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ISOLATION AND IDENTIFICATION OF VIRULENT *Klebsiella* spp FROM POULTRY IN CHHATTISHGARH

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Abstract: The study focused on investigating *Klebsiella* spp infections in poultry, a significant pathogen in both human and animal health. Fecal samples were collected from poultry exhibiting symptoms such as diarrhea and sudden death in Chhattisgarh state. Through comprehensive bacteriological and molecular analyses, 29 isolates of *Klebsiella* spp were identified and characterized. Biochemical tests confirmed typical *Klebsiella* spp traits, including positive catalase and negative oxidase tests, and specific growth patterns on selective media like MacConkey agar and Eosin Methylene Blue agar. Molecular identification using PCR targeting the *Klebsiella gyr*A gene further validated the presence of *Klebsiella* spp. The findings underscored the prevalence and diversity of *Klebsiella* spp in poultry, emphasizing the need for continued surveillance and management strategies to mitigate potential health risks associated with these pathogens.

Keywords: *Klebsiella*, identification, media, gyrA

2. INTRODUCTION

Klebsiella pneumoniae is an opportunistic pathogen and is among the common causes of nosocomial infections. Together with five other bacteria, it is known as the ESKAPE pathogens (Santajit and Indrawattana, 2016). While there are many species and subspecies of *Klebsiella*, *Klebsiella pneumoniae* is considered clinically the most important species (Franklin-Alming *et al*., 2021). Recently, Dong *et al*. (2022) reported on the taxonomy of hypervirulent and MDR *Klebsiella* spp and reported that the *Klebsiella* genus consists of the *Klebsiella pneumoniae* species complex (KpSC), *Klebsiella aruginosa*, and *Klebsiella oxytoca* species complex (such as *Klebsiella pasteurii*, *Klebsiella terrigena*, *Klebsiella oxytoca*, *etc*.).

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Extensive research has been conducted on *Klebsiella* spp. from humans to characterize the bacteria's phenotypes and genotypes. *Klebsiella* infection in poultry has been reported to cause high mortality in balady chicks (Sarakby, 1979; Karaman, 1980). *Klebsiella pneumoniae* can cause localized or systemic infection in poultry and other birds (Shivaprasad, 1998).

The diagnosis of *Klebsiella* infection can be confirmed through microbial culture analyses of wound swabs, urine, or other relevant clinical samples, based on the specific context of the illness. The presence of red color, rod shaped bacteria in Gram- stained smear is indicative of *Klebsiella*, which may present as capsulated and non- sporing. Samples are cultured on MacConkey and EMB agar medium and aerobically incubated for further identification. Molecular diagnostic techniques such as PCR may be necessary for further confirmation.

The present study aimed to investigate the impact of *Klebsiella* spp. infections on chickens. The research included confirming the pathogenicity of the organism through tests.

MATERIAL AND METHODS

3.1. Collected samples:

Fecal samples were collected from 150 poultry birds suffering from diarrhea and sudden death. The birds were obtained from 35 farms in different localities of three districts (Balod, Durg and Rajnandgaon) in Chhattishgarh state from 2023 to 2024. Individual cases were subjected to clinical examination by registered veterinary practitioner. Samples underwent clinical, post-mortem, and bacteriological examinations.

Table 1: Details of the total number of samples collected from three districts of Chhattishgarh during the study

S. No	Species	Total No of size	Sample type	Districtwise No.			Total
				Balod	Durg	Rajnandgaon	
3.	Poultry	150	Anal swabs	49	53	48	150

3.23.2. Bacterial isolation:

Each of appropriately processed samples were inoculated first in the Nutrient broth and then a loopful cultured broth showing turbidity was streaked in the MacConkey agar plates and incubated at 37^0C for 24 hr. The *Klebsiella* growth was distinguished by its mucoid colony that appears as a pink colony on MacConkey medium due to lactose fermentation. Thus, the isolates identified as *Klebsiella* spp was subcultured on selective media agar plates, like

MacConkey and/or Eosin methylene Blue (EMB) agar plates using the streak plate method. A single colony showing characteristic morphology was transferred to nutrient agar slant in duplicate and maintained as a pure culture throughout the study

3.3. Bacterial Identification:

3.3.1. Cultural and Biochemical characteristics:

Bacterial isolates obtained as pure culture were presumptively identified based on characteristic colony morphology as well as biochemical characteristics. Biochemical tests like the catalase test, oxidase test, IMViC tests (Indole, Methyl Red, Voges- Proskeur and Citrate test) and TSI (Triple Sugar Iron) agar test were performed as per standard procedure described by MacFaddin, (2000).

3.3.2. Polymerase Chain Reaction (PCR):

Each of isolates suspected for *Klebsiella* was confirmed by PCR amplification using *Klebsiella gyr*A genus- specific primers. Genomic DNA of phenotypically suspected isolates was extracted using either the heating lysis method or boiling- snap chill method (Dashti *et al.*, 2009). The genomic DNA of each isolate was used as a template for PCR amplification of *Klebsiella* genus-specific primer and for further molecular analyses. PCR amplification reactions were optimized using positive controls.

Gene	Primer sequence	Lenth (bp)	Reference
*Klebsiella gyr*A	F-CGCGTACTATACGCCATGAACGTA-3' R- ACCGTTGATCACTTCGGTCAGG-3'	441	(Okafor and Nwodo, 2023)

3.3.2.1. PCR protocol

The PCR assay used the BIO RAD T100 Thermal Cycler PCR system to confirm the presumptive isolates. The assay was performed under the following conditions: an initial denaturation of 94°C for 5 minutes; 35 cycles of 30 seconds at 94°C, 45 seconds at 55°C for annealing, extension at 72°C for 45 seconds, and a final extension cycle of 72°C for 10 minutes. The total reaction mixture of 25 µl consisted of 12.5 µl industrially synthesized master mix, 1 µl each of forward and reverse primers (Eurofins, Genomics India Pvt. Ltd., Bangluru), 6.5 µl PCR grade water, and 4 µl template DNA. Electrophoresis was carried out

using a 1.5% agarose gel and 0.5x TBE buffer, set at 100 V for 45 minutes. The gel was stained with 4 µl ethidium bromide. A 100 bp DNA marker was used to estimate the DNA band, which was then examined with a UV transilluminator.

4. RESULTS & DISCUSSION

Members of the genus *Klebsiella* have rapidly evolved within the past decade. They have generated organisms that simultaneously exhibit both multidrug resistance and hypervirulence (MDR-hv) phenotypes. These organisms are associated with severe hospital- and community-acquired infections. Carbapenem-resistant infections with an unknown optimal treatment regime were of particular concern among the MDR-hv *Klebsiella* strains.

Poultry samples, primarily anal swabs, were predominantly collected from Balod and Durg, with symptoms largely centered on gastrointestinal disturbances such as diarrhea, including severe cases with bloody diarrhea. A significant number of isolates were from poultry in Rajnandgaon, with similar symptoms of diarrhea collected.

The table 2 provides an overview of the total suspected isolates and cultural characteristics of *Klebsiella* spp isolated from various samples, detailing their growth patterns across three different media: Nutrient Broth, MacConkey Agar, and Eosin Methylene Blue (EMB) Agar. A total of 29 suspected isolates of *Klebsiella* spp were obtained. In Nutrient Broth, bacteria produced turbidity, indicating significant bacterial growth. The presence of turbidity in the broth signifies active proliferation of bacteria, leading to a cloudy appearance of the medium due to the accumulation of bacterial cells. On MacConkey Agar, *Klebsiella* spp, *Escherichia coli*, and *Enterobacter* spp formed pink colonies.

This color change occurs because these bacteria ferment lactose, producing acidic byproducts that alter the pH indicator in the agar, resulting in pink colonies (figure: 1). This characteristic indicates lactose fermentation, a key feature of this bacterial genus. *Klebsiella* spp form mucoid pink color colonies and bacteria show non-motility under the microscope, whereas *Enterobacter aerogenes* showed motility.

Figure 1: Mucoid colonies suspected for *Klebsiella* spp in MacConkey agar

In Eosin Methylene Blue (EMB) Agar, *Klebsiella* spp exhibited colonies with a red coloration and a non-metallic sheen (figure 2).

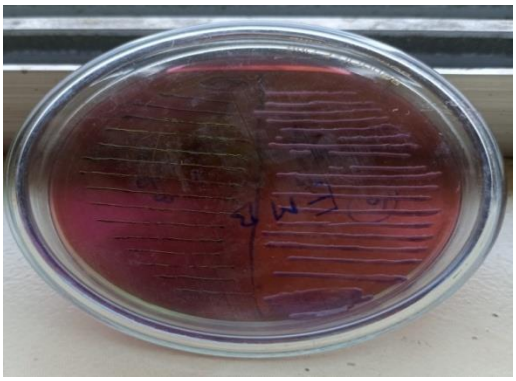

Figure 2: Two distinct cultures in EMB agar. On the left side, *E. coli* showed a metallic sheen colony, whereas on the right side, *Klebsiella* spp showed a mucoid red colored culture.

EMB Agar is used to differentiate *Escherichia coli* from other lactose fermenting gram-negative *Enterobacteriaceae* bacteria based on their capability to form metallic sheen colonies. While *Klebsiella* spp does not produce the metallic green sheen typical of more vigorous lactose fermenters, such as *Escherichia coli*. Instead, the red colonies with a non-metallic sheen confirm moderate lactose fermentation.

These findings collectively confirm the identification of *Klebsiella* spp based on its characteristic growth patterns and colony morphology in different microbiological media.

Table 2: Profile of isolates to be identified for *Klebsiella* spp

Serial No	Id of isolates suspected for *Klebsiella* spp	Sample type	District	Clinical symptom
1	KPB-1	Poultry,anal swab	Balod	Diarrhea
2	KPA-3	Poultry, Anal swab	Rajnandgaon	Diarrhea
3	KPA-3'	Poultry, Anal swab	Durg	Diarrhea
4	KPA-4	Poultry, Anal swab	Durg	Diarrhea
5	KPR-7	Pouitry, Anal swab	Rajnandgaon	Diarrhoea
6	KPD-8	Poultry, Anal swab	Durg	Dirrhoea
7	KPR-9	Poultry, Anal swab	Rajnandgaon	Dirrhoea
8	KPD-10	Poultry, Anal swab	Durg	Dirrhoea
9	KPD-11	Poultry, Anal swab	Durg	Dirrhoea
10	KPD-12	Poultry, Anal swab	Durg	Dirrhoea
11	KPD-13	Poultry, Anal swab	Durg	Dirrhoea
12	KPB-14	Poultry, Anal swab	Balod	Dirrhoea

13	KPB-15	Poultry, Anal swab	Balod	Bloody dirrhoea
14	KPB-16	Poultry, Anal swab	Balod	Dirrhoea
15	KPR-18	Poultry, Anal swab	Rajnandgaon	Deability
16	KPB-19	Poultry, Anal swab	Balod	Diarrhea
17	KPB-24	Poultry, Anal swab	Balod	Diarrhea
18	KPB-25	Poultry, Anal swab	Balod	Diarrhea
19	KPR-29	Poultry, Anal swab	Rajnandgaon	Diarrhea
20	KPR-41	Poultry, Anal swab	Rajnandgaon	Diarrhea
21	KPR-42	Poultry, Anal swab	Rajnandgaon	Diarrhea
22	KPB-43	Poultry, Anal swab	Balod	Diarrhea
23	KPR-49	Poultry, Anal swab	Rajnandgaon	Diarrhea
24	KPD-54	Poultry, Anal swab	Durg	Diarrhea
25	KPB-58	Poultry, Anal swab	Rajnandgaon	Diarrhea
26	KP-1	Poultry, anal swab	Durg	Diarrhea
27	KP-2	Poultry, anal swab	Durg	Diarrhea
28	KP-3	Poultry, anal swab	Durg	Diarrhea
29	KP-4	Poultry, anal swab	Durg	Diarrhea

Each isolate underwent a series of diagnostic tests to determine their enzymatic activities and metabolic properties. The results reveal uniformity in the bacterial profile across all tested isolates.

All *Klebsiella* isolates exhibited a positive catalase test, indicating the presence of the enzyme catalase, which decomposes hydrogen peroxide into water and oxygen. Conversely, all isolates showed a negative result in the oxidase test, confirming the absence of the enzyme oxidase.

In the Triple Sugar Iron (TSI) test, all *Klebsiella* isolates demonstrated a yellow slant and yellow butt, with gas production and no hydrogen sulfide (H2S) production. The yellow coloration in both the slant and butt suggests that the bacteria ferment glucose and possibly sucrose or lactose, leading to acid production throughout the medium. The presence of gas indicates the fermentation of sugars with the production of carbon dioxide or other gases, while the lack of H2S production is consistent with the characteristics of *Klebsiella* spp.

The IMViC tests revealed consistent results across the isolates: all *Klebsiella* tested negative for indole and methyl red, and positive for Voges-Proskauer (VP) and citrate tests. The negative indole test indicates that the isolates do not produce indole from tryptophan. The negative methyl red test suggests that the isolates do not produce large amounts of acidic end-products from glucose fermentation. The positive VP test indicates the production of acetoin, a neutral end-product of glucose fermentation. The positive citrate test confirms the ability of the isolates to utilize citrate as their sole carbon source. The biochemical profiles of the isolates are characteristic of *Klebsiella* spp, with consistent results in catalase, oxidase, TSI, and IMViC tests. These findings provide a comprehensive biochemical fingerprint, confirming the identity of the isolates as *Klebsiella* spp.

All isolates, however, tested positive for urease activity. This uniform positive result signifies that each strain is capable of hydrolyzing urea to produce ammonia and carbon dioxide, a defining characteristic of *Klebsiella* spp. The consistent urease activity across all isolates underscores their metabolic capability and supports their identification as *Klebsiella* spp.

Figure 3 presents the results of testing the *Klebsiella* genus specific primer *Gyr*A gene in 29 bacterial isolates. Of these, 22 isolates showed the presence of the *Gyr*A gene, indicating its presence in the majority of the samples. On the other hand, 7 isolates were negative for the *Gyr*A gene, suggesting that this gene is absent or undetectable in these specific isolates. The negative result isolates were KPD-9, KPD-11, KPD-12, KPD- 13, KPB-14, KPB-15 and KPB-16. This result confirmed that the 22 isolates were indeed *Klebsiella* spp. The widespread presence of the gyrA gene in the tested isolates highlights its frequent occurrence in the studied population. This distribution offers valuable insights into the genetic traits of the isolates and may have implications for understanding the genetic diversity and potential resistance mechanisms within the bacterial strains being investigated.

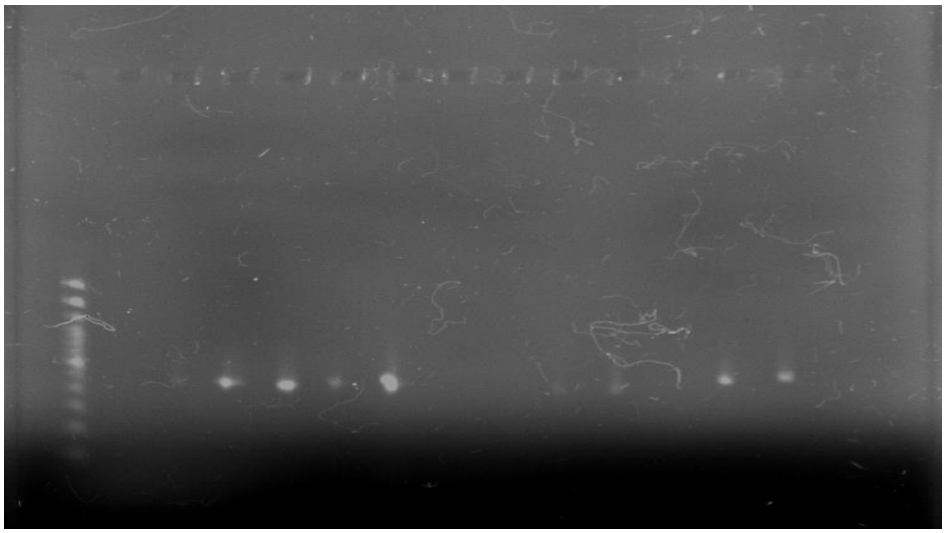

Figure 3: Amplification of *gyrA* (441 bp) gene. Lane M: 100 bp DNA ladder, Lanes 3,4,5,6 and 11- positive test, Lane 2,7,8,910- negative test, Lane 12- positive control, Lane 1- Negative control.

All samples were taken from diseased birds, so the isolates from these birds were virulent and pathogenic. Out of total samples, 29 tested positive for *Klebsiella* spp phenotypically, while 22 isolates tested positive genotypically using the *Klebsiella* gyrA primer. The same primer was used by Okafor and Nwodo (2023) [9] for genus level identification of *Klebsiella* spp. These findings were among 150 samples collected from poultry herds where mortality was observed. The 29 phenotypically positive samples accounted for 19.33% of the total, whereas the 22 genotypically positive samples had a prevalence rate of 14.67%. Tantyawy *et al.* (2018), Frankline- Alming *et al*. (2021) and Kowalczyk *et al.* (2022) also isolated *Klebsiella* spp from clinical cases in poultry.

CONCLUSION

Klebsiella spp have been previously ignored in the case of poultry diseases, but their incidences have increased over the last few decades. Our research findings have observed its impact on poultry gastrointestinal mortality, indicating the need for extensive related studies.

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