in fecal samples of calves having age below 30 days. Herrera-Luna *et al.*, 2009 [30] reported 3.3% *Klebsiella* in diarrheal calves aged 0 to 6 weeks. In contrast to our finding Bobbadi *et al.*, (2019) [23] reported 29.4% samples of animal intestines to harbor *Klebsiella*. Montso *et al.*, (2019) [24] found *K. pneumoniae* species in 32% samples of cattle faeces. Sousa *et al.*, (2019) [25] isolated *K. pneumoniae* from 15% faecal samples of domestic and wild animals. Cheng *et al.*, 2018 [26] reported 15.5% *Klebsiella* spp. in nasal swabs collected from 1 to 2 old cattle calves.

Cheng *et al.*, 2018 [26] reported that, in *Klebsiella* resistance rates to ampicillin, amoxicillin, ciprofloxacin, cefotaxime, ceftriaxone, gentamicin and ceftazidime were 93.9%, 81.8%, 60.6%, 57.6%, 33.3%, 27.3% and 18.2% respectively, which

is in close relationship with our findings. Manikandan and Asmath (2013) ^[1] reported least susceptibility for the ampicillin (16.7%) and cotrimoxazole (29.2%). While, imipenem, gentamycin, ciprofloxacin, cefotaxime, ceftriaxone and ceftazidime were showing 86.1%, 80.6%, 76.4%, 66.7%, 66.7% and 54.2% sensitivity, respectively. Nirwati *et al.* (2019)^[3] studied the AST of *K. pneumoniae* and found resistance for ampicillin, gentamycin, ceftriaxone, ciprofloxacin and trimethoprim as 97.70%, 38.46%, 56.64%, 44.62% and 53.13%, respectively. Abebe *et al.*, 2020 reported resistance against ampicillin 63.2%. Chaudhary *et al.*, 2020 ^[5] reported 41.6%, 25%, 16.6% and 16.6% towards ceftriaxone, gentamycin, ciprofloxacin and trimethoprim, respectively. Lee *et al.*, 2021 ^[7] reported highest resistance i.e. 87.5% towards ampicillin in *Klebsiella* followed by 50%, 53.6%, 39.3%, 32.1% and 50% resistance in ceftriaxone, ciprofloxacin, ceftazidime, ceftazidime plus clavulanic acid and gentamycin, respectively. Kashefieh *et al.*, 2021 ^[6] reported ciprofloxacin, gentamycin, cefotaxime, ceftriaxone, ceftazidime, ampicillin, tetracycline and aztreonam to be resistance as 67%, 51%, 68%, 67%, 68%, 25%, 77% and 65%, respectively. EL-Tawab *et al.*, 2021 ^[8] tested trimethoprim, cefotaxime, amoxiclav, meropenem, and reported 100%, 5%, 19% and 0% resistance, respectively. Arbab *et al.*, 2021 ^[10] found *Klebsiella* showing 95% resistance against ampicillin and tetracycline. Carvalho *et al.*, 2021 ^[9] reported ciprofloxacin, gentamycin and tetracycline to be resistant as 100%, 75% and 66.67%, respectively. Zhang *et al.*, 2021 ^[11] reported very low resistance against colistin, imipenem and meropenem. Junaid *et al.*, 2022 ^[12] also reported the co-trimoxazole was highly sensitive with the colistin and tigecycline while ceftazidime and cefotaxime were showing higher degree of resistance.

Although *Klebsiella* is a member of the normal gut microflora of animals, the prevalence of the bacteria in the intestines and, consequently, faeces, may vary with age, season, the bacteriological quality of the feed and water at farms, the type of bedding material (organic versus inorganic), the use of antibiotics as a growth promoter, and any prevailing clinical diseases at the time of sample collection. (Xiong *et al.*, 2018) ^[27].

Therefore, proper and necessary attention should be paid to the management of the calf by making sure that the environment where calving occurs is properly cleaned to remove bacteria from previous calving's, that the calf consumes colostrum in the first few hours of life, and that the calf is hand fed with feeders in case feeding is difficult. Inadequate ventilation and crowding of calves are additional risk factors that should be avoided wherever possible.

Dairy cows intermittently shed *Klebsiella* in their feces, and this is linked to the organism's temporary rather than long-term stay in the digestive tract. (Munoz and Zadoks, 2007) ^[28]. Because it has an influence on both hospital and community settings, antibiotic resistance is currently a highly severe issue that has drawn a lot of attention from the greater scientific community (Shaikh *et al.*, 2015) ^[2]. This suggests that prompt detection of resistant determinants in bacteria inside diagnostic laboratories and awareness with the antibiotic resistance traits of circulating strains are crucial for the prudent assessment of these organisms' potential harm to animals and people in a specific place.



Plate 1: Mucoïd pink colour colonies of *Klebsiella* on MacConkey agar



Plate 2: Mucoïd colonies of *Klebsiella* on Nutrient agar

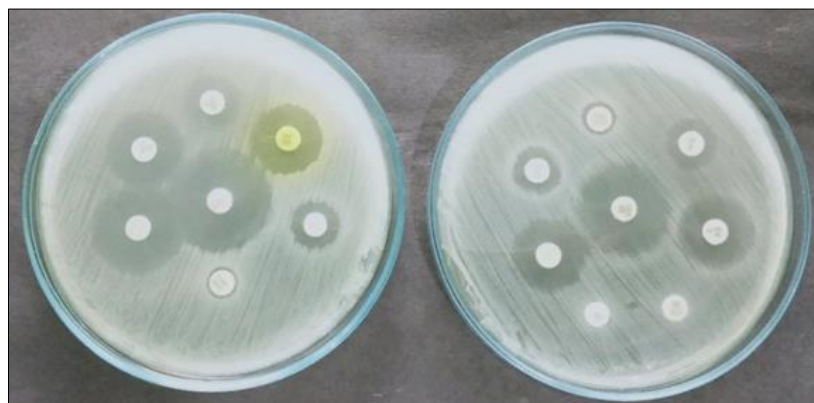
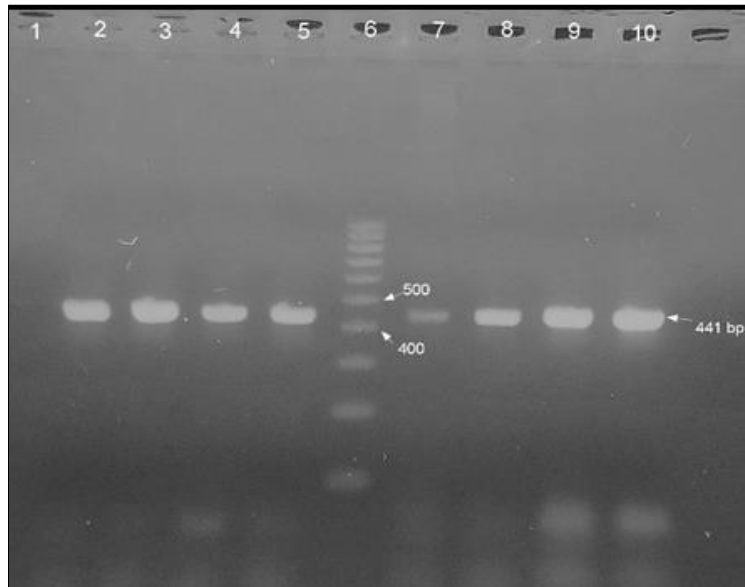


Plate 3: Results of Antibiotic sensitivity test



Lane 6: 100bp ladder, Lane 1: negative control, Lane 2: Positive control (441 bp), Lane 3,4,5,7 to 10: Positive samples (441 bp)

Plate 4: Gel electrophoresis of amplicon (441 bp) of *Klebsiella* isolates

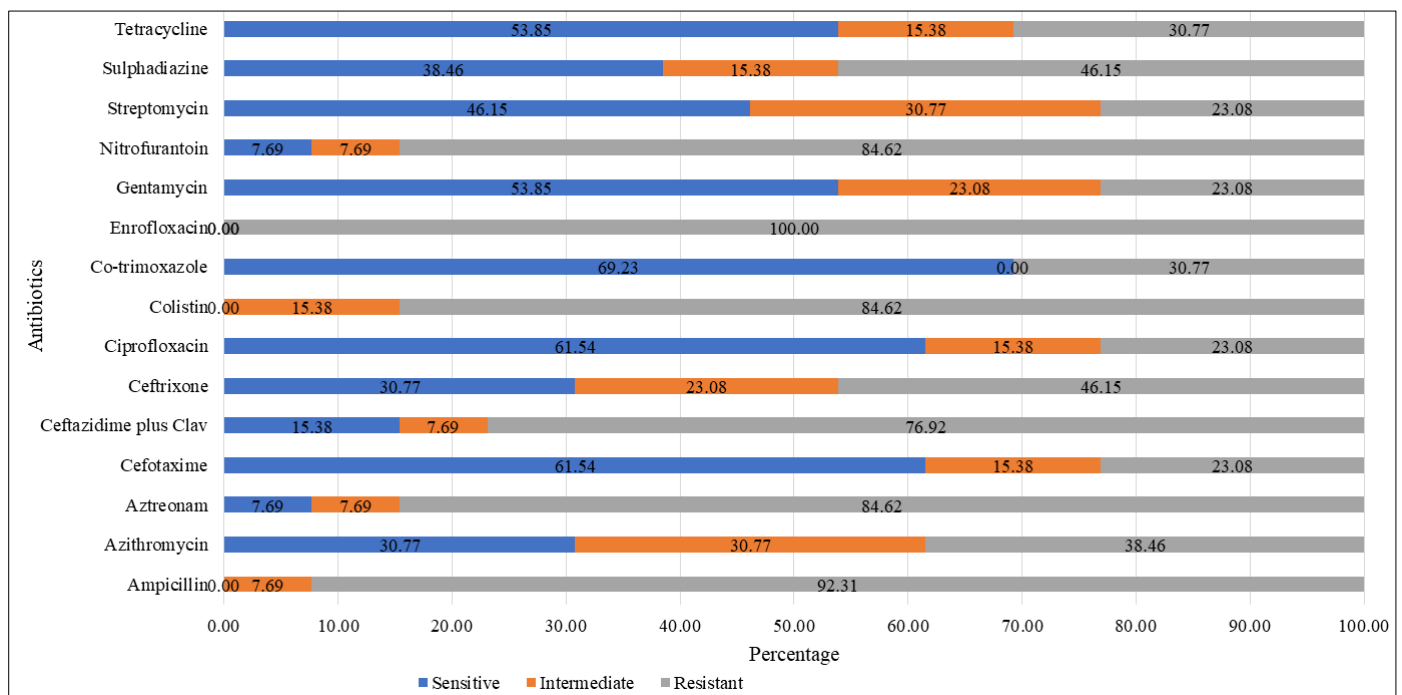


Fig 1: Comparative antibiotic resistance patterns of *Klebsiella* isolated from diarrheic cattle calves.

Conclusion

Numerous *Enterobacteriaceae* species have been identified from the feces of animals and are found in their intestines. However, the study's weakness was that its primary focus was on isolating *Klebsiella* only. The study was also limited by the fact that just one district's worth of cattle calf feces was gathered. Despite this, it is recommended that additional research be done to identify other *Enterobacteriaceae* members present in animals in order to address variables that contribute to the effective spread of antibiotic resistance to humans.

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