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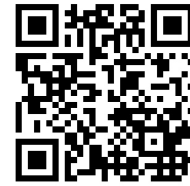

Research Paper

GENOTYPIC CHARACTERIZATION OF ANTIBIOTIC RESISTANT SALMONELLA ISOLATES RECOVERED FROM LOCAL AND IMPORTED POULTRY

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Abstract

Seventeen *Salmonella*serovars were isolated from 260 imported and 140 local poultry sources. The samples were obtained from 58 chickens, 319 ducks and 23 turkeys. The *Salmonella* isolates were tested for their antibiotic resistance by using antibiograms and genotypically. The isolates were screened for the ability to grow in the presence of antibiotics Amoxicillin, Penicillin, Ciprofloxacin, Doxycycline hydrochloride, Nalidixic acid, Norfloxacin, Streptomycin and Trimethoprim /Sulphamethazole) and for the presence of the following genes: *dfrA,aadA2,*bla_{TEM} and *qnrS*. 17 *Salmonella* isolates were recovered from the examined samples, 7 isolates from chickens, 9 isolates from ducks and 1 isolate from turkey.The recovered *Salmonella* isolates belonged to 17 different serovars.*Salmonella* isolates (5.8 to 70.5%) were resistant to the tested anti-microbial agents. The maximum resistance was observed against Trimethoprim (70.5%) followed by Penicillin (41%), Amoxicillin and Streptomycin (29.5% for each), Nalidixic acid (23.5%), Norfloxacin (11.7%), Doxycycline and Ciprofloxacin (5.8% for each). A high multiple antibiotic resistances (MAR) index in a range of 0.25 to 1 was observed in the tested *Salmonella*serovars.

Key words: *Salmonella*, Resistance, MDR, Poultry.

INTRODUCTION

The demand for the production of high quality livestock meatis increasing. However, the poultry livestock production system,despite being the top livestock industry in the Egypt, isconstantly challenged with various microbial diseases suchas salmonellosis that lead to morbidity-linked reduction in productivity and increased cost of disease treatment

Salmonellosis is a zoonotic bacterial disease of national and international importance. The worldwide distribution of salmonellosis often parallels the patterns of trading animal products and food, and the migration patterns of humans and animals [1, 2]

Avian *Salmonella*infections are important as they cause of clinical disease in poultry and constitute a source of foodborne illness to human. Moreover, foodborne *Salmonella*outbreaks can lead to severe economic losses to poultry producers as a result of regulatory actions, market restrictions, or reduced consumption of poultry products.

Non-typhoid *salmonellae*have a broad host range in poultry and mammals, and *Salmonella*Typhimurium is a threat to public health [3].*Salmonella* serotype distribution can give insight in contamination routes and persistence along a production chain. Therefore, it is important to determine not only *Salmonella* prevalence but also to specify the serotypes involved at the different stages of the supply chain [4]
In recent years, an increase in antibiotic resistance has been observed in salmonellae isolated from foods of animal origin. In addition, several authors have reported an increase in the emergence of drug-resistant *Salmonella* strains. Since antibiotics are widely used for growth promotion and disease treatment in commercial poultry production systems, they are now recognized as a potential risk in disseminating multidrug-resistant (MDR) Salmonella spp. [5 – 7]

Information on the antimicrobial resistance profile is applied in combination to the detection of the relevant resistance genes.This characterization can reveal a certain variation if the resistance is due to the acquisition of DNA carried by mobile elements **[8 - 11]**Therefore, for many authors, the preferred approach is to combine phenotypic and biomolecular methodologies to guarantee thecorrect typing of the antibiotic resistance of the different strains**[12, 13]**
The aim of this study was to investigate the correlation between the existed antibiotic resistance phenotype and related resistance genes on *S. enterica* isolates from (local and imported) poultry through the application of antibiograms and the Polymerase Chain Reaction screen on resistant genes. Each antimicrobial was chosen as a representative of its corresponding antibiotic class (penicillins, aminoglycosides, sulfonamides and tetracyclines). Therefore, the screened genes were selected for their assumed capacity to determine resistance specific mechanisms toward the tested antimicrobials.

MATERIALS AND METHODS

SAMPLING:

Total 400 different samples (liver- gall bladder -yolk sac-cecum)were aseptically collected from native and imported chicken, ducks and turkey poultsfor *salmonella* isolation (Table 1). The samples were collected from apparently healthy and diseased poultry that were submitted to the reference Laboratory for Veterinary Quality Control on Poultry Production (RLQP), Dokki .

Bacteriological examination

Isolation of *salmonellae* from food and animal faeces was conducted according to ISO-6579:2002 standard[14] .Suspected Salmonella colonies were identified biochemically according to [15].

Serological identification of salmonellae

Typing of *Salmonella* isolates was performed in the Reference Laboratory for Veterinary Quality Control on Poultry Production using poly- and monovalent specific *Salmonella* antisera [16].

Antibacterial sensitivity test

The antibiotic susceptibility was determined according to the recommendations set by the Clinical and Laboratory Standards Institute **(Clinical and Laboratory Standards Institute, CLSI, 2007)**[17]for the disk diffusion technique. The antimicrobials and concentrations tested were (Amoxicillin25 μg, Penicillin10 μg, Ciprofloxacin5 μg, Doxycycline hydrochloride30 μg, Nalidixic acid30 μg, Norfloxacin10 μg, Streptomycin10 μg and Trimethoprim /Sulphamethazole25 μg) (Oxoid, United Kingdom)The inhibition zones were measured and scored as sensitive, intermediate susceptibility or resistant according to the CLSI recommendations. *E. coli* ATCC 25922 was used as a reference strain for antibiotic disc control.

Multiple antibiotic resistance indexing of isolates (MAR)

The multiple antibiotic resistance (MAR) index is defined as a/b where ‘a’ represents the number of antibiotics to which the particular isolate is resistant and ‘b’ the number of antibiotics to which the isolate was exposed [18]. MAR index values higher than 0.2 are

considered to have originated from high risk sources of contamination like humans, commercial poultry farms, swine and dairy cattle, where antibiotics are often used. MAR index values of less than or equal to 0.2 indicate a strain originated from animals in which antibiotics are seldom or never used.

Genotypic characterization
DNA extraction:
DNA extraction for *Salmonella* isolates was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly,200 µl of the bacterial suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56OC for 10 min.,then 200 µl of 100% ethanol were added to the lysate, followed washing and centrifugation as recommended by the manufacturer. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

Molecular identification of Salmonellaspp by *inv*A gene:
InvasionproteinA(*invA*) genewas thoughttobehighlyconservedinalmost all*Salmonella*serovars andoftenselectedas atargetgene in PCRassaysto differentiate *Salmonella*fromnon-*Salmonella*(Daum*etal*.,2002 andHsuetal.,2011) . The oligonucleotide primers for *invA*gene specific detection were 5´-GTG AAA TTA TCG CCA CGT TCG GGC AA-3´and 5´-TCA TCG CAC CGT CAA AGG AAC C-3´(Rhan et al. 1992).

Detection of Resistance Genes
PCR detection and confirmation of resistance to ampicillin, streptomycin, sulfonamides, and tetracycline were carried out using published primer sequences (Table 3). A uniplex-PCR was used for each of the primer sets of resistant genes *dfrA*, *aadA2*,*bla*$_{TEM}$and*qnrS*. The condition for PCR was an initial denaturation at94 ^{o}C for 3 min, followed by 30 cycles of 94°C for 30 s, 53°C for30 s, and 72 ^{o}C for 1 min, with a single final extension at 72 ^{o}C for7 min. The PCR amplification was performed in a thermal cycler (Swift MiniPro, ESCO.) by using a 2X DreamTaq DNA PCR master Mix (Thermo Scientific).The reaction mixture consisted of 12.5 µl master mix, 3 µl of bacterial DNA, 0.25 µl of each primer in concentration (25 pmol) and nuclease free water up to 25 µl.

RESULTS
The incidence of salmonellae among the examined samples:
Totally, 400 samples were tested for *Salmonella* spp. Out of the tested samples, 4.25% (17/400) were positive for salmonellae In addition to identification of the isolates by biochemical characterization, we further identified them by detecting *inv*A gene, which is commonly used as the PCR diagnostic targets for Salmonella in the food industry and research fields [19, 20]. The results indicated that all the 17 isolates were positive for *inv*A gene. These isolates were serotyped into 17 serovars (Table 4),namely, *Salmonella* Jedburgh, *Salmonella* Harrisanburg, *Salmonella* Braenderup, *Salmonella* Newlands, *Salmonella* Southbank, *Salmonella* SekondiII, *Salmonella* Sinchew, *Salmonella* Brandenburg, *Salmonella* Ruzizi, *Salmonella* Noyao, *Salmonella* Give, *Salmonella* Colindale, *Salmonella* Enteritidis, *Salmonella* Lamberhurst, *Salmonella* Newport, *Salmonella* Grampian *and Salmonella* Nigeria.

Phenotypic resistant pattern in relation to antimicrobial groups:
In vitro sensitivity of 17 isolates of different *Salmonella* serovarswas done against 8 different chemotherapeutic agents representing five antimicrobial groups. The tested isolates were resistant to Trimethoprim (TMP) 70.6 %, followed by beta-lactam antibiotic group Penicillin (P) & Amoxicillin (Amx) 47 % & 41.2, respectively while, the resistant percent detected in aminoglycoside antibiotic groups as Streptomycin (Str) was 29.5 % . In quinolones antibiotic members, the resistance for Ciprofloxacin (Cip) &Nalidixic acid (Na) was 23.5 % but,

Norfloxacin (NOR) achieved the lowest resistant percent (17.6%). Doxycycline representing tetracycline antibiotic group was 23.5% (Table 5)

Phenotypic resistant pattern in relation to *Salmonella*serovars:

Salmonella Nigeria demonstrated highest resistance to all tested antimicrobial agents at a rate of 100 % (Table 4). Five other *Salmonella*serovarsexhibited multi drug resistance against more than two antimicrobial agents including *Salmonella*Noyao (87.5%), *Salmonella*Grampian (75.0 %), *Salmonella* Brandenburg&*Salmonella* Braenderup (50.0%) and finally *Salmonella* Harrisonburg (37.5%)

Gene pattern resistant in relation to *Salmonella*serovars:

According to the tested five antimicrobial groups, beta-lactam, aminoglycosides, quinolones, tetracycline and trimethoprime, the selected antibiotic resistance genes werebla_{TEM} (beta-lactamase), *qnrS* (quinolones), *aadA2* (streptomycin) and *dfrA* (trimethoprime.).The obtained results revealed that, the tested isolates contained gene sequences encoding the beta-lactamase resistance: bla_{TEM} (41.2%),. All the isolates lacked *qnrS*; however, the isolates exhibited 23.5 % resistance against Nalidixicacid (Table 7). In spite of The highest resistance percent detected for Trimethoprim antimicrobial but the resistance gene detection was the lowest (11.8%).Streptomycin was 29.5% with presence of *aadA2* 47.0%.

DISCUSSION

*Salmonella*spp. areamong the very important bacterial pathogen of poultry in the world that cause high economic losses in poultry rearing and food industries. It has been reported that in addition to mishandling of poultry product and raw poultry carcasses, uncooked poultry meat is also one of the most frequent cause of human infection by *Salmonella* spp. [21]

In this study 17Salmonellaserovars were isolatedfrom 400 examined poultry samples with recovery rate (4.25%). This isolation percent comes in accordance with global recovery rates for salmonellae from avian sources [22 – 24]**.**

Imported Day Old Ducklings (DOC) act as potential source of *Salmonella*serovars, as they were isolated from DOC and duckling boxes by 2.8 %. The recovery of salmonella from DOC varied according to study regions as it reached 16.6% from ducks in Iraq [25]or 19.3% in Egypt [26]

The percentage of isolation of *Salmonella* spp. from turkeyin this study was (4.3%), while **Osman *et al.* (2010) recorded** a higher rate (12.6%), and **in (2014) they published** an almost similar isolation rate (4%).of salmonellae from turkey[7, 26].

The isolated 17*Salmonella*serovars were*Salmonella*Jedburgh*, Salmonella*Harrisanburg*, Salmonella*Braenderup*, Salmonella*Newlands*, Salmonella*Southbank*, Salmonella*SekondiII*, Salmonella*Sinchew*, Salmonella*Brandenburg*, Salmonella*Ruzizi*,Salmonella*Noyao*, Salmonella*Give*, Salmonella*Colindale*, Salmonella*Enteritidis*, Salmonella*Lamberhurst*,Salmonella*Newport*, Salmonella*Grampian and*Salmonella*Nigeria (Table 7) did not completely differ from many other studies in the same region [26 , 27].

The isolated serovars results of these study are nearly similar to that of **Adziteye*t al.*, (2012a)**in Malaysia as they isolated 115 *Salmonella*serovars comprising of (37) *Salmonella*Typhimurium*,* (26) *Salmonella*Hadar*,* (15) *Salmonella*Enteritidis*,* (15) *Salmonella*Braenderup, (14) *Salmonella*Albany, and (8) *Salmonella*Derby from ducks and their environmental sources[28].

Antimicrobial agents are valuable tool to treat clinical diseases and to maintain healthy and productive animals. in addition to the human health concerns. While antimicrobial resistant pathogens pose a severe and costly animal health problem [29].Unfortunately , data on the prevalence of antimicrobial resistance in veterinary pathogens are sparse , particularly in developing countries , where antimicrobials are overused in veterinary medicine and food animals [30].

As shown in Table (4),most of *Salmonella*serovars were sensitive to quinolone antimicrobial agents (Ciprofloxacin , Nalidixic acid &Norfloxacin) as 14 from 17 strains were sensitive to Ciprofloxacin with percentage 82%., 13 strains were sensitive to Norfloxacinand Nalidixic acid

with percentage 76.5%. The highest resistance was detected to Trimethoprime with percentage 70.5%. Beta-lactamase members in this work (Penicillin and Amoxicillin)were active against tested *Salmonella*serovars by 53.0 and 58.5 %, respectively.
The resistance phenotype to TMP, P and Amx is exhibited in the majority of the 17 isolates from the three types of avian origin (Chicken, Duck & Turkey) (Table 5). *Salmonella* Nigeria, which was isolated from chickens, showed a complete resistance toward all tested antimicrobial agents.
In this study, the serovar*Salmonella*Enteritidis isolated from the chicken sample did not display any resistance to the tested antibiotics; this result comes in agreement with **Ghosh*et al.* (2002)**[31], but completely differedfrom other authors[32 , 33]. Ahmed and Shimamoto (2012) found that a large number of isolates were resistant to Am, Sxt and Te in *Salmonella*Enteritidis isolated.
In recent years, an increase in antimicrobial drug resistance, including resistance to nalidixic acid, among *Salmonella* spp. has been observed in many countries particularly in Asia[34 - 37].but this is opposite to our result as most of our strains are sensitive to nalidixic (61.5%).
The emergence of multidrug resistance was a matter of concern and it was observed in our result as 7 strains (41.1%)., namely serovars: *Salmonella* Harrisonburg, *Salmonella*Braenderup, *Salmonella*Brandenburg, *Salmonella*Noyao,*Salmonella*Give,*Salmonella*Grampian and *Salmonella*Nigeria. This result agreed with many authors[39, 40].
The gene sequence *qnrS* was absent in all of the isolates. In particular, *Salmonella*Jedburgh&*Salmonella* Colindale did not exhibit any of the gene investigated. All tested isolates showed 47.0% resistance against Penicillin representing beta-lactamase antibiotic group and possessed gene of *bla*$_{TEM}$, 41.2% (Table 7). A resistance gene for Streptomycin was found in 47.0 %, a percentage higher than resistance percent (29.5%).
A high percentage of the *Salmonella spp.* exhibited resistance to the various test antibiotics. The high prevalence of multiple drug resistant bacteria in this region is of epidemiological concern, as this will restrict the choices of antibiotics in the treatment of typhoid fever to a few compounds. A number of virulence factors have been identified and characterized in *Salmonella* species that contribute to bacterial virulence [41– 45].

Table (1): Number, source and typesof examined samples:

	Chickens	Ducks	Turkeys	Total
Local	**35**	**21**	**0**	**56**
Imported	**23**	**288**	**23**	**334**
Total	**58**	**319**	**23**	**400**

The isolation of S. enterica was conducted according to the EN ISO

Table (2) Uniplex PCR primers used for identification of genes and corresponding antimicrobials

Gene designation	Oligonucleotide sequences (5'-3')	Corresponding antimicrobial	Amplicon size(in bp)	Reference
dfrA	AGC ATT ACC CAA CCG AAA GT	Trimethoprim	817	Huovinen *et al*., 1995
	TGT CAG CAA GAT AGC CAG AT			
aadA2	TGTTGGTTACTGTGGCCGTA	Streptomycin	622	Walker *et al*., 2001
	GATCTCGCCTTTCACAAAGC			
***bla*$_{TEM}$**	ATCAGCAATAAACCAGC	Beta- lactamase	516	Colom *et al*., 2003
	CCCCGAAGAACGTTTTC			
qnrS	ACGACATTCGTCAACTGCAA	Quinolones	417	Robicsek *et al*., 2006
	TAAATTGGCACCCTGTAGGC			

Table (3): The incidence of salmonellaeamong the examined samples:

Types of flocks	No. of Examined samples	No. of Positive samples	%
Chickens	58	7	**12**
Ducks	319	9	**2.8**
Turkeys	23	1	**4.3**
Total	400	17	4.25 *

[*] *The percentage was calculated according to the total number of samples examined.*

Table (4): Multidrug resistance pattern for salmonella isolated from poultry

	Amoxicillin	**Penicillin**	**Doxycycline**	**Naldixic acid**	**Streptomycin**	**Ciprofloxacin**	**Norfloxacin**	**Trimethoprim**	**Total Resistant antimicrobial**	**Resistant (%)[b]**
***Salmonella* Jedburgh**	S	S	S	S	S	S	S	R	1	12.5
***Salmonella* Harrisonburg**	R	R	S	R	S	S	S	S	3	37.5
***Salmonella* Braenderup**	R	R	I [a]	S	R	S	S	R	5	63.0
***Salmonella* Newlands**	S	S	S	S	S	S	S	R	1	12.5
***Salmonella* Southbank**	S	S	S	S	S	S	S	R	1	12.5
***Salmonella* SekondiII**	S	S	S	S	S	S	S	R	1	12.5
***Salmonella* Sinchew**	S	S	S	S	S	S	S	R	1	12.5
***Salmonella* Brandenburg**	R	R	S	S	R	S	S	R	4	50.0
***Salmonella* Ruzizi**	S	I [a]	S	S	S	S	S	R	2	25.0
***Salmonella* Noyao**	R	R	I [a]	R	R	R	R	S	7	87.5
***Salmonella* Give**	I [a]	S	S	S	S	I [a]	S	R	3	37.5
***Salmonella* Colindale**	S	S	S	S	S	S	S	R	1	12.5
***Salmonella* Enteritidis**	S	S	S	S	S	S	S	S	0	0
***Salmonella* Lamberhurst**	R	R	S	S	S	S	S	S	2	25.0
***Salmonella* Newport**	S	S	S	S	S	S	S	R	1	12.5
***Salmonella* Grampian**	S	R	I [a]	R	R	I [a]	R	S	6	75.0
***Salmonella* Nigeria**	I [a]	R	R	R	R	I [a]	I [a]	R	8	100

[a] **I:** Intermediate antibiotic profile considered as resistant **-** [b] **resistant (%):** Total number of resistant antimicrobial agents / total number of tested antimicrobial agents

Table (5): The percentage of sensitive and resistance of 17 *salmonella* serovars isolated against 8antibiotic discs.

	Antibiogram Phenotypic Pattern					
	Resistance				Sensitive	
	Resistant	Intermediate	Total	**%***	**No.**	**%***
Amoxicillin	5	2	7	41.2	10	58.5
Penicillin	7	1	8	47.0	9	53.0
Doxycycline	1	3	4	23.5	13	76.5
Nalidixic acid	4	0	4	23.5	13	76.5
Streptomycin	5	0	5	29.5	12	70.5
Ciprofloxacin	1	3	4	23.5	13	76.5
Norfloxacin	2	1	3	17.6	14	82%
Trimethoprim	12	0	12	70.6	5	29.4%

*% according to the total number of examined isolates (17 isolates)

Table (6): MARS index analysis of *salmonella* isolates.

isolates	**No. of antibiotics to which the isolate was resistant (a)**	**MAR index(a/b*)**
***Salmonella* Nigeria**	8	1
***Salmonella*Noyao**	7	0.88
***Salmonella*Grampian**	6	0.75
***Salmonella*Braenderup**	5	0.63
***Salmonella*Brandenburg**	4	0.5
***Salmonella*Harrisanburg**	3	0.37
***Salmonella*Give**	3	0.37
***Salmonella*Lamberhurst**	2	0.25
***Salmonella.* Ruzizi**	2	0.25

*No. of antibiotic to which the isolates were subjected= 8(b)

Table (7) antibiotic resistance and resistance gene in salmonella isolates

	Serovar	**Penicillin (P)**			**Nalidixic acid (Na)**			**Streptomycin (Str)**			**Trimethoprim (TMP)**		
		Phenotypic		R-Gene	Phenotypic		R-Gene	Phenotypic		R-Gene	Phenotypic		R-Gene
		S	R	*BlA*$_{tem}$	S	R	*qnrS*	S	R	*aadA2*	S	R	*dfrA*
DUCK	*Salmonella* Jedburgh	+	-	-	+	-	-	+	-	-	-	+	-
	*Salmonella*Harrisonburg	-	+	+	-	+	-	+	-	-	+	-	-
	Salmonella. Braenderup	-	+	+	+	-	-	-	+	-	-	+	+
	Salmonella Southbank	+	-	+	+	-	-	+	-	-	-	+	-
	Salmonella SekondiII	+	-	+	+	-	-	+	-	-	-	+	-
	Salmonella Sinchew	+	-	+	+	-	-	+	-	-	-	+	-
	Salmonella Brandenburg	-	+	+	+	-	-	-	+	-	-	+	+
	Salmonella Ruzizi	-	I(+)	-	+	-	-	+	-	+	-	+	-

	Salmonella Give	+	-	-	+	-	-	+	-	+	-	+	-
Chicken	*Salmonella*. Noyao	-	+	-	-	+	-	-	+	+	+	-	-
	Salmonella Colindale	+	-	-	+	-	-	+	-	-	-	+	-
	Salmonella Enteritidis	+	-	-	+	-	-	+	-	+	+	-	-
	Salmonella Lamberhurst	-	+	-	+	-	-	+	-	+	+	-	-
	Salmonella. Newport	+	-	-	+	-	-	+	-	+	-	+	-
	Salmonella. Grampian	-	+	-	-	+	-	-	+	+	+	-	-
	Salmonella Nigeria	-	+	-	-	+	-	-	+	+	-	+	-
T	*Salmonella* Newlands	+	-	+	+	-	-	+	-	-	-	+	-
Total		**10**	**8**	**7**	**13**	**4**	**0**	**12**	**5**	**8**	**5**	**12**	**2**
(%)		**53.0**	**47.0**	**41.2**	**76.5**	**23.5**	**0**	**70.5**	**29.5**	**47.0**	**29.4**	**70.6**	**11.8**

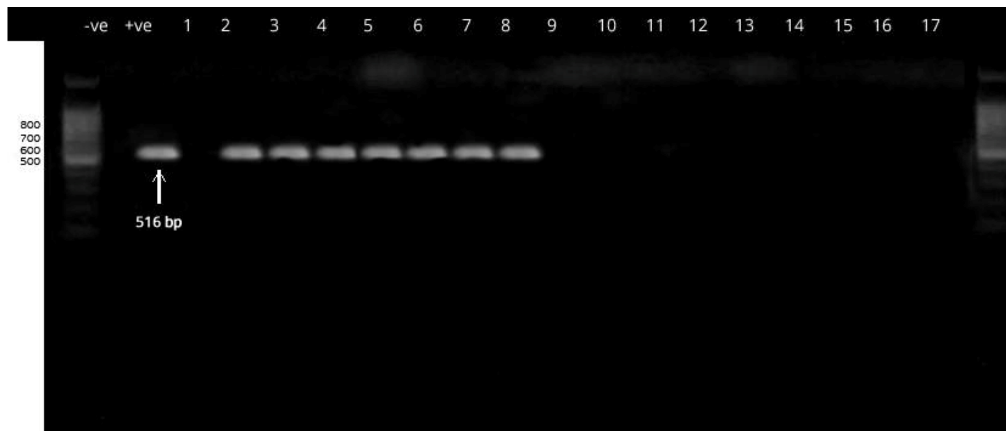

Photo no. (1)Amplification of 516 bp fragments of primers specific for *bla*$_{TEM}$ gene

Photo no. (2) Multiplex PCR allowed no amplification product for the 417 bp of qnrS gene.

Photo no. (3) Amplification of 622 bp fragments of primers specific for aadA2gene.

Photo no. (4)Amplification of 817 bp fragments of primers specific for dfrA gene.

REFRENCE

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