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Molecular Detection of *Klebsiella* spp. from Poultry Meat

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ABSTRACT

The Poultry are quite often implicated as a major reservoir of human enteric pathogens, and several human infections were traced after consumption of food products of animal origin. Commensal and opportunistic Enterobacteriaceae present in the poultry gut can be indirectly transmitted to humans through the food chain. In this study a total 150 raw Poultry meat samples (poultry muscle) was collected in sterile test screw capped vials and immediately transferred to the laboratory at 4˚C for processing and bacteriological investigation which include the morphological characterisation, culture characterisation, Biochemical test and genotypic confirmation. 17 (11.33%) samples out of 150 raw chicken samples were positive for Klebsiella species.

***Keywords:** Klebsiella, Food borne disease, Raw Poultry Meat.*

INTRODUCTION

The Poultry sector in India is valued at about Rs. 80,000 crores (2015-16) broadly divided into two sub-sectors-one with a highly organized commercial sector with about 80% (Rs. 64,000 crore) of the total market share and the other being unorganized with about 20% of the total market share of Rs. 16,000 Crore (National Action Plan for Egg & Poultry, 2019). Poultry are quite often implicated as a major reservoir of human enteric pathogens, and several human infections were traced after consumption of food products of animal origin. Most commonly Enter obacteriaceae found in the poultry gut can be indirectly transmitted to humans through the food chain and *Klebsiella* spp.is one of the most common infectious disease affecting chicks causing great economic losses. (Paterson & Bonomo, 2005).

The isolation, identification and characterization of *Klebsiella spp.* in chicken have been accomplished from the retail Poultry meat market in Anand district. The genus *Klebsiella* was named by Trevisan (1885) to honour the clinical microbiologist Edwin Klebs.

The first *Klebsiella* species ever described was a capsulated bacillus from patients with Rhinoschleoma (Brisse et al., 2009). *Klebsiella* species are Gram-negative, non-motile, usually encapsulated rod-shaped bacteria belonging to the family Enterobacteriaceae. *Klebsiella* species often occur in mucoid colonies. The genus consists of 77 capsular antigens (K antigens), leading to different serogroups (Janda et al., 2007).

Biochemically *Klebsiella* positive reaction for catalase test, Voges Proskauer, Urease and Citrate test, meanwhile were negative for Oxidase, Indole and Methyl red tests. Cultivation of isolate on Mac-Conkey agar gives lactose fermenting colonies, more or less dome shaped. Three major species of *Klebsiella viz. Klebsiella pneumoniae, Klebsiella oxytoca* and *R. ornithinolytica* are ubiquitous in nature and are often detected in foods, sewage, soil, plants and the gastrointestinal tracts of animals (Sękowska, 2017). *K. pneumoniae* has been isolated from meats, vegetables and pastries (Babu et al., 2013).

Klebsiella pneumoniae is widely distributed in the gastrointestinal, urinary, and respiratory tracts of healthy people. It causes opportunistic infections mainly nosocomial infections; it is a common hospital acquired pathogen causing severe respiratory infections such as pneumonia. (Sękowska, 2017). Phenotypic-based methods for identification of *Klebsiella pneumoniae* are time consuming with low sensitivity hence; the various molecular-based approaches were used to identify *Klebsiella pneumoniae*. PCR has been successfully identified among these methods as a valuable method that provides rapid, sensitive and accurate.

MATERIALS AND METHODS

This research was conducted at the Department of Veterinary Public Health and Epidemiology, College of Veterinary Science and Animal Husbandry, Anand Agricultural University, Anand. A total 150 raw Poultry meat samples (poultry muscle) were collected from retail meat market in and around Anand under aseptic precautions in sterile screw lid sample vials and immediately transferred to the laboratory at 4°C for further processing and microbiological analysis.

Isolation & identification

Sample was inoculated into 5 ml of nutrient broth for bacterial growth at 24 hours at 37°C. The growth in Nutrient broth was transferred to Mac-Conkey agar and again incubated at 37°C for 24 hours for selective growth of Gram negative bacteria, for specific isolation of *Klebsiella* spp., the pink, mucoid coloured colonies was picked up for further overnight incubation in HiCrome *Klebsiella* Selective Agar base supplemented with *Klebsiella* Selective Agar supplement (Carbenicillin 25mg/500 ml) at 37°C. Magenta coloured individual colonies was picked up as instructed by manufacturer and pure cultured in Nutrient broth and simultaneously streaked in MHA (Mueller-Hintonagar) plates for further study. All the pure cultures were kept at 4°C for further use.

Morphological characterization: Glass slides were stained with Gram's stain and examined microscopically for morphological characteristics of the isolates.

Cultural characteristics: The colonial morphology onto Mac-Conkey's agar was studied.

Biochemical characterization: Catalase test, Oxidase test, Indol test, Methyl red test, Vogasproskauer test, Citrate utilization test and Urease test, Triple sugar iron test were performed.

Genotypic identification: Identification of *Klebsiella* genus by *Klebsiella* specific gene (GyrA gene) and *Klebsiella* pneumonia species identified by KP-16S (NM3) gene using polymerase chain reaction (PCR).

DNA extraction for *Klebsiella* Genus and *Klebsiella* pneumonia isolates: The DNA from *Klebsiella* Genus was extracted by boiling method. A loopful of pure culture was suspended in 100 μl of DNAse and RNAse free milliQ water in a sterilized micro centrifuge tube. The suspension was vortexed and then heated at 95°C for 10 mins in thermal cycler. This was then centrifuged at 10000 rpm

for 6 mins so that the cell debris settle down. The upper aqueous phase was transferred to another PCR tube and this was used as a DNA template for PCR.

Oligonucleotide primers set GyrA: Templete DNA (5µl), forward and reverse primers (1µl), 12.5µl of master mix (2 times) (Fermentas, India) and 5.5µl of DNase free water (Fermants, India) in a total volume of 25 µl. The melting temperature ™ of each oligonucleotide using the formula Tm = 4(G+C) + 2 (A+T), where G, C, A and T indicate the number of corresponding nucleotides in the oligonucleotide. Razmyar et al. (2015).

Oligonucleotide primers set KP-16S (NM3) for *Klebsiella pneumonia*: Templete DNA (5µl), forward and reverse primers (1µl), 12.5µl of master mix (2 times) (Fermentas, India) and 5.5µl of DNase free water (Fermants, India) in a total volume of 25 µl. The melting temperature ™ of each oligonucleotide using the formula Tm = 4(G+C) + 2 (A+T), where G, C, A and T indicate the number of corresponding nucleotides in the oligonucleotide. Aurna et al. (2017).

PCR protocol:

1) The reaction was included in a total volume of 25µL in 0.5 ml Eppendorf tube containing templete DNA (5µl), forward and reverse primers (1µl), 12.5µl of master mix (2 times) and 5.5µl of DNase free water.

2) The tubes were placed in the thermal cycler previously programmed.

3) At the end of cycling the tubes were stored at - 20°C until needed for electrophoresis.

Cyclic condition for detection of GyrA gene:
The thermal cycler was programmed as follows: (i) one cycle for 5 minutes at 94°C for initial denaturate the template DNA followed by (ii) 30 cycles of denaturation, annealing and extension at 94°C for 45 sec. and, 55° for 30 sec and 72° C for 45 sec. The 30 cycles were followed by a final cycle of extension at 72°C for 4 minutes to ensure that the entire PCR product was double strand DNA.

Cyclic condition for detection of gene KP-16S (NM3):
The thermal cycler was programmed as follows:(i) one cycle for 3 minutes at 94°C for initial denaturate the template DNA followed by (ii) 35 cycles of denaturation, annealing and extension at 94°C for 30 sec. and, 58° for 30 sec and 72° C for 30 sec. The 35 cycles were followed by a final cycle of extension at 72°C for 10 minutes to ensure that the entire PCR product was double strand DNA.

RESULTS

Results of poultry chicken:
A total 150 raw Poultry meat samples (poultry muscle) revealed 17 isolates of *Klebsiella* with percentage of 11.33%. **(Table 1).**

Identification of suspected isolates:

Morphological identification of *Klebsialla*: Gram negative bacilli, non-spore forming, capsulated, non-motile and arranged singly. **(Fig 1).**

Culture character: on mac-Conkey Rose Pink Mucoid coloured colonies. **(Fig 2)** On selective media of *Klebsiella* selective agar base supplemented with Carbenicillin (25 mg/500 ml) produce magenta coloured colonies. **(Fig3).**

Biochemical identification:
Gave positive reaction for catalase test, Vogasproskauer test, citrate test and urease test. Meanwhile the isolates were negative for indole, oxidase and methyl red tests, and Triple sugar iron test produce acid slant and acid butt. **(Table 1) (Fig 4) (Fig 5) (Fig 6).**

Results of PCR:
Result of polymerase chain reaction for detection of *Klebsiella* genus specific GyrA gene were 12 isolates give positive results. **(Table 2) (FIG 7).**

Result of polymerase chain reaction for detection of *Klebsiella pneumonia* by using KP-16S (NM3) gene were 9 isolates give positive results. **(Table 3) (FIG 8).**

DISCUSSION

Results demonstrated by examination of 150 raw chicken samples revealed the percentage of *Klebsiella* infection among the examined

chicks was found to be 11.33%. The obtained results agree with that of Guo et al. (2016) 11.4%, Veeraselvam et al. (2019) 15.13%, Wu et al. (2016) 17.0% and are differed with the result received by Gundogan et al. (2011) 80%, Fielding et al. (2012) 40%, Mahanti et al. (2017) 65%, Eibach et al. (2018) 29%, Von Tippelkirch et al. (2018) 31.8% who recorded higher isolation rate of *Klebsiella* associated with heavy contamination, unhygienic processing of poultry meat. Also lower prevalence rate was recorded by Aly et al. (2014) 10.66%, Hayati et al. (2019) 9.21% and Hermana et al. (2020) 2.5%. Among the positive *Klebsiella* spp. 52.94% strains were confirmed of *Klebsiella pneumoniae* to be positive by PCR. The obtained results agree with that of Rajaa, et al. (2011) who recorded higher isolation rate of *Klebsiella pneumoniae* (40.4), Kurupati et al. (2004) 100%.

The selected isolation media used in this test was Mac-Conkeys agar media after incubation 24 hrs colonies appear rose pink mucoid coloured colonies after overnight incubation at 37°c. This result agree with that of Aher et al. (2012). Total of 17 positive isolates were confirmed as *Klebsiella* spp. on the basis of standard biochemical tests. All the purified *Klebsiella* spp. isolates were exhibited standard biochemical results like catalase (+ve), oxidase (-ve), Indole - Methyl red - Voges Proskauer – (Citrate utilization on Simmon's citrate medium), (IMViC) (- or +, -, +, +) and H2S production on TSI agar and this result agree with Alves et al. (2006). In the present study, GyrA (*Klebsiella* genus) and KP-16S (NM3) (*Klebsiella pneumonia*) genes were detected by PCR using specific primer sequences which yielded product sizes of 441 bp and 657 bp respectively. Out of total 17 isolates, 12 isolates (70.58%) was positive for GyrA gene this result concised with result of Aly et al. (2014) and (Younis et al., 2016) and 9 isolates (52.94%) was positive for KP- 16S (NM3) gene this result concised with result of (Kurupati et al., 2004).

Table 1: Prevalence of *Klebsiella* spp.

Types of samples	Total No. of samples collected	No. of samples positive	Prevalence percentage
Poultry Muscle	150	17	**11.33**

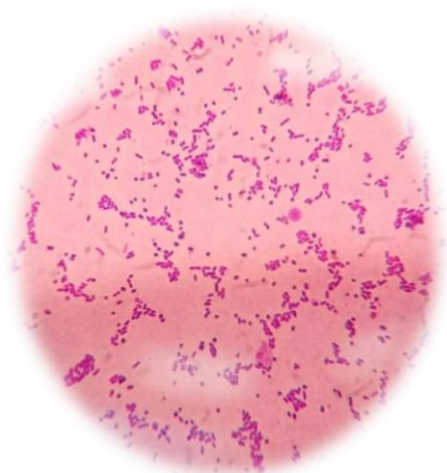**Table 2: Results of the biochemical tests performed for all *Klebsiella spp.* Isolates**

Sr.no	Biochemical tests performed for the confirmation of culturally isolated *Klebsiella spp.*	Results for all *Klebsiella* spp. isolates
1	Indol Production	-ve
2	Methyl-red test	-ve
3	Voges-Proskauer test	+ve
4	Citrate utilization on Simmon's citrate medium	+ve
5	Catalase test	+ve
6	Oxidase test	-ve
7	Urease test	+ve
8	Triple sugar ion test H2S production on TSI agar	Yellow slant, Red butt, H2S production

Table 3: Detection of *Klebsiella* spp. by PCR

Sr. No.	Name of Genes	No. of isolates found positive	Prevalence Percentage
1	*gyrA* (441 bp)	1 2	70.58 %

Table 4: Molecular Detection of *Klebsiella pneumoniae gene* by PCR

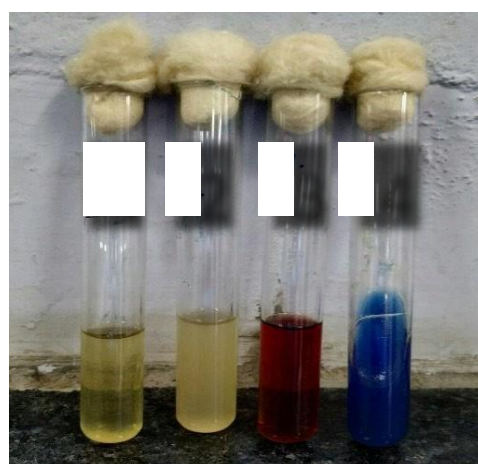| Sr. No.	Name of Genes	No. of isolates found positive	Prevalence Percentage
1	KP-16S (NM3) 657bp	9	52.94 %

Figure 1: Gram negative, Non- motile, Encapsulated and Rod shaped bacterium

Figure 2: Rose pink Mucoid coloured colonies on MacConkey agar

Figure 3: Magenta coloured colonies on *Klebsiella* selective agar

Figure 4:
1) **Indole negative**
2) **Methyl red negative**
3) **Voges-Proskauer positive**
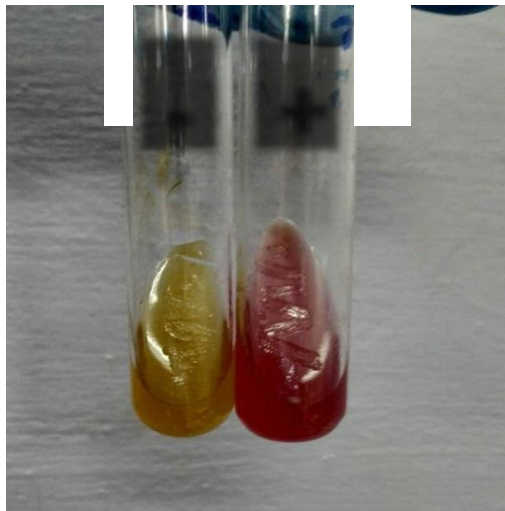4) **Citrate positive**

Figure 5: Urease test
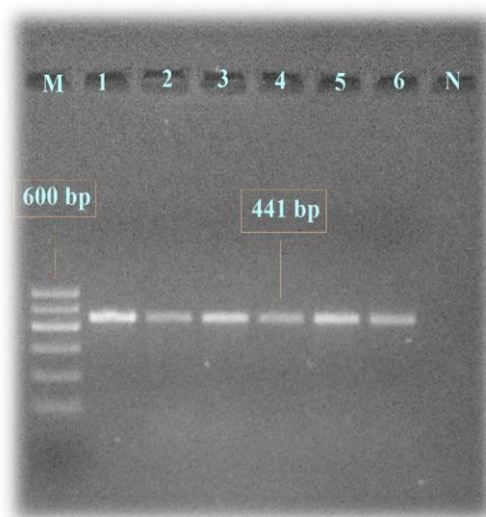

Figure 6: TSI test

Figure 7: PCR amplification of gyrA(441 bp) genes
Lane M: 100 – 600 bp plus DNA ladder (Qiajen, Germeny)
Lane 1: Positive control (MTCC 7407 *Klebsiella Pneumoniae*)
Lane 2: 2-6: Samples positive for *gyrA* gene of *Klebsiella spp.*
Lane 3: 7: Samples negative for gyrA gene of *Klebsiella spp.*(Negative control)
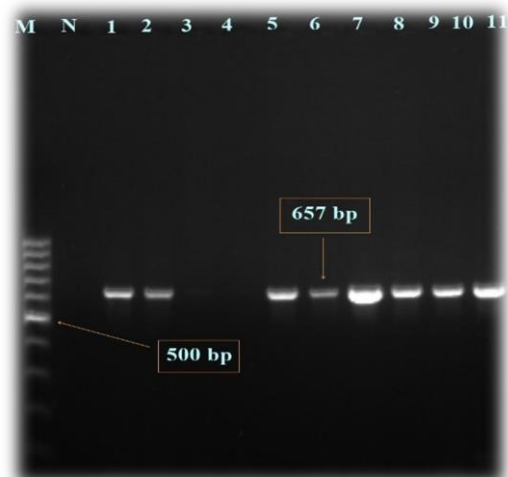

Figure 8: PCR amplification of KP-16S (NM3) (657bp) genes Lane M: 100 bp plus DNA ladder (Genei, Banglore)
Lane N: Negative control
Lane 1, 2, 5-10: Samples positive for *KP-16S* gene of
Klebsiella spp.
Lane 3, 4: Samples negative for   *KP-16S* gene of *Klebsiella spp.*
Lane 11: Positive control (MTCC 7407 *Klebsiella Pneumoniae*)

CONCLUSIONS

Out of total 150 chicken samples (Raw chicken), 17 (11.33%) samples were found positive for *Klebsiella species* by cultural isolation. All the *Klebsiella* spp. isolates showed mucoid pink colonies on Mac-Conkey's Agar medium and were further processed in *Klebsiella* Selective Agar which showed colonies with Maganta colour and all the17 *Klebsiella* spp. isolates exhibited similar IMViC pattern of - - + +, produced negative result for Indole and Methyl Red (MR) and positive for Vogasproskauer (VP), citrate utilization test. Out of 17 isolates investigated, *Klebsiella* spp. isolates were found a prevalence rate of 12(70.58%) gyrA (441bp) gene and 9(52.94%) KP-16S (NM3) (657bp) by PCR.

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