Molecular characterization of *Salmonella* species isolated from pigeon

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Abstract

A total of 109 samples from cloacal swabs from apparently healthy adult Pigeons, pooled organs (liver, lung, and intestine) from apparently healthy and diseased squabs, litter and unhatched eggs were collected from different pigeon houses to investigate the presence of *Salmonella spp.*, an overall rate of 9% was detected. Serotyping of isolates identified *2* Serotypes*, Salmonella* Abortus equi and *Salmonella* Doncaster with a percentage of 60 % and 40 % , respectively. Isolates were tested for antimicrobial susceptibility using disc diffusion method against 16 different chemotherapeutic agents. Using PCR technique for testing the presence of three virulence genes revealed that all examined strains harbored two of targeted virulence genes; *invA, stn* with an incidence rate of 100% for both , and a percentage of 80*%* of isolates have *pef A* gene. Investigating the presence of antibiotic resistance genes by PCR technique demonstrated the existence of *par C* quinolone resistance gene with an incidence rate of 70% , on the other hand, PCR proved total absence of *qnrA* quinolone resistance gene. Testing the existence of *tetA* and *tet B* tetracycline resistance genes by PCR revealed incidence rates of 90% and 40%, respectively.

Key words: *Salmonella spp*., *feral pigeon, PCR, Silent genes*.

Introduction

Pigeon may serve as a reservoir for several pathogenic agents that can be transmitted to poultry, wildlife, domesticated pets, and/or humans via excreta, secretions, or dust from feathers. In addition, ingestion of infected pigeons by wild and domestic animals can also transmit these pathogenic agents to it. ***(*Sousa *et al.,* 2010)**

Feral birds are ones that have escaped from domestication and have managed to establish breeding population in the wildlife as pigeons, doves, parrots, water fowls, ducks, geese and many species of Song birds ***(*Ehrlich *et al.,* 1988).** Two hundred feral pigeons were examined for salmonellosis and considered that the wild birds may constitute a reservoir for important bird pathogens and zoonotic agents ***(*Lillehaug *et al.,* 2005).** *Salmonella* Serovars are one of the primary food-borne pathogens that cause infections in both animals and humans ***(*Akbarmehr, 2010*).*** Many researchers examined free-ranging birds and isolated *Salmonella* as ***(*Brittingham and Temple 1986; Hiltonet *al.,* 1997 and Pennycott *et al.,* 2002*)*** who isolated *Salmonella* from wild free living Pigeon and Sparrows in a garden, and ***(*Hudson *et al.,* 2000*)*** who isolated *Salmonella* from free living pigeons.

Free-ranging birds can be sub-clinical carriers for about 2000 *Salmonella species*, and so they simply are a reservoir of *Salmonella*, it's also recorded that *Salmonella species* vary with geographic location and the types of food consumed, **(Diseases & Treatments, 2009).**

The increase in antibiotic resistance rates is of

concern and constitutes a threat to public health **(Álvarez *et al.,* 2012).** One of the earliest steps in the pathogenic cycle of the facultative intracellular pathogen *Salmonella spp*. was the invasion of the intestinal epithelium, ***inv A*** was a member of this locus, and it was the first gene of an operon consisted of at least two additional invasion genes **(Galan *et al.,* 1992).** The pathogenesis of salmonellosis depends upon a large number of factors controlled by an array of genes that synergizes into the actual virulence of *Salmonella*, namely *Salmonella* enterotoxin (***stn***), and plasmid encoded fimbrial ***(pef****A)* genes **(Murugkar *et al.,* 2003).** Resistance to ampicillin, chloramphenicol, streptomycin, sulphonamides and tetracycline (abbreviated **ACSSuT**) is common, although resistance to other antibiotics and other resistance patterns occur. **(Ridley *et al.,* 1998 and Boyed *et al.,* 2001*).*** High level multidrug resistance is normally associated with mobile genetic elements (e.g. plasmids, transposons, integrons, phages, etc.) that encode specific resistance **(Recchia and Hall., 1995 and Hall, 1997).**

Thus the aim of this study was to investigate and characterize multidrug resistant virulent *Salmonella spp.* Isolated from feral pigeon flocks and high-lightened the importance of applying surveillance in pigeon for better understanding the role of pigeon in transmitting pathogens to environment, human and animals.

Materials and Methods

Samples.

A total of **109** samples from different feral pigeon houses, **45** cloacal swabs from apparently healthy adult birds, pooled organ samples from **31** apparently healthy and diseased squabs (liver, lung and intestine), **29** litter samples and **4** unhatched eggs were collected under aseptic conditions.

Isolation and Identification.

The samples were examined for *Salmonella* isolation and identification according to **ISO 6579:2002 COR. 2004.**

Serotyping.

Salmonella isolates were serotyped in Reference Laboratory for Veterinary Quality Control on Poultry Production by slide agglutination test using commercially available kits (**Test Sera Enteroclon, Anti –*Salmonella*, SIFIN Berlin, Germany).**

Antibiogram.

It was done by using disc diffusion technique, the antibiotic susceptibility was tested against 16 antibiotic discs which are commonly used. Judgment was done according to ***(*Bauer *et al.,* 1966).**

Conventional PCR technique.

Extraction:

Salmonella DNA was extracted using commercially available kit, QIAamp DNA Mini Kit, Catalogue no.51304.

Amplification.

invA gene, *pef*A gene and *stn*gene were amplified according to **(Olivera, 2003 and Murugkar *et al.,* 2003*).*** *qnrA, ParC* quinolone resistance genes were amplified according to ***(*Lunn*et al.,* 2010*),*** *tet A* and *tet B* tetracyclines resistant genes were amplified according to (**Randall *et al.,* 2004).**

Table (1). Annealing temperatures of primers and the size of amplified products required for detecting the tested genes .

Target gene	Annealing Temp.	Size of amplified product bp
invA	55° C	284
pef A	55°° C	700
Stn	59° C	600
ParC	57° C	374
qnrA	55° C	516
Tet A	56° C	576
Tet B	56° C	633

Results

Salmonella spp. was isolated from 10 (22%) out of 45 examined cloacal swabs while no *Salmonella* was detected from neither 29 examined litter samples nor 31 examined birds as pooled samples (liver, intestine, lung), nor 4 eggs with an overall incidence rate of 9% out of the total 109 examined samples .

Table (2). Incidence of *Salmonella* isolates among examined samples.

Type of samples	No. of examined samples	Incidence of *Salmonella* isolation		
		Negative	Positive	
		No.	No.	Percent
Cloacal swabs	45	35	10	22%
Litter	29	29	0	0%
Pooled organs (liver, intestine, lung)	31	31	0	0%
Eggs	4	4	0	0%
Total	109	99	10	9 %

Table (3). Serotypes of *Salmonella* isolates.

Serotype	Antigenic formula			Number of isolates	Percentage of serotypes among iso-
	O	H 1st Phase	H 2nd phase		
Salmonella Abortusequi	4,12	---	E, n, x	6	60%
Salmonella Doncaster	6,8	A	1, 5	4	40%

Serotyping of isolates revealed 6 (60%) *Salmonella* Abortusequi isolates and 4 (40%) *Salmonella* Doncaster out of total 10 detected *Salmonella* isolates.

Table (4). Antibiotic sensitivity and resistance pattern for Ten *Salmonella* Isolates.

Antibiotic group	SN	Chemotherapeutic agent	Symbol	Conc. of disc	Resistant	Sensitive
Penicillins	1	Penicillin	P	5 ug	100%	0%
	2	Amoxicillin	AMX	25 ug	0%	100%
	3	Ampicillin	AMP	10 ug	0%	100%
Nitrobenzene drevatives	4	Chloramphinicol	C	30 ug	16.67%	83.33%
Peptides	5	Colistin	CT	10 u	0%	100%
Macrolydes	6	Erythromycin	E	15 ug	100%	0%
1st Generation Quinolones	7	Flumequine	UB	30 ug	0%	100%
2nd Generation Quinolones	8	Ciprofloxacin	O	5 ug	0%	100%
	9	Norfloxacin	NOR	10 ug	16.67%	83.33%
Aminoglycosides	10	Neomycin	N	30 ug	100%	0%
	11	Gentamycin	GM	10 ug	16.67%	83.33%
	12	Streptomycin	S	10 ug	100%	0%
Tetracyclines	13	Doxycycline	DFX	30 ug	0%	100%
	14	Tetracycline	TE	30 ug	0%	100%
	15	Oxytetracycline	OT	30 ug	0%	100%
Diaminopyrimidine	16	Trimethoprime	SXT	1.25 ug	66.67%	33.33%

The antibiotic susceptability pattern of *Salmonella* isolates for 16 various chemotherapeutic agents were tested, it was observed that amoxicillin, ampicillin, colistin, ciprofloxacin, doxycyclin, tetracycline and oxytetracyclin was 100% effective as 100 % of isolates were sensitive to the aforementioned antibiotics, on the other hand multidrug resistance pattern has been recorded among all isolates. Out of total 10 detected isolates, 100% resistance rate was observed against 4 types of the tested antibiotics which were penicillin, erythromycin, neomycin and streptomycin, 66.67% of isolates were resistant to trimethoprime, and 16.67% of isolates were resistant to 3 tested antibiotics which were chloramphinicol, norfloxacin and gentamycin .

Investigating the presence of *invA, pefA*, and *stn* virulence genes are by PCR technique are shown in figure 1, 2, and 3, respectively.

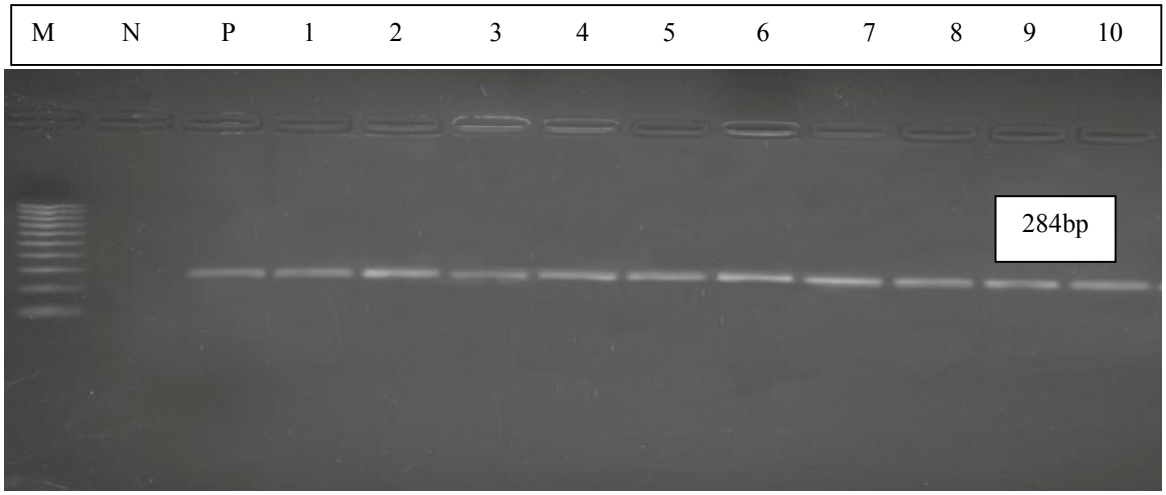

Figure (1). PCR results for *invA* gene ,from left to right, lane (M) 100 bp ladder *(QIAGEN, Gmbh) (100-1000 bp),* lane N (negative control), lane P (positive control), lane (1) to lane (10) are ten positive isolates for *invA* gene specific PCR product with 284 bp.

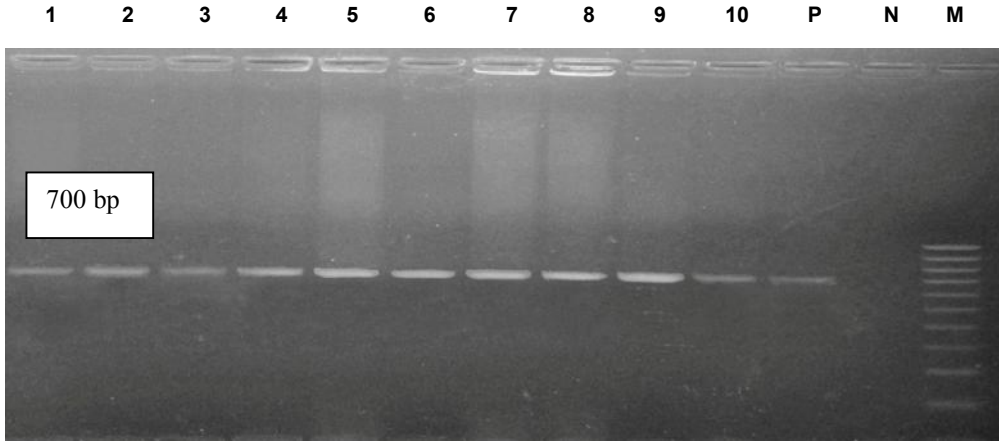

Figure (2). PCR results for *pefA* gene, from left to right, specific amplicon of 700 bp was detected from 1st to 10th lanes . Lane 11: positive control, lane 12: negative control, while lane (M) 100 bp ladder *(QIAGEN, Gmbh) (100-1000 bp).*

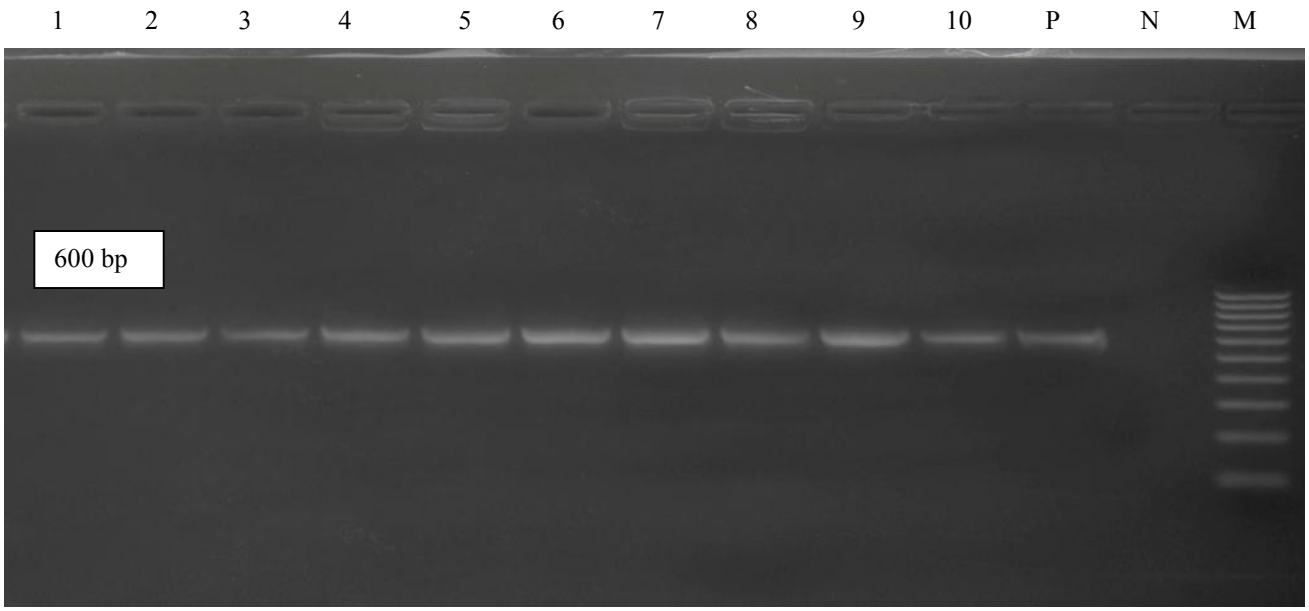

Figure (3). PCR results for *stn* gene, from left to right 1st lane to 10th lane are the positive specific PCR product of 600bp for the ten examined *Salmonella* isolates. Lane 11: positive control, lane 12: Negative control and lane M: 100 bp ladder *(QIAGEN, Gmbh) (100-1000 bp).*

Salmonella isolates were tested by PCR for detection of three virulence genes *invA, pefA* and *stn* genes and detection of the specific PCR products for each which is 284 bp, 600 bp and 700 bp, respectively. PCR results revealed 100 % positive results for both *invA* and *stn* genes, and 80% positive results for *pefA* gene.

Table (5): PCR results for antibiotic resistance genes.

Antibiotic group	Resistance genes	Incidence rate of detection
Quinolones	*ParC*	70%
	qnrA	0%
Tetracyclines	*tetA*	90%
	tetB	40%

PCR technique detected the presence of *ParC* with an incidence rate of 71% while demonstrated the total absence of *qnrA* gene, for tetracycline resistance genes PCR detected *tetA* and *tetB* with an incidence rates of 90 % and 40 %, respectively.

Discussion

In the present study 10 *Salmonella* isolates were recovered out of total 109 examined samples from pigeon with an incidence rate of 9%, higher incidence rates were reported by **(Faddoul and fellows, 1965; Muller, 1965 and Osman *et al.,* 2013)** who recorded incidence rates of 88% and 30%, and 33.3%, respectively. Lower incidence rates were recorded by **(Georgiades and Iordanidis, 2002; Lillehaug *et al.,* 2005*;* Tanaka *et al.,* 2005*;* González *et al.,* 2007*;* and Sousa *et al.,* 2010*)*** who recorded incidence rates of 3.9%, 0%, 3.9%, 4% and 7.9% , respectively.

Host specificity of *Salmonella* was discussed by (**Timothy, 2006**) who reported that *Salmonella* Serovars can be subdivided into three groups on the basis of host prevalence, the first group is host-specific Serovars, these typically cause systemic disease in a limited number of phylogenetically related species for example, *Salmonella* Gallinarum which is almost exclusively associated with systemic disease in fowl, the second group is host-restricted strains, these are primarily associated with one or two closely related host species but may also infrequently cause disease in other hosts for example, *Salmonella* Dublin which is generally associated with severe systemic disease in ruminants, however is potentially capable of infecting other animal species and humans, the third group consists of the ubiquitous *Salmonella enteric* Serovars, such as *salmonella* Typhimurium that usually induce gastroenteritis in a broad range of unrelated host species.

In the pre*sent study Salmonella* Abortusequi was identified with a percentage of 6 (60%) *and Salmonella* Doncaster with a percentage of 4 (40%) from isolated *Salmonella*, The detection of the non-motile *Salmonella* Abortusequi *is* recorded by ***(*Madić *et al.,* 1997**), who isolated the non-motile *Salmonella* Abortusequi with antigenic formula 4,12:- as the single causative agent in each case in an abortion outbreak occurred in a herd of 38 horses. Variation among detection studies that revealed different serotypes was justified in (**Diseases & Treatments, 2009**) which reported that *Salmonella species* vary with geographic location and the types of food consumed. The results also agreed with that of ***(*Tanaka *et al.,* 2005**) who indicated that pigeon feces are a source of several zoonotic agents for birds, animals and humans. Serotypes detection rates vary greatly between studies for example what was recorded by **(Sousa *et al,* 2010*)*** who recorded that 80% were *Salmonella* Typhimurium, 10% was *Salmonella enterica* subsp. *Enterica* serotype 4,12 and 10% was *Salmonella enterica subsp. enterica serotype 4, 12, i,* and ***(*Georgiades and Iordanidis, 2002*)*** who stated that *Salmonella* Typhimurium was the most frequently isolated serotype in pigeons (75.5% of isolates), followed by *Salmonella* Enteritidis (11.3%). *Salmonella* Gallinarum and *Salmonella Hadar* (3.8%), as well as S*almonella* Abony *(1.9%)* were less frequent. The results also agreed with that of ***(*Tanaka *et al.,* 2005)** indicated that pigeon feces are a source of several zoonotic agents for birds, animals and humans.

Antibiogram test revealed that 50% of Salmonella isolates possess multidrug resistance against 4 tested antimicrobials, 33.33% of isolates possess resistance against 6 antibiotics and 16.67% showed resistance against 5 types of antibiotics. This results agreed with that of ***(*Economides *et al., 2*012 and Leonard *et al.,* 2012)** that detected multidrug resistant *Salmonella* isolates against two or more drug classes from pigeon with an incidence rate of 50% and 29.3% respectively.

In this study the detection of multidrug resistant *Salmonella* isolates from pigeon disagreed with different authors as **(Charlene *et al.,* 2000; Kriz *et al.,* 2011)** who stated that all *Salmonella* isolates were sensitive to all 15 and 16tested antimicrobials, respectively and with ***(*Busani*et al.,* 2004)** who reported that multi-resistance was rare in strains from pigeon.

The study detected 100 % resistance rate against penecillin this rate is higher than those detected by ***(*Charlene *et al.,* 2000; Chiu *et***

al.,* 2006; Benacer *et al.,* 2010 and Economides *et al.,* 2012)** who detected resistance rates of 18.2%, 49%, 53.1% and 39%, respectively. But the present study is almost agreed with ***(*Kilonzo *et al.,* 2013*)who recorded resistance rate of 89% against pencillin.

The study detected 100% resistance rate against erythromycin, this result disagreed with that observed by **(Kimpe *et al,* 2002)** who detected resistance rate of 45%.

The result of sensitivity rate to ampicillin was100%, this result agreed with that recorded by **(Kimpe *et al.,* 2002*)**, but higher resistance rates against ampicillin were detected by ***(*Benacer *et al.,* 2010 and Leonard *et al.,* 2012*)*** who recorded 29.7% and 13.3%, respectively.

The study observed 100% sensitivity rate to amoxicillin, almost similar result was recorded by ***(*Leonard *et al.,* 2012)** who observed a sensitivity rate of (86.7%) against amoxicillin.

The study detected100% sensitivity rate to ciprofloxacin, this result agreed with that of **(Busani *et al.,* 2004 and Leonard *et al.,* 2012*)*** who both reported 100% sensitivity to ciprofloxacin.

The present study detected that 100% of examined *Salmonella* isolates were sensitive to colistin this result didn't go with that of ***(*Morales *et al.,* 2012*)*** who reported a resistance rate of 21% to colistin.

The Antibiogram detected 100% sensitivity to tetracyclines, this result almost agreed with that of **(Charlene *et al.,* 2000 and Kimpe *et al.,* 2002*)*** who recorded that sensitivity of *Salmonella* strains to tetracyclines were **81.8%** and **85%,** respectively. Lower sensitivity rates were detected by ***(*Benacer *et al.,* 2010 and Economides *et al.,* 2012*)*** who detected 70.2% and 69% to the same antimicrobial agents, respectively.

The results of antibiotic susceptibility of our study are in variance with some studies and in accordance with others, indicating that antibiotic pattern varies with different isolates, time and development of multiple drug resistant isolates ***(*Holmberg *et al.,* 1984 and Sharada *et al.,* 2010).**

The importance of understanding the association between human salmonellosis cases and animal sources as an important epidemiological factor is needed to successfully control the spread of the infection within communities and to determine the extent to which pigeons might harbor this pathogen and pose a risk to the human population in Egypt, **(Osman *et al.,* 2013)**. Pigeons had *Salmonella spp.* and could disseminate this pathogen in the environment, **(Sousa *et al,* 2010*)***.

It is interesting to investigate the virulence of the **10** detected *Salmonella* isolates by using different sets of primers **as *invA, stn, pefA***, many authors as ***(*Galan *et al.* 1992, Tuchili *et al.,* 1995 and Darwin and Miller, 1999)** selected the *invA* and explained its use, as this gene was necessary for the invasion of the host cell. PCR detected *invA* gene with an incidence rate **of 100% this** result agreed with that of (**Lampel *et al.,* 2000; Ferretti *et al.,* 2001 and Salehi *et al.,* 2005)** who supported the use of **i*nvA*** primer due to its accuracy and uniform distribution among *Salmonella*, thus increased the sensitivity of test. The study detected ***stn*** gene with an incidence rate of **100%** this result agreed with that of (**Prager *et al.,* 1995 and Rozila *et al.,* 2007)** who found that all strains and Serovars of *Salmonella* enterica **(100%)** such as Serovar Typhimurium, Enteritidis, Dublin, Typhi, etc. carried the *Salmonella* enterotoxin determinant *stn* as far as examined in PCR and hybridization studies. PCR result for *stn*gene is also in agreement with that of (**Murugkar *et al.,* 2003*)*** who reported that *stn* gene was widely distributed among *Salmonella* irrespective to Serovars and source of isolation. **Clouthier *et al.,* 1993**mentioned that some genes were known to be involved in adhesion and invasion as *pef*genes. **Baumler *et al.,* 1996,** investigated the role of *pef*operon containing genes for plasmid-encoded fimbria of *Salmonella* Typhimurium, and reported that *pefA*, B, C and D resulted in increasing the virulence of *Salmonella* and caused fluid accumulation in infant mice. **Rahmane *et al.,* 2000** observed that *Salmonella* Typhimurium harbor only genes of *pefA* genes and not *sef.* In the

present study , PCR detected *pef* gene with an incidence rate of 100% this result is similar to that of (**Gulig *et al.,* 1993)** who stated that every isolate of *Salmonella* Typhimurium was found to possess all the virulence genes that were screened for a part from *pefA* and *sopE*, the *pefA* gene which located on a virulence plasmid rather than the bacterial chromosome . The use of Antimicrobial therapy is rarely necessary for *Salmonella* Infection, but in cases of systemic salmonellosis, fluoroquinolones are used for treatment **(Stoycheva and Murdjeva, 2006). Tran and Jacoby. 2002,** stated that Plasmid-mediated quinolone resistance is also emerging and that *qnr, qepA* and *aac (6')-Ib-cr* are plasmid- encoded genes that confer quinolone resistance, *qnr* comprises a group of pentapeptide repeat proteins that protect bacteria against quinolones in a dose-dependent manner**.** Resistance to quinolones is mainly due to: (i) mutations in the quinolone resistance determining regions (QRDRs) of the target genes (*gyrA* and *gyrB,* which encode DNA gyrase, and p*arC* and *parE*, which encode topoisomerase IV), and (ii) low accumulation of the antimicrobial within the cell, mostly with increased efflux due to over expression of the AcrABTolC efflux pump **(Fàbrega *et al.,* 2009).** The PCR result for detection of *parC* gene and *qnrA* gene revealed an incidence rate of 70% and 0 %, respectively, this result is-compatible to that obtained by antibiogram as sensitivity rate detected to flumequin and ciprofloxacin was 100 %**. Meakins *et al.,* 2008,** mentioned that despite wide use of fluoroquinolones such as ciprofloxacin, the levels of resistance to these antimicrobials remain low. PCR results for quinolone resistance genes agreed with that of **(Okusu *et al.,* 1996)** who reported that despite the *qnr*genes have been detected worldwide, the prevalence of the *qnr* genes is still low in *Salmonella spp.* (0.2–3%, reaching 9.8%).Several different *tet* genes have been described as conferring resistance to tetracyclines in *Salmonella* ,the most frequent types of *tet* genes belong to classes A, B, C, D, and G. The *tet* gene of class A was found on plasmids as well as on the chromosome, whereas the genes *tet*(B), *tet*(C), and *tet*(D) were detected on the chromosomes of *S. enterica* bacteria of different Serotypes, the *tet* (A) gene is often part of transposon Tn*1721* has also been described to occur in *Salmonella* **(Cristina *et al.,* 2004).** The incidence rates detected in this study for *tetA* and *tetB* genes by PCR was 90% and 40% , respectively, this result disagreed with that of antibiogram which detected 100 % sensitivity rates against tetracyclines, on the other hand this result agreed to those reported by **(Deekshit *et al.,* 2004**) who observed the existence of a high rate of antibiotic resistance in the *Salmonella* populations prevailing in environmental sources as well as an absence of correspondence between the presence of antibiotic resistance genes, and the exhibition of a the corresponding phenotypic trait of resistance against the respective antibiotic compound. This result also agreed with those of (**Randall *et al.,* 2004** and **Enne and Bennett, 2010)** who reported that a threat may be posed by isolates with silent, but intact, antibiotic resistance genes, such isolates possess wild-type genes that are not expressed, but may convert to resistance by activating expression of the silent genes, they detected silent resistance genes by PCR and DNA sequencing ,of those silent acquired resistance genes; *aadA*, *bla* (OXA-2), *strAB*, *sul1*, and *tet(A*). The present study confirmed that *tetA* gene determinant is present in a higher incidence than that of *tetB*, this result agreed with that recorded by (**Hamada *et al.,* 2003; Asai *et al.,* 2006 and Shahada *et al.,* 2006*)* who stated that the most common tetracycline resistance determinant in chickens belonged **to *tetA*** gene. It was concluded that multidrug resistant virulent strains of *Salmonella spp*. which harbored silent tetracyclines resistance genes were disseminated throughout environment by feral pigeons and may pose a public health threat on man and animals.

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