Multidrug Resistance and Molecular Characterization of *Klebsiella spp.* Isolated from the Cloacal Samples of Broiler Chickens in Bangladesh

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Abstract

Klebsiella spp. poses a significant zoonotic threat, capable of direct and indirect transmission from poultry farms to humans, resulting in severe conditions such as pneumonia, bloodstream infection, enteric fever, meningitis and urinary tract infections. The escalating use of antibiotics in the poultry industry is contributing to the rise of multidrug-resistant (MDR) microorganisms. Thus, a study was undertaken to comprehend the multidrug resistance and virulence gene profile of *Klebsiella spp.* isolated from poultry cloacal samples sourced from local markets of North Dhaka, Bangladesh. Following aseptic collection, the samples were transported to the laboratory for pure culture isolation. Employing standard microbiological methods such as mucoid colony characteristic, Gram-negative rod-shaped bacteria, biochemical tests and lactose fermentation in selective media, the isolated colonies were presumptively identified. Subsequently, an antibiotic susceptibility test, utilizing the disk diffusion assay, was conducted to assess drug sensitivity in these isolates. A PCR assay was performed to determine the presence or absence of *KPC* gene of *Klebsiella*. Among the isolates, ten colonies were identified as *Klebsiella spp*. based on the colony characteristics, Gram staining, biochemical tests and growth on selective media. Notably, eight isolates (excluding 2 and 4) were incapable of producing indole from tryptophan, indicating the presence of two different types of species. All isolates exhibited resistance to at least three antibiotics, namely ceftazidime, ampicillin and cefoxitin. Isolate 2 and 5 were demonstrated additional resistance to azithromycin, while isolate 9 and 10 exhibited further resistance to streptomycin and doxycycline. Molecular analysis indicated that 40% of these isolates harbor *KPC* virulent gene (880bp), with the most multidrug resistant isolate 9 and 10 possessing this gene. This study is pivotal for assessing the prevalence of virulent and MDR *Klebsiella spp.* in chicken cloacal samples, providing insights into shedding and transmission risk on soil and water environment, as well as to humans.

Keywords: Bangladesh, *Klebsiella spp.*, PCR, Poultry, Multidrug resistance.

Significance | Public awareness for multidrug-resistant and virulent Klebsiella spp.

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1. Introduction

Bangladesh stands out among the nations in the North-Eastern part of South Asia as country marked by success and development, boosting a remarkable story of poverty reduction and rapid growth. A significant portion of the Bangladeshi population relies on agriculture, farming, poultry, and various resources for their

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livelihoods (Hamid et al., 2016). Poultry serves as one of the most sustainable sources of protein globally and is a key player in Bangladesh's economic growth and development, ranking as the second significant contributor (Nourish Poultry and Hatchery Ltd., Dhaka, Bangladesh et al., 2021). With its vital role in providing employment opportunities and offering relatively affordable animal proteins, the poultry industry in Bangladesh produces a substantial 570 million tons of meat and 7.334 billion eggs annually (Haque et al., 2020). The geographical structure and favorable weather conditions in Bangladesh make it conducive for agriculture and poultry farming, involving the processing of different bird types for meat, eggs, and feathers used in various accessories. However, despite its rapid growth, this sector faces ongoing challenges, including economic challenges on a global scale, such as insufficient production compared to consumer demands. The insufficient production has led to concerns about meeting the nutritional needs of the population. According to the national health strategy, adults require 120 grams of meat daily and 104 eggs per year. Presently, the availability is only at 67.17% and 63.65%, falling significantly below the minimum requirements for meat, eggs, and other protein items (Hannan et al., 2020). The progress of production faces challenges from the spread of microbial diseases and a lack of efforts to control them.

Poultry farms are closely associated with transmissible pathogens and zoonotic diseases, presenting a major challenge for eradication and control strategies. Pathogenic bacteria, such as *Klebsiella spp.*, are commonly found in the environment and contaminated poultry products (Agyare et al., 2019; Salaheen et al., 2015; Thorpe et al., 2022). In the current scenario of poor sanitation, these pathogenic *Klebsiella spp.* can easily be expelled to other poultry birds, promoting bacterial shedding. Transmission can occur through contact with contaminated food or water, waste disposals, contact with contaminated surfaces, or even through direct contact with contaminated tools used in poultry (Kowalczyk et al., 2022; Mondal, 2022; Podschun & Ullmann, 1998).

Additionally, the frequent use of antibiotics in poultry farms has led to multidrug resistance in the normal microbial flora of broiler chickens, posing severe health risks as opportunistic pathogens in the gastrointestinal tracts of both humans and animals (Abreu et al., 2023; Hedman et al., 2020; Kousar et al., 2021). Concerns over the rise of multidrug-resistant (MDR) *Klebsiella spp.* bacteria have increased, as these strains are challenging to treat due to their resistance to numerous antibiotics (Awoke et al., 2021; Nirwati et al., 2019). The aggressive approach towards disease control, driven by the increased number of livestock or poultry, necessitates a large amount of prophylactic and therapeutic antibiotic use.

Klebsiella spp. exhibit genetic diversity, impacting their contagiousness, antibiotic resistance, and virulence. Some strains contain the virulent *Klebsiella pneumoniae* carbapenemase (*KPC*) gene, enhancing their pathogenicity (Fatima et al., 2021; Lavigne et al., 2013). The global outbreak of carbapenem-resistant Enterobacteriaceae (CRE) infections is attributed to the virulent *KPC* gene of *Klebsiella*, which is a bacterial plasmid-mediated carbapenemase gene. This gene, being easily transferred between bacteria, has contributed to the rapid proliferation of CRE infections (Chen et al., 2014; Suay-García & Pérez-Gracia, 2019; Wyres & Holt, 2018). The *KPC* gene can be transferred through contact with contaminated surfaces or bodily fluids, and humans can contract it from animals by consuming contaminated food or water, leading to life-threatening infections such as pneumonia, diarrhea, urinary tract infections, sepsis, meningitis, and bloodstream infections (Paczosa & Mecsas, 2016). The presence of virulent *KPC*-containing multidrug-resistant *Klebsiella* spp. in poultry is alarming and warrants careful consideration (Odari & Dawadi, 2022). Therefore, our present study aims to the isolate, identify and evaluate MDR, and virulence gene profile of *Klebsiella spp.* isolated from poultry cloacal samples in North Dhaka, Bangladesh.

Materials and Methods

Reagents and equipments

All the media, equipment, chemicals and reagents were used for the isolation, identification, characterization, antibiotics sensitivity, and virulence gene profiling of microorganisms were sourced from the Department of Biochemistry and Microbiology, North South University, Bangladesh. PCR primers for the molecular identification of *KFC* gene in *Klebsiella spp.* were obtained from the Department of Microbiology, Jashore University of Science & Technology, Jashore, Bangladesh.

Study period, area and sample collection

This study spanned from July 2022 and October 2023, with the laboratory works were conducted in Microbiology and Molecular Biology laboratories of the Department of Biochemistry and Microbiology, North South University, Dhaka-1229, Bangladesh. Aseptic collection of broiler chicken cloacal samples (n=3) was carried out in sterile 15mL falcon tubes containing buffer peptone water (BPW) from various locations (Aziz sharak, Ghatpar, and Dhali corner) near Bashundhara R/A, Dhaka-1229, Bangladesh (Figure 1). The samples were then transported to the laboratory for further processing and investigation to identify the *Klebsiella spp.* following to the guidelines outlined in Bergey's manual of systematic bacteriology. The detail workflow, encompassing from sample collection through molecular characterization, is illustrated in Figure 2.

Sample processing and preservation

The samples underwent serial 10-fold dilution, followed by spreading on nutrient agar (NA) media, and incubation at 37°C overnight. Subsequently, a four-quadrant streak plate method on NA media was employed for subcultures and isolation of pure

cultures. Selection of random single colonies for stock preparation and preservation was based on the colony characteristics such as shape, texture, pigmentation, and appearances. To prepare the stocks for preservation, the isolated pure cultures were grown in LB medium at 37°C overnight. The following day, 700µl bacteria-containing sample was mixed with 300µl of 50% glycerol in sterile 1.5mL Eppendorf tubes and stored at -20°C.

Gram staining and biochemical tests

Preserved samples were incubated in NA media at 37°C overnight, followed by Gram staining to assess the size, shape, arrangement. Additionally, this staining aimed to differentiate bacteria into large two broad categories: Gram-positive and Gram-positive. Subsequently, seven distinct biochemical tests were conducted to ascertain various characteristics: citrate utilization; sugars (glucose, fructose and lactose) fermentation and production of H_2S gas; motility, indole production and urease activity; presence of cytochrome c oxidase enzyme, presence of catalase enzyme, glucose fermentation ability; 2,3-Butanediol (product of glucose fermentation) fermentation by the Simmons citrate test; triple sugar iron (TSI) test, motility-indole urease (MIU) test, oxidase test, catalase test, methyl red (MR) test and voges-proskauer (VP) test, respectively (Alves et al., 2006; Hansen et al., 2004; Patel et al., 2017; Salauddin et al., 2019).

Selective and differential media

Gram-positive and Gram-negative bacteria were cultured on various selective and differential media including m-FC, MacConkey Agar, Eosin Methylene Blue (EMB), Endo agar, Tryptic Soy Agar (TSA) and Xylose-Lysine Deoxycholate (XLD) agar. These media were employed for the presumptive identification of the *Klebsiella* isolates based on their capacity to utilize lactose and distinctive colony pigments (Rahman et al., 2023; Saha et al., 2013; Salauddin et al., 2019).

Disk diffusion assay

The Kirby-Bauer disk diffusion assay (Yao et al., 2021) was employed to assess the spectrum of antibiotic resistance and susceptibility. To execute this assay, pure cultures for each isolate were aseptically inoculated into Muller Hinton broth (MHB) and incubated for 3 hours. To ensure organisms were in the log phase of growth, samples in MHB were adjusted to an absorbance at OD_{600} ~ 0.5 and utilized for the preparation of bacterial lawn on Mueller Hinton agar media. A sterile cotton swab was dipped into the bacterial suspension and used to create a bacterial lawn on a freshly prepared and dried MHA plate. The plate was allowed to dry, zones were marked for the placement of each antibiotic disc. With the help of sterile forceps, the discs were carefully positioned at the center of each designated segment and incubated up-right for 24 hours at 37°C. Clear zone diameters were measured using a metric ruler after incubation for interpretation. The results were interpreted according to Clinical and Laboratory Standards Institute (CLSI, 2020) guidelines. The antibiotics disc used in this study were azithromycin (AZM), streptomycin (S), doxycycline (DO), tobramycin (TOB), ceftriaxone (CTR), amikacin (AK), chloramphenicol (C), ceftazidime (CAZ), norfloxacin (NOR), ampicillin (AMP) and cefoxithin (CX).

DNA extraction

For DNA extraction, a 1.5mL overnight (O/N) bacterial culture was transferred to an eppendorf tube and centrifuged (Himac, Ibaraki, Japan) at 10,000 rpm for 10 minutes. The resulting pellets were washed with 750µl 1X PBS (Thermo, Waltham, MA USA) at 10,000 rpm for 2 minutes. After discarding supernatants, pellets were resuspended in 500µl of 5% chelex (Bio-rad, Hercules, CA, USA) using a pipette. Subsequently, the chelex-sample mixture was incubated in a heat block (Biometra, Göttingen, Germany) at 95°C for 20 minutes and then cooled at -20°C for 5 minutes. Following this, the samples were centrifuged for 10,000 rpm for 10 minutes, and the supernatants were carefully transferred to a fresh eppendorf tube. Quantification of double-stranded DNA (dsDNA) was conducted using a spectrophotometer (Eppendorf, Hamburg Germany) at OD_{260}. Purity of DNA was assessed by checking the OD_{260} / OD_{280} ratio.

PCR

To prepare a 10µl PCR master mixture, the following components were utilized: 200 ng (1~3µl) of template DNA, 5µl of 2X GoTaq® G2 Green Master Mix (Promega, Madison, WI, USA), 1µl of forward primer (KPC F primer: 5′-TGTCACTGTATCGCCGTCTAG-3′), 1µl of reverse primer (KPC R primer: 5′-TTACTGCCCGTTGACGCCCAATCC-3′) and nuclease free water (Thermo, Waltham, MA USA) as needed (Gootz et al., 2009). The samples were thoroughly mixed by vortexing, briefly centrifuged and then placed in the MiniAmp Plus PCR machine (Applied Biosystem by Thermo Fisher Scientific, Waltham, MA, USA). The amplification of *KPC* gene was carried out under the following conditions: initial incubation at 72°C for 7 minutes followed by 35 cycles of denaturation at 94°C for 1 minute, primer annealing at 59.5°C for 2 minutes and extension at 72°C for 3 minutes. The final extension was performed for 7 minutes at 72°C. Samples were stored at 4°C for immediate analysis or -20°C for future analysis.

Agarose gel electrophoresis

A 10µl of PCR sample was loaded onto a 1% (w/v) agarose gel (GeneDireX Inc., Taoyuan City, Taiwan) in 1X Tris-Borate-EDTA (TBE) buffer. Electrophoresis was conducted at 100volts for approximately 30 minutes. Following electrophoresis, gel was stained with ethidium bromide (EtBr) for 10 minutes, washed and visualized on a UV trans illuminator. A 100bp ladder (GeneDireX Inc., Taoyuan City, Taiwan) was used as molecular weight marker.

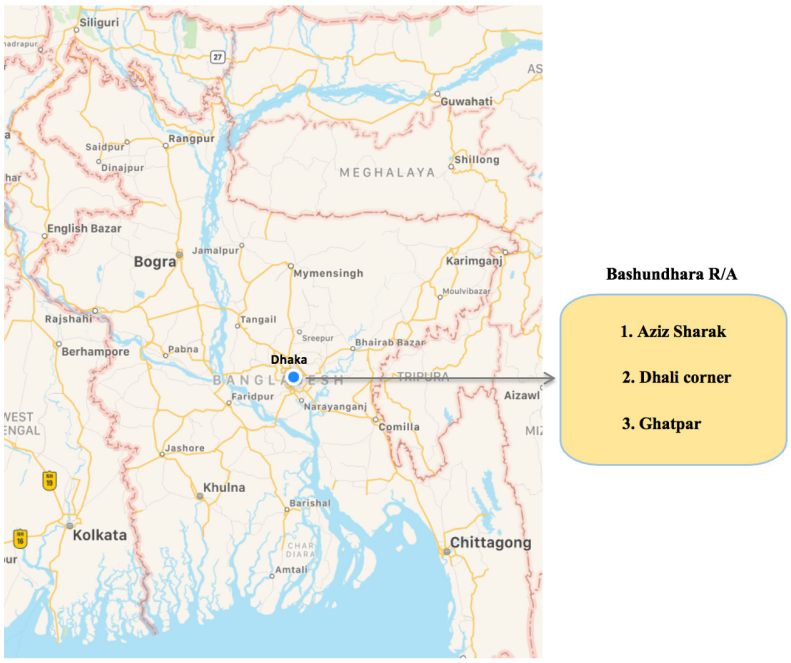


Figure 1: Sample collection locations: Samples were collected from North Dhaka near the Bashundara R/A, Dhaka-1229, Bangladesh. The arrow indicates the specific locations from where the broiler chicken cloacal samples were collected.

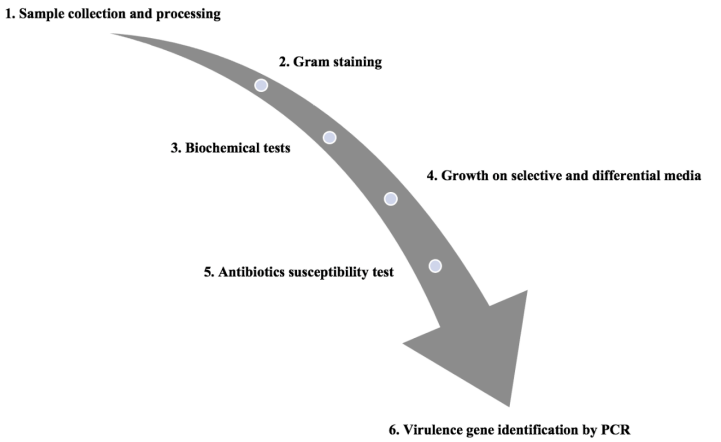
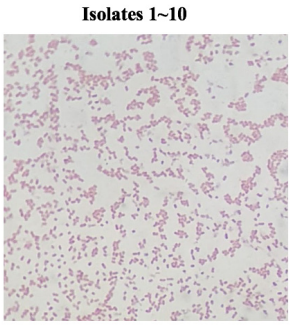
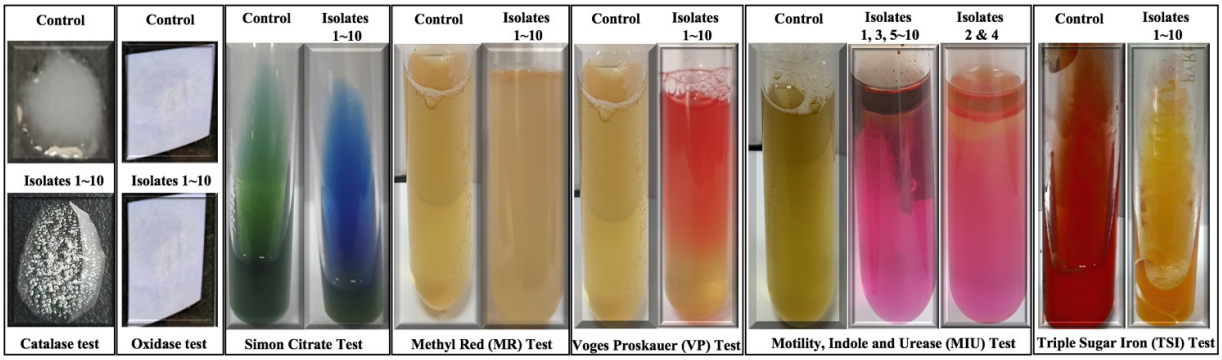


Figure 2: Schematic flowwork from sample collection and processing to presumtive identification, MDR screening, and virulence gene profiling by polymerase chain reaction (PCR).

a. Gram staining



b. Biochemical test



C. Growth on nutrient and differential agar media.

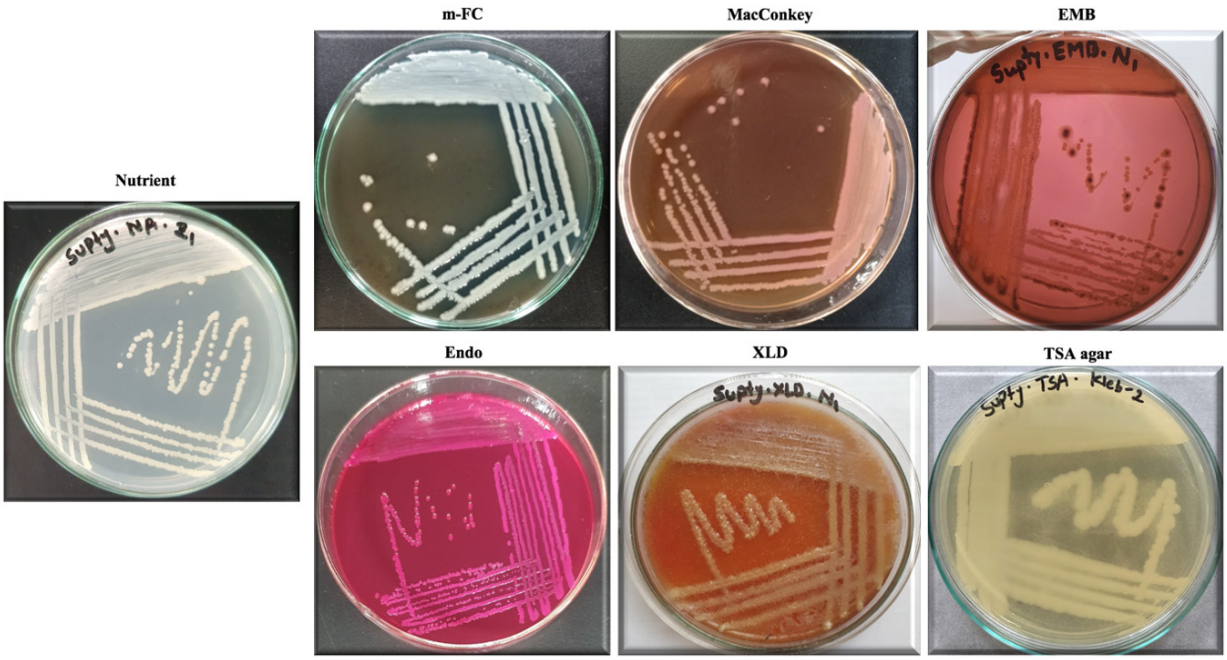


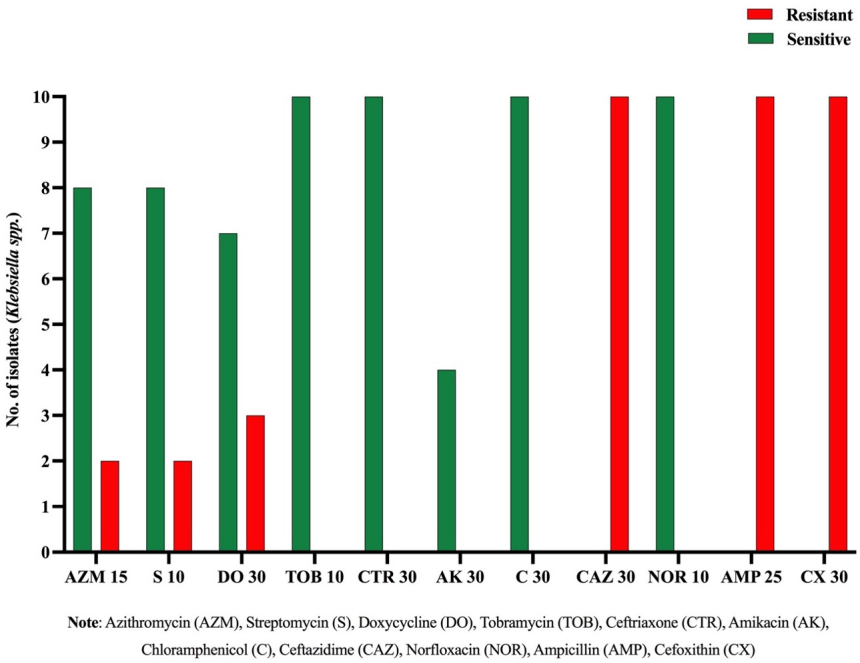
Figure 3. Presumtive identification of *Klebsiella spp.* from poultry cloacal samples. **a.** Gram staining: Isolates were examined under oil immersion microscope (100X) after staining. Pink coloration indicates Gram-negative bacteria. **b.** Biochemical test: These tests serve presumptive identification method of microorganisms. In the catalase test, bubble formation and appearance of blue color in Simon citrate test indicate a positive catalase test and positive citrate utilization test, respectively. Isolates containing urease gene demonstrated the ability to hydrolyze urea. The indole test measures tryptophan degradation, and a change in slant and butt color to yellow in the TSI test indicates fermentation of sugars such as the glucose, sucrose and lactose. Media break down signifies lactose and sucrose fermentation. **c.** Growth on nutrient, selective and differential media: The growth pattern on selective and differential agar media is observed for a more accurate confirmation of presumptive identification of unknow microorganisms. Parameters such as forms, size, texture and pigmentation were recorded.

Table 1. Cultural characteristics *Klebsiella spp.* isolates on nutrient, selective and differential media.

Agar media	Forms	Size	Texture	Pigment	Appearance
Nutrient	Circular	Large	Smooth	Non-pigmented	Shiny
m-FC	Circular	Large	Smooth & Mucoid	Bluish- Gray	Shiny
MacConkey	Circular	Large	Smooth & Mucoid	Pink colonies	Shiny
EMB	Circular	Large	Smooth & Mucoid	Mucoid pink and purple	Shiny
Endo	Circular	Large	Smooth & Mucoid	Pink mucoid	Shiny
XLD	Circular	Large	Smooth	Yellow	Shiny
TSA	Circular	Large	Smooth	Creamy White	Shiny

Table 2. Biochemical tests result of *Klebsiella spp.* isolates from poultry cloacal samples. In this context, the "+" sign indicates a positive result, the "-" indicates a negative result and the "A" indicates acid.

Isolates	Catalase	Oxidase	Simon citrate	MR	VP	Motility	Indole	Urease	TSI (stunt / butt)
Klebsiella spp. isolate 01	+	-	+	-	+	-	-	+	A/A, gas
Klebsiella spp. isolate 02	+	-	+	-	+	-	+	+	A/A, gas
Klebsiella spp. isolate 03	+	-	+	-	+	-	-	+	A/A, gas
Klebsiella spp. isolate 04	+	-	+	-	+	-	+	+	A/A, gas
Klebsiella spp. isolate 05	+	-	+	-	+	-	-	+	A/A, gas
Klebsiella spp. isolate 06	+	-	+	-	+	-	-	+	A/A, gas
Klebsiella spp. isolate 07	+	-	+	-	+	-	-	+	A/A, gas
Klebsiella spp. isolate 08	+	-	+	-	+	-	-	+	A/A, gas
Klebsiella spp. isolate 09	+	-	+	-	+	-	-	+	A/A, gas
Klebsiella spp. isolate 10	+	-	+	-	+	-	-	+	A/A, gas



Note: Azithromycin (AZM), Streptomycin (S), Doxycycline (DO), Tobramycin (TOB), Ceftriaxone (CTR), Amikacin (AK), Chloramphenicol (C), Ceftazidime (CAZ), Norfloxacin (NOR), Ampicillin (AMP), Cefoxithin (CX)

Figure 4: Antibiotics succeptibility profile of *Klebsiella spp.* isolates. The antibiotics susceptibility profile of *Klebsiella spp.* isolates from poultry cloacal samples is presented in this graph. The bar diagram was created using Prism 9 (Prism Grape Pad Software, La Jolla, CA, USA).

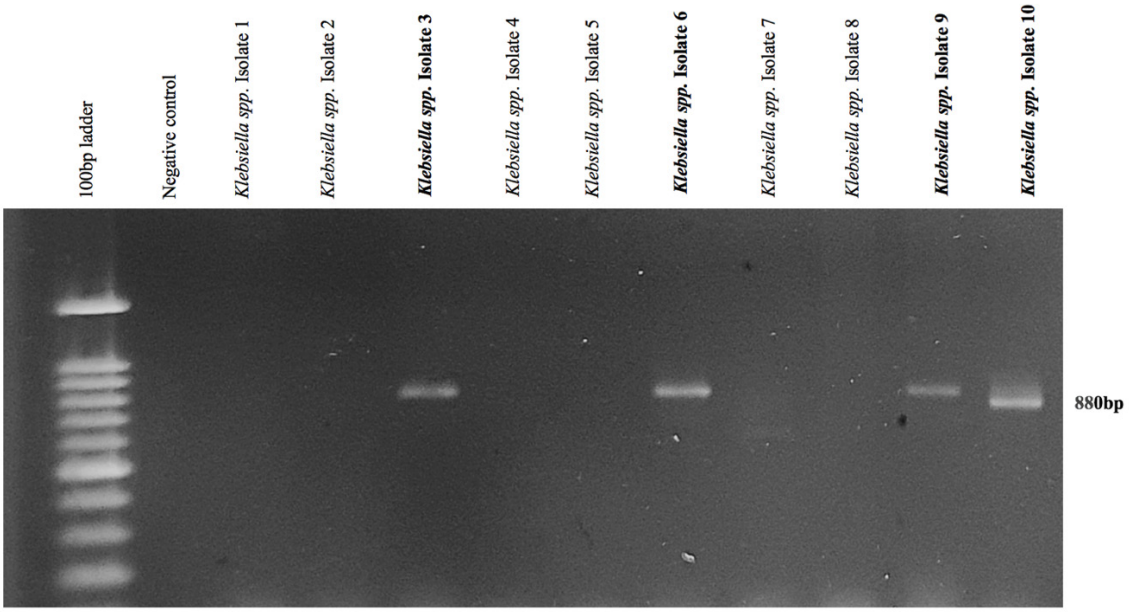


Figure 5. Molecular profiling of *KPC gene* in *Klebsiella spp.* isolates. The presence of a band near 880bp indicates the presence of virulent KPC genes among those isolates. A negative control and a 100bp ladder were also used in this experiment.

Results

Klebsiella spp. identification and characterization

Investigation of broiler chicken cloacal samples collected near Bashundhara R/A, Dhaka, Bangladesh, revealed that 10 out of approximately 30 colonies were positive for *Klebsiella spp.* The identification process involved Gram staining, biochemical tests, as well as assessing growth and colony characteristics on selective and differential media (Figure 3c and SI Table 1). All ten isolates, examined though Gram staining, were identified as Gram negative and rod-shaped bacteria (Figure 3a) as reported in the previous studies (Patel et al., 2017; Rahman et al., 2023). Subsequent biochemical tests were conducted, and the results are presented in Figure 3b and Table 2. Notably, all the isolates exhibited catalase positive, oxidase negative, Simon citrate positive, MR negative, VP positive, nonmotile and urease positive. Interestingly, two isolates (Isolate 2 and isolate 4) out of the ten isolates were indole positive, suggesting the the presence of two different types of species and their inability to produce indole from tryptophan. Additionally, TSI test indicated the yellow slant and yellow butt with gas formation, a characteristic often observed during the identification of *Klebsiella spp.* (Iqbal et al., 2021). The isolates were streaked on nutrient, m-FC, MacConkey, EMB, Endo, XLD, and TSA agar media. In all cases, the isolates displayed a circular, large and shiny appearance. The texture was smooth on nutrient, XLD and TSA agar media, while it was mucoid smooth on m-FC, MacConkey, EMB and Endo agar media. In terms of pigmentation, the isolates were non-pigmented in Nutrient agar media, bluish-gray on m-FC, pink on MacConkey, indicative of lactose fermentation and differentiation among lactose fermenters in *Enterobacteriacae,* mucoid pink and purple on EMB, indicating sucrose or lactose fermentation and the degree of acid production, pink mucoid on Endo, differentiating lactose fermenters from non-lactose fermenters of Gram negative bacteria, creamy white on TSA agar media and yellow on XLD, distinguishing lactose fermenting (yellow colonies) Gram negative bacteria indicating an acidic state of the medium.

Antibiotics susceptibility test

We conducted the Kirby Bauer disk diffusion assay to assess the antibiotics susceptibility of the *Klebsiella* isolates (Yao et al., 2021). In this study, all isolates exhibited resistance to at least three antibiotics commonly used in poultry farms, namely Doxycycline, Ceftazidime, Ampicillin and Cefoxitin. Notably, isolate 2 and isolate 5 demonstrated additional resistance to Azithromycin, while isolate 9 and isolate 10 exhibited further resistance to Streptomycin and Doxycycline. It is important to highlight that these isolates did not display resistant to Tobramycin, Amikacin and Chloramphenicol (Figure 4 and Table 1).

Molecular identification of KPC virulent gene in Klebsiella isolates

A PCR test was performed using KPC-specific primers to detect the presence of KPC virulence gene in the ten isolates. Following gel electrophoresis and staining with EtBr, we successfully identified the desired band 880bp fragment corresponding to the *KPC* virulent gene (Gootz et al., 2009). This molecular assay revealed that 40% of these isolates carried *KPC* virulent gene, with notable inclusion of the most MDR isolates, isolate 9 and isolate 10 as shown in Figure 5.

Discussion

Klebsiella spp. ranks as the second leading cause of pneumonia, following *Escherichia coli,* posing a significant a major threat to both public health and environment (Juan et al., 2020). The transmission of *Klebsiella Spp*. can occur through contaminated food or waste disposal of poultry in water, a factor strongly linked to community-acquired infections in humans. The consumption of poultry products contaminated with MDR and virulent *Klebsiella spp.* can lead to serious conditions such as bacteremia and sepsis (Riwu et al., 2022). While there are numerous reports about MDR *Escherichia coli,* cases involving *Klebsiella spp.* are less frequently reported, despite their ability to rapidly develop resistance to commonly used antibiotics in the poultry farms. Additionally, the prevalence of virulence genes in antibiotic-resistant strains heightens the risk of contracting serious infections (Brendecke et al., 2022; Jain et al., 2021; Poirel et al., 2018; Sivaraman et al., 2020). Hence, consuming contaminated or undercooked broiler chicken poses potential health hazards.

One objective of this study was to isolate and characterize *Klebsiella spp.* from the poultry cloacal samples, as the majority of gut microbiota is shed into the environment in Dhaka, Bangladesh. Differentiation of *Klebsiella spp.* from other *Enterobacteriaceae* was achieved through Gram staining, biochemical tests and growth on selective and differential media. All ten isolates in our study were identified as *Klebsiella spp.* based on Gram staining, lactose fermentation capability and cultural characteristics on various selective and differential media.

To assess the antibiotic sensitivity or resistance of these ten isolates, we utilized a panel of commercially available antibiotics commonly used in Bangladeshi poultry farms. The Kirby Bauer disc diffusion method was employed for the antibiotic susceptibility assay, revealing a notable number of *Klebsiella spp. isolates* resistant to more than three antibiotics, likely attributed to the deliberate use of antibiotics in poultry farms.

Subsequently, a molecular assay by PCR was conducted to investigate the prevalence of KPC virulent genes in these isolates, considered a definitive test for the identifying *Klebsiella* spp. -

specific virulent genes. Our data indicates a significant number of *Klebsiella* isolates harboring this virulent gene in conjunction with resistance to more than three antibiotics.

The significance of this study is not limited to tracking the multidrug resistance and virulence forms of *Klebsiella spp.*, but it also provides a deep insight of public health concerns. This includes the possibility of the transmission of these pathogenic microorganisms from poultry farm handlers and shops to the mass population, as well as the environment. In addition, this study also provides insight into the current antibiotics resistant profile in Bangladesh, which will guide selection of future treatment strategies. Furthermore, from the perspective of One Health approach, these types of studies help recognizing the interconnection of human, animal and environmental health. Hence, understanding the dynamics of resistance and virulence in *Klebsiella spp*. in different settings contributes to a holistic approach to health management. Finally, this study will help to reform the public health policies and guidelines, as well as develop strategies to mitigate the impact of multidrug-resistant and pathogenic strains of *Klebsiella spp.* on healthcare systems.

While our study provides valuable insights, it has some limitations. We did not assess the presence of capsules in the ten isolates, a crucial indicator of bacterial virulence. Additionally, further investigation may involve 16s rRNA sequencing to identify specific *Klebsiella* species. We aim to perform whole genome sequencing of *Klebsiella spp.* Isolate 3, Isolate 6, Isolate 9 and Isolate 10 for a comprehensive understanding of their phylogenetic analysis, metabolic and virulence genes profiling.

Conclusion

This study holds significance in assessing the prevalence of virulent and multidrug resistance *Klebsiella spp.* in the chicken's cloacal samples, highlighting the potential risk of shedding and transmission to the soil and water environment as well as humans. Poultry industries, being the largest animal stock in Bangladesh, necessitate strategic planning to curb disease spread for sustainable future development. In Bangladesh, poultry farms are closely linked to contagious diseases and various zoonotic infections, posing a significant challenge in the current scenario and for strategic planning aimed at eradication and regulation (Hennessey et al., 2021). The widespread presence of MDR bacterial strains, attributed to indiscriminate antibiotic treatments, increases the likelihood of life-threatening illness. It is imperative for the government to issue stricter instruction and implement necessary actions to control the unnecessary use of antibiotics in the poultry farms across Bangladesh.

Author Contributions

S.M.S. conducted experiments and analyzed data, as well as contributed to writing the original draft, reviewing, and editing. T.M.F.S. also conducted experiments, analyzed data, and contributed to writing the original draft, reviewing, and editing. K.T.I. participated in experiments and data analysis. R.N.R. was involved in experiments and data analysis. K.K. contributed to experiments and data analysis. S.M.B.U.I played a role in conceptualization, validation, project management, writing the original draft, reviewing, editing, and supervision.

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Competing financial interests

The author has no conflict of interest.

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