RESEARCH ARTICLE

Molecular Detection of *Klebsiella* spp. from Buffalo Milk and Farm Environment

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Abstract

Mastitis caused by Gram-negative bacteria is becoming more common in both well-run and unorganized dairy farms. Coliforms are the major environmental pathogens in the etiology of bovine mammary infections. Among these coliforms, the opportunistic pathogens, *Klebsiella* spp. may cause the severe mastitis. *Klebsiella* spp. was isolated from 15 (5.00%) out of total 300 samples. The prevalence of *K. pneumoniae* in buffaloes and farm environment was 4.66% and 5.33%, respectively. *Klebsiella* and *K. pneumoniae* were recovered from all type of samples, *viz.,* mastitic milk, faeces, bedding material and floor, drinking water and soil sample. The prevalence of virulence gene (*fim*H) in *Klebsiella* spp. isolated from buffaloes and farm environment was 53.33%.

Key words: Buffalo, Farm environment, *fimH, Klebsiella,* Mastitis.

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Introduction

Buffalo occupies a prominent place in the social, economic and cultural life of rural communities in India. However, its production is hampered by certain diseases. Gram-negative bacterial mastitis is becoming more common in both organized and unorganized dairy farms. Coliforms are the major environmental pathogens in the etiology of bovine mammary infections. They contaminate milk through faeces, water and soil. Among these coliforms, the opportunistic pathogens, *Klebsiella* spp. may cause the severe mastitis. *K. pneumoniae* and *K. oxytoca* are frequently isolated from domestic livestock and dairy environment (Ahmed *et al*., 2016). *Klebsiella* species are Gram-negative, non-motile, facultative anaerobic, encapsulated and rod-shaped bacteria of family *Enterobacteriaceae* and belongs to the ESKAPE group of pathogens capable of 'escaping' the biocidal action of antibiotics and mutually representing new paradigms in pathogenesis, transmission and resistance (Omoya and Ajayi, 2016). Therefore, the present research was planned to study of molecular detection of *Klebsiella* spp. from buffalo milk and farm environment.

Materials and Methods

Source of Samples and Collection

The work was conducted in the Department of Veterinary Microbiology, College of Veterinary and Animal Husbandry, NDVSU, Mhow (India). A total number of 300 samples, 150 from buffalo (mastitic milk, faeces) and 150 from farm environment (bedding material and floor, drinking water and soil) were collected from various organized and unorganized buffalo farms located in Indore and Ujjain districts of Madhya Pradesh. The samples were collected aseptically in sterile test tubes or polythene bags, and transported on ice to laboratory for further processing. The California mastitis test was performed for milk samples to detect subclinical mastitis as per Schalm *et al*. (1971).

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Isolation and Identification of *Klebsiella*

The samples were inoculated in BHI broth, and incubated at 37°C for 12 h for enrichment. Subsequently a loopful of bacterial growth from BHI broth was inoculated by streak plate method on MacConkey agar (MCA) and incubated at 37ºC for 24-48 h. Single, mucoid, isolated pink bacterial colony, indicative of lactose fermentation, was inoculated on Eosin-Methylene Blue (EMB) agar to rule out the presence of *E. coli* according to Ammar *et al*. (2021). Lactose fermenting mucoid bacterial colony on MCA, which did not produced metallic green sheen on EMB agar, was stained by Gram's method and negative staining, and if showed presence of Gram negative rods and capsule, were further cultured on selective media m-Kleb agar base according to Geldreich and Rice (1987). The growth from the single colony was

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and Rice (1987). The growth from the single colony was inoculated on Nutrient agar slant. The slants were incubated aerobically at 37ºC for 24 h and after checking the purity by Gram's staining was preserved at 4ºC for further studies. Also, the samples were preserved in glycerol stock. The presumptive identification of bacterial isolates as *Klebsiella* was accomplished by colonial and bacterial morphology, motility and biochemical tests (Barrow and Feltham, 1993).

Molecular Detection of *Klebsiella* Isolates by PCR

Klebsiella isolates, thus identified by phenotypic methods, were further subjected to confirmation by PCR. Extraction of DNA was done by using readymade kit (GenElute Bacterial Genomic DNA Kit, Sigma Life Science) according to the manufacturer's instructions.

The PCR was standardized for the detection of *Klebsiella*, targeting genus specific *Klebsiella gyr A* gene and species-specific gene *16S-23S ITS* for *K. pneumoniae* using published primers. PCR was performed in thermocycler (Applied Biosystems, USA). The product size of gene *Klebsiella gyr A* was 441 bp (Table 1) and the first denaturation step was carried out under thermal cycling conditions of 94°C for 5 min, then 35 cycles each of cyclic denaturation at 94°C for 30 sec, annealing temperature at 60°C for 45 sec, and extension temperature at 72°C for 45 sec. The temperature of final extension was 72°C for 10 min (Younis *et al.,* 2017). Whereas, the product size of gene *K. pneumoniae 16S-23S ITS* was 130 bp (Table 1) and the thermal cycling conditions were performed as an initial step of denaturation at 94°C for 5 min, followed by 35 cycles each at 94°C for 30 sec (cyclic denaturation), annealing temperature at 58°C for 30 sec and extension temperature at 72°C for 40 sec. The final extension was done at 72°C for 10 min (Younis *et al.,* 2017).

The PCR was also standardized for the detection of virulence gene *fim*H in Klebsiella isolates using published primers and the product size of 508 bp (Table 2). The amplification cycles for *fimH* gene were initial step of denaturation at 94°C for 5 min, followed by 35 cycles each of denaturation at 94°C for 45 sec, annealing at 60°C for 45 sec, extension at 72°C for 60 sec, and final extension at 72°C for 7 min (Fernandes *et al.,* 2011). The amplified products were electrophoresed in 1.5% agarose gel (in TBE buffer), stained with ethidium-bromide solution and visualized by a UV transilluminator (Alpha-Innotech) and digitally recorded by gel documentation system (AlphaImager, USA).

Table 1: Details of primers used for molecular detection of *Klebsiella* for PCR reaction

Target gene	Primer Sequence	Product size (bp)	Reference
Klebsiella gyr A	F:CGCGTACTATACGCCATGAACGTA R: ACCGTTGATCACTTCGGTCAGG	441	Younis *et al.* (2017)
K. pneumoniae 16S- 23S ITS	F: ATTTGAAGAGGTTGCAAACGAT R:TTCACTCTGAAGTTTTCTTGTGTTC	130	Younis *et al.* (2017)

Table 2: Details of primer used for molecular characterization of *fim*H gene

Target gene	Primer Sequence	Product size (bp)	Reference
*fim*H	F: TGCAGAACGGATAAGCCGTGC R: GCAGTCACCTGCCCTCCGGTA	508	Fernandes *et al.* (2011)

Results and Discussion

Out of 300 samples including CMT positive milk samples obtained from buffaloes and farm environment, 133 samples produced lactose fermenting pink colonies on MCA following incubation at 37°C for 24 h. These 133 isolates were subsequently streaked on EMB agar to rule out the presence of *E. coli.* Only 15 out of 133 isolates did not produce metallic sheen and dark centre on EMB agar. These 15 isolates were found to possess a thick capsule on negative staining, produced highly mucoid, viscous, pink colored or pinkish dome shape colonies on MCA and formed a large string when tried to be lifted vertically by bacteriological loop (Fig. 1) and produced bluish or bluish green color colonies on selective media m-Kleb agar (Fig. 2). A pure colony suggestive of *Klebsiella* was further inoculated on nutrient agar for biochemical tests. Thus, on the basis of colonial and morphological characteristics, these 15 isolates were tentatively presumed to be *Klebsiella* spp. Similar type of colonial and morphological characters were observed by various scientists (Li *et al*., 2014; Osman *et al*., 2014; Cheng *et al*., 2019; Arya *et al*., 2020; Ammar *et al*., 2021).

Fig. 1: Hypermucoid colonies of bacteria on Mac-Conkey agar forming string on lifting.

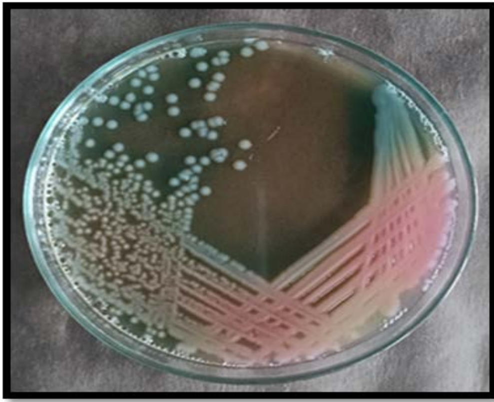

Fig. 2: Bacterial growth on m-Kleb agar base showing purple bluish or bluish green in colour.

Identification of *Klebsiella* spp.

All the *Klebsiella* isolates were non-motile and positive for catalase and negative for oxidase test. On TSI medium these *Klebsiella* isolates produced acidic butt and slant with gas production, but no H_2S was observed. In IMViC reactions, all *Klebsiella* isolates were negative for indole production and methyl red test, but were positive for VP reaction and citrate utilization test. The results of biochemical tests were in agreement with the reports of various workers (Zadoks *et al*., 2011; Ahmed *et al.,* 2016; Badri *et al.,* 2017; Cheng *et al.,* 2019; Arya *et al.* 2020; Ammar *et al*., 2021).

Molecular Detection of *Klebsiella* spp. and their Prevalence

Klebsiella isolates identified phenotypically as above were confirmed genotypically by PCR using primers for genus specific gene *gyr A*. All 15 isolates produced a desired PCR product of 441 bp size (Fig. 3), thus were confirmed to be *Klebsiella*. Ahmed *et al*. (2016), Younis *et al*. (2016) and Ammar *et al*. (2021) also used same set of primers to confirm *Klebsiella* genus. All the 15 *Klebsiella* isolates (100%) were confirmed as *K. pneumoniae* by detection of 130 bp product of species-specific gene *16S-23S ITS* (Fig. 4). Younis *et al*. (2017) and Cheng *et al*. (2019) also targeted this gene for molecular detection of *K. pneumoniae*. Osman *et al*. (2014) and Ammar *et al*. (2021) also reported use of same primers *gyr A* and *16S-23S ITS* to confirm isolates as *Klebsiella* and *K. pneumoniae* by PCR. Ahmed *et al*. (2016) reported that genotypic methods are more sensitive for detection of *Klebsiella* species.

In present research, the genus *Klebsiella* was isolated from buffaloes and farm environment with an overall prevalence of 5%. All the 15 *Klebsiella* isolates (100%) were confirmed as *K. pneumoniae*. The sample wise prevalence of *Klebsiella* isolated from buffaloes and farm environment was 4.66% (7/150) and 5.33% (8/150), respectively. In this research pursuit, the highest prevalence of *Klebsiella* among 15 positive cases was recorded in water 10.00% (5/50), followed by 6.00% (3/50) in faeces, 4.00% (4/100) in mastitic milk, 4.00% (2/50) in bedding material and floor, and 2.00% (1/50) in soil sample. Our results are in close agreement with Sharma and Sindhu (2007), Botrel *et al.* (2010) and Ali *et al.* (2011), who reported prevalence of *Klebsiella* in mastitic milk as 2.03%, 2.1% and 5.2 %, respectively. Osman *et al*. (2014) and Ammar *et al.* (2021) reported 4.3% and 4.0% prevalence of *K. pneumoniae* in milk samples which is very similar to our data. Contrary to our findings, high *Klebsiella* prevalence of 34.3% and 62.0 % in milk was reported by Ahmed *et al.* (2016) and Badri *et al.* (2017), respectively, and excessively high percentage of *Klebsiella* prevalence in faeces (67%), water (89%) and bedding (68%) by Zadoks *et al.* (2011), and in faeces (81%) and bedding material (78%) by Munoz *et al*. (2006).

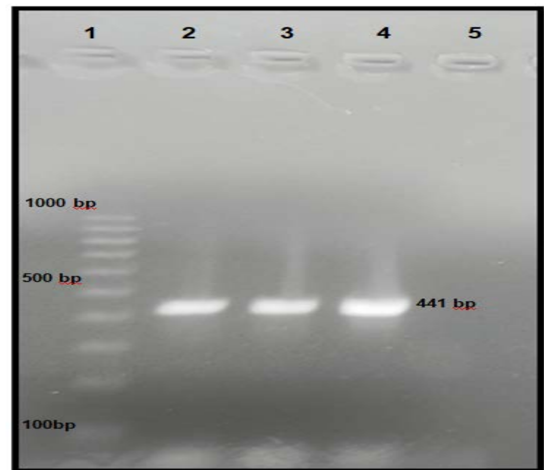

Fig 3: Agarose gel electrophoresis showing amplified product (441 bp) of *Klebsiella gyr A* gene. Lane 1: Ladder (100bp), Lane 2: Positive control, Lane3,4: Samples, Lane 5: Negative control.

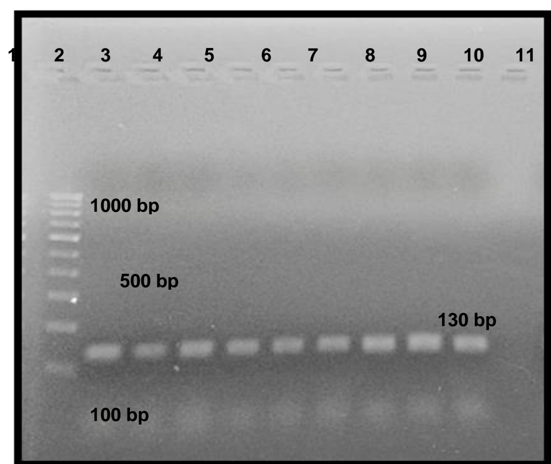

Fig. 4: Agarose gel electrophoresis showing amplified product (130 bp) of *16S-23S ITS* gene of *K. pneumonia.* Lane 1: Ladder (100bp), Lane 2: Positive control, Lane 3,4,5,6,8,9,10: Samples, Lane 7: Negative control.

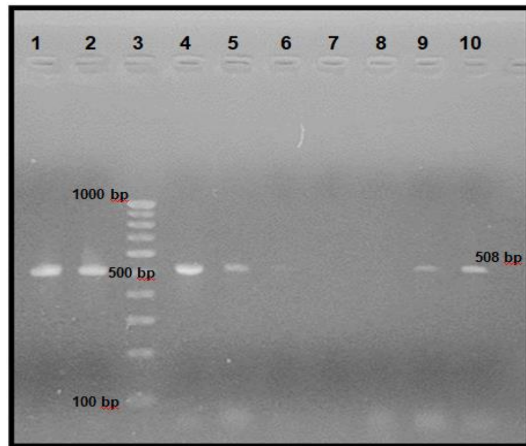

Fig. 5: Agarose gel electrophoresis showing amplified product (508 bp) of *fim*H gene. Lane 3: Ladder (100 bp), Lane 1: Positive control, Lane 2, 4, 5, 9, 10: Samples, Lane 6,7,8: Negative control.

Prevalence of Virulent Gene in *Klebsiella* Isolates

An amplicon size of 508 bp was considered positive for *fim*H in isolates (Fig. 5). Out of 15 isolates, 8 (53.33%) isolates were positive for *fim*H gene. In *Klebsiella* isolates the gene *fim*H was recovered in 4 sources of samples but not detected in samples of bedding material and floor. Out of total 8 isolates, the *fim*H gene detection was maximum in mastitic milk samples (4) followed by drinking water (2), feces (1) and in soil (1). In our study, the total prevalence of *fim*H gene in *Klebsiella* isolates was 53.33%. Prevalence of *fim*H gene detected (55%) by Gao *et al.* (2019) was almost similar with the overall prevalence of the present research. Gao *et al.* (2019) reported 55% of *fim*H gene prevalence in *Klebsiella* which corroborates with our findings but contrast to this, Xu *et al.* (2022) reported higher, *i.e.* 100% prevalence of *fim*H gene in *K. pneumoniae* of dairy animals. Cheng *et al.* (2020) also reported *K. pneumoniae* strains to be positive for *fim*H gene. *FimH* is a protein found at the tip of the fimbriae in *Klebsiella* and plays role in the initial stages of infection and in the production of biofilms which indicates that *fimH* has important role in the pathogenesis.

Conclusions

Klebsiella pneumoniae, exist as normal flora in the gastrointestinal tract of animals and humans, resides in the mucosal surfaces of mammals and the environment (soil, water, etc.). Despite this, they are opportunistic pathogens and can cause serious nosocomial infections like pneumonia, septicemia, meningitis, urinary tract infections, soft tissue infections and pyogenic liver abscesses in mammals besides bovine mastitis and other infections in livestock. Prevalence of *Klebsiella* and *K. pneumoniae* in buffaloes and farm environment was recorded although at a lower rate. The bacteria were found to be present in all type of samples*, viz.,* mastitic milk, faeces, bedding material and floor, drinking water and soil sample. The prevalence of virulence gene, *i.e., fim*H was also recorded.

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