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Antibiotic resistance and virulence profiles of *Salmonella* serovars isolated from broiler chickens

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ABSTRACT

This study investigated *Salmonella* serovars' antibiotic sensitivity and virulence of *Salmonella* isolated from 86 freshly dead chicks showed white diarrhea. A total of 7 strains out of 334 samples of (liver, spleen, heart blood, intestinal content) were detected. The sensitivity tests were examined against different eight antibiotics: ampicillin, amikacin, gentamicin, cefotaxime, vancomycin, erythromycin, ciprofloxacin, and cefepime. All *Salmonella* strains resisted ampicillin, gentamicin, vancomycin, and erythromycin. *S.* Ruiru exhibited the most extensive resistance profile, being resistant to all antibiotics except cefotaxime. Ciprofloxacin was ineffective against *S.* Typhimurium from liver samples and *S.* Stanleyville. All strains except *S.* Ruiru were susceptible to cefepime. Virulence testing revealed that only *S.* Typhimurium (one strain) and *S.* Ruiru produced a hemolytic zone. *S.* Stanleyville, *S.* Cerro, and *S.* Ruiru displayed lipolytic and starch hydrolysis activity. None of the strains exhibited proteolytic or DNAse activity. Molecular analysis for the resistance genes "*bla*TEM , *ere*A , *aad*B , *qnr*A, *van*A" and virulence gene "*inv*A" gene showed presence of "*inv*A" gene, *bal*TEM gene, and *qnr*A gene in all examined salmonella serovars. While the *ere*A gene was found in all samples except *S.* Typhimurium from the intestine and the *aad*B gene was present in all samples except *S.* Ruiru Although, the *van*A gene was detected only in *S.* Typhimurium from the intestine, *S.* Ruiru and *S.* Cerro. In conclusion, the isolated *Salmonella* serovars exhibited varying degrees of antibiotic resistance and virulence.

1. INTRODUCTION

Salmonella, a rod-shaped, facultative anaerobic, Gram-negative bacteria, is part of the Enterobacteriaceae family (Ryan *et al.*, 2017). This bacterium, which is harmful to humans, is predominantly found in animals used for food, especially chickens (Cosby *et al.*, 2015). Pullorum disease and fowl typhoid are the two most significant bacterial infections affecting chickens. This occurs by *Salmonella* Pullorum (*S.* Pullorum) and *Salmonella* Gallinarium (*S.* Gallinarium*)* which are responsible for pullorum disease and fowl typhoid, respectively (Tadele *et al.*, 2014; Sannat *et al.*, 2017). Both diseases have an incubation period of 4-6 days. Whereas fowl typhoid mainly affects older birds, while pullorum is a septicemic disease that affects young hens (Kwon *et al.*, 2000).

The transmission of *Salmonella* can occur either vertically or horizontally. Vertical transmission occurs through trans-ovarian transmission, which arises from either a systemic illness in the mother bird that causes infection of the ovaries and subsequent egg production in the oviduct, or through the migration of bacteria from the cloaca into the reproductive organs (Dos Santos *et al.*, 2018). Horizontal transmission occurs fecal-oral route through ingestion of contaminated drinking water, contaminated feed, and unclean cages by the feces of infected chicks (Zamora-Sanabria and Alvarado 2017; Yanestria *et al.*, 2019).

Infection of chicken with *Salmonella* may be clinical with apparent signs on birds or subclinical without exhibiting any symptoms, and the bird acts as a carrier for infection mainly occurs in birds over 22 weeks of age, and act as a source for infection for many farms by *salmonella* through their regularly and continuously expelling in feces (Gow *et al.*, 2009; Jajere, 2019). The signs of *salmonella* infection mainly include higher mortality, low-quality hatched chicks, depression, weakness, loss of appetite, and diarrhea that causes feces to adhere to the vent (Wigley *et al.*, 2005). The postmortem examination of the affected birds showed an enlarged, dark, and friable liver with a bronze appearance in case of infection with *Salmonella* Gallinarium (Shivaprasad, 2000).

The global increase in poultry production and output, provided attention of the farmers to the excessive use of antibiotics to improve growth and protect the health of the bird, this led to the appearance of resistant strains of bacteria that may withstand different antibiotics (Page and Gautier 2012; Tiseo *et al.*, 2020). According to The World Health Organization (WHO) there is a list of antibiotic-resistant bacteria that may navigate the research to develop new antibiotics. Among these, *Salmonella* has been identified as a high-priority organism, signaling the urgent need for advancements in antimicrobial treatments (WHO, 2017). These appear as a result of great diversity in *Salmonella* serovars; it may resist to single antibiotics, such as ampicillin and chloramphenicol, or to more than one

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antibiotic as occurs in multi-drug resistance (MDR) *Salmonella* spp. that has been reported worldwide (Ricke and Calo, 2015; Raji *et al.*, 2021). The resistance occurs either through changes in the microorganisms' genetic material or by acquiring resistance genes, that spread between bacteria through different methods such as conjugation, transduction, or transformation, which involve the movement of genetic elements. This transferability of resistance genes is a significant factor in the propagation of AMR (Reygaert, 2018).

So, the current study aimed to examine the antimicrobial sensitivity test for the isolated *Salmonella* strains isolated from freshly dead chickens suffered from white diarrhea collected from different farms in Al-Qalyubia Governorate, Egypt, with a special characterization of virulence and drug resistance genes of the isolated *Salmonella* serovars.

2. MATERIAL AND METHODS

2.1. Bacterial strains:

Seven serotypes of *Salmonella* strains "3 *S.* Typhimurium, 2 *S.* Stanleyville, 1 *S.* Cerro, 1 *S.* Ruiru previously isolated by the same authors according to ISO 6579-1 (2017) and obtained from 86 freshly dead bird collected from different farms in Al Qalyubia Governorate, Egypt, during the period from October 2023 to January 2024. Ethical approval number is (BUFVTM 03-11-23)

2.2. In-Vitro antimicrobial Sensitivity test for Salmonella isolates.

The in-vitro sensitivity test was done for each isolated *Salmonella* strain according to Koneman et al. (1997). *Salmonella* serovars were examined for their susceptibility to 8 antimicrobial discs (Table,1) using disk diffusion assay according to instructions described by CLSI (2023).

Table 1 Antimicrobial standardized discs (Himedia), concentrations and interpretation of their effect (CLSI, 2023)

Antimicrobial disks (Himedia)		Disk Conc.	Zone of inhibition(mm) Resistant ≤ mm (R)	Intermediate mm range (IS)	Sensitive ≥ mm (S)
Ampicillin	AMP/10	10µg	13	14-16	17
Amikacin	AK/30	30µg	16	17-19	20
Gentamicin	CN/10	10 µg	14	15-17	18
Cefotaxime	CTX..30	30 µg	22	23-25	26
Vancomycin	VA/30	30 µg	15	-	16
Cefepime	CPM/30	30 µg	18	19-24	25
Ciprofloxacin	CIP/5	5 µg	20	21-30	31
Erythromycin	E/10	10 µg	11	12-14	15

2.3. Virulence activities of the isolated Salmonella species:

The isolated serovars were examined for their capability to make the following virulence activities: Hemolytic activity, Lipolytic activity, Proteolytic activity, Starch activity (amylase activity), and DNAse activity (Beshiru *et al.*, 2018).

2.4. Polymerase chain reaction for the isolated strains.

The isolated *Salmonella* strains were screened for the presence of five antibiotic resistance genes (*aad*B, *ere*A, *van*A, *bla*TEM, *qnr*A) and one virulence gene (*inv*A) as a common gene for *Salmonella* by using PCR.

DNA extraction was performed using the QIAamp DNA Mini Kit as follows added 200 µl of the sample suspension with 20 µl of proteinase K and 200 µl of lysis buffer and incubated at 56°C for 10 min. After incubation, 200 µl of 96% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations, and the nucleic acid was eluted with 100 µl of elution buffer.

4.4.1. Oligonucleotide primer

Primers used are scheduled in Table (2), supplied from Metabion (Germany)

Table 2 Sequences of the used oligonucleotide primers.

Primers	Sequence ⁻5……3⁻	Amplified product	Reference
inv A	F: GTGAAATTATCGCCACGTTCGGGCAA R: TCATCGCACCGTCAAAGGAACC	284 bp	Oliveira *et al.*, 2003
aadB	F: GAGCGAAATCTGCCGCTCTGG R: CTGTTACAACGGACTGGCCGC	319 bp	Frana *et al.*, 2001
bla$_{TEM}$	F: ATCAGCAATAAACCAGC R: CCCCGAAGAACGTTTTC	516 bp	Colom *et al.*, 2003
ereA	F: GCCGGTGCTCATGAACTTGAG R: CGACTCTATTCGATCAGAGGC	420 bp	Nguyen *et al.*, 2009
qnr A	F: ATTTCTCACGCCAGGATTTG R: GATCGGCAAAGGTTAGGTCA	516 bp	Robicsek *et al.*, 2006
vanA	F: GGCAAGTCAGGTGAAGATG R: ATCAAGCGGTCAATCAGTTC	763 bp	Maharjan *et al.*, 2021

4.4.2. PCR amplification

PCR amplification: The PCR mixture was prepared in a 25 µl reaction containing 12.5 µl of Emerald Amp Max PCR Master mix, 1 µl of each 20 pmol primer, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in applied bios system 2720 thermal cycler with specific cyc ling conditions for each target gene (Table 3). The evaluation of the PCR products was done through using agarose gel electrophoresis and the results was read by the aid of a gel documentation system. (Sambrook *et al.*, 1989).

Table 3 Cycling conditions for each target gene.

Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
aadB	94˚C 5 min.	94˚C 30 sec.	58˚C 30 sec.	72˚C 30 sec.	35	72˚C 10 min.
invA	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 30 sec	35	72˚C 7 min.
bla$_{TEM}$	94˚C 5 min.	94˚C 30 sec.	54˚C 40 sec.	72˚C 45 sec	35	72˚C 10 min.
ereA	94˚C 5 min.	94˚C 30 sec.	60˚C 40 sec	72˚C 45 sec	35	72˚C 10 min.
qnrA	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	35	72˚C 10 min.
vanA	94˚C 5 min.	94˚C 30 sec	54˚C 40 sec.	72˚C 45 sec.	35	72˚C 10 min.

3. RESULTS

3.1. *Invitro* antibiotic sensitivity tests for the isolated strains:

The susceptibility of various isolated *Salmonella* serotypes to a spectrum of antibiotics was evaluated, where–all identified strains demonstrated sensitivity to cefepime, with the exception of *S.* Ruiru. Similarly, all strains except for *S.* Cerro and *S.* Ruiru were sensitive to cefotaxime. amikacin showed effectiveness against *S.* Typhimurium from liver samples and *S.* Stanleyville while showed variable resistance against the other serovars. Resistance was noted in all *Salmonella* strains against gentamicin, vancomycin, and erythromycin. All strains were resistant to ampicillin, *except for S.* Typhimurium from intestinal samples. Only *S.* Ruiru showed resistance to amikacin and cefepime. Ciprofloxacin was ineffective against *S.* Typhimurium from liver samples and *S.* Stanleyville (Fig 1)

3.2. Virulence activities of the isolated strains:

The virulence activity for the isolated *Salmonella* strains showed the ability of *S.* Typhimurium "only one strain" and *S.* Ruiru to produce a clear hemolytic zone in sheep blood agar. While strains *S.* Stanleyville, *S.* Cerro, and *S.* Ruiru showed lipolytic activity and starch activity. And none of them showed proteolytic activity or DNAse activity (Table 4).

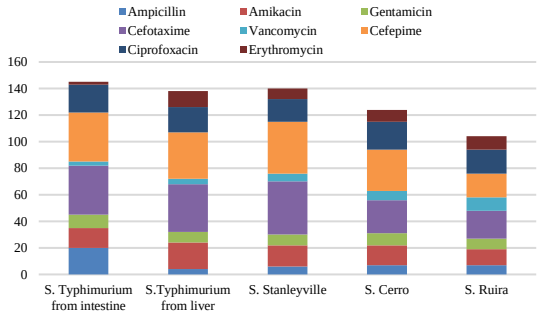

Fig. 1 Antibiotic sensitivity test for isolated serovars.

Table 4 Virulence activity for isolated strains.

Activities	Strains							
	S. Typhimurium		*S.* Stanleyville		*S.* Cerro		*S.* Ruira	
	No.	%	No.	%	No.	%	No.	%
Haemolytic activity	1	14	0	00	0	00	1	14
Lipolytic activity	0	00	2	28.5	1	14	1	14
Proteolytic activity	0	00	0	00	0	00	0	00
Starch activity	0	00	2	28.5	1	14	1	14
DNAse activity	0	00	0	00	0	00	0	00

% is related to the total no. of isolated Salmonella serovars.

3.3. Molecular detection of *Salmonella* resistance genes and virulence genes:

The molecular examination of the resistance genes "*bal*TEM , *ere*A , *aad*B , *qnr*A, *van*A" and virulent gene "*inv*A" in five isolated strains "2 strains of *S.* Typhiymurium isolated from intestine and liver, *S.* Stanleyville, *S.* Ruiru and *S.* Cerro*"* (Table 5) showed the ability of virulent gene "*inv*A" gene, which is associated with *Salmonella* pathogenicity to amplified in 284 bp and *bla*TEM gene, which confers resistance to beta-lactam antibiotics, that amplified in 516bp in all examined samples (Fig. 2a). While *ere*A gene, conferring resistance to erythromycin, able to be amplified in 420bp in all examined samples except *S.* Typhiymurium from intestine and *aad*B gene, conferring resistance to aminoglycosides, intensified at 319bp in all examined samples except *S.* Ruiru (Fig.2b). Moreover, for *van*A, conferring resistance to vancomycin, that amplified in 763bp and appear in *S.* Typhimurium from intestine, *S.* Ruiru, and *S.* C*erro* while absent in *S.* Typhimurium from liver and *S.* Stanleyville and for *qnr*A gene, conferring resistance to quinolones, that intensified at 516bp. and present in all examined samples (Fig. 2c).

Table 5 Resistant genes analysis by PCR

Sample no.	Strain	*inv*A	*bla*TEM	*ere*A	*an*A	*aad*B	*qnr*A
1	*S. typhimurium from intestine*	+	+	-	+	+	+
2	*S.typhimurium* from liver	+	+	+	-	+	+
3	*S. ruiru*	+	+	+	+	-	+
4	*S. cerro*	+	+	+	+	+	+
5	*S. stanleyville*	+	+	+	-	+	+

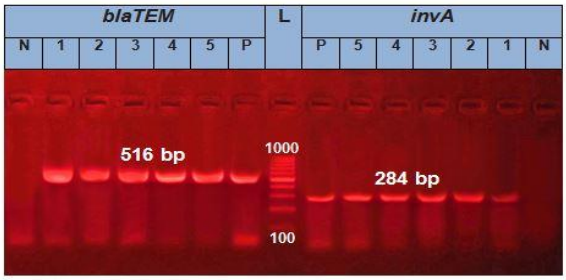

Fig 2a Agarose gel electrophoresis for *inv*A gene (284bp) and *bla*TEM gene (516bp). L: Ladder 100-1000. Lane (P): positive control (for *inv*A gene *Salmonella* ATCC 14028 while for resistance gene the positive control represented by field sample that were previously confirmed to be positive or negative by PCR for the related genes in the Reference laboratory for veterinary quality control on poultry production, Animal health research institute). Lane (N): negative control. Samples 1-5: positive samples for the examined genes

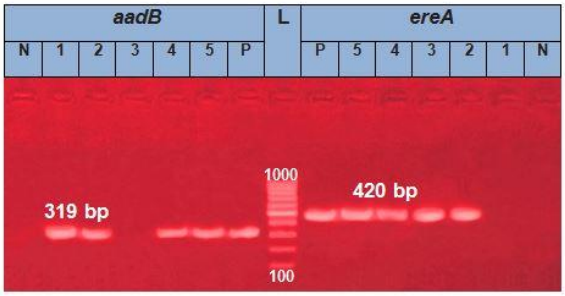

Fig 2b Agarose gel electrophoresis for *ere*A gene (420bp) and *aad*B gene (319bp). L: Ladder 100-1000 Lane (P): positive control (the positive control represented by field sample that were previously confirmed to be positive or negative by PCR for the related genes in the Reference laboratory for veterinary quality control on poultry production, Animal health research institute). Lane (N): negative control. Samples no. 2-5: examined isolates showed positive results for *ere*A gene. Samples no. 1,2,4,5: positive for *aad*B gene

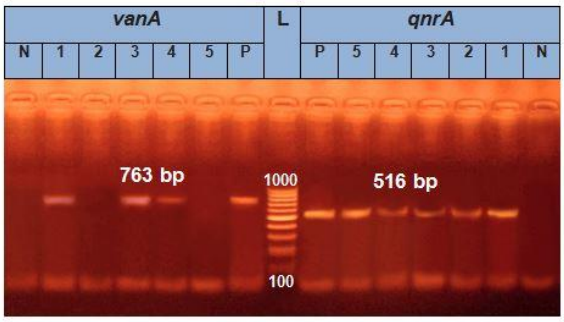

Fig.2.c: Agarose gel electrophoresis for *qnr*A gene (516bp) and *van*A gene (763bp). L: Lader 100-1000. Lane (P): positive control (the positive control represented by field sample that were previously confirmed to be positive or negative by PCR for the related genes in the Reference laboratory for veterinary quality control on poultry production, Animal Health Research Institute). Lane (N): negative control. Samples no. 1-5: examined isolates showed positive results for *qnr*A gene. Samples no. 1,3,4: positive for *van*A gene

4. DISCUSSION

Salmonellosis ranks as one of the most prevalent infectious diseases globally affecting both poultry and humans. It is caused by bacteria known as *Salmonella*. It caused a significant health concern due to its widespread nature and potential impact on public health and the poultry production (Selvaraj *et al.*, 2010; Castiglioni Tessari *et al.*, 2012). To reduce their hazardous effects in poultry production, several farmers tend to use excessive antibiotics to overcome their danger or as a growth promoter, this led to the appearance of multidrug-resistant strains of *salmonella* (Antunes *et al.*, 2016).

The present study focused on the examination of *in-vitro* antimicrobial sensitivity test for the *Salmonella* strains "*S.* Typhimurium, *S.* Stanleyville, *S.* Cerro, *S.* Ruiru. That were isolated from 86 freshly dead chicken, samples represented as "liver, heart blood, spleen, and intestine". All the obtained isolates showed complete resistance to gentamicin, vancomycin, erythromycin, and ampicillin except for *S.* Typhimurium isolated from the intestine showed sensitivity to ampicillin only and resist to others. And showed complete susceptibility to cefotaxime and cefepime except for *S.* Ruiru showed intermediate susceptibility to them. While for ciprofloxacin, the isolates showed varying antimicrobial activity vary from resistance to intermediate resistance. This is nearly similar to Moawad *et al.* (2017) who determined that the majority of *S.* Enterica isolates were resistant to ampicillin (87.0%) and cefotaxime (80.0%), and Tarabees *et al.* (2017) who found that *S.* Typhimurium isolates were resistant to ampicillin, amoxicillin, penicillin, neomycin, ofloxacin, doxycycline, and chloramphenicol, also Awad *et al.* (2020) declared that the isolates from chicken carcasses in Mansoura, Dakahlia Province, were resistant to erythromycin (96.78%), doxycycline (93.55%), streptomycin (80.65%), and amoxicillin 67.8%. In addition, Abd El-Mohsen *et al.* (2022) showed that the obtained

isolates exhibited complete antibacterial resistance to amoxicillin and ampicillin (100%), but (88.88%) resistance to cefotaxime and oxytetracycline (83.33%) to erythromycin, (72.22%) to doxycycline, (66.66%) to neomycin and (61.11%) resistance to amikacin. Somewhat, antibacterial sensitivity was noticed to colistin (55.55%), spectinomycin (44.44%) and norfloxacin (33.33%). while our result contrasted with the data obtained by Helal *et al.* (2019) who determined that the examined *Salmonella* serovars were highly sensitive to doxycycline, chloramphenicol, followed by amoxicillin, ampicillin, gentamycin, and sulfamethoxazole plus trimethoprim. All examined strains showed multidrug resistance as they were resistant to more than one antibiotic, "4 to 5 different antibiotics" This was similar to Elkenany *et al.* (2019), who showed that MDR strains showed resistance against three or more antibiotics in 76.7% of isolates. While Abdeen *et al.* (2018); Sharma *et al.* (2019) and Abd El-Mohsen *et al.* (2022) determined that their isolates showed multidrug resistance to more than five antibiotics.

Regarding the phenotypic virulence activity for the pathogenic strains mainly gives a little picture about the pathogenicity of the examined strains but this isn't helpful for the examined *Salmonella* strains as most of them don't show the activity. For instance, hemolytic activity represents one of virulence activity that measures the ability of the bacteria to produce hemolysins, toxins that damage RBC membranes. However, hemolysis isn't a perfect indicator of virulence. Some highly pathogenic strains might not be hemolytic (Igbinosa *et al.,* 2021). In the present study only one strain of *S.* Typhimurium and *S.* Ruiru was able to make hemolytic activity when cultured into sheep blood agar this is nearly similar to the results previously detected by Singh et al. (2004) who studied the ability of 175 strains of *S.* Enterica isolated from different sources to make hemolysis to 11 different blood agar media made with either non-washed horse/sheep erythrocytes or with washed erythrocytes of cattle, sheep, horse, goat, rabbit, guinea pig, and human A, O and B blood groups. And their results declared that all host restricted *Salmonella* namely, *S.* Enterica serovar Gallinarum, *S.* Enterica serovar anatum, *S.* Enterica serovar abortusequi and *S.* Enterica *serovar paratyphi* B could be divided into different haemolysin types based on their inability to produce hemolysis on one or more types of blood agar, while strains of all zoonotic *Salmonella* serovars induced hemolysis on all the 9 types of blood agar made of washed erythrocytes. None of 175 *Salmonella* could produce hemolytic colonies on blood agar made of non-washed horse/ sheep erythrocytes. The lipolytic activity helps *Salmonella* to obtain nutrients from the host cell but it's not a virulent test for it (Igbinosa *et al*., 2021). According to the current results strains *S.* Stanleyville, *S.* Cerro, and *S.* Ruiru showed lipolytic activity while other strains were unable to utilize it. This disagreed with the previous result of Igbinosa *et al.* (2023) who displayed hemolytic activity and lipase production from *Salmonella* Typhimurium isolate. The ability of the strain to produce protease enzyme and so utilization of protein known as proteolytic activity, all the examined strains unable to produce protease or utilize protein, this was contrasting the results of Beshiru *et al.* (2018) who reported that *Salmonella* Typhimurium exhibits proteolytic activity. The ability of the tested bacteria to degrade DNA is known as DNase activity and this isn't directly linked to virulence but can be used for differentiation among *Salmonella* serovars (strains) (Igbinosa *et al.,* 2021). All the examined strains are unable to produce DNase activity.

The development of resistance could be due to carrying antibiotic-resistance genes (Jian *et al.,* 2021). That is produced by pressure use of antibiotics, even at a very low level (Duarte *et al.,* 2019). In the recent study, we focused on molecular detection of the virulence gene *inv*A gene and the following resistance genes "*bla*TEM, *ere*A, *aad*B, *qnr*A, *van*A" " Table 5". The *inv*A gene, which encodes for protein responsible for epithelial cell invasion of intestine, was detected in all examined *Salmonella* strains. This came in the same line with the previous recorded results of Helal *et al.,* (2019); Roshdy *et al.,* (2020); Tiwari *et al.* (2022) and Ndlovu *et al.* (2023). The *inv*A is specific for *Salmonella* serovars and is considered as a 'gold standard' for genetic typing of *Salmonella*, as pointed out by O'Regan *et al.* (2008).

The generation of beta-lactamase, bacterial enzymes capable of hydrolyzing third-generation cephalosporines, is mediated by plasmid confer resistant mechanism (Rhouma and Letellier, 2017). The Presence of *bla*TEM gene in *Salmonella* poses a serious problem in the treatment and management of salmonellosis in poultry (Wu *et al.,* 2015). In the present study, such gene have been seen in all the examined isolates, this came in harmony with Ammar *et al.* (2019); Yang *et al.* (2019); Zhao *et al.* (2020); Abd El Tawab *et al.* (2021) and higher than that reported by Ahmed *et al.* (2014); Ibrahim *et al*. (2014); Elkenany *et al.* (2019) who reported presence of *bla*TEM gene in 41.5%, 40%, 96% from examined *salmonella* strains respectively.

However, quinolones are mainly used in veterinary medicine for the treatment of *salmonella* (Mehdi *et al.,* 2018). Several studies demonstrated the appearance of resistance genes against it in *salmonella* isolates like *qnr*A, *qnr*B and *qnr*S (Yang *et al.,* 2013). In the present study, quinolone resistance gene *qnr*A was reported in all examined strains, this is higher than that detected by Yang *et al.* (2013); Moawad *et al.* (2017) which reported the presence of *qnr*A gene in 46.6%, 33.3% respectively from examined *salmonella* strains.

The resistance gene for erythromycin *ere*A gene was also examined and according to the detected results, it appears in all strains except *S.* Typhimurium isolated from intestine, this came nearly like Algammal *et al.* (2023) and was higher than that mentioned by Shalaby *et al.* (2022). While the gene confers vancomycin resistant *van*A gene present in all isolates except *S.* Typhimurium and *S.* Stanleyville. And that for aminoglycoside resistant *add*B gene was detected in all examined strains except *S.* Ruiru its part of add family resistance gene coding for aminoglycoside resistance, this is agreed with the study of Shen *et al.* (2023) who determined the presence of aminoglycoside resistance genes in 93.3% from examined *salmonella* strains. Although, the presence of resistance genes in bacteria not always equate to the degree of drug resistance that is found in an isolate. This is because bacteria have various strategies by which they neutralize antibiotics: the efflux pumps, drug-resistance gene mutations, and biofilm formation (Christaki *et al.,* 2020).

5. CONCLUSIONS

The results highlight the widespread antimicrobial resistance among *Salmonella* serovars and the need for effective antimicrobial management policies to mitigate the risk of salmonellosis in the poultry industry.

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