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Microbiological and molecular studies on *Salmonella spp.* isolated from broilers in Kafr El-Sheikh governorate, Egypt

Mohamed T. Shaaban [1], Hegazy , A.M [2],Sami I.Menisy [3]

1,3-Botany Department , Faculty of Science , Menoufia University, Egypt
2- Animal Health Research Institute , Kafr El -Sheikh Regional Lab ,Egypt
Corresponding author e-mail. dr.sami_lab@yahoo.com

Abstract

Prevalence of *Salmonella* pathogen in poultry in Kafr El - Sheikh governorate, Egypt were analyzed in 100 pooled samples from poultry (liver, spleen , cloacal swab, gall bladder), using culture and PCR based methods. The results showed that *S. enteric* was detected in 10 samples (10%), *S. enterica* serovar Enteritidis , *S. enterica* serovars Typhimurium and non-typable serovars were detected in 4(40%) , 2(20%) and 4(40%) respectively . All isolates were multi-drug resistant .Also, all isolates had drug resistant genes except for only one isolate was *S. enterica* serovar Enteritidis . No integrons were detected.

Keywords. Poultry, Salmonella spp., MDR (multidrug resistance) ,PCR, resistance gene.

1 Introduction

The genus *Salmonella* is phylogenetically clustered in the family of Enterobacteriaceae (Bennasar et al., 2000). Most *Salmonella* are motile, with the exception of the poultry-specific serotypes of *S.gallinarium* and S. pullorum (Grimont et al., 2000). *Salmonella* spp are typically found in soil, water, food, and the gastro-intestinal tract of humans and other animals (Anderson and Ziprin, 2001). *Salmonella* is Gram-negative, intracellular, straight rod shaped, facultative, non-spore forming, and generally motile with peritrichous flagella (Molbak et al., 2006). The bacterium has a width of 0.7 to 1.5 µm and a length of 2.0 to 5.0 µm (Holt et al., 1994).

Most *Salmonella* ferment glucose and produce hydrogen sulfide gas with or without acid; however, *S. typhi* are incapable of producing gas from fermentation of glucose**.** Furthermore, most *Salmonella* are unable to attack lactose and sucrose. *Salmonella* are non-tolerant to oxidase and can convert nitrate to nitrite**.** In addition, lysine and ornithine are decarboxylated by *Salmonella*. *Salmonella* nomenclature is credited to early pioneers such as White, Borman,Kauffmann, Edwards, and Le Minor (Hanes, 2003).

Over the years, the *Salmonella* nomenclature system has been revised several times (Euzeby, 1999). Started by White and followed by Kauffmann, a one serotype-one species concept, known as the Kauffmann and White system, was created based on the somatic " O ", flagella " H " and surface envelope (Vi) antigens (Brenner et al., 2000**;** Grimont et al., 2000). Not long after its creation, the one serotype-one species concept was discontinued, since most serotypes were closely related (Andrews and Baumler, 2005).

There are several methods of pathogen typing systems. They are divided into two categories: phenotypic (conventional, traditional) and genotypic (molecular, PCR-based). Phenotypic systems include serotyping, phage typing, antibiotic resistance (R-type), biotyping, antibiogram, and bacteriocin (Cooke et al., 2007).

Antimicrobial agents have been widely used in poultry to treat infections caused by a variety of bacterial pathogens. However, this widespread use of large quantities of antimicrobials in poultry in some countries, including Egypt, often without professional consultation or supervision, is problematic. Furthermore, the use of

antibiotics as growth promoters in poultry feeds has been permitted worldwide for the last 60 years (Aarestrup, 2005). However, concerns about development of antimicrobial resistance and zoonotic transfer ofantibiotic resistance genes led to withdrawal of approval for antibiotics as growth promoters in European poultry feeds (Castanon, 2007).Antibiotics have long been the first line of defense to prevent bacterial infection, but have lost their potency as bacteria have grown increasingly resistant to treatment (Singer and Hofacre, 2006). Bacterial antimicrobial resistance is a serious emerging public health concern because of the compromised efficacy of antimicrobial agents used in the treatment of infectious diseases (Martinez and Baquero,2002). Intense animal and bird farming, in which antibiotics are routinely used as growth promoting and therapeutic agents, could be a source for development of antimicrobial resistance (Singer and Hofacre, 2006). Multidrug-resistant bacteria carried by animals and birds can enter the human food chain through the consumption of meat or other animal or bird products (Collignon et al., 2005).The present work was aimed to isolate and identify *Salmonella* bacteriologically and by molecular method , detection of resistance pattern phenotypically and characterizing important antimicrobial resistance genes by using PCR .

2 Materials and Methods

2.1. Samples for surveying *Salmonella*

A total of 100 broiler of varying ages (one-day old till 45 day old) were collected from 19 farm in kafr El-sheikh governorate. From each farm 3-5 live and freshly dead birds were taken for isolation and identification of *Salmonella sp.*Pooled samples from liver, spleen, gall bladder and cloacal swabs were collected in sterile containers , and were transported directly to the laboratory as soon as possible on the same day of collection to be cultured .

2.2.Microbiological and molecular analysis (Isolation and identification)

2.2.1-Microbiological methods

Samples were cultured in Rappaport Vassiliadis Broth at 37◦C for 18 hrs. and then subcultured on XLD agar at 37◦C for 24-48 hrs. Isolates were identified as *Salmonella spp.* based on their colony morphology on selective media, biochemical testing (Edwards et al., 1986) and serologically.

2.2.2-Antimicrobial susceptibility testing

The antimicrobial sensitivity phenotypes of *Salmonella spp.* were determined using the Kirby–Bauer disk diffusion method according to the standards and interpretive criteria described by the Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute, 2002).The following antibiotics(Oxiod) were used: amoxicillin–clavulanic acid (AMC), 20/10 µg , Ampicillin (AMP), 10 µg , Cefotaxime (CTX), 30 µg , Ceftriaxone (CRO), 30µg , Ciprofloxacin (CIP), 5 µg , Gentamicin (GEN), 10 µg , Streptomycin (STR), 10 µg; Sulfamethoxazole-trimethoprim (SXT), 23.75/1.25 µg , Tetracycline (TET), 30 µg , Enrofloxacin (ENR), 5 µg , Cefoperazone/sulbactam (SCF), 105 µg , Norfloxacin (NOR) , 5µg, Nalidixic acid (NAL), 30 µg , Ceftazidime (CAZ), 30 µg.

2.2.3-Molecular methods

2.2.3.1-Bacterial DNA preparation for, polymerase chain reaction :

An overnight bacterial culture (200 µL) was mixed with 800 µL of distilled water and boiled for 10 min. The resulting solution was centrifuged and the supernatant used as the DNA template. Amplification reactions were carried out with 10 µL of boiled bacterial suspensions, 250 mM deoxynucleoside triphosphate, 2.5 mM $MgCl_2$, 50 pmol of primers and 1 U of AmpliTaq Gold DNA Polymerase (Applied Biosystems, Roche, NJ, USA). Distilled water was added to bring the final volume to 50 µL.

2.2.3.2-Screening for class 1 and class 2 integrons

The class 1 integron primers, 5′-CS and 3′-CS, which amplify the region between the 5′-CS and 3′-CS of class 1 integrons, were used as previously described (Table 1) (Ahmed et al., 2007). For detection of class 2 integrons, PCR was performed with the primer pair hep 74 and hep 51, which are specific to the conserved regions of class 2 integrons (Ahmed et al.,2007). The reaction products were subjected to electrophoresis in a 1.0% agarose gel, stained with ethidium bromide and visualized under ultraviolet light.

2.2.3.3-Screening for antimicrobial resistance genes

The *Salmonella* isolates were tested for each of bla resistance genes and qnr genes(TEM, SHV, CTXM, OXA and CMY β-lactamase-encoding genes),(genes qnr-A , qnr-B , qnr-S) by PCR using universal primers as described previously (Table 1) (Ahmed et al., 2007).

3 Results

The results are summarized in tables (1- 5). From table(5) it is clear that 10 isolates are belonging to *Salmonella spp.* Results in table (2)showed the incidence of both the positive samples (10%) and farms(36.8%).Ten isolates were confirmed to be *Salmonella* by Multiplex PCR by using primers specific for genus *Salmonella*, OMPCF and OMPCR, (with a target PCR amplicon size of 204 bp). By using other pairs of primers specific for the most common *Salmonella* serovars including *enteritidis* (target size 304 bp) and *typhimurium* (target size 401 bp), the result revealed that 4 isolates belonged to *Salmonella enteritidis* (40 %) , 4 isolates un-typable (40%) and 2 isolates (20%) *Salmonella typhimurium*(Fig.1).

Identified *Salmonella* isolates were subjected to antibiotic sensitivity test. The results are presented in tables 3 and 4.Testing for antimicrobial susceptibility using disk diffusion method revealed that ten isolates with multidrug resistance phenotypes (Table 5). The most commonly observed resistance phenotypes were recorded against ampicillin, ceftriaxone, ceftazidime, cefotaxime , nalidixic acid, norfloxacin , enrofloxacin and ciprofloxacin.

Furthermore, eight isolates showed resistance to multiple extended-spectrum β-lactam antibiotics such as cefotaxime,ceftazidime, ceftriaxone.(Table 5).

PCR identified CTX-M, a narrowspectrum β-lactamase gene, which confers resistance to penicillins and first-generation cephalosporins, in three MDR *Salmonella* isolates ,(Table 5), CTX-M was identified in one isolate of *S. enterica* serovar Enteritidis, two isolates of other *Salmonella spp.*(Table 2).

These isolates were resistant to multiple extended-spectrum β-lactams such as cefotaxime, ceftazidime and ceftriaxone, (Table 5). Also, the extended-spectrum β-lactamase-encoding genes, blaTEM-1w is identified in one isolate of *S. enterica* serovar *typhimurium,* one isolate of *S. enterica* serovar *enteriditis*, two isolates of other *Salmonella* serovar (Table 5).

These four isolates were resistant to multiple extended-spectrum β-lactams such as cefotaxime, ceftazidime and ceftriaxone, (Table 5) none of the tested *Salmonella* isolates were positive for OXA , SHV-12 and CMY-2 β-lactamase encoding genes (Table 5).

Multiplex PCR-screening and DNA sequencing identified the plasmid-mediated quinolone resistance genes, qnrS , qnrB and qnrA in four isolates of *S. enterica* serovar *enteritidis* ,*typhimurium* and other *Salmonella* (Table 5). In addition ,most isolates that were found to harbor quinolone resistance genes were also shown to be resistant to multiple quinolones such as nalidixic acid, ciprofloxacin , norfloxacin and enrofloxacin (Table 5).

4 Discussion

Antimicrobial agents have been widely used in poultry to treat infections caused by a variety of bacterial pathogens. However, this widespread use of large quantities of antimicrobials in poultry in some countries, including Egypt, often without professional consultation or supervision, is problematic.

In this study, 100% of the tested isolates showed multidrug resistance phenotypes, mainly against ampicillin, and its derivatives. Most of theseantimicrobial agents are regularly used in treatment of poultry diseases in Egypt. Similar resistance phenotypes have been detected and isolated from diseased chickens (Yang*et* et al., 2004 ;Johnson et al., 2005 ;Kim*et* et al., 2007;Randall et al., 2011 and Obeng et al., 2012).In this study, microbiological examination confirmed that all isolates were of the genus *Salmonella* .Also, in this study, PCR examination confirmed that all *Salmonella* isolates were of the genus *Salmonella.* Further multiplex PCR analysis identified 2 isolates as *S. enterica* serovar Enteriditis and 4 as *S. enterica* serovar Typhimurium.

Antimicrobial susceptibility testing showed that *Salmonella* isolates displayed multidrug resistance phenotypes, particularly against AMP, CRO, CAZ, and CTX (Table 5). Most of these antimicrobial agents are regularly used in poultry farming (Singer and Hofacre 2006). Similar multidrug-resistance phenotypes of *Salmonella* isolated from poultry have been reported worldwide (Ahmed et al., 2009).

Interestingly, in this study, many isolates of both *S. enterica* serovar *enteritidis* and *S. enterica* serovar *typhimurium* showed resistant phenotypes to the extended-spectrum β-lactam antibiotics such as CTX, CRO and CAZ (Table 5)

These multidrug resistant phenotypes of *Salmonella* are of great clinical significance because β-Lactamases and quinolones and third-generation cephalosporins are considered frontline therapeutic drugs for treatment of typhoidal and other *Salmonella* infections in hospitals (Bradford , 2001; Hohmann , 2001). The ability of bacteria to acquire and disseminate exogenous genes via mobile genetic elements such as plasmids and transposons has been a major factor in the development of multiple drug resistance over the last 50 years.

The increasing incidence of antimicrobial resistance in bacteria has led to great interest in the genetics and mechanisms of resistance evolved by these bacteria to overcome the effects of antimicrobial agents. A substantial proportion of the resistance genes are present on mobile genetic elements, called integrons, which are integrated into the bacterial DNA .Integrons are capable of capturing individual gene cassettes, which mostly encode antibiotic resistance, by a site-specific recombination system. Hence, integrons play a crucial role in the spread of antibiotic resistance genes in bacteria (Mazel, 2006). Class 1and class 2 integrons are widespread among multi-resistant Gram-negative bacteria (Mazel , 2006). In this study, not class 1 nor class 2 integrons can be identified in *S. enterica* serovars (*enteritidis* ,*typhimurium* and non-typable)

β-lactams belong to a family of antibiotics, the members of which have a β-lactam ring. Penicillins, cephalosporins, clavams (or oxapenams), cephamycins and carbapenems are members of this family.In Gram-negative bacteria, resistance to β-lactam antibiotics is primarily mediated by β-lactamases, which hydrolyze the β-lactam ring and thus inactivate the antibiotic (Bradford , 2001). Penicillin derivatives (β-lactams) are broad spectrum antibacterial agents widely used in human and veterinary medicine. Resistance to β-lactams in Gram-negative bacteria is primarily mediated by β-lactamases. In this study, β-lactamase-encoding genes were detected in (60 %) of isolates.

Many different β-lactamases have been described, but TEM-, SHV-, OXA-, CMY- and CTX-M-β-lactamases are the most predominant in Gram-negative bacteria (Livermore and Woodford , 2006). Here, not all groups of β-lactamases were identified, but only bla_{TEM-1} and bla_{CTX-M} were only identified (Table 5). bla_{TEM} was detected in 32.4%of strains isolated from chickens in Korea (Kim et al., 2007), and also from strains isolated in Spain (Mora et al., 2012). Furthermore, bla_{TEM} and bla_{CTX-M} were reported in strains isolated from broiler chickens and turkeys in the United Kingdom (Randall et al., 2011), while bla_{TEM} was identified in strains isolated from poultry in Australia (Obeng et al., 2012).

Table(1): Primers used for PCR and DNA-sequencing

Primer	Sequence(5'to3')	Amplicon size	Target	Reference
***Salmonella* serotyping**				
OMPCF	ATCGCTGACTTATGCAATCG	204**(bp)**	*Salmonella*	**Alvarez *et al*.(2004)**
OMPCR	CGGGTTGCGTTATAGGTCTG			
ENTF	TGTGTTTTATCTGATGCAAGAGG	304	*Enteritidis*	**Alvarez *et al*.(2004)**
ENTR	TGAACTACGTTCGTTCTTCTGG			
TYPHF	TTGTTCACTTTTTACCCCTGAA	401	*Typhimurium*	**Alvarez *et al*.(2004)**
TYPHR	CCCTGACAGCCGTTAGATATT			
Integrons				
5'-CS	GGCATCCAAGCAGCAAG	Variable	Class 1 integron	**Ahmed *et al*.(2007)**
3'-CS	AAGCAGACTTGACCTGA			
hep 74	CGGGATCCCGGACGGCATGCACGA TTTGTA	Variable	Class 2 integron	**Ahmed *et al.*(2007)**
hep 51	GATGCCATCGCAAGTACGAG			
B-lactamases				
TEM-F	ATAAAATTCTTGAAGACGAAA	1080	bla_{TEM}	**Ahmed *et al*.(2007)**
TEM-R	GACAGTTACCAATGCTTAATC			
SHV-F	TTATCTCCCTGTTAGCCACC	795	bla_{SHV}	**Ahmed *et al*.(2007)**
SHV-R	GATTTGCTGATTTCGCTCGG			
OXA-F	TCAACTTTCAAGATCGCA	591	bla_{OXA}	**Ahmed *et al*.(2007)**
OXA-R	GTGTGTTTAGAATGGTGA			
CTX-M-F	CGCTTTGCGATGTGCAG	550	bla_{CTX-M}	**Ahmed *et al*.(2007)**
CTX-M-R	ACCGCGATATCGTTGGT			
CMY-F	GACAGCCTCTTTCTCCACA	1007	bla_{CMY}	**Ahmed *et al*.(2007)**
CMY-R	TGGAACGAAGGCTACGTA			
Plasmid-mediated quinolone resistance				
qnrA-F	ATTTCTCACGCCAGGATTTG	516	*qnr*A	**Robicsek *et al*. (2006c)**
qnrA-R	GATCGGCAAAGGTTAGGTCA			
qnrB-F	GATCGTGAAAGCCAGAAAGG	469	*qnr*B	**Robicsek *et al*. (2006c)**
qnrB-R	ACGATGCCTGGTAGTTGTCC			
qnrS-F	ACGACATTCGTCAACTGCAA	417	*qnr*S	**Robicsek *et al.* (2006c)**
qnrS-R	TAAATTGGCACCCTGTAGGC			

Table (2).Incidence of infection in different farms and samples.

Examined farm	+ sample	Tot. sample	%	+ farm	total	%
Kafr el-sheikh	2	15	13.3	1	4	25.0
Sedi salim	2	23	8.7	2	5	40.0
Desouke	2	17	11.8	1	3	33.3
Mahalet el kasab	1	10	10	1	2	50.0
Sedi ghazy	2	11	18.2	1	2	50.0
Sakha	1	24	4.2	1	3	33.3
total	10	100	10	7	19	36.8

Table (3). The result of different antibiotics against 10 isolates of *Salmonella sp.*

Used antibiotic	**Isolate no.**									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Spectinomycin (SPX)	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**
Tetracycline (TET)	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**
Enrofloxacin (ENR)	**S**	**R**	**S**	**R**	**R**	**S**	**R**	**S**	**R**	**R**
amoxicillin–clavulanic acid (AMC)	**S**	**S**	R	**S**	**S**	**S**	**S**	R	**S**	**S**
Ampicillin (AMP)	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**
Sulfamethoxazole/trimethoprim (SXT)	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**
Ceftriaxone (CRO)	**R**	**S**	**R**	**R**	**R**	**R**	**S**	**R**	**R**	**R**
Nalidixic acid (NAL)	**S**	R	**S**	**R**	**R**	**S**	R	**S**	**R**	**R**
Ceftazidime (CAZ)	**R**	**S**	**R**	**R**	**R**	**R**	**S**	**R**	**R**	**R**
Gentamicin (GEN)	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**
Norfloxacin (NOR)	**S**	**R**	**S**	**R**	**R**	**S**	**R**	**S**	**R**	**R**
Cefoperazone/sulbactam (SCF)	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**
Cefotaxime (CTX)	**R**	**S**	**R**	**R**	**R**	**R**	**S**	**R**	**R**	**R**
Ciprofloxacin (CIP)	**S**	**R**	**S**	**R**	**R**	**S**	**R**	**S**	**R**	**R**

S= sensitive R= resistant

Table (4).Summarized results of antimicrobial sensitivity test

	Degree of sensitivity			
	resistant		**sensitive**	
antimicrobial agent	**no.**	**%**	**no.**	**%**
Spectinomycin (SPX)	0	0	10	100
Tetracycline (TET)	0	0	10	100
Enrofloxacin (ENR)	6	60	4	40
amoxicillin–clavulanic acid (AMC)	2	20	8	80
Ampicillin (AMP)	10	100	0	zero
Sulfamethoxazole/trimethoprim (SXT)	0	0	10	100
Ceftriaxone (CRO)	8	80	2	20
Nalidixic acid (NAL)	6	60	4	40
Ceftazidime (CAZ)	8	80	2	20
Gentamycin (GEN)	0	0	10	100
Norfloxacin (NOR)	6	60	4	40
Cefoperazone/sulbactam (SCF)	0	0	10	100
Cefotaxime (CTX)	8	80	2	20
Ciprofloxacin (CIP)	6	60	4	40

Table(5).Resistance phenotype and prevalence of integrons and resistance genes in *Salmonella* isolated from diseased broilers

No.	Serovar	Resistance phenotypes	Resistance gene
1	***Salmonella sp.***	AMP, CAZ , CRO, CTX	bla_{TEM-1} , bla_{ctx}
2	***S. typhimurium***	AMP, CIP ,ENR,NA,NOR	qnr_{B}
3	***S . enteriditis***	AMC , AMP, CAZ , CRO, CTX	bla_{TEM-1}
4	***S. enteriditis***	AMP, CAZ,CIP,CRO,CTX,ENR,NA,NOR	qnr_{A}
5	***Salmonella sp.***	AMP, CAZ,CIP,CRO,CTX,ENR,NA,NOR	qnr_{S}
6	***Salmonella sp.***	AMP, CAZ , CRO, CTX	bla_{ctx}
7	***S. typhimurium***	AMP, CIP ,ENR,NA,NOR	bla_{TEM-1} , bla_{ctx} , qnr_{S}
8	***S. enteriditis***	AMC , AMP, CAZ , CRO, CTX	-
9	***S. enteriditis***	AMP, CAZ,CIP,CRO,CTX,ENR,NA,NOR	qnr_{B}
10	***Salmonella sp.***	AMP, CAZ,CIP,CRO,CTX,ENR,NA,NOR	bla_{TEM-1}

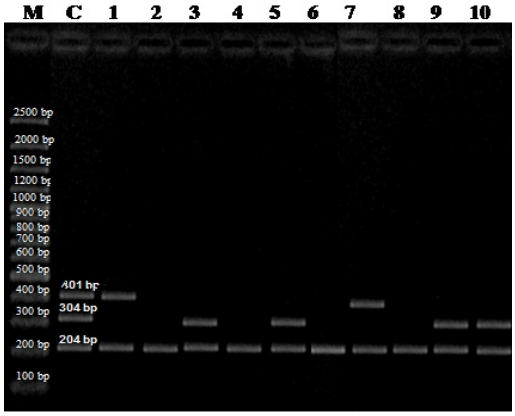

Fig. (1). Agarose gel electrophoresis for PCR results of serotyping of all isolates for detecting genus Salmonella , Salmonella enteric. M : is 100 base-pair size Marker Ladder, Lane C :204 bp control Positive refers to *Salmonella sp.*304 bp control positive refers to the species enteritidis, Lane 1&7refers to the species typhimurium (401 bp control positive), Lane 3,5,9 &10 refers to the species enteritidis,Lane 2,4,6&8 refers to the untyped *Salmonella*e. It is clear from this figure that All isolates were positive *Salmonella sp.* at 204 bp positive control ,4 isolates were positive

In this study, we identified bla_{TEM} in one isolate of *S. enterica* serovar Enteritidis , one isolate of *S. enterica* serovar Typhimurium and two isolates of other *Salmonella spp*.(Table 5). bla_{TEM-1} mediates resistance to AMP and CEF, which is clearly reflected in the resistance phenotypes of these isolates (Table 5). TEM β-lactamase has been previously detected in *Salmonella* serovars in animals in Japan (Ahmed et al., 2009), Egypt (Ahmed et al., 2009) and Korea (Yang et al., 2002). bla_{CTX-M} arise resistance to penicillins, extended-spectrum cephalosporins, and monobactams, and the enzymes are inhibited by clavulanate, sulbactam, and tazobactam. Typically, the CTX-M-ases hydrolyze cefotaxime more efficiently than ceftazidime, which is reflected in substantially higher MICs to cefotaxime than to ceftazidime.

bla_{CTX-M} was detected in one isolate of *S. enterica* serovar Enteritidis and in 2 isolates of other *Salmonella* serovar (Table 5). bla_{CTX-M} has previously been identified and reported increasingly in gram-negative rods (Bradford, 2001,Bonnet, 2004, Eckert, et al., 2004,Woodford, et al., 2004 , Hernandez, et al., 2005, Lartigue, et al., 2005, Naas, et al*.,* 2005, Pitout, et al., 2005) and was characterized and isolated in Germany and Italia(Barthelemy et al., 1992, Bauernfeind et al*.,* 1996)

Quinolones are used extensively to combat bacterial poultry pathogens worldwide. In this study, plasmid-mediated quinolone resistance genes qnr was identified in 40 % of the tested isolates. qnrB was previously reported in a multidrug-resistant strains in Portugal (Pomba et al., 2009),and in Australia (Platell et a*l*., 2010). In Egypt, qnr has been detected in 6.9% of Gram-negative bacteria isolated from farms (Ishida et al*.,*2010). In conclusion, in this study we isolated and identified multidrug-resistant strains of*S. enterica* serovar *enteritidis* and *S. enterica*serovars*typhimurium* from diseased broilers in Kafr El-Shiekh.

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