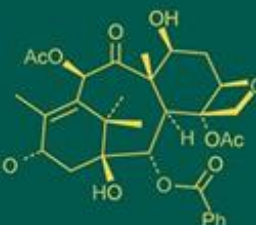
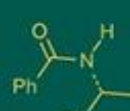
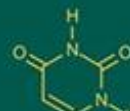
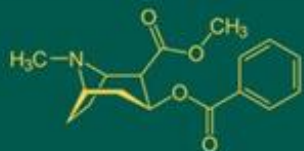


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Isolation and molecular identification of *klebsiella* spp. from poultry and agricultural settings: A morphological and genotypic approach

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Abstract

Klebsiella is an emerging pathogen of One Health importance. *Klebsiella* species comprises of normal intestinal flora of poultry, but could be opportunistic whenever the immune system of affected bird is compromised. The increasing emergence of antimicrobial-resistant *Klebsiella* strains in poultry presents significant concerns for animal health, food safety, and public health due to the zoonotic potential of these bacteria. This study aims to investigate the prevalence, of *Klebsiella* spp. in poultry, with a focus on their isolation from gastrointestinal, respiratory, and farm environmental samples. Both phenotypic and genotypic methods were employed for identification, including selective culturing, biochemical profiling, and molecular confirmation using PCR. One hundred fifty samples were collected for bacteriological isolation and confirmation of *Klebsiella* and *K. pneumoniae* by phenotypic and genotypic methods. *Klebsiella* spp were isolated from 17 (11.3%) out of 150 samples. The prevalence of *K. pneumoniae* in poultry birds and farm environment was 8.0%.

Keywords: *Klebsiella*, poultry, opportunistic pathogens, phenotypic methods, genotypic methods

Introduction

Klebsiella species, is a Gram-negative, non-motile, encapsulated bacteria that are commonly found as commensals in the gastrointestinal tract of poultry and other animals. In healthy birds, these bacteria generally coexist harmlessly within the gut microbiota, playing no apparent pathogenic role. However, under certain conditions such as stress, immunosuppression, or disruption of the normal microbiota *Klebsiella* can become an opportunistic pathogen. In poultry, this may lead to respiratory infections, septicemia, or localized infections such as yolk sac infections in chicks. Moreover, *Klebsiella* spp. isolated from poultry environments have increasingly shown resistance to multiple classes of antibiotics, including β -lactams and fluoroquinolones, raising significant concerns about their role as reservoirs of antimicrobial resistance (AMR). Although the prevalence of *Klebsiella* in poultry is relatively low compared to other pathogens, its capacity to acquire and disseminate resistance genes underscores the importance of routine monitoring and molecular surveillance in poultry production systems to mitigate potential zoonotic transmission and safeguard both animal and public health.

Among the most concerning pathogens linked to antibiotic resistance is the ESKAPE group a collection of clinically significant bacteria. This group includes *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Staphylococcus aureus*, *Enterococcus faecium*, *Pseudomonas aeruginosa*, and *Enterobacter* species, all of which are major contributors to hospital-acquired infections and are increasingly resistant to multiple drug classes. *Klebsiella pneumoniae* is particularly concerning due to its role in severe infections, especially in immunocompromised and critically ill patients, and its increasing resistance to antibiotics. It also poses significant risks in animal populations, particularly poultry (Singh 2018; Kot B, Witeska, 2024)^[1, 2].

Global antibiotic consumption in animals is estimated to be twice that of humans. The improper and excessive use of these drugs in veterinary practices both for therapeutic purposes and as growth promoters has played a major role in the emergence of multidrug-resistant (MDR) microorganisms. This concern is especially pronounced in modern intensive poultry farming systems, where high-density bird populations kept in confined spaces create ideal conditions for the rapid spread of antibiotic-resistant bacteria (Chaudhry et al., 2020)^[3].

In poultry, *Klebsiella* species are commonly found in the intestinal flora, but they can become opportunistic pathogens when the birds' immune systems are weakened. *K. pneumoniae* has been isolated from birds with respiratory infections, where it exacerbates symptoms such as gasping, sinus swelling, and respiratory inflammation. The spread of *Klebsiella* within poultry populations can lead to significant economic losses, due to cost of treatment or culling.

Considering the significance of *Klebsiella* in poultry and the limited research on this pathogen in the Madhya Pradesh region, this study was undertaken to identify and confirm *Klebsiella* in poultry and farm environmental samples. The investigation utilized both phenotypic and genotypic methods, employing published primers to ensure precise detection and characterization.

Materials and Methods

The study aimed to isolate *Klebsiella* species from poultry and the farm environment. A total of 150 samples were collected from various poultry farms around Mhow and Indore. The samples included nasal exudate, cloacal swabs, feed, water and litter as given in Table 01. The samples were streaked on MacConkey agar and bacterial isolates were identified as *Klebsiella* based on their cultural, morphological, and biochemical characteristics like Gram staining and catalase, oxidase, IMViC and urease tests. Genotypic confirmation of isolates was done by PCR using primers targeting the genus-specific *gyrA* gene for genus *Klebsiella* and the species-specific

Table 1: Sources of samples

Sr No.	Sources of sample	Total no of samples
1	Cloacal swabs	65
2	Nasal exudate	10
3	Lung tissue	15
4	Heart tissue	12
5	Liver tissue	12
6	Poultry feed	11
7	Drinking water	12
8	Farm soil	4
9	Litter material	9
	Total	150

Phenotypic Isolation of *Klebsiella*

Isolation of *Klebsiella*:

After collection, samples were transported on ice to the lab, inoculated into BHI broth, and incubated at 37 °C for 12 hours for enrichment. A loopful of growth from BHI was streaked onto MacConkey agar and incubated at 37 °C for 24-48 hours. Mucoid pink colonies, indicating lactose fermentation, were transferred to EMB agar to exclude *E. coli*. Colonies that fermented lactose but lacked a metallic green sheen on EMB were Gram-stained. Gram-negative rods were selected for further bacterial identification.

Identification of *Klebsiella*:

Klebsiella identification was initially based on colony and bacterial morphology. Biochemical tests were then performed according to the procedure described by Barrow and Feltham (1993)^[4].

Gram staining

For Gram staining, a single bacterial colony was picked, smeared on a glass slide, and fixed by gentle heating. Crystal violet was applied for 2 minutes, followed by washing with running water. Lugol's iodine was added for 1 minute, and then washed off. Acetone alcohol was briefly applied, and after washing, safranin was added for 1 minute. The slide was

washed, blotted, air-dried, and examined under a 100X oil immersion lens.

Capsular staining

A single bacterial colony was stained using the negative staining method to reveal the capsule. A drop of nigrosin was placed on one end of a clean slide, and a loopful of inoculum was mixed in the stain. A thin smear was made by spreading the culture at a 45° angle, air-dried, and examined under a 100X oil immersion lens.

Motility examination

A loopful of 6-8 hour old BHI broth culture was placed in the depression of a concavity slide, and a coverslip was added, sealing the edges with paraffin. The slide was examined under a 40X high-power objective lens.

Catalase test

A loopful of 24-hour old culture from a nutrient agar slant was mixed with 3% hydrogen peroxide on a clean slide. A catalase-positive strain exhibited immediate effervescence.

Oxidase test

A loopful of bacterial growth was rubbed on a moistened oxidase disc. A deep purple or mauve color within 10 seconds indicated a positive reaction, while no color change was considered negative.

Triple sugar iron test (TSI)

A well-isolated colony was inoculated into Triple Sugar Iron agar by stabbing the medium and streaking the slant. The slants were incubated at 35 °C for 18-24 hours. A positive reaction was indicated by gas formation, yellowing of both the slant and butt (acidic reaction), while no change signified a negative result.

Methyl Red test (MR)

A loopful of test culture was inoculated into 2 ml sterile glucose phosphate broth and incubated at 37 °C for 48 hours. After incubation, 2 drops of methyl red were added. A positive reaction was indicated by a pink-red color, while a negative reaction showed yellow.

Voges - Proskauer test (VP)

After the methyl red test, the same cultures were used for the Voges-Proskauer (V.P.) test. Two drops of creatine solution and 1 ml of 40% potassium hydroxide were added, and the tubes were gently shaken and kept at room temperature in a slanting position for up to 4 hours. A positive reaction was indicated by eosin-pink color development, while no color change indicated a negative result.

Indole test

A loopful of bacterial culture was inoculated into the medium and incubated at 37 °C for 48 hours. Afterward, 0.5 ml of Kovac's Indole reagent was added and shaken gently. A red color ring indicated a positive reaction, while a yellow ring indicated a negative reaction.

Citrate utilization test

The organisms were grown aerobically on Simmon's citrate agar slopes at 37 °C for up to 7 days. A positive reaction was indicated by the development of a blue color, while no color change was negative. Positive tubes were subcultured onto another slant and reincubated at 37 °C for 7 days; redevelopment of blue confirmed the positive result.

Urease test

The cultures were heavily inoculated into Christensen's urea broth and incubated at 37 °C for 24-48 hours. The tubes were observed after 4 hours and up to 4 days. A negative result showed no color change or yellow, while a positive result displayed a purple-pink color.

Glucose utilization test

Peptone water with glucose was inoculated and examined daily for 7 days. A positive reaction was indicated by a yellow color, while a negative reaction showed an orangered color.

Genotypic detection of *Klebsiella* isolates

Klebsiella isolates, thus identified by phenotypic methods, were further subjected to confirmation by polymerase chain reaction as follows.

Preparation of broth culture of *Klebsiella*

The bacterial isolates were cultivated by inoculating in BHI (Hi Media) and incubating at 37 °C for 12-18 hours (overnight grown culture).

Extraction of DNA

DNA extraction was performed using the GenElute Bacterial Genomic DNA Kit, following the manufacturer's instructions:

- Centrifuged 1.5 mL of overnight bacterial culture at 12,000-16,000 × g for 2 minutes, discarding the culture medium.

- Resuspended the pellet in 180 µL of Lysis Solution T/Buffer.
- Added 20 µL of Proteinase K, mixed, and incubated at 55 °C for 30 minutes.
- Added 200 µL of Lysis Solution C, vortexed, and incubated at 55 °C for 10 minutes.
- Added 500 µL of Column Preparation Solution to a pre-assembled binding column and centrifuged at 12,000 × g for 1 minute; discarded the eluate.
- Added 200 µL of ethanol (95-100%), vortexed, and mixed thoroughly.
- Transferred the lysate into the binding column, centrifuged at ≥6500×g for 1 minute, and discarded the eluate.
- Added 500 µL of Wash Solution 1, centrifuged at ≥6500×g for 1 minute, and discarded the eluate.
- Added 500 µL of Wash Solution, centrifuged at 12,000-16,000 × g for 3 minutes to dry the column.
- Added 200 µL of Elution Solution, centrifuged at ≥6500×g for 1 minute to elute the DNA.
- Stored the extracted DNA at -20 °C.

Polymerase chain reaction (PCR)

PCR was standardized for detecting *Klebsiella* by targeting the genus-specific *Klebsiella gyrA* gene and two species-specific genes: 16S-23S ITS and *pehX* for *K. pneumoniae* respectively using published primers (Table 02). The PCR mix was prepared as shown in Table 03.

Table 2: Details of primers used for molecular detection of *Klebsiella* for PCR reaction.

Target gene	Primer Sequence	Product size (bp)	Reference
<i>Klebsiella gyrA</i>	F:CGCGTACTATACGCCATGAACGTAR: ACCGTTGATCACTTCGGTCAGG	441	Younis et al., 2017 ^[5]
<i>K. pneumoniae</i> 16S- 23S ITS	F: ATTTGAAGAGGTTGCAAACGATR:TTCACTCTGAAGTTTCTTGTGTTC	130	Younis et al., 2017 ^[5]
<i>K. oxytoca</i> <i>pehX</i>	F: GATACGGAGTATGCTTTACGGTGR:TAGCCTTTATCAAGCGGATACTGG	343	Younis et al., 2017 ^[5]

Table 3: Components used in PCR mixture

Component	Volume
Master mix	12.5µl
NFW	8.5µl
Forward primer	1µl
Reverse Primer	1µl
DNA Template	2 µl
Total	25 µl

Thermocyclic conditions for identification of *Klebsiella* genus

PCR was performed in an Eppendorf thermocycler for *Klebsiella* identification targeting the *gyrA* gene. The protocol included: initial denaturation at 94 °C for 5 minutes, annealing at 60 °C for 45 seconds, and extension at 72 °C for 10 minutes.

Thermocyclic conditions for identification of *K. pneumoniae*

PCR conditions for identification of *K. pneumoniae* targeting I6S-23SITS gene included initial denaturation at 94 °C for 5 minutes, annealing at 58 °C for 30 seconds and extension at 72 °C for 10 minutes.

Agarose gel electrophoresis

The amplified PCR products were electrophoresed in a 1.5% agarose gel (Sigma Aldrich) prepared in 1X TBE buffer (Genie) and stained with ethidium bromide. The gel was set with a gel comb, allowed to solidify, and placed in an electrophoresis tank

with TBE buffer. PCR samples, mixed with 6X bromophenol blue dye, were loaded with a 100+ bp DNA ladder. Electrophoresis was run at 90 volts/cm for 1 hour. Bands were visualized using a UV transilluminator and digitally recorded with a gel documentation system (Alphamager, USA).

Results and Discussion

A total of 150 samples were collected from poultry birds and farm environments, 38 produced lactose-fermenting pink colonies on MCA after incubation. Of these, 17 isolates produced highly mucoid, viscous, pink colonies on MacConkey agar (Fig 1), forming a large string when lifted with a bacteriological loop (Fig 4). These isolates were identified as Gram-negative rods (Fig 2) and exhibited a thick capsule on negative staining (Fig 5). The colonies were streaked onto EMB agar, where no metallic sheen was observed (Fig 3). Based on these colonial and morphological characteristics, the 17 isolates were tentatively identified as *Klebsiella* spp.

On biochemical characterization these 17 strains were found catalase positive (FIGURE 6), oxidase negative (FIGURE 7), produced acidic butt and slant with gas but no H₂S production in TSI slants (FIGURE 8), and $\cdot$, $\cdot$,+,+ in IMViC

reactions(FIGURE 9 to 12) . Additionally, all isolates were urease producers (FIGURE 13) and glucose fermenters (FIGURE 14), consistent with *Klebsiella* spp.



Fig 1: Muroid pink bacterial colonies on Mac-Conkey agar

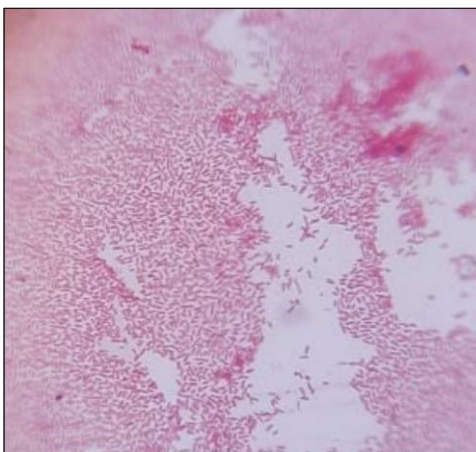


Fig 2: Gram negative pink bacilli

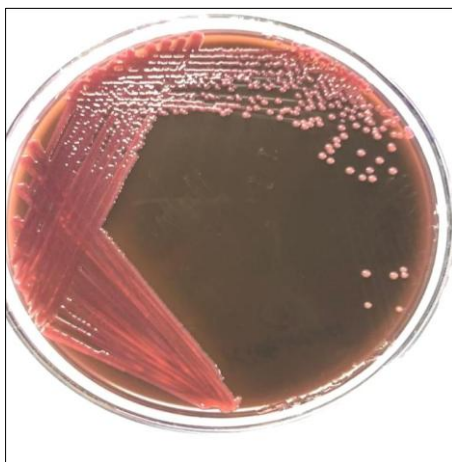


Fig 3: Bacterial growth without metallic sheen on EMB agar

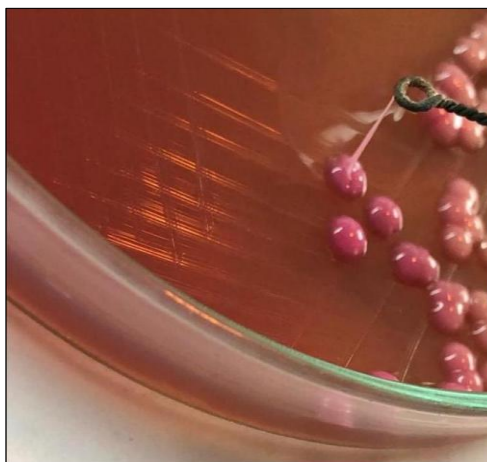


Fig 4: Hypermucoid colonies of bacteria Mac- Conkey agar forming string on lifting

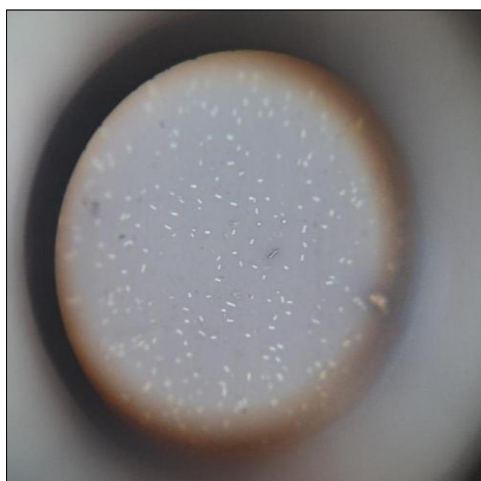


Fig 5: Staining of capsule by negative staining technique



Fig 6: Catalase test

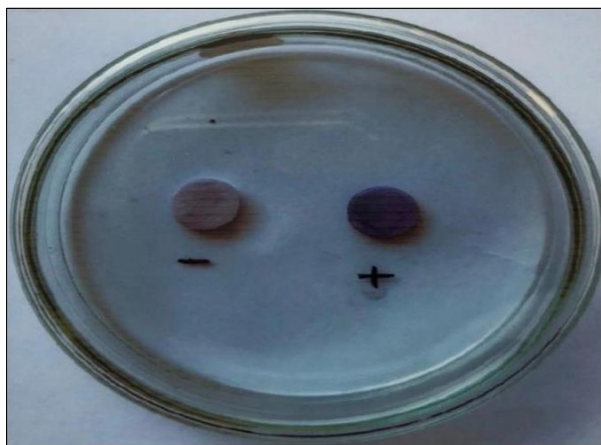


Fig 7: Oxidase test



Fig 8: Triple sugar iron test.



Fig 9: Indole test.

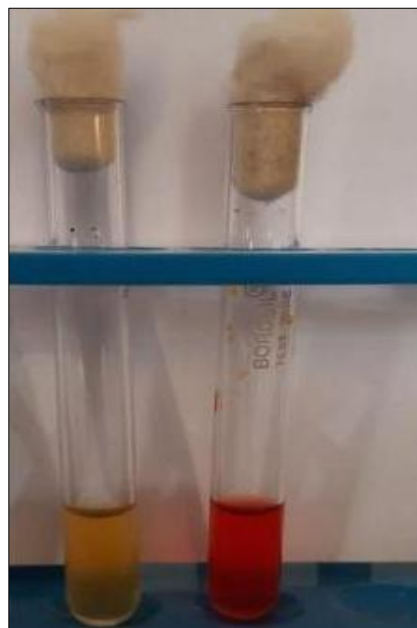


Fig 10: Methyl red test



Fig 11: Voges Proskauer test.



Fig 12: Citrate utilization test.

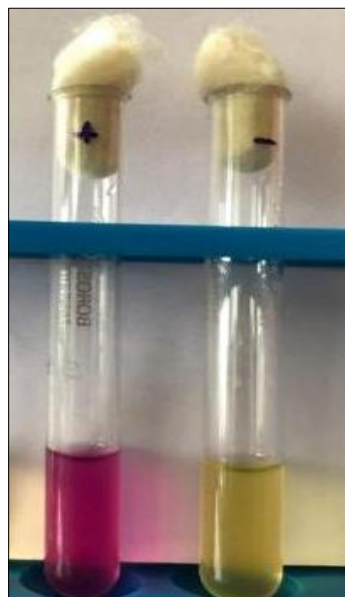


Fig 13: Urease test



Fig 14: Glucose utilization test

Genotypic Identification of *Klebsiella* Isolates

The phenotypically identified *Klebsiella* isolates were confirmed genotypically via PCR with *gyrA* primers targeting the genus-specific gene. All 17 isolates yielded the expected 441 bp product (FIGURE 15), consistent with the methods of Aly *et al.* (2014)^[6], Younis *et al.* (2016)^[7], and Modh *et al.* (2021)^[8]. Additionally, 12 of the 17 isolates (70.58%) were

identified as *K. pneumoniae*, producing the 130 bp PCR product for the species-specific 16S-23S ITS gene (FIGURE 16).

Klebsiella, a natural inhabitant of the gastrointestinal tract in humans, animals, and birds, was isolated from cloacal swabs of healthy birds in this study, highlighting its role as a constituent of the gut microbiome. The prevalence of *Klebsiella* is presented in Table 04.

Table 04: Prevalence of *Klebsiella* in different samples from poultry birds and farm environment

Sr. No.	Sources of sample	Total no. of samples collected	Samples positive for <i>Klebsiella</i>	Percentage (%)
1	Cloacal swabs	65	10	15.38
2	Nasal exudate	10	0	0.0
3	Lung tissue	15	0	0.0
4	Heart tissue	12	0	0.0
5	Liver tissue	12	0	0.0
6	Poultry feed	11	2	18.8
7	Drinking water	12	2	16.6
8	Soil sample	4	2	50
9	Litter material	9	1	11.11
	Total	150	17	11.3

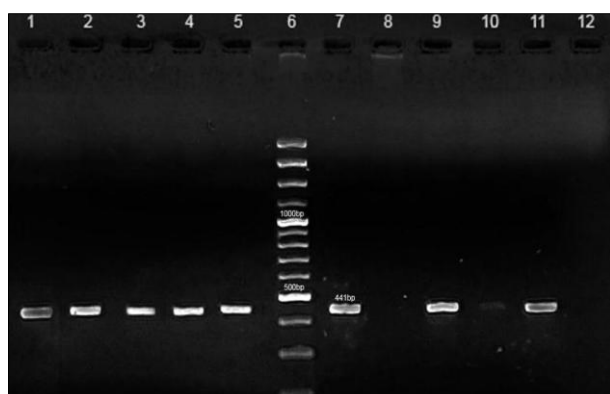


Fig 15: Agarose gel electrophoresis showing amplified product (441bp) of *Klebsiella gyr A* gene; Lane 1-5, 9-11: Positive samples; Lane 6: Ladder (100bp); Lane 7: Positive control; Lane 8: Negative control



Fig 16: Agarose gel electrophoresis revealed the 130 bp product of the 16S-23S ITS gene specific to *K. pneumoniae*, with positive samples in lanes 1-5, 11, and 12. Negative samples were in lanes 6, 10, and 13, while lane 7 contained a 100 bp DNA ladder. Lane 8 was the positive control and lane 9 the negative control.

Discussion

The phenotypically identified *Klebsiella* isolates were confirmed genotypically by PCR using *gyrA* primers. All 17 isolates produced the expected 441 bp product (Plate 15), confirming them as *Klebsiella*. Aly *et al.* (2014)^[6], Younis *et al.* (2016)^[7], and Modh *et al.* (2021)^[8] also used the same primers for genotypic confirmation. Of the 17 isolates, 12 (70.58%) were identified as *K. pneumoniae*, producing the 130 bp product with primers for the species-specific 16S-23S ITS gene (Plate 16). This method was also employed by Younis *et al.* (2017)^[5], Tantawy *et al.* (2018)^[9], and Elgaos *et al.* (2019)^[10] for the molecular detection of *K. pneumoniae*.

In our study, 100% of *Klebsiella* strains identified phenotypically were confirmed by genus-specific PCR. In contrast, Younis *et al.* (2017)^[5] found less than 100% correlation between phenotypic and genotypic methods, with genotypic methods proving to be more sensitive.

Klebsiella is an emerging One Health pathogen, widely distributed in the environment and associated with humans and animals (Holt *et al.*, 2015)^[11]. *K. pneumoniae* and *K. oxytoca* are the most clinically significant species, capable of causing both primary and secondary infections in humans, livestock, and poultry (Effah *et al.*, 2020)^[12]. In this study, *Klebsiella* was isolated from poultry and farm environments with an overall prevalence of 11.3%, aligning with previous reports (Aly *et al.*, 2014; Elgaos *et al.*, 2019; Modh *et al.*, 2021)^[6, 10, 8] that observed a prevalence of 10-14%. In contrast, Gundagon *et al.* (2011)^[13], Fielding *et al.* (2012)^[14], Mahanti *et al.* (2018)^[15], and Eibach *et al.* (2018)^[16] reported a higher prevalence of 40-80%, while Abdulrazzaq *et al.* (2014)^[18] found it to be only 7%. *Klebsiella* was isolated from 8.7% of poultry samples and 11.11% of farm environment samples, with the highest prevalence in farm soil (50.50%), followed by poultry feed (18.18%), drinking water (16.67%), cloacal swabs (15.38%), and litter (11.11%) (Table 18). Tripathi *et al.* (2017) also detected *Klebsiella* in poultry litter and farm soil. Among poultry samples, *Klebsiella* was isolated solely from cloacal swabs (8.7%), which contradicts Mahanti *et al.* (2018)^[15] and Tewari *et al.* (2019)^[17], who reported isolation rates of 40-75%. No *Klebsiella* was isolated from visceral organs of dead poultry, contrasting with the findings of Younis *et al.* (2017)^[5], Tantawy *et al.* (2018)^[9], and Elgaos *et al.* (2019)^[10].

Conclusions

The variation observed in prevalence of *Klebsiella* spp in different studies is obvious as it depends on various factors, such as geographical location of the farm, season, breed, age, hygienic conditions and source of feed and water. The presence of *Klebsiella* in contaminated farm environment and gut of healthy birds could serve as potential reservoir for this bacteria and may result in direct or indirect transmission of infection to the human beings.

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