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Isolation and molecular identification of *Klebsiella* spp. in raw meat from Mandla district of Madhya Pradesh

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Abstract

Meat is an essential part of human nutrition; still high nutrient load also makes it a probable carrier for foodborne pathogen. *Klebsiella* spp., an emerging food borne pathogen alarming public health because of its virulence nature and growing anti-microbial resistant strains. The present study examined 51 retail raw meat samples i.e. poultry meat, pork and goat meat samples collected from retail shops of 12 markets in tribal-dominated Mandla district of Madhya Pradesh. *Klebsiella* spp. specific morphological characteristics colonies were recovered by bacteriological isolation method and further identified by biochemical tests. Thirty-eight *Klebsiella* spp. presumptive isolates i.e. 74.5% were identified. Raw goat meat exhibiting the highest exposure to *Klebsiella* spp. (76.47%) followed by poultry meat (74.07%) and pork (71.4%). Market wise variation in *Klebsiella* contamination of raw meat was observed with six markets showed 100% exposure rate. Presumptive isolates were confirmed at genomic level by PCR using *Klebsiella* genus specific *gyrA* gene primers and *Klebsiella pneumoniae* specific *rpoB* gene primers. Out of 38 presumptive *Klebsiella* isolates; 22 and 8 isolates were found positive for *gyrA* gene and *rpoB* gene respectively. Most of *K. pneumoniae* isolates recovered from goat meat samples collected from Narayanganj market. Overall retail raw meat samples collected from Mandla district showed higher *Klebsiella* contamination highlighting need of hygienic slaughtering and trading practices to ensure food safety.

Keywords: *Klebsiella* spp., *Klebsiella pneumoniae*, Raw meat, Mandla district, *gyrA* gene and *rpoB* gene

Introduction

Meat represents an important component of human food and contamination of raw meat with pathogenic microbes poses high risk of food borne illness [1]. Microbial load of raw meat depends on infrastructure of slaughter house, slaughtering practices, sanitation of meat processing, handling, quality of water sources, availability of cold storage facility etc. [2]. During slaughtering of animals and processing of meat, there are high chances of pathogen contamination of the raw meat from contaminated animal skin, infected internal organs, contaminated faecal material etc. [3]. Monitoring of microbial contamination in raw meat becomes a critical public health-safety requirement. The members of *Klebsiella* genus are often considered an agent of foodborne illness and reported can be found in seafood, frozen foods, and fresh meat [4]. The members of genus *Klebsiella* belong to *Enterobacteriaceae* family are gram negative and encapsulated bacteria, causes severe infections such as septicemia, liver abscesses, urinary-tract infections, and pneumonia in humans. *Klebsiella pneumoniae* (*K. pneumoniae*) is the medically most important member of this genus and leading cause of nosocomial *Klebsiella* infections in human [5]. *Klebsiella* spp. are opportunistic pathogens and mostly cause infections in immune-compromised and hospitalized individuals [6, 7, 8]. The species under *Klebsiella* genus are known as one of the most critical multi-drug resistance (MDR) organisms globally and increasingly emergence of hyper-virulent strains posing significant public health threat [9]. *K. pneumoniae* is ubiquitous in environment and found in soil, water bodies, sewage, animal bedding material,

vegetation *etc.* and reason for being the common contaminant of food items [10]. In humans and animals, the *K. pneumonia* localises in upper respiratory tract and gastrointestinal tract and excreted in faeces along with other enterobacteria [11]. Domesticated and commercial farm animals and poultry get *K. pneumonia* infection when come in contact with contaminated water bodies, soil, vegetation *etc.* The infected animals and poultry transmit the organism to humans due to cohabitation, routine handling of infected animals, handling of contaminated raw meat, consumption of undercooked meat and all such activities that facilitate exposure possibilities to *K. pneumonia*. Many studies have reported contamination of *K. pneumonia* in raw meat and other animal origin foods and genetic relatedness between *K. pneumonia* isolates recovered from retail meat and human clinical samples [12, 13, 14, 15, and 16]. Therefore, the present study was undertaken to isolate *Klebsiella* spp. in retail meat sample (poultry meat, goat meat, and pork) collected from different markets of Mandla district of Madhya Pradesh and their identification by morphological, biochemical tests and molecular techniques. The district represents significant tribal population where meat consumption is a daily nutrition and deeply associated to cultural practices and religious beliefs. In this area, the retail shops follow traditional open-air slaughtering of animals. No studies on microbial contamination of raw meat meant for human consumption have been available in public domain.

Materials and Methods

Area of study

The present study was conducted in Mandla district, located in the eastern part of Madhya Pradesh, India (22.60°N latitude and 80.37°E longitude). Mandla is a predominantly tribal region characterized by tropical climate with hot summers, moderate rainfall, and mild winters.

Sample Collection

A total of 51 raw meat samples were collected from shops of 12 retail markets of Mandla district including Madhupuri, Mandla, Hirdenagar, Ramnagar, Maharajpur, Udaipur, Badianjaniya, Badikheri, Niwas, Padmi, Bicchiya and Narayanganj between December 2024 to August 2025. The samples included 27 poultry meat, 17 goat meat, and 7 pork meat. Samples were collected aseptically in sterile tubes and transported to laboratory in thermo-insulated boxes. The meat samples were processed promptly in the laboratory (Animal Biotechnology Centre, NDVSU, Jabalpur).

Bacteriological isolation and identification

The 10 gram of each meat sample was pre-enriched in 90 ml sterile buffered peptone water (HiMedia) and incubated overnight at 37 °C. Diluted pre-enriched culture was spread onto Eosin Methylene Blue (EMB) agar (HiMedia) and incubated overnight. The mucoid pink or purple coloured colonies presumed of *Klebsiella* spp. were picked up and further streaked on Hichrome *Klebsiella* selective agar (HiMedia). The purple-magenta coloured colonies were picked up and inoculated nutrient broth for growth. Both agar plates and broth culture were stored for further study. Both Gram staining and capsule staining were performed for morphological identification of *Klebsiella* isolates. Capsule staining was performed by negative staining method. Briefly, a loopful of the bacterial culture was mixed with a

drop of nigrosin on a clean glass slide and a thin smear was made by using edge of a second slide. The thin smear was air dried, followed by flooded with 1% crystal violet for one minute. The excess amount of crystal violet stain was drained off by tilting the slide and the smear was air dried. The slide was then observed under an oil immersion microscope.

Biochemical tests

Presumptive *Klebsiella* isolates were subjected to a series of biochemical tests including catalase, oxidase, indole, methyl red (MR), Voges-Proskauer (VP), citrate utilization, urease, triple sugar iron (TSI), oxidase and catalase tests for further identification.

Genomic level identification of *Klebsiella* spp.

Genomic DNA extraction

Genomic DNA was extracted using the boiling method^[17] with some modifications. A loopful of overnight-grown presumptive *Klebsiella* colonies were suspended in 500 µL of trypsin-EDTA buffer and centrifuged at 3,000 rpm. The bacteria pellet was resuspended in fresh 100 µL trypsin-EDTA buffer and boiled at 100°C for 10 minutes followed by snap chilling in ice for 5 minute. The bacteria lysate was centrifuged at high speed and the upper clear fraction was transferred to a new micro-centrifuge tube. The clear fraction, contained genomic DNA and plasmid, was then used as DNA templates in subsequent polymerase chain reaction (PCR) experiments.

Molecular identification by PCR

Reported primers for the *gyrA* and *rpoB* genes (Table 1) were used to carry out PCR for molecular confirmation of *Klebsiella* isolates. Fifty microliter of PCR reaction mixture consisted of 25 µL of master mix (Dss Takara Bio India), 0.2µM of each forward and reverse primers, 2.5 µL of template DNA and nuclease-free water. The thermal condition followed for PCR as follows; initial denaturation at 98 °C for 5 minutes, followed by 30 cycles of denaturation (98°C / 1 minute), primer annealing (56°C / 1 minute), and extension (72 °C / 1 minute) and final extension step (72 °C / 5 minute). The PCR amplified products were separated on 1.5% agarose gel containing DNA-staining dye and visualized under UV light in gel documentation system.

Results and Discussion

Isolation, morphological characterization and biochemical characterization

A total of 51 raw meat (poultry meat, goat meat, and pork) samples collected from twelve retail markets in Mandla district were subjected to bacterial isolation, morphological and molecular identification to assess the contamination rate of raw meat with *Klebsiella* spp. (Fig.1). In microbiological isolation process, included pre-enrichment followed by plating on EMB agar, the mucoid and pink/purple coloured colonies were picked up. The colonies were streaked on *Klebsiella* selective agar for selective isolation and easy detection of *Klebsiella* spp. The bacteria colonies exhibited classic round, mucoid and purple to magenta-coloured morphology (Fig. 2) were presumed to represent *Klebsiella* spp. The morphological identification by capsule staining of the selected colonies confirmed the presence of a thick polysaccharide capsule, observed as a clear halo

surrounding violet-stained bacilli (Fig.3). This capsule is a critical virulence determinant, enhancing environmental persistence and resistance to phagocytosis [20]. The Table 2 represents summary of biochemical tests performed for all presumptive *Klebsiella* isolates. All isolates were found positive for catalase and oxidase reactions. However, *Klebsiella* spp. isolates showcased variable results in TSI, urease, citrate, MR-VP and indole tests. As it was already reported that different *Klebsiella* spp. metabolised a particular substrate differently and produced non-result in biochemical tests. This variation in metabolism exploited as epidemiological tool in differentiation of isolates of *Klebsiella* spp [21].

Molecular confirmation by PCR

Molecular identification of all presumptive isolates at genomic level by PCR further substantiated the preliminary cultural, morphological and biochemical characteristics of pure *Klebsiella* isolates. The *Klebsiella* genus specific gene *gyrA* and *K. pneumonia* specific gene *rpoB* were used for screening all presumptive *Klebsiella* isolates. Observed PCR product bands of approximate size 441bp and 108bp on agarose gel confirmed the amplification of target region of *gyrA* gene and *rpoB* gene respectively (Fig. 4).

Market-wise and meat type-wise isolation of *Klebsiella* spp.

The table 3 represents the summary of bacteria culture isolation and morphological identification, biochemical test results and molecular identification by PCR results. The table 3 also shows the market-wise and meat type-wise *Klebsiella* contamination in raw meat samples. Results of culture based isolation as well as phenotypic characterization demonstrated that 38 meat samples out of 51 (*i.e.* 74.50%) were presumed to be positive for *Klebsiella*. Market-wise assessment analysis indicated that highest *Klebsiella* spp. contamination (*i.e.* 100%) level was observed in Hirdenagar, Narayanganj, Madhupur, Padmi, Udaipur and Mandla markets. Meat samples collected from Ramnagar, Niwas, Bicchiya, Badikheri and Maharajpur showed variable *Klebsiella* contamination (*i.e.* 0-75%). The variation in *Klebsiella* load in raw meat samples demonstrated the influence of market-specific factors such as sanitation, vendor hygiene, and environmental exposure.

Among the different meat types, goat meat exhibited the highest contamination rate (13/17 *i.e.* 76.47%), followed by chicken (20/27 *i.e.* 74.07%) and pork (5/7 *i.e.* 71.42%). Among the 38 presumptive *Klebsiella* spp. isolates; 22 and 8 isolates were found positive for *gyrA* gene and *rpoB* gene respectively. Analysis of *gyrA* gene PCR-positive *Klebsiella* spp. isolates across meat types demonstrated that the chicken samples exhibiting the highest positivity (12 out of 20), followed by goat meat (6 out of 13) and pork (4 out of 5). Similarly, analysis of *rpoB* gene PCR-positive *Klebsiella* spp. isolates revealed that only 05 chicken and 03 pork raw meat samples were found positive for *K. pneumonia*. All *rpoB* positive isolates were showed conformity with results of *gyrA* gene PCR and standard biochemical tests for *K. pneumonia*. Out of 05 *Klebsiella* isolates from pork samples, 03 samples were found *K. pneumonia* positive (*i.e.* 60%). Out of 20 *Klebsiella* isolates from chicken samples, 05 samples were found *K. pneumonia* positive (*i.e.* 25%). Surprisingly, all six *K. pneumonia* isolates were obtained from 08 meat samples collected from Narayanganj retail market (*i.e.* 75%). The Narayanganj retail market was surrounded by tribal habitats and the market vendors procured backyard domesticated pigs, poultry and goats from the local tribes for raw meat retailing purpose. Many previous studies done at different locations of India were also reported occurrence of *Klebsiella* spp. in retail raw meats. One study reported occurrence of *Klebsiella* spp. in 70.58% (cultural identification) and 11.33% (molecular identification) of poultry meat samples collected from retail markets of Anand, Gujarat [12]. Another study reported presence of *Klebsiella* spp. in 37.50% of beef samples collected from retail shops at Thrissur and Palakkad districts of Kerala [13]. Mahanti A *et al.* (2017) isolated *Klebsiella* spp. from 10.7% of clonal swabs samples collected from indigenous and broilers poultry bird in West Bengal [22]. Many studies undertaken abroad such as Ghana [23], Germany [24], Turkey [14] *etc.* reported higher occurrence of *Klebsiella* spp. in poultry meat. Collectively, the present study and previous studies demonstrated that the raw meat act as significant reservoirs of *Klebsiella* spp., and exposure rate may likely be influenced by geographic location, sample type, and hygienic practices followed during raw meat processing.

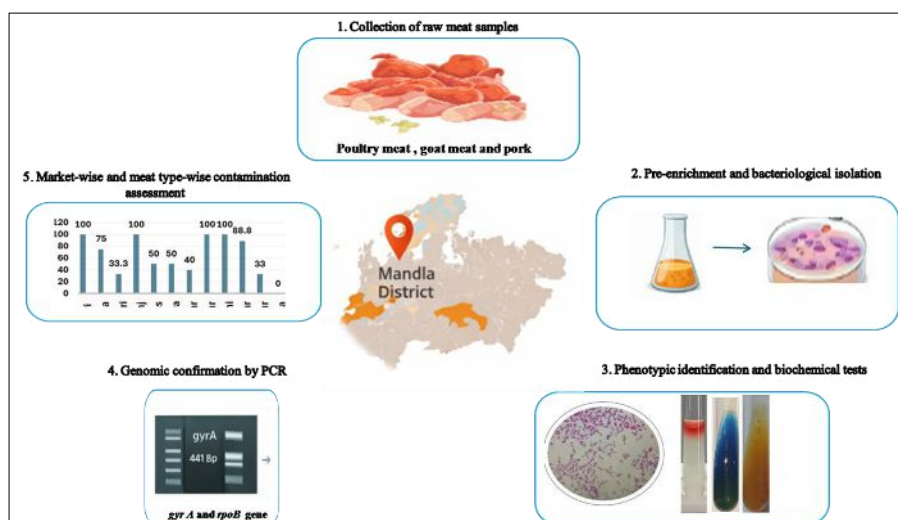


Fig 1: Overview of the present study designed for detection of *Klebsiella* spp. in raw meat samples collected from retail markets of Mandla district, Madhya Pradesh (India)

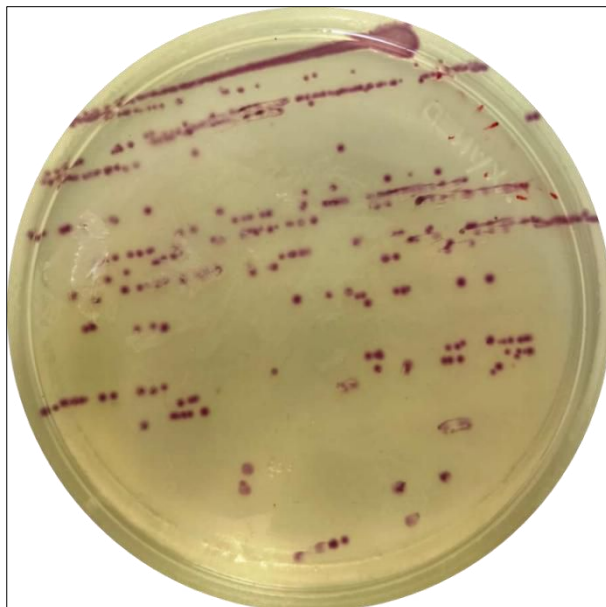


Fig 2: Purple coloured colonies of *Klebsiella* spp. grown on *Klebsiella* specific agar (Himedia)

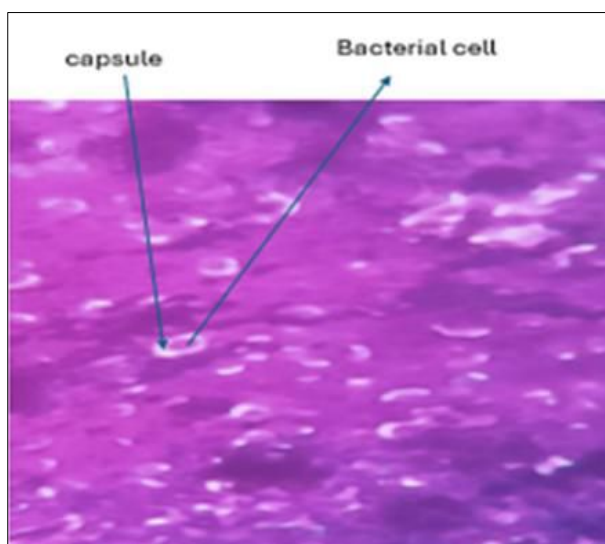


Fig 3: Capsule staining of *Klebsiella* spp. isolates. Capsules appeared as colourless halos surrounding purple *Klebsiella* bacilli

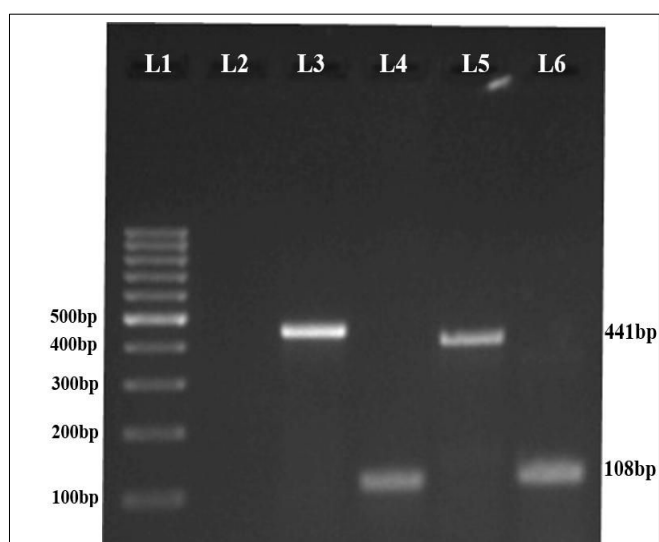


Fig 4: Agarose gel electrophoresis of polymerase chain reaction amplified products. L1: 100 bp ladder, L2: No- template control, L3 and L4: Positive control for *gyrA* gene and *rpoB* gene respectively, L5: Amplified product of *gyrA* gene from presumptive *Klebsiella* spp. isolate and L6: Amplified product of *rpoB* gene from presumptive *Klebsiella pneumonia* isolate. Size of amplified product for *gyrA* gene and *rpoB* gene are 441bp and 108bp respectively

Table 1: Primers used for molecular identification of *Klebsiella* isolates

Target genes	Primer sequence (5'-3')	Amplicon size	References
<i>gyrA</i>	F: CGCGTACTATACGCCATGAACGTA R: ACCGTTGATCACTTCGGTCAGG	441bp	[18] and [19]
<i>rpoB</i>	F: CAACGGTGTGGTTACTGACG R: TCTACGAAGTGGCCGTTTT	108bp	

Table 2: Results of motility and biochemical tests for presumptive *Klebsiella* spp. isolates and *rpoB* gene PCR confirmed *Klebsiella pneumoniae*

Tests	Presumptive <i>Klebsiella</i> spp. isolates	Biochemical Characteristics of <i>Klebsiella pneumoniae</i>
Motility	Negative i.e non-motile	Non-motile
TSI	Variable	Negative
Catalase	Positive	Positive
Oxidase	Negative	Negative
Urease	Variable	Positive
Indole	Variable	Negative
Methyl red	Variable	Negative
Citrate	Variable	Positive
VP	Variable	Positive

Table 3: Market-wise and raw meat type-wise assessment for *Klebsiella* contamination based on morphological, biochemical and molecular methods of identification

Markets	<i>Klebsiella</i> spp. morphological and biochemical test results (Presumptive <i>Klebsiella</i> isolates /number of samples collected)				<i>Klebsiella</i> spp. genomic identification by PCR
	Poultry meat	Goat meat	Pork	Market-wise assessment	<i>gyrA</i> (Number of Positive) and <i>rpoB</i> (Number of positive)
Ramnagar	1/3	0	1/2	2/5 (40%)	Nil
Mandla	0	4/4	0	4/4 (100%)	3 and 0
Bicchiya	3/ 4	0	0	3/4 (75%)	1 and 0
Udaipur	2/2	0	0	2/2 (100%)	2 and 1
Narayanganj	5/5	0	3/3	8/8 (100%)	6 and 6
Madhupuri	3/3	0	0	3/3 (100%)	3 and 0
Maharajpur	2/4	0/2	0	2/6 (33.33%)	2 and 1
Badikheri	2/ 4	0	0	2/4 (50%)	Nil
Hirdenagar	0	9/9	0	9/9 (100%)	3 and 0
Padmi	2/2	0	0	2/2 (100%)	1 and 0
Niwas	0	0	1/2	1/2 (50%)	1 and 0
Badianjaniya	0	0/2	0	0/2 (nil)	Nil
	20/27 (74.07%)	13/17 (76.47%)	5/7 (71.42%)	38/51 (74.50%)	<i>gyrA</i> positive: 22/38 (57.89%) and <i>rpoB</i> positive: 8/38 (21.05%)

Conclusion

The present study demonstrated higher contamination rate of raw meat with *Klebsiella* spp., sold across different markets in Mandla district. Thirty-eight presumptive *Klebsiella* spp. (i.e.74.5%) isolates were identified by traditional culture based isolation, phenotypic characterization by capsule staining and biochemical tests. However, 57.89% of presumptive isolates were confirmed at genomic level by *Klebsiella* genus-specific *gyrA* gene PCR. Only 8 *K. pneumoniae* pure isolates were identified by species- specific *rpoB* gene PCR. Raw meat samples procured from Narayanganj market demonstrated highest *Klebsiella* spp. contamination. The tribes primarily follow cost-effective traditional approaches like backyard rearing and unhygienic management practices. Additionally lack of slaughtering facilities and unhygienic open market meat handling practices could be the reason for higher environmental exposure of retail meat to *Klebsiella* spp. However, higher surrounding temperature in central India may significantly facilitate bacterial proliferation. The pure isolates of *K. pneumoniae* will be studied extensively at genomic level to identify multi drug resistant and hypervirulent strains.

Contribution by Authors

All the authors contributed equally to writing the manuscript. The final manuscript was read by all authors and consented to publication.

Conflict of Interests

The authors have declared no conflict of interest.

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