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Pathogenicity of bacterial isolates associated with high mortality in duckling in Behira province

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ABSTRACT

During 2020–2022, a survey of 25 farms of ducklings of various breeds (Pekini and Muscovy), that ranged from one day up to a month of age suffering from various symptoms and mortality rates varied from 10 to 35 % revealed isolation of *Klebsiella pneumonae* 36 % whereas the prevalence of *E coli, Staph aureus and Salmonella Typhimurium* were 32%, 28% and 16% respectively. Following PCR confirmation *of Klebsiella pneumonae,* the presence of the its virulence genes were surveyed and results showed that uge (44%) and rmpA (22%), as well as an antimicrobial susceptibility profile, which revealed 100% resistant to Ampicillin, Amoxicillin and Penicillin G followed by77 % toward tetracycline, 22% toward streptomycin . Contrarily, they were extremely sensitive to ciprofloxacin and enrofloxacin, as well as 77% to cefotaxime and 66% to gentamycin. The surveillance of class 1 integron (intl1) and genes of antimicrobial resistance demonstrated that (66%) of isolates harbor Int1,whereas only 33% of the isolates involved blaSHV, they all had the tetA and blaTEM genes. *Salmonella* and *E. coli* isolates were serotyped, and it was discovered that the *E. coli* isolates belonged to six different O-serogroups, including O55:H7, O148:K25, O86: K61, O114:H21, O26:H11 and O127:H6 while *Salmonella* isolates were recognized as *S. Typhimurium*. . In our investigation, invA gene is present in 100% of the *S. Typhimurium* isolates, while the mgtC gene is present in 75% of them.

INTRODUCTION

Bacterial diseases are the leading cause of mortality globally, and due to haphazard use of antibiotics, antimicrobial resistance has become an emerging threat **(Bhattarai et al. 2021).** *Klebsiella pneumonia* infects both humans and animals worldwide, and these infections are associated with resistance to crucial antibiotics **(Marques et al. 2019),** it consider a major zoonotic bacteria of the Enterobacteriaceae family **(Wang et al. 2020).** In poultry, *K. pneumoniae* is one of the respiratory patho-

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gens causing high mortality in chicks and broilers **(Hamza et al. 2016).** *K. pneumoniae* exhibits variety of virulence elements such as capsules, mechanisms of iron removal, endotoxins, binders, and antibiotic resistance, which have been found to be important in pathogenesis (**Zhang et al. 2018).** The establishment of *K. pneumoniae* strains carrying diverse resistance genes has significantly expanded over the previous few decades **(Wu et al. 2019).** Antibiotic resistance genes can be acquired by bacteria from mobile components, which aid in their distribution throughout various bacteria (**Blair et al. 2015).** Due to their presence on plasmids, pathogenicity islands and transposons, integrons are potent mobile genetic components that can migrate between various bacteria. (**Firoozeh et al. 2019).**

Class 1 integrons are particularly prevalent in gram-negative bacteria, like *K pneumoniae,* and are often documented. In integrons antimicrobial resistance genes were encoded by internal gene cassettes and have two conserved areas, including the 3′ and 5′ conserved segments (3′ CS and 5′ CS), in their structure **(Lima et al. 2014).** In *K. pneumoniae* the expression of ESBL is one way by which bacteria become cephalosporins and monobactams resistant while expression of carbapenemase is the second pathway, that aids in the development of resistance to all obtainable β - lactams **(Riwu et al. 2020).**

One of the most serious infections affecting ducks, salmonella infection which has a huge impact on both public health and the economy **(Yang et al. 2019).** A wide variety of clinical symptoms in poultry, such as septicemic lesions, suppurative dermatitis, and suppurative arthritis, are linked with *Staphylococcus aureus* infection (**Elfeil 2012).** Pathogenicity of it was investigated in an experiment by **Amen et al. (2019)** on 7-day-old chicks and resulted in mortality of 100%, 100% and 26.7% of chicks through subcutaneous injection, oral and intra nasal route.

The goal of this study was to look into the main bacterial infection linked to high duckling mortality rates as well as its virulence characteristics.

MATERIAL AND METHODS

1. Ethical approval:

No experimentation on animals was done as part of this research. Freshly dead and diseased duckling were euthanized then samples were collected in accordance with the regulations of Animal Health Research Institute and the General Organization for Veterinary Services.

2. Sample Collection

A total of 25 farms of ducklings of different breeds (Pekini and Muscovy), range from one day up to a month of age suffering from mortality rates varied from 10 to 35% and different symptoms (depression, lethargy, body weight loss, inactivity, pale combs, reduced water and feed consumption, ruffled feathers,, diarrhea, omphalitis, lameness and arthritis) during 2020-2022 from different localities in Behira province were tested to detect the incremented bacterial isolates.

3. Bacterial Isolation and Identification

The internal organs (liver, heart, lung, kidney, spleen) and blood were collected. The samples initially incubated in buffer peptone water, after that cultivated on different specific media. *E coli* isolation was performed on both Eosin methylene blue and MacConkey agar and identified by (IMVIC) (**Quinn et al. 2002).** *K. pneumoniae* isolation was done on MacConkey and Blood agar and identified based on Gram's staining, and biochemical **(Kumar Arya et al. 2020)** then confirmed by PCR. (ISO 6579 2002) was used for isolation of *Salmonella. Staph aureus* isolation was performed on both Baird parker agar and Mannitol salt agar then subsequently recognized using biochemical tests **(Quinn et al. 2002).**

5. *E. coli* and *Salmonella* Serotyping:

Salmonella serotyped according to (Grimont and Weill, 2007), whereas *E. coli* isolates were serotyped in accordance with **Neter (1973)** in RLQP Animal Health Research Institute using antisera (Sifin diagnostics GmbH, Germany).

6. In-Vitro anti-microbial sensitivity test:

The bacterial resistance profile was evaluated using the agar diffusion technique according to the Clinical Laboratory Standard Institute (**Wayne, 2010**). *K.pneumoniae* isolates were subjected to antibiotic sensitivity test against nine commonly used antibiotics Aminoglycosides (gentamycin10 µg, streptomycin100 µg (high concentration), Cephalosporines (Cefotaxime 30 µg), Fluroquinolones (Ciprofloxacin 5 µg, Enrofloxacin 5 µg), Penicillines (ampicillin 10 µg, amoxicillin10 µg and Penicillin G 10 µg) Tetracyclines (tetracycline 30 µg) .

7. Detection of Resistance Genes and Virulence-Associated Genes

I-DNA extraction

DNA was extracted from samples using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH), according to manufacture method.

II-Amplification of PCR.

For *K. pneumonia* Table (1) illustrates the used primers (supplied from Metabion (Germany).

A 25 µl reaction containing 12.5 µl of Emerald Amp Max PCR Master Mix (RR310A) (Takara, Japan), 1 µl of each primer at a concentration of 20 pmol, 5.5 µl of water, and 5 µl of DNA template was used. The reaction was carried out in a thermal cycler by Applied biosystem 2720.

For Salmonella Table (2) illustrates Salmonella typhimurium's target genes, cycling conditions, amplicon sizes, and primer sequences.

A 25- µl reaction including 12.5 µl of Emerald Amp Max PCR Master Mix(RR310A) (Takara, Japan), 1 µl of each primer at a concentration of 20 pmol, 4.5 µl of water, and 6 µl of DNA template was used. Thermal cycler T3 Biometra was used to carry out the reaction.

III-PCR Products analysis.

The PCR products were separated using 5V/cm gradient electrophoresis on a 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature. Each gel slot had 20 µl of the PCR products put in it for the gel analysis. To estimate the sizes of the fragments, a generuler 100 bp ladder (Fermentas, Thermo, Germany) was employed. A gel documentation system (Alpha Innotech, Biometra) took pictures of the gel, and computer software was used to analyse the information.

Table 1. *K. pneumonia* target genes, Primers sequences, and cycling conditions.

Target gene	Sequences of Primers	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
K. pneumonia 16S-23S ITS	ATTTGAAGAGGTTGCAAACGAT TTCACTCTGAAGTTTTCTTGTGTTC	130	94˚C 5 min.	94˚C 30 sec.	55˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	(Turton et al., 2010)
rmpA (virulence gene)	ACTGGGCTACCTCTGCTTCA CTT-GCATGAGCCATCTTTCA	535			50˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	(Yeh et al., 2007)
uge (virulence gene)	TCTTCACGCCTTCCTTCACT GATCATCCGGTCTCCCTGTA	534			55˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	(Osman et al., 2014)
Int1	CCTCCCGCACGATGATC TCCACGCATCGTCAGGC	280			50˚C 30 sec.	72˚C 30 sec.	72˚C 10 min.	(Kashif et al., 2013)
TetA(A) (AB resist. gene)	GGTTCACTCGAACGACGTCA CTGTCCGACAAGTTGCATGA	576 bp			50˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	(Randall et al., 2004)
blaTEM (AB resist. gene)	ATCAG-CAATAAACCAGC CCCCGAAGAACGTTTTC	516 bp			54˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	(Colom et al., 2003)
blaSHV (AB resist. gene)	AG-GATTGACTGCCTTTTTG ATTT-GCTGATTTCGCTCG	392 bp			54˚C 40 sec.	72˚C 40 sec.	72˚C 10 min.	

Table 2. Salmonella typhimurium's target virulence genes, cycling conditions, amplicon sizes, and primer sequences

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
invA (virulence gene)	GTGAAATTATCGC CACGTTCGGGCAA TCATCGCACCGTC AAAGGAACC	284	94˚C 5 min.	94˚C 30 sec.	55˚C 30 sec.	72˚C 30 sec.	72˚C 10 min.	(Oliveira et al., 2003)
mgtC (virulence gene)	TGA CTA TCA ATG CTC CAG TGA AT ATT TAC TGG CCG CTA TGC TGT TG	677	94˚C 5 min.	94˚C 30 sec.	58˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	(Huehn et al., 2010)

RESULTS

1. Serotypes and Bacterial Incidence

Overall, 36% of cases involved *K. pneumonae*, whereas 32%, 28%, and 16% of cases involved *E coli, S. aureus,* and *S. Typhimurium*, respectively. The majority of the O-serogroups that were identified by serotyping the *E. coli* isolates were O55:H7, O148:K25, O86:K61, O114:H21, O26:H11, and O127:H6, while *Salmonella* isolates identified as *S. typhimurium*

2. Screening of Genes Associated with Virulence

PCR was used to confirm *K. pneumoniae* isolates (fig. 1). Screening these isolates for presence of virulence genes *uge and rmpA* revealed that 4/9 (44%) contain uge (fig.2) and 2/9(22%) contain rmpA (fig.3). All of the *S. Typhimurium* isolates in our study have the invA gene (100%) (Fig.4) while 75% of them have the mgtC gene (fig.4).

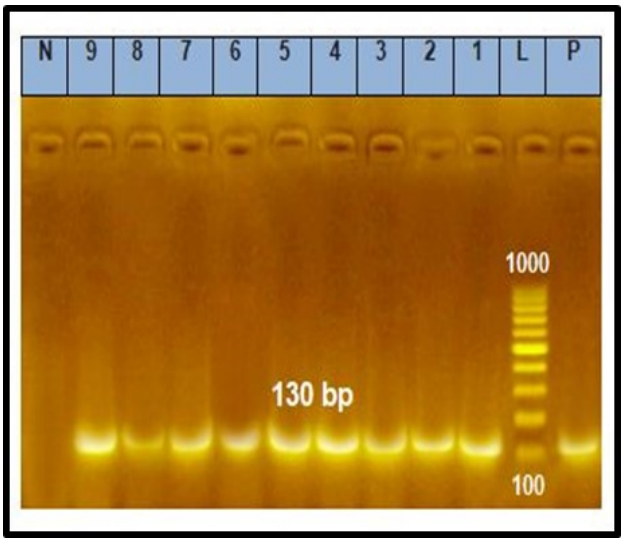

Fig. 1: Electrophoretic pattern *K.pneumoniae*: (L) : ladder, (P) control +ve, (N): control -ve, Lane(1,2,3,4,5,6,7,8,9) True *K. pneumonia 16S-23S ITS*(130 bp).

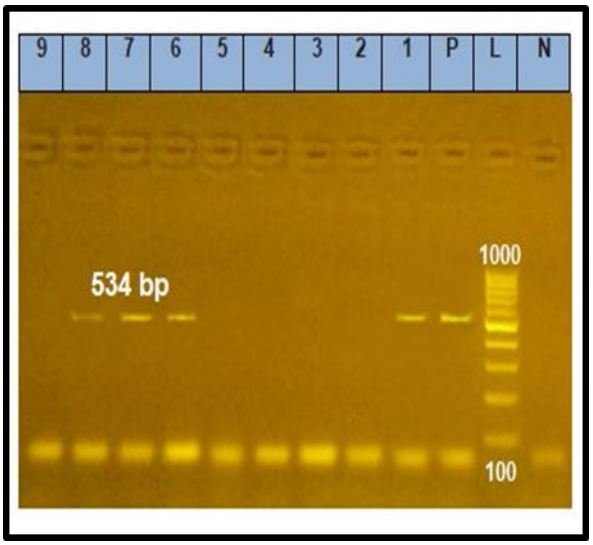

Fig. 2: Electrophoretic pattern *K.pneumoniae uge* virulence gene: (L) : DNA ladder, (P) control + ve, (N): control - ve, Lane(1, ,6,7,8,9) +ve *uge gene*(534 bp).

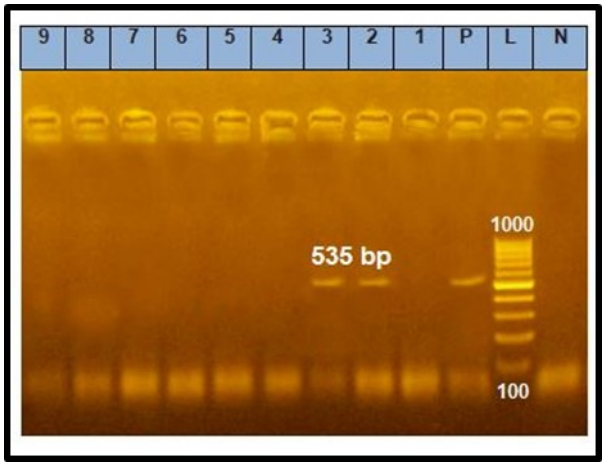

Fig. 3: Electrophoretic pattern *K.pneumoniae rmpA* virulence gene: (L) : ladder, (P)+ve control, (N): -ve control , Lane(2,3) *rmpAgene*(535 bp) positive

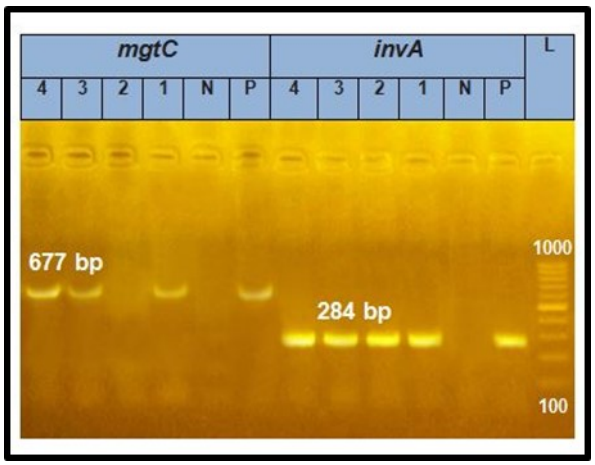

Fig. 4: Electrophoretic pattern *S. Typhimurium* : (L) : ladder, (P) control +ve, (N): control -ve, Lane(1,2,3,4) +ve *S. Typhimurium invA gene* (284bp).lane (1,3,4) positive mgtC gene (677bp).

3. Antibiotic Susceptibility

In-vitro susceptibility testing of *K. pneumoniae* isolates showed varying degrees of resistance to tested antimicrobial table (3) as all isolates (100%) were resistant to Ampicillin, Amoxicillin and Penicillin G followed by77 % toward tetracycline, 22% toward streptomycin . However, they were extremely sensitive to ciprofloxacin and enrofloxacin, as well as77% to cefotaxime and 66% to Gentamycin

Table 3 Percentage (%) Susceptibility Pattern of *K. pneumonae*

	AMP	AMX	Penicillin G	T	S	CIP	ENR	CTX	G
Susceptibility(%)	-	-	-	-	33	100	100	77	66
Intermediate (%)	-	-	-	22	44	-	-	22	33
Resistance (%)	100	100	100	77	22	-	-	-	-

Note: AMP: ampicillin; AMX: amoxicillin; T:tetracycline S: streptomycin; CIP: ciprofloxacin; ENR: Enrofloxacin; CTX: cefotaxime; G: Gentamycin

4. Detection of Antimicrobial Resistance Genes

Screening these isolates for presence of Int1, tet A, blaTEM and blaSHV revealed that 6/9(66%) of isolates involve Int1 (fig.5) and table (4), all the isolates harbor *tetA* (fig.7) and blaTEM genes (fig.6) while only 3/9 (33%) involve blaSHV (fig.8).

Table 4 Association between the occurrence of integrons and AMR phenotype in *K.pneumonae*

Antibiotic	AMP	AMX	Peni-cillin G	T	S	CIP	ENR	CTX	G
No. of resistant isolates, integrons positive (%)	6(66%)	6(66%)	6(66%)	5(55%)	1(11%)	0(0%)	0(0%)	0(0%)	0(0%)
No. of resistant isolates, integrons negative (%)b	3(33%)	3(33%)	3(33%)	2(22%)	1(11%)	0(0%)	0(0%)	0(0%)	0(0%)
No. of sensitive isolates, integrons positive (%)	0(0%)	0(0%)	0(0%)	0(0%)	2(22%)	6(66%)	6(66%)	5(55%)	4(44%)
No. of sensitive isolates, integrons negative (%)	0(0%)	0(0%)	0(0%)	0(0%)	1(11%)	3(33%)	3(33%)	2(22%)	2(22%)
No. of moderately resistant isolates, integrons positive (%)	0(0%)	0(0%)	0(0%)	1(11%)	3(33%)	0(0%)	0(0%)	1(11%)	2(22%)
No. of moderately resistant isolates, integrons negative (%)	0(0%)	0(0%)	0(0%)	1(11%)	1(11%)	0(0%)	0(0%)	1(11%)	1(11%)

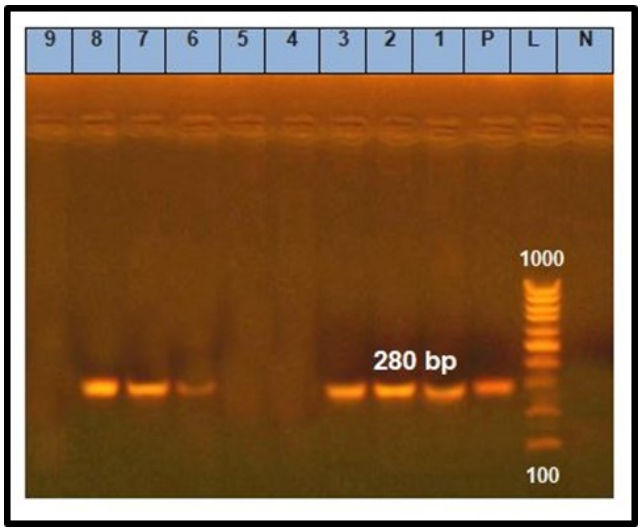

Fig 5: Electrophoretic pattern *K.pneumoniae int 1* gene: (L) : ladder, (P)+ve control, (N): -ve control, Lane (1,2,3,6,7,8) positive *int 1*(280 bp).

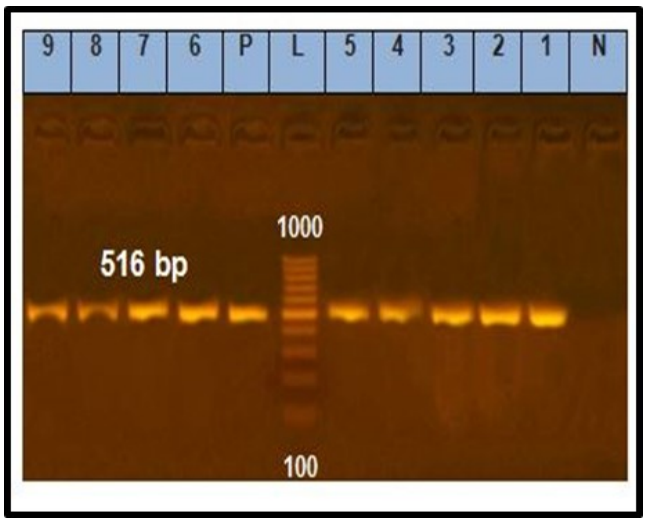

Fig. 6: Electrophoretic pattern K.pneumoniae blaTEM gene: (L) : ladder, (P) control +ve, (N): control-ve, Lane(1,2,3,4,5,6,7,8,9) positive bla TEM (516 bp**).**

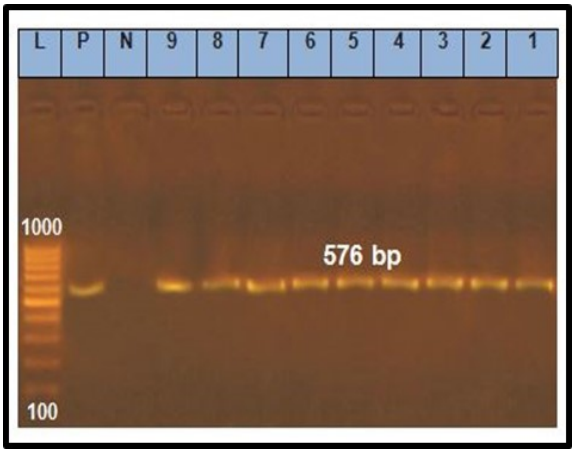

Fig. 7: Electrophoretic pattern K.pneumoniae tet A gene: (L) : ladder, (P)+ve control, (N): -ve control, Lane1,(2,34,5,6,7,8,9) positive tetA gene(576 bp**).**

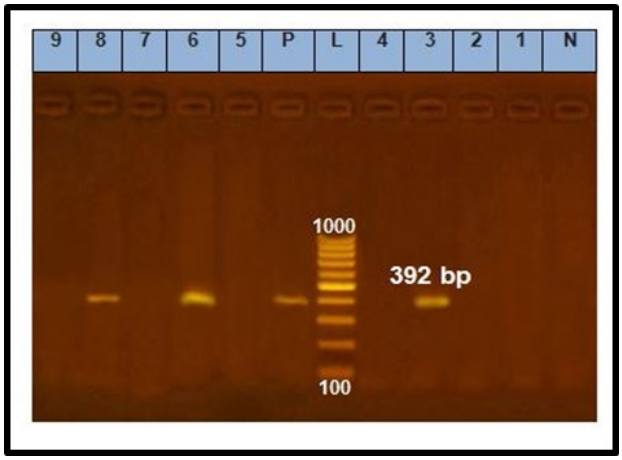

Fig. 8: Electrophoretic pattern K.pneumoniae blaSHV: (L) : ladder, (P)+ve control, (N): -ve control, Laneb (3,6,8) positive bla SHV(392 bp**).**

DISCUSSION

One of the challenges to duck breeding is the early death of ducklings. This investigation revealed the presence of *Klebsiella pneumonae* in 36 % of examined farms (confirmed by PCR fig. 1), compared to the results obtained by **Banerjee et al. (2019) (14.85%) and Mondal et al. (2022)**(16.75%).It reported with *E. coli* as a cause of variable diseases that can lead to death rates of up to 20–30% **(Khelfa and Morsy, 2015).**

One of the many virulence components that contribute to *K. pneumoniae's* pathogenicity is its capsule, which guards it from phagocytosis and potentially fatal serum factors **(Hsu et al. 2011).** Surveying of Uge gene and rmpA revealed their presence in 44% and 22% respectively (Fig.2) and (fig 3), this finding demonstrated that the rmp gene was less prevalent than the uge gene, which may be related to what was mentioned by **Yu et al. (2006)** the prevalence of rmpA has been shown to be less frequent in strains from bacteremic cases than that from liver abscesses. These genes are responsible for invasion, pathogenicity and colonization for *K. pneumoniae* **(Aher et al. 2012)** , they also play a significant role in synthesis of capsule, resistance of phagocytosis, liver abscess, and infection of blood.

Li et al. (2019) attributed high mortality rates of *Klebsiella pneumonae* infection to its antibiotic resistance, making them challenging to treat and manage. In-vitro sensitivity testing *of K. pneumoniae* isolates showed varying degrees of susceptibility to tested antibiotics as all isolates (100%) were resistant to Ampicillin, Amoxycillin and Penicillin G highlighting these antibacterials' poor therapeutic effects for treatment of ducks against *K. pneumoniae*, followed by77% to tetracycline which is lower than that recorded by **Safika et al. (2022)**(95%) and higher than that recorded by **Zaghloul et al. (2021)** (34.1) and **Permatasari et al. (2020)**(35.72%),while his result for resistance toward streptomycin (21.4%) similar to our result (22%) . However, they had a high sensitivity to ciprofloxacin and Enrofloxacin (100% for each) and 77% to cefotaxime compared to the 64.3% reported by **Nnachi et al. (2015),** as well as 66% sensitivity to Gentamycin and the remaining 33% showed moderate sensitive.

Multidrug resistance in bacteria has been mostly disseminated via class 1 integrons **Cambray et al. 2010**, which has been documented in Gram-negative bacteria with its role in the distribution and spread of antimicrobial resistance (**Deng et al. 2015).** Surveying *K. pneumonae* isolates for Int 1 reveal its presence in 67% (6/9) (Fig.5) and table .4 which revealed the spreading of antibiotic resistance among isolated *K. pneumonae* as result of a significant association between the existence of class 1 integrons and the emergence of

MDR (**Li et al. 2013).** Furthermore, analysis of isolated strains for genes associated with antibiotic resistance found that 100, 100, and 33% *K. pneumoniae* possessed the tet A , bla TEM (ampicillin resistance), and blaSHV (responsible for penicllinase hydrolysis) genes respectively. Compared with that reported by **Mondal et al. (2022),** 8.3% and 37.5% of *K. pneumoniae* isolates from ducks were found to possess *bla*SHV and *blaTEM,* while 13.33% and 33.33% were found to be positive, according to **A. Banerjee et al. 2019**. The results illustrated that all bla SHV genes are present in strains positive for the class 1 integron, suggesting that intl1 and blaSHV genes may be carried on the same plasmid. The same observation was reported by **Jones et al. (2005),** while a low rate of association between integrons and ESBL genes was found by **Machado et al. (2007).** Also there was a co-existence of two different ESBL genes (blaTEM and bla SHV) in the same strain in 33.3% of isolates. Resistance to tetracycline is governed by *tet* genes, which are involved in either active efflux of the drug, ribosomal protection or enzymatic drug modification **(Giovanetti et al. 2003).** In spite of presence of tetA gene in all *k. pneumonae* there is 77% resistant and the remnant 22% were moderate sensitive and this result in accordance with that recorded by **Xu et al. (2021),** who examined isolates carried the wild-type tet A gene and found 75.8% of these tet A bearing isolates exhibited a tigecycline (one member of tetracycline) resistant phenotype and attributed this to tet A mutants are often located in different types of plasmids, and these plasmids have different promoter and regulatory sequences that may result in different expression levels of tet A, in addition to occurring mutation of tet A. The examples of these promoters reported by other author as **Zhang et al. (2019)** reported the coexistence of mcr-1 and the tet A variant on the same plasmid from a *K. pneumoniae* isolate in human and **Yao et al. (2020)** also reported coexistence between blaIMP and tet A variant on IncFII plasmid in a clinical *K. pneumoniae* isolates.

E coli was prevalent in 32% of cases, which highlighted its significant involvement as a cause of duckling death. It can cause a wide range of issues, but the most serious sickness strikes them between the ages of 2 and 6 weeks, when fatality rates can approach 43% **(Punnoose et al. 2021).** Numerous authors connected *E. coli* infection with mortality in ducklings, such as **Islam et al. (2004)**(11%), **Bariha et al. (2019)(**55%), Roshdy et al. (2012) (30.8%) in infected ducks that were more than a week old and still alive, while it was (28.4%) in recently dead ducks and **Khelfa and Morsy (2015)(**up to 20-30%) of mortality due to *E coli* with *K. pneumoniae*. The detected *E coli* in this investigation belonged to a variety of serotypes including (EPEC) O55:H7, (isolated from three farms), O86: K61, O114:H21 and O127:H6 (Ørskov and Ørskov, 1992), (EHEC) O26:H11 (Mainil, 1999) and Shiga toxin-producing *E. coli* (STEC) O148:K25.

The findings of this investigation revealed that *Staph aureus* was present in 28% of the samples that were tested, which is greater than the percentages noted by **Eid et al. (2019) (**12.2%) and Amen et al. (2019)(6.6) , drawing attention to its significance as a cause of duckling mortality. *Staph aureus* was identified by **Meyer et al. (2021)** in the event of severe mortality outbreaks in layer flocks, as the predominant isolate from several organs, such as lungs, liver, bone marrow and spleen, in addition to its recovery from 64.1% diseased and dead chickens by **Bakeet and Darwish (2014).** Also an outbreak of omphalitis was reported by **Mondal and Sahoo (2014)** in the week-old ducklings with different clinical (swollen abdomen septicaemia, reduction in feed and water consumption) which resulted in gradual severely dehydrated carcass.

The current study found that the isolation of *salmonella* was 16%, which is greater than the 9.3% reported in **Zhao et al. (2017)'s** study , but virtually in agreement with EL-GAOS et al. (2020) (18.5%), **Abdelaziz et al. (2020)**(14.1) and Lam et al., 2002 (18%). It is consistent with the findings of **Martelli et al. (2016) and Eid et al. (2019)** that *S. Typhimurium* is the most common serotype in ducks that all *salmonella* isolates in this experiment were serologically identified as being. According to report of **Punnoose et al. (2021),** *S.*

typhimurium is responsible for duckling disease and death, particularly during the age of two weeks. It consider one of the main reasons of duckling death in Pekin duck farm suffering 95% mortality According **to (Badr and Nasef, 2016).**

A pathogenic bacteria's ability to infect hosts is enabled by its virulence factors. Pathogenic *Salmonella* have several virulence components that enable them to invade the host, persist there, and eventually spread diseases **(Marcus et al., 2000).** One of the initial stages in the *Salmonell*a spp. pathogenic cycle is intestinal epithelial cell invasion **(Galan et al., 1992).** Invasion of host epithelial cells requires the bacterial membrane protein encoded by the invA gene **(Darwin and Miller, 1999).** All of the *S. Typhimurium* isolates in our study have the invA gene (100%) Fig.4 , which is identical to that found by **Hamed et al. (2023), EL-GAOS et al. (2020) and Abdelaziz et al. (2020),**while 75% of them have the mgtC gene, which, according to Alix and **Blanc Potard (2008),** is essential for intra-macrophage survival and proliferation in magnesium-depleted media as well as for organisms that enter host cells.

Conflicts of Interest

There are no conflicts of interest in this work, according to the authors.

CONCLUSSION

This study highlights the prevalence of pathogenic bacteria linked to duckling mortality, as outlined in *K. pneumonae, E coli, Staph aureus,* and *S. Typhimurium,* together with their virulence factors and patterns of reactivity to antimicrobial agents.

Conflict of interest

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