



Molecular characterization of Aminoglycoside and Tetracycline resistant *Salmonella* isolates causing new born ruminants diarrhea

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ABSTRACT

Ten isolates of *Salmonella* species were obtained from sporadic cases of profuse diarrhea in new born ruminants from El-Menofiya and El-Kalubia Governorates in Egypt. These isolates were as follow 7 isolates from calves (*Salmonella* Typhimurium, *Salmonella* Enteritidis, two were *Salmonella* Saintpaul, two were *Salmonella* Langeveld and *Salmonella* Havana), two isolates from lambs (*Salmonella* Typhimurium, *Salmonella* Bardo) and one isolate from goat-kids (*Salmonella* Enteritidis). *Salmonella* isolates resistance to aminoglycosides was 0.00% for Amikacin, 30% for tobramycin and 50% for streptomycin. Susceptibility of isolates to tetracycline was 50% for doxycycline. Results showed a high incidence of aminoglycosides resistance gene *aadB* in 100% of the isolates while *aadA2* genes in 40% of the isolates. Incidence of tetracycline resistance genes was 0.00% for *tetA(B)* and 80% for *tetA(A)*. The difference between the results of this study and those from other regions in Egypt necessitate a complete survey overall the country to make a complete and clear map of salmonella serovars, their antibiotics susceptibility and in molecular characterization of resistance determinants in each region.

Key words: Diarrhea, newborn ruminants, *Salmonella*, aminoglycosides, tetracycline.

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1. INTRODUCTION

With the extensive and widespread application of antibiotic as a therapeutic agent in animals and human, and as a growth promoter in livestock, bacteria have been exposed to sub-inhibitory (non-lethal) dose of antibiotics (Singh et al., 2015). This has played critical role in the evolution of antibiotic resistance (Andersson and Hughes 2014), and selection of antibiotic resistant bacteria (Gullberg et al., 2011). The efficacy of many antimicrobials drugs for treating clinical cases is decreasing as more antimicrobial resistant *Salmonella* subtypes emerge (Angulo et al., 2000 and Winokur et al., 2000). Studying of resistance determinants with molecular and genetic methods have a critical role in understanding, controlling, and spreading

of resistant pathogens. Aminoglycosides are the most frequently used antibiotic agents in the treatment of infections by both Gram-negative and Gram-positive bacteria (Samadi et al., 2015). This group of antibiotics acts by binding to the ribosomes of bacteria and in turn interferes with protein synthesis (Mingeot et al., 1999). The aminoglycoside-bound bacterial ribosome is unavailable for translation of mRNA during protein synthesis thereby causing bactericidal effects (Le Goffic et al., 1979). Aminoglycoside resistance of *Salmonella* strains is generally associated with expression of aminoglycoside-modifying enzymes (Alcaine et al., 2007). Beside binding to ribosomal 30S subunit, Streptomycin has been shown to perturb the structure of the decoding centre, while other

aminoglycosides such as paromomycin affect conformations of the 16S rRNA bases directly involved in decoding (Carter *et al.*, 2000; Demirci *et al.*, 2013). Bacterial resistance to aminoglycosides can be acquired through different mechanism including decreased uptake of aminoglycosides into the cell via decreased cell-membrane permeability, reduction of the drug concentration in the cell by efflux pumps development, alteration of the drug-binding site by mutation or chemical modification of the 16S RNA or ribosomal proteins, and enzymatic modification of aminoglycosides, leading to drug inactivation and diminished binding (Azucena & Mobashery, 2001, Llano-Sotelo *et al.*, 2002). Enzymatic modification of aminoglycosides is mediated by three types of modifying enzymes, aminoglycoside O-nucleotidyltransferases (ANTs), aminoglycoside N-acetyltransferases (AACs) and aminoglycoside O-phosphotransferases (APHs). In *salmonella enteric A*adA was recently characterized to be an ANT (3") (9) streptomycin / spectinomycin adenylyl transferase encoded by the *aadA* gene that adenylylates the 3-hydroxyl group of the streptomycin glucosamine ring and the 9-hydroxyl group of the spectinomycin actinamine ring (Chen *et al.*, 2015). Tetracycline act by inhibiting protein synthesis. They enter bacterial cells by an energy-dependent process and bind reversibly to the A site of the 30S ribosomal subunits of the bacteria. (Mascaretti 2003). This process blocks the access of aminoacyl-tRNA to the RNA-ribosome complex, preventing bacterial polypeptide synthesis therefore it inhibits protein synthesis (Chopra *et al.*, 1992). Tetracycline resistance results from acquisition of exogenous DNA encoding proteins involved in active efflux of tetracycline or in protection of the ribosome. Resistance to tetracycline is prominent among *S. Typhimurium* isolates in humans (34%), chickens (39%), cattle (59%), and swine (88%) according to a ten-

year average from the National Antimicrobial Resistance Monitoring System CDC (2012) and FDA (2011). In Gram-negative bacteria, six classes of *tet* efflux pumps including *tetA*, *tetB*, *tetC*, *tetD*, *tetE*, *tetG*, are of clinical importance. These tetracycline resistance genes conferred efflux of tetracycline from the cell and encoded the first of the three different types of tetracycline resistance mechanisms to be found in bacteria (Roberts 1996). These efflux pumps use an antiport mechanism of transport involving the exchange of a proton for tetracycline-ation complex (Lyras and Rood. 1996). Isolates of various *Salmonella enterica* subsp. *enterica* serovars of zoonotic or veterinary importance showed high levels of tetracycline resistance. Among *S. Dublin* (Ferris *et al.* 1992) and *S. Choleraesuis* (Weide-Botjes 1998) from the USA, 65% and 89% of the isolates proved to be resistant to tetracyclines. Tetracycline resistance among *S. Typhimurium* isolates seemed to vary according to the animal source and the geographical origin of the isolates (Gnanou 1998).

This study aimed to characterize the prevalence, serotypes and genetic determinants of aminoglycosoides and tetracycline resistant salmonella serovars causing diarrhea in new born animal in El-Menofiya and El-Kalubia Governorates, Egypt.

2. MATERIAL AND METHODS:

2.1. Samples Collection

A Total of 236 fecal swabs were collected from diarrheic Calves (150 cases), lamb (55cases) Goat kids (31cases) as sporadic cases presented in Governmental Veterinary Clinics in El-Menofiya and El-Kalubia Governorates, Egypt.

2.2. Isolation and Identification of salmonella

Swabs were suspended in buffered peptone water (1:10 dilution) then incubated at 37°C (16-20 hours). Then incubated buffered

peptone water (0.1 ml) was transferred with a pipette into a tube containing (10 ml) of Rappaport-Vassiliadis soy peptone (RVS) broth and incubated at 42°C (20-24 hours). After that, a loopfull of RVS broth was inoculated and streaked separately onto selective agar plates as Xylose Lysine Desoxycholate (XLD) agar, Brilliant Green agar (BGA), MacConkey's agar then incubated at 37°C for 24-48h. Typical colonies of Salmonella on XLD agar were pale pink with black center and on MacConkey's agar appear pale, colorless smooth and transparent. Suspected colonies were identified as Salmonella spp. based on their colony characters on selective media, and the biochemical testing using TSI agar, Urea agar, L-lysine decarboxylase, Voges Proskauer, Methyl red tests, Simmons citrate and Indole tests. Also, Salmonella spp. were confirmed biochemically by using API 20E system (BioMérieux, Marcy-l'Étoile, France). Finally, Salmonella isolates were serotyped based on slide agglutination for O and H antigens according to Kauffmann-White (1974) and using the antisera from (Mast Salmonella diagnostic antisera) (UK).

2.3. Antimicrobial sensitivity

The antimicrobial sensitivity phenotypes of Salmonella were determined by agar disc diffusion method as described by Finegold and Martin (1982), and according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2014), using antibiotic disc from Oxoid (Thermo Fisher Scientific, Inc. (NYSE: TMO, UK). The discs used were amikacin (30 µg), tobramycin (10µg), streptomycin (10µg), doxycycline (30µg) (Oxid, UK).

2.4. Bacterial DNA preparation for PCR

Bacterial culture DNA was extracted using the (QIAamp DNA extraction mini kit) (QIAGEN. Duesseldorf, Germany, (Egypt branch) according to the mini kit instructions (200 µl) of an overnight bacterial culture was mixed with 200 µl buffer plus 20 µl QIAGEN protease in 1.5 ml tube, mixture was incubated at 56°C for

10 min, then 200 µl ethanol (96%) were added, vortexing for 15 seconds, applied to the QIAamp mini spin column, centrifugated at 8000 rpm for 1 min, washed two time with washing buffer and DNA was eluted from the column with 150 µl buffer AE elution buffer.

2.5. Amplification of Antibiotic Resistant genes in salmonella serovars

PCR mixture was prepared According to Emerald Amp GT PCR mastermix (Takara, Co., Japan). Code No. RR310A kit: In PCR tube a 6µl bacterial DNA template, 12.5µl Emerald Amp GT PCR master mix (2x premix), 1µl of forward and reverse primers (20 pmol) and 4.5 µl PCR grade water to bring total volume to 25. was primary denaturation cycle at 94°C for 5 min followed by 35 cycle of 94°C for 30 secs, 53°C for 45 The amplification condition sec and 72°C for 45 sec followed by one final extension cycle at 72°C for 10 min using the primers sequence and amplification conditions listed in table (1)

3. RESULTS

A total of 236 fecal samples collected from diarrheic newborn ruminants were screened for the presence of Salmonella species. Identification procedures (cultivation, isolation, biochemical and serological tests) identified ten isolates of Salmonella species as 7 isolates from calves (Salmonella Typhimurium, Salmonella Enteritidis, two were Salmonella Saintpaul, two were Salmonella Langeveld and Salmonella Havana), 2 isolates from lambs (Salmonella Typhimurium, Salmonella Bardo) and one isolate from goat-kids (Salmonella Enteritidis). Salmonella isolates showed 0.00 %, 30% and 50% resistance to amikacin, tobramycin and streptomycin respectively. The resistance to doxycycline was 50% (Table 2). Aminoglycoside resistance gene (*aadB*) was detected in all Salmonella isolates (Table 3) showing amplification of 319bp

(Figure 1) while the (*aadA2*) was detected only in 40% of isolates (*S. Saintpaul* No.73, *S. Langeveld* No.67 and 96 and *S. Typhimurium* No.10,) Figure (2) showing amplification of 622bp. Tetracycline resistance gene *tetA* (A) was detected in

80% of isolates but was not detected in *S. Typhimurium* No.99 and *S. Havana* No.133 Figure (3) showing amplification of 576bp. Tetracycline resistance gene *tetA* (B) was not detected in any of the isolates Figure (4).

Table (1). Primers used to amplify aminoglycosoides and tetracycline resistance genes

Primer	Sequence	Product size	Reference
<i>aadB</i>	GAGCGAAATCTGCCGCTCTGG CTGTTACAACGGACTGGCCGC	319 bp	Frana et al., 2001
<i>aadA2</i>	TGTTGGTTACTGTGGCCGTA GATCTCGCCTTTCACAAAGC	622 bp	Walker et al., 2001
<i>tetA</i> (A)	GGTTCACCTCGAACGACGTCA CTGTCCGACAAGTTGCATGA	576 bp	
<i>tetA</i> (B)	CCTCAGTTTCTCAACGCGTG GCACCTTGCTCATGACTCTT	633 bp	Randall et al., 2004

Table (2). Sensitivity of the Salmonella Serotypes isolated from diarrheic calves, lamb and goat kids to aminoglycosides and Tetracycline

<i>Salmonella</i>	isolate	Zone of inhibition due to aminoglycoside discs in each <i>S.</i>			
		Amikacin	Tobramycin	Streptomycin	Doxycycline
Calves serovar					
<i>S. Typhimurium</i>	99	(24) S	(18) S	(19) S	(19) S
<i>S. Enteritidis</i>	103	(18) S	(14) I	(19) S	(19) S
<i>S. Saintpaul</i>	62	(20) S	(10) R	(17) S	(17) S
<i>S. Saintpaul</i>	73	(26) S	(18) S	(10) R	(10) R
<i>S. Langeveld</i>	67	(20) S	(18) S	(10) R	(10) R
<i>S. Langeveld</i>	96	(23) S	(17) S	(8) R	(8) R
<i>S. Havana</i>	133	(18) S	(14) I	(10) R	(10) R
Lambs serovar	10	(20) S	(9) R	(-) R	(-) R
<i>S. Typhimurium</i>					
<i>S. Bardo</i>	27	(21) S	(12) R	(17) S	(17) S
Goats kids serovar	25	(17) S	(18) S	(19) S	(19) S
<i>S. Enteritidis</i>					
Number of resistant serovars		0	3	5	5
Percentage of resistant Serovars		0.00	30%	50%	50%

-Number in brackets: size of inhibition zone with mm. -R: resistant, S: sensitive, I: intermediate. according to (CLSI 2014)

Aminoglycoside and Tetracycline Resistant Salmonella

Table (3) Incidence of aminoglycosides and tetracycline resistance genes in Salmonella isolates

S. Serovars	Isolate number	Genes			
		<i>aadB</i>	<i>aadA2</i>	<i>tetA(A)</i>	<i>tetA(B)</i>
Calves	99	+	-	-	-
<i>S. Typhimurium</i>					
<i>S. Enteritidis</i>	103	+	-	+	-
<i>S. Saintpaul</i>	62	+	-	+	-
<i>S. Saintpaul</i>	73	+	+	+	-
<i>S. Langeveld</i>	67	+	+	+	-
<i>S. Langeveld</i>	96	+	+	+	-
<i>S. Havana</i>	133	+	-	-	-
Lambs	10	+	+	+	-
<i>S. Typhimurium</i>					
<i>S. Bardo</i>	27	+	-	+	-
Goats kids	25	+	-	+	-
<i>S. Enteritidis</i>					

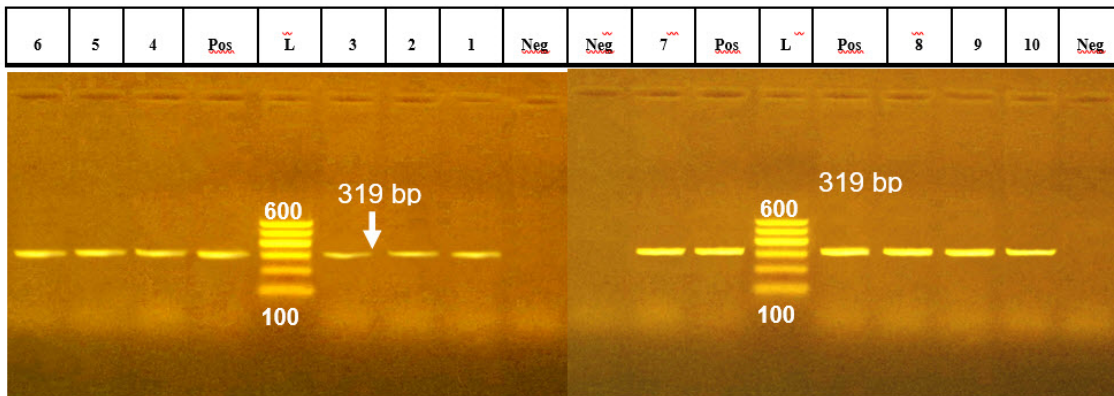
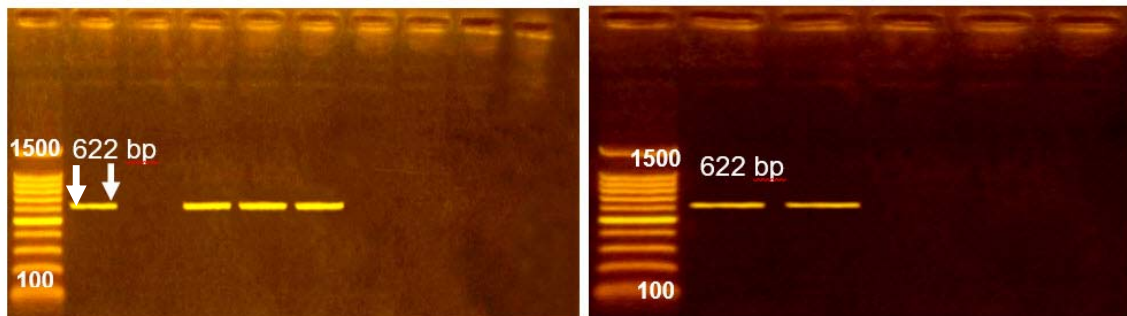


Figure (1):PCR detection of aminoglycosoides resistance gene (*aadB*) in different salmonella isolates

L (DNA ladder), Neg (Negative control), Pos (Positive control), (1-7) Salmonella isolates from Diarrheic calves: 1,3 (*S. Saintpaul*), 2,4 (*S. Langeveld*) ,5 (*S. Typhimurium*) ,6 (*S. Havana*), 7 (*S. Enteritidis*), (8, 10) Salmonella isolates from diarrheic lambs :8 (*S. Typhimurium*), 10 (*S. Bardo*), 9 (*S. Enteritidis*) from diarrheic goat kids.



Figure(2):PCR detection of aminoglycosoides resistance gene (*aadA2*) in Salmonella isolates

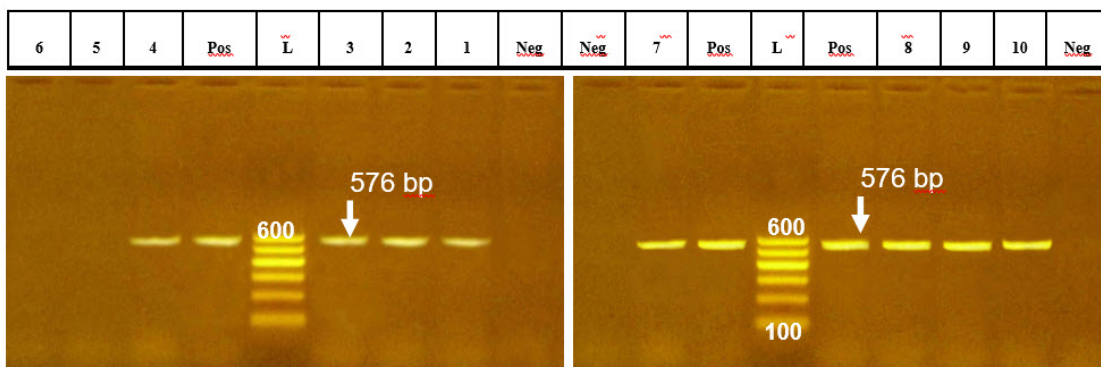


Figure (3): PCR detection of tetracycline resistance gene (*tetA*) in salmonella isolates

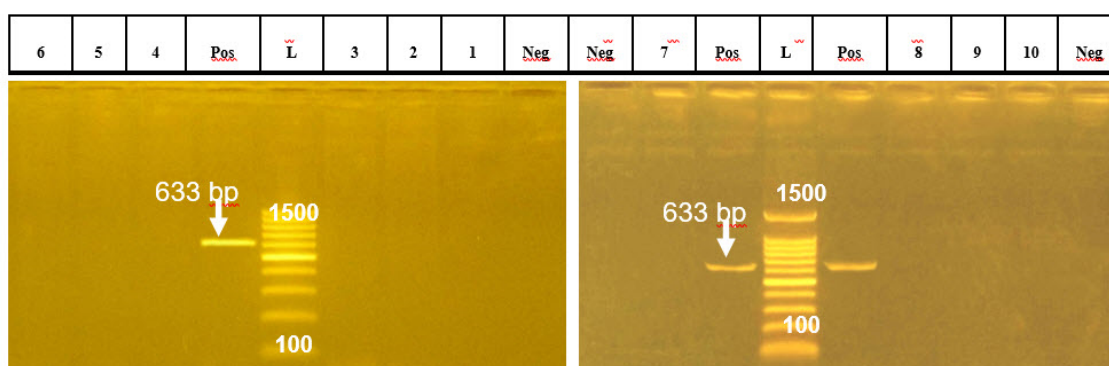


Figure (4): PCR detection of one tetracycline resistance gene (*tetB*) in salmonella isolates. L (DNA ladder), Neg (Negative control), Pos (Positive control), (1-7) Salmonella isolates from Diarrheic calves: 1, 3 (*S. Saintpaul*), 2,4 (*S. Langeveld*), 5 (*S. Typhimurium*), 6 (*S. Havana*), 7 (*S. Entritidis*), (8, 10) Salmonella isolates from diarrheic lambs :8 (*S. Typhimurium*), 10 (*S. Bardo*), 9 (*S. Entritidis*) from diarrheic goat kids.

4. DISCUSSION

The use of antibiotics based on earlier report of their effectiveness may not be effective at all times (Kumar *et al.*, 2012). Given the increasing prevalence of *Salmonella* isolates resistant to antibiotics, make it mandatory to investigation over different geographical region and continuous periodical search for the prevalence of newly emerged drug-resistant salmonella strains and their MDR determinants. Based on historical evidence, continued emergence of AMR and/or virulent subtypes can be expected. Therefore, additional epidemiologic and molecular research should be directed towards the entire population to understand

the epidemiology, ecology and evolution associated with changes in the prevalence of AMR *Salmonella* within livestock populations (Habing 2012). This study aimed to investigate the prevalence of salmonella species in the sporadic cases of diarrheic calves, lambs and goat kids in the middle of Egypt and to identify their serotypes in addition to determination of their sensitivity or resistant to aminoglycoside and tetracycline and to characterizes the determinant of their resistance to these antibiotics.

Susceptibility of different salmonella to members of aminoglycosides showed that the resistance varies between 0.00% to Amikacin, 30% to Tobramycin and 50% to Streptomycin Table (2). This result is

completely different from that of a previous result showed that 100% of *Salmonella enterica* isolated from clinically diarrheic human in Egypt are resistance to Streptomycin (Osman et al., 2014). Moreover, other study in a different region on chicken meat and clinical human cases in Egypt, 25% and 66.7% of salmonella isolates were resistance to Streptomycin and gentamicin respectively (Gharieb et al 2015). There is a close relation between animal serovars and that isolated from human in each region as considerable number of serotypes frequently isolated from humans have been isolated from sick or clinically healthy cattle (Hoelzer et al., 2011). Therefore the difference in the susceptibility to aminoglycosides could be related to the difference in sampling region and the previous antibiotic stress facing the salmonella serovars in our sampling region than others'. Indeed, selective pressure favors the emergence of antimicrobial resistance pathogens such as *Salmonella*, which is frequently harbored in the animal intestinal tract (Aarestrup 2000). In this study, this difference in the susceptibility of salmonella isolates may be also due to the incidence of the aminoglycoside resistance genes as *aadB* genes was detected in 100% of the isolates while *aadA2* was detected only in 40% of the isolates Table (3). The *aadA2* is a one of the gene cassettes containing aminoglycoside acetyltransferase which confers resistance against streptomycin and spectinomycin (Gharieb et al 2015). Therefore, the detection of the *aadA2* is in consistence with the susceptibility of the salmonella isolates to streptomycin. In a recent study in Ghana, bacterial isolates including salmonella showed resistance levels of <50% for amikacin and gentamicin (Opintan et al., 2015). However, in the current study, salmonella isolates resistance to amikacin was 0.00%. This difference may be attributed to the difference of the previous use of amikacin between the two countries. Parallel to our finding a recent study showed salmonella isolates

susceptibility to amikacin and gentamicin was 98-99% (Reshetneva et al., 2015). This results is further strengthened by report from Turkey showing *Salmonella Enteritidis* isolated from human feces susceptibility is 100% to amikacin and gentamicin (Acar et al., 2015).

Susceptibility of different salmonella isolates to the tetracycline (Doxycycline) showed that the resistance 50 % to Doxycycline table (2). In antimicrobial susceptibility tests of salmonella isolates from farm animals in China, *Salmonella* spp. resistance to doxycycline were (28.22%), and to were tetracycline (27.58%) (Kuang et al.,2015). Resistance to tetracycline of isolates from gastroenteritis human patients from Minia governorate, Egypt was 77% which is higher than our finding (Mahmoud et al., 2015). While in a study of salmonella Typhi cultured from blood from patient from Cairo governorate, Egypt, it was reported that salmonella Typhi resistance to tetracycline was 35% (Wasfy et al., 2002). Thus the resistant pictures vary from region to region in Egypt that may reflect the general or specific antibiotic previous exposure and stress on the microorganism and emergence of different levels of resistance in each region. In spite of the 50% resistance to tetracycline the expression of *tetA* gene was detected in only 40% of the isolates while *tetB* could not be detected in any isolates Table (3). Indeed, it was reported that not all resistant isolates of non typhoid salmonella express the *tetB* genes (Mahmoud et al., 2015). This may imply that *tetA* or *tetB* are not the only genes responsible for salmonella resistance to tetracycline. The tetracycline resistance genes are *tetA*, *tetB*, *tetC*, *tetD*, *tetE*, *tetG*, *tetK*, *tetL*, *tetM*, *tetO*, *tetS*, *tetA(P)*, *tetQ*, and *text* (Andrew et al.,2004). *tetB* gene was only detected by PCR in 64.7% of tetracycline resistant non typhoid isolates from gastroenteritis human patients ((Mahmoud et al., 2015).

5. CONCLUSION

In this study, the susceptibility of the isolates to aminoglycosides is higher than that to Tetracycline. This makes amikacin the drug of choice to treat suspected or confirmed salmonella infection at least in the region of this study. The difference between the results of the current study and those from other regions in Egypt necessitate a complete survey overall the country to make a complete and clear map of the reservoirs and their antibiotics susceptibility in addition molecular characterization of resistant determinants in each region.

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