Kafrelsheikh Vet. Med. J. Vol. 13 No. 2 (2015) (129-148)

OCCURRENCE OF QUINOLONE RESISTANCE GENES AMONG *SALMONELLA* SPECIES ISOLATED FROM CHICKENS

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ABSTRACT

Resistance to antimicrobial agents within nontyphoidal Salmonella serotypes is considered a serious problem worldwide so this study was aimed to investigate the wide spread Plasmid-mediated quinolone resistance between different Salmonellae isolated from chickens .The antimicrobial susceptibility was applied on all isolates then PCR was applied for most resistant isolates to quinolones to detect the Plasmid-mediated quinolone resistance genes (qnrA, qnrB, qnrS). Forty one Salmonella isolates (9.1%) were obtained from 450 samples. All isolates exhibit resistance against all antimicrobial agents except amikacin in which give no resistance. All isolates showed resistance with 100% against nalidixic acid and flumequin but 4 isolates showed the lowest resistance (9.7%) to levofloxacin. Five isolates were sensitive to amoxicillin giving the lowest percentage (12.1%). Twenty two Salmonella isolates from a total of 41 isolates were examined by PCR for Plasmid-mediated quinolone resistance genes (qnrA, qnrB, and qnrS). while 19 Salmonella isolates not examined as they were less resistant to quinolones. Five isolates (5/22) (22.7%) were positive for qnrA, 2 isolates (2/22) (9%) were positive for qnrB and 4 isolates (4/22) (18%) were positive for qnrS.

Keywords: *Salmonella,*Plasmid-mediated resistance genes,chickens, Egypt**.**

INTRODUCTION

Avian salmonellosis is a problem of economic concern to all phases of poultry industry from production to marketing. As a result of extensive use of antibiotics in human and veterinary medicine, serious increase in the spreading of multiple antibiotic resistant *Salmonella* has occurred ***(Cruchaga et al., 2001)***. Antimicrobial resistance is a global public health problem. Although all countries are affected, the extent of the problem in the developing nations is unknown ***(Ang et al., 2004)***. First, *qnr*A1 from a clinical strain of *Klebsiella pneumoniae* isolated in Alabama was described. This strain carried plasmid pMG252, which contained the gene encoding quinolone resistance, later named *qnr*A1 ***(Martinez et al., 1998)***. Another *qnr* gene from a *Shigella flexneri* isolated during an outbreak of food poisoning in Japan. This strain contained a plasmid, designated pAH0376, containing a gene with high similarity to *qnr,* which was designated *qnr*S. The *qnr*S protein was also a 218-amino acid protein with 59% similarity to *qnr*A that conferred low level resistance to fluoroquinolones ***(Hata et al., 2005)*.** In 2006, Jacoby and colleagues described a third gene encoding quinolone resistance, *qnr*B. This gene was first found in a *Klebsiella pneumoniae* isolate from India and encoded a 214- amino-acid protein of the pentapeptide repeat family, which had 41% amino acid identity with *qnr*A and 39% amino acid identity with qnrS ***(Jacoby et al., 2006)*.** To date, a total of 6 *qnr*A, 4 *qnr*S, and 20 *qnr*B variants have been described in the literature and are listed in the database maintained at the website

http://www.lahey.org/qnrStudies ***(Jacoby et al., 2008)***. Later, other transferable resistance genes were found to cause reduced susceptibility to quinolones. The aminoglycoside acetyltransferase variant *aac*(6_)Ib-cr is able to modify ciprofloxacin and norfloxacin. Moreover, in 2007, in Japan, but also in Belgium, another gene, *qep*A, was discovered to encode a putative specific efflux pump, which is able to reduce susceptibility to hydrophilic quinolones. ***(Cavaco et al., 2008)***. Furthermore, a second variant, named *qep*A2, from France was recently described ***(Cattoir et al., 2008)*****.** Recently, Wang and colleagues described another *qnr* gene, *qnr*C, which was found in Proteus mirabilis; however, its sequence is not yet publicly available, but it was found that *qnr*C encodes a 221-amino-acid protein with different amino acid identities from *qnr*D, which indicates that the gene is different from *qnr*D ***(Wang et al., 2008)*****.** *qnr*D, which has been found to cause reduced susceptibility to fluoroquinolones in isolates of *Salmonella enterica serovar* Bovismorbificans and Kentucky strains isolated from humans in the Henan province of China. The complete plasmid was sequenced, and the novel *qnr*D gene was cloned along with both the *qnr*A1 and *qnr*S1 genes, which were cloned for comparisons of the susceptibility patterns in vitro. The novel *qnr*D gene shares similarities with the previously described *qnr* genes and encodes a putative pentapeptide repeat protein that is able to confer reduced susceptibility to fluoroquinolones. A phylogenetic analysis shows that it clusters separately from the known *qnr* genes and variants**.** ***(Cavaco et al., 2008)*** Here, we investigated the

presence of Plasmid-mediated resistance genes of *Salmonella* isolated from chickens in Egypt.

MATERIAL AND METHODS

2.1. Sampling and isolation of *Salmonella:*

A total of 450 samples from apparently healthy and diseased chickens were collected and transferred immediately in ice box to bacteriology laboratory under aseptic condition. The samples were first pre-enriched in buffered peptone water at 37◦C for 18–24 h. Then, a 0.1 ml of pre-enriched broth was transferred to 10ml of Rappaport Vassiliadis broth and incubated at 37 ◦C for 18 h, subcultured on MacConky and xylose lysine deoxycholate (XLD) agars, and incubated at 37 ◦C for 24–48 h. Suspected Salmonella spp., based on colony morphology on the selective media, were identified biochemically ***(according to ISO 6579 (2002) method*** and also confirmed by API 20E system (BioMérieux, Marcy-L'Etoile, France).

2.2. Serological typing of Salmonella:

The isolates that were identified biochemically as *Salmonella* were subjected to serological identification according to Kauffman-White Scheme ***(Kauffman, 1974)*** for determination of somatic (O) and flagellar (H) antigen.

2.3. Antimicrobial susceptibility testing:

The antimicrobial susceptibility testing was done according to ***Finegold and Martin (1982)*** using the agar disc diffusion method on

Mueller Hinton agar (Oxoid) plates. The used antimicrobial agents were Nalidixic acid (30µg), Flumequine (30µg), Ciprofloxacin (5µg), Enrofloxacin (5µg), Norfloxacin (10µg), Levofloxacin (5µg), Amoxicillin (10 µg), Ampicillin – sulbactam (20µg), Cefotaxime (30µg), Ceftriaxone (30µg), Ceftazidime (30µg), Neomycin (30µg), Amikacin (30 µg), Gentamycin (10µg), Streptomycin (10µg), Oxytetracyclin (30µg) and Sulfamrthoxazole- Trimethoprim (25µg). The zones of inhibition that formed were measured to assess resistance or susceptibility according to the interpretation criteria established by ***(CLSI) standard (2005).***

2.4. Detection of resistance genes in isolated Salmonellae using PCR

2.4.1. Bacterial DNA preparation for PCR

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 was used as the DNA template. Amplification reactions were carried out with 10 µl of boiled bacterial suspensions, 250 µm deoxynucleoside triphosphate, 2.5 µm MgCl2, 50 pmol of primers and 1U of AmpliTaq Gold DNA Polymerase (Applied Biosystems, Roche, NJ, USA). Distilled water was added to bring the final volume to 50 µl. After PCR reactions, the reaction products were subjected to electrophoresis in a 1.0% agarose gel, stained with ethidium bromide and visualized under UV light.

2.4.2. Screening for plasmid mediated quinolone resistance genes

The *Salmonella* isolates were tested for plasmid-mediated quinolone resistance genes *qnr*A, *qnr*B, and *qnr*S with primers described in **Table (1)**

RESULTS AND DISCUSSION

3.1. Isolation and identification of *Salmonella*

Salmonella spp. are important food-borne pathogens that are demonstrating increasing antimicrobial resistance rates in isolates obtained from food animals and humans. Increased antimicrobial resistance has been attributed to various animal and human sources via clonal dissemination and direct selection pressure through the use of antimicrobial agents ***(Angulo and Griffin, 2000).*** In this study 450 samples were examined for presence of *Salmonella*. Forty one (9.1%) out of 450 birds were found positive for *Salmonella* isolation. The results in this study agreed with ***El-Morsi (1998)*** who isolated *Salmonella* species from liver samples of chicks with an incidence of 12%, ***Rehan (2004)*** who isolated *Salmonella* species from broiler chickens with an incidence of 12% and ***Ammar et al., (2010)*** who identified the most common *Salmonella* serovars in broilers and laying breeding reproducers in Eastern Algeria and isolated *S.* Typhimurium with incidence of (12%) but the results in this study nearly in coordinated with ***Schluter et al., (1994)*** who isolated *Salmonella* from laying chicken flocks 13.3% and ***Dahal (2007)*** who examined 400 chicken samples to detect the prevalence of *Salmonella* in them which was 13%. On the other hand the results of this study differ from ***Osman (1992)*** who collected 150 random samples from different broiler farms, and isolated 45 *Salmonella*

strains with an incidence of 30%, ***Schluter et al., (1994)*** who isolated *Salmonella* from chicken broiler flocks with an incidence of 6.2%, ***Mohamed et al., (1999)*** who isolated *Salmonella* from 200 faecal samples from living chickens at Kafr El- Sheikh Governorate, the incidence of isolation was 2.5%, ***Jafari et al., (2007)*** who isolated *Salmonella* from 422 apparently healthy chickens in Iran, five samples 5.8% out of 85 pooled samples were positive for Salmonella and ***Islam et al., (2006)*** who recorded 33 *Salmonella* isolates from layer chickens in Dhaka and Gazipur regions of Bangladesh.

3.2. Salmonella serotyping

Salmonella isolates were serotyped using poly and monovalent "O" and "H" antisera and the results of this study revealed that 18 strains were isolated(8) (19.5%) *S.* Typhimurium, (1) (2.4%) *S.* Apeyeme, (4) (9.7%) *S.* Kentucky, (1) (2.4%) *S.* Daula, (6) (14.6 %) *S.* Newport, (3) (7.3%) *S.* Tamale, (3) (7.3%) *S.* Molade, (1) (2.4%) *S.* Colindale, (1) (2.4%) *S.* Lexington, (2) (4.8%) S. Bargny, (2) (4.8%) S. Enteritidis, (1) (2.4%) *S.* Papuana, and (1) (2.4%) *S.* Labadi, (2) (4.8%) *S.* Santiago (2) (4.8%) *S.* Magherafelt, (1) (2.4%) *S.* Rechovot (1) (2.4%) S. Takoradi, and (1) (2.4%) S. Angers. The results in this study reported that *S.* Typhimurium participated with the higher percentage from the isolated serotypes (19.5%) by 8 isolates while *S.* Apeyeme, *S.* Daula, *S.* Colindale, *S.* Lexington, *S.* Papuana, *S.* Labadi, *S.* Rechovot *S.*Takoradi, and *S.* Angers participated with the lower percentage (2.4%) by one isolate for each of them. These results agreed with ***Verma and Gupta (1995)*** who isolated *S.* Typhimurium with a percentage of (18.10%)

While these results differ from ***Edel et al., (1991)*** who recorded S. Entertidis with an incidence of 1.4% for laying flocks and 1.1% for broiler breeder flocks in Netherland in 1990, ***Osman (1992)*** who isolated 45 Salmonellae from different broiler farms with an incidence of 30% and the serological typing revealed 21 (46.7%) *S.* Pullorum, 9(20%) *S.*Gallinarum, 7 (15.6%) *S.* Typhimurium and 8 (17.8%) *S.* Entertidis, ***Ibrahim (1995)*** who isolated 55 strains of *Salmonella* species, 22 were from chicks, 18 from broilers and 15 from layers. The isolated serovars were *S.* Montevideo, *S.* Typhimurium, *S.* Entertidis, *S.* Lexington, *S.* Infantis, S. Reading, S. Cerro, S. Hadar and S. Tennesee, ***Toh et al., (1996)*** who reported 608 isolates of *Salmonella* at the Central Veterinary Laboratory Singapore. A total of 560 of the isolates belonged to 72 serotypes and 14 serogroup while other 48 were untypable. The commonest serotypes isolated were *S.* Typhimurium (23.8%), *S.* Weltevereden (10%), *S.* Bockly (5.1%), *S.* Brraenderup (3.9%) and *S.* Entertidis (3.3%), ***EL-Zeedy et al., (2007)*** who isolated 17 isolates of *S.* Typhimurium and one isolate of *S.* Kentucky, ***Bonyadian et al., (2007)*** who determined the prevalence of Salmonellae contamination of chicken carcasses in slaughterhouses in central Iran (Yazd province). Serological tests showed that *S.* Typhimurium was the main contaminant of the samples (52.2%). solated serotypes were *S.* Newport (15.6%), *S.* Enteritidis (12.2%), *S.* Havana (8.9%), *S.* Dublin (5.6%) and *S.* Paratyphi-B (5.6%).

3.3. *Antimicrobial susceptibility testing*

All isolates exhibit resistance against all antimicrobial agents except amikacin which give no resistance and these results agreed with ***Snow et al., (2011)*** who reported that all *Salmonella* isolates from commercial layer flocks in UK were sensitive to amikacin with (100%). Forty one chicken isolates showed resistance with 100% against nalidixic acid and flumequin but four isolates showed the lowest resistance (9.7%) to levofloxacin. Thirty seven isolates were sensitive to levofloxacin with (90.2%) while 5 isolates were sensitive to amoxicillin giving the lowest percentage (12.1%) **Table (2)**. The obtained results differ from ***Boris et al., (2012)*** who reported that all *Salmonella* isolates were sensitive to chloramphenicol and streptomycin (100%) while 92 isolates (58%) were sensitive to nalidixic acid and of 66 (41.7%) isolates were sensitive to all antibiotics. Contrary to these results ***Cardoso et al., (2006)*** who reported that *Salmonella* showed sensitivity to doxycycline hydrochloride with 100%. However ***Khan et al., (2010)*** stated that all Salmonella isolates exhibit (100%) resistant to cephalexin and rifampicin while about 90% and 88% of the isolates were resistant to ampicillin and tetracycline. The results in this study differ from ***Zdragas et al., (2012)*** who reported (5%) resistance to streptomycin (highest resistance rates) and (2%) to tetracycline and nalidixic acid (lowest rate), ***Munawwar et al., (2010)*** who reported (100%) resistance to cephalexin and rifampicin in *Salmonella* isolated from chicken meats in Dubai, while 87.88% of these Salmonellae were sensitive to ciprofloxacin and amikacin.

3.3. *Polymerase Chain Reaction technique for different resistance genes in the examined isolates*

Fluoroquinolones are broad-spectrum antimicrobial agents widely used in clinical medicine. Resistance to this class of antibacterials is

usually caused by mutations in the chromosomal genes that code for DNA gyrase and/or DNA topoisomerase IV, the target enzymes, and/or mutations resulting from alterations in drug accumulation ***(Ruiz, 2003).*** Quinolone resistance encoded by the plasmid-mediated quinolone resistance gene, *qnr*, was first discovered in a clinical strain of *Klebsiella pneumoniae* isolated in 1994 ***(Martinez et al., 1998)***. The *qnr* gene confers nalidixic acid and low-level fluoroquinolone (e.g., ciprofloxacin) resistance ***(Robicsek et al., 2006a).*** Plasmid-mediated quinolone resistance is of concern because the resistance determinants are potentially disseminated among bacteria because of plasmid mobility. There are at least three main types of qnr gene, *qnr*A, *qnr*B and *qnr*S ***(Robicsek et al., 2006a).*** In this study twenty two *Salmonella* isolates out of 41 isolates that showed more resistance to quinolones in antimicrobial susceptibility tests were examined by PCR for *qnr*A, *qnr*B and *qnr*S (Plasmid-mediated quinolone resistance genes) while 19 *Salmonella* isolates not examined by PCR for these genes as they were less resistant to quinolone in antimicrobial susceptibility tests. Five isolates (5/22) (22.7%) were positive for *qnr*A gene from the 22 examined isolates giving amplification of 516 bp fragments. Also the positive control showed 516 bp fragments whereas no amplification could be observed with the negative control **(figures 1) Table (3)**. Two isolates (2/22) (9%) were positive for *qnr*B gene from the 22 examined isolates giving amplification of 469 bp fragments. Also the positive control showed 469 bp fragments whereas no amplification could be observed with the negative control **(figures 2) Table (3).** Four isolates (4/22) (18%) were

positive for *qnr*S gene from the 22 examined isolates giving amplification of 417 bp fragments. Also the positive control showed 417 bp fragments whereas no amplification could be observed with the negative control **(figures 3) Table (3)**. While ***(Ahmed et al., 2009b)*** reported that *qnr*S and *qnr*B were identified in two isolates of *S. enterica* serovar Enteritidis and one isolate of *S.enterica* serovar Typhimurium respectively. *qnr*S was identified previously in S. *enterica* serovar Typhimurium isolated from animals in Japan ***(Ahmed et al., 2009a)*** and S. *enterica* serovar Infantis of avian origin in Germany ***(Kehrenberg et al., 2006).*** Also, qnrB was previously identified in many S. *enterica* serovars, such as Mbandaka and Berta isolated from patients in the United States ***(Gay et al., 2006)***, and Stanley, Typhimurium, Virchow and Virginia from patients in the United Kingdom ***(Hopkins et al., 2007).*** No Plasmid-mediated quinolone resistance gene was identified in 224 *Salmonella* spp. isolates**.** ***(Xiang et al., 2011).***

Table (1): Primers used for gene amplification

Primers	Nucleotide Sequence	Annealing temperature	References
Plasmid-mediated quinolone resistance			
qnrA	F(5'- ATTTCTCACGCCAGGATTTG -3') R(5'- GATCGGCAAAGGTTAGGTCA -3')	53°C	Robicsek et al., 2006b
qnrB	F (5'- GATCGTGAAAGCCAGAAAGG -3')	53°C	Robicsek et al.,

	R(5'- ACGATGCCTGGTAGTTGTCC -3')		2006b
qnrS	F(5'- ACGACATTCGTCAACTGCAA -3') R(5'- TAAATTGGCACCCTGTAGGC -3')	53°C	Robicsek et al., 2006b

Table (2): Numbers and percentages of Salmonella isolates exhibiting resistance to various antimicrobial agents.

Antimicrobial Agent	Resistant Isolates		Senstive Isolates	
	No	%*	No	%*
Nalidixic acid	41	100	0	0
Flumequin	41	100	0	0
Ciprofloxacin	13	31.7	28	68.2
Enerofloxacin	20	48.7	21	51.2
Norfloxacin	8	19.5	33	80.4
Levofloxacin	4	9.7	37	90.2
Amoxicillin	36	87.8	5	12.1
Ampicilliln+ sulb	33	80.4	8	19.5
Cefotaxime	7	17	34	82.9
Ceftriaxone	9	21.9	32	78
Ceftazidime	9	21.9	32	78
Neomycin	17	41.4	24	58.5
Amikacin	0	0	41	100
Gentamycin	12	29.2	29	70.7
Streptomycin	23	56	18	43.9
Oxytetracyclin	32	78	9	21.9
Sulfa+trimethoprim	28	68.2	13	31.7

Table (2): Results of Polymerase Chain Reaction technique for Plasmid-mediated resistance genes from the examined isolates.

Code No	*qnr*A	*qnr*B	*qnr*S	Serotype
9	-	-	-	*S.* Daula
10	+	-	-	*S.* Newport
11	-	-	+	*S.* Tamale
12	+	-	-	*S.* Molade
13	-	-	-	*S.*Typhmurium
14	+	-	-	*S.* Newport
17	-	-	-	*S.* Lexington
18	-	+	-	*S.* Bargny
19	+	-	+	*S.* Rechovot
21	-	-	-	*S.* Enteritidis
22	-	-	+	*S.* Magherafelt
23	-	-	-	*S.*Typhmurium

25	-	-	-	*S.* Labadi
26	-	-	+	*S.* Apeyeme
27	-	+	-	*S.* Tamale
28	-	-	-	*S.*Enteritidis
32	-	-	-	*S.* Molade
33	-	-	-	*S.* Kentucky
34	-	-	-	*S.* Newport
35	-	-	-	*S.* Kentucky
36	+	-	-	*S.* Newport
41	-	-	-	*S.* Bargny

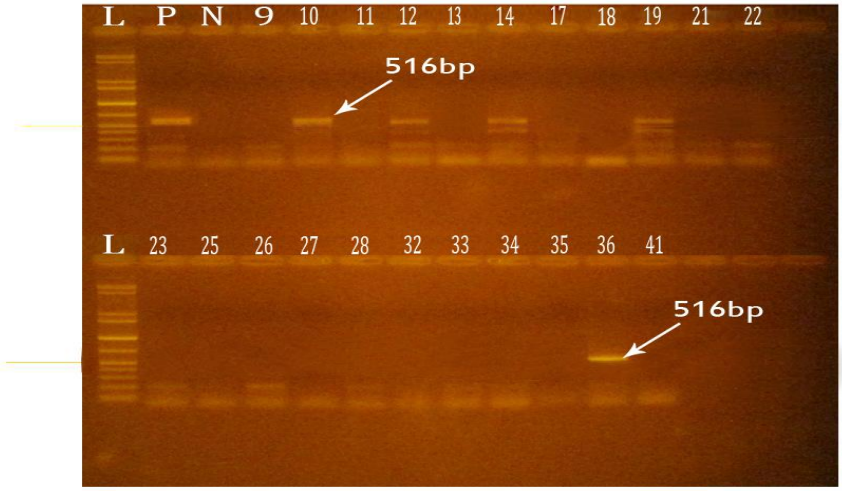

Fig. (1): Agarose gel electrophoresis showing specific PCR of *Salmonella* isolates using primer set for *qnr*A gene (516 bp) - L= ladder& lane P= positive control& lane N= negative control and lanes (10,12,14,19,36) were positive for this gene.

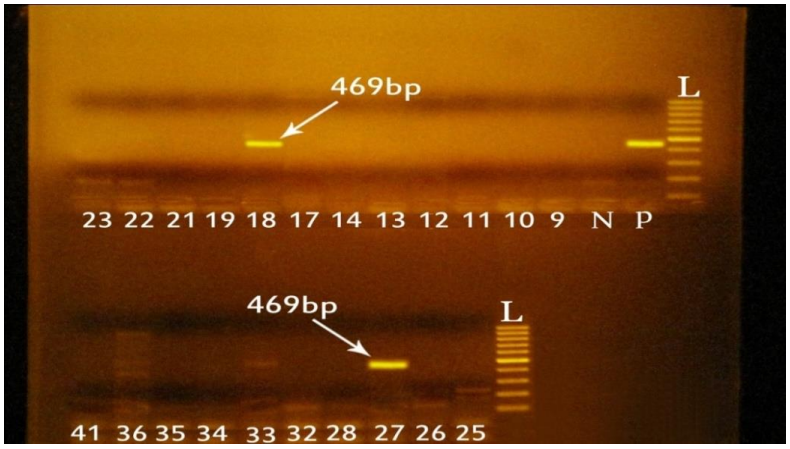

Fig. (2): Agarose gel electrophoresis showing specific PCR of *Salmonella* isolates using primer set for *qnr*B gene (469 bp) - L= ladder& lane (1)= positive control& lane (2)= negative control and lanes (18 and 27) were positive for this gene.

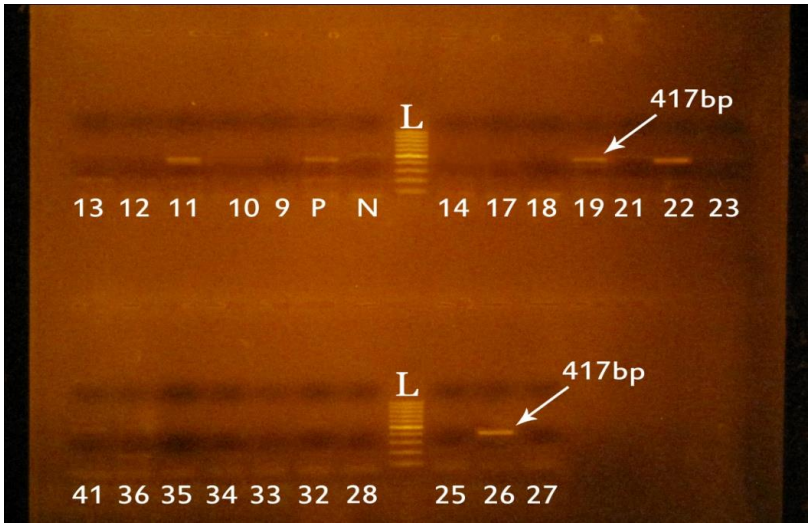

Fig. (3): Agarose gel electrophoresis showing specific PCR of Salmonella isolates using primer set for *qnr*S gene (417 bp) - L= ladder& lane (1)= negative control& lane (2)= positive control and lanes (11,19,22 and 26) were positive for this gene.

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