Global Journal of Molecular Sciences 6 (1): 15-21, 2011
ISSN 1990-9241
© IDOSI Publications, 2011

Molecular Cloning and Characterization of Streptomycin and Spectinomycin Resistance Gene (aadA) in *Salmonella typhimurium* Isolate from Egypt

[1]*Maymona A. Kord,* [1]*El-Sayed T. Abdel-Salam Sayed and* [2]*Youssry S. El-Sadi*

[1]Botany Department, Faculty of Science, Cairo University, Giza, Egypt
[2]Scientific Office, Trust Medical Company, Giza, Egypt

Abstract: The bacterial gene *aad*A encodes the enzyme aminoglycoside-3'-adenyltransferase considered as one of the plastid transformation markers. *aad*A is a gene bound to play a fatal role in inactivating streptomycin and spectinomycin antibiotics, protecting the bacterial cell. We succeeded to isolate and characterize the full sequence of *S. typhimurium aad*A gene from the bacterial genome at 789bp approximately. We also cloned the *aad*A gene and expressed the aminoglycoside 3'-adenyltransferase protein in *Escherichia coli*. The DNA fragment was sequenced and aligned with the annotated *aad*A gene sequences from *Salmonella* species in the GenBank. The alignment result showed that the sequenced *aad*A gene scores maximum identity with *aad*A gene sequence from *Salmonella typhimurium* LT2 strain with conserved 98% identity. The molecular weight of the recombinant protein found to be approximately 29.17 kDa of 262 amino acids. The alignment of recombinant protein sequence and annotated protein sequence in database revealed variability in some amino acids residues.

Key words: *Salmonella typhimurium* · *aad*A Gene · Streptomycin · Spectinomycin · Chloroplast Transformation · Marker Gene

INTRODUCTION

Salmonella typhimurium is a Gram-negative, facultative anaerobic, flagellated, non-spore forming bacterium with diameters 0.7 - 1.5 µm and lengths 2 - 5 µm [1]. It is the one of the pathogenic agents of salmonellosis, a major cause of enteric illness, leading to many hospitalizations and a few rare deaths if no antibiotics are administered. *Salmonella typhimurium* has been studied over the past thirty years on the worldwide emergence as multi-drug resistant pathogen [2, 3]. The bacterium can be isolated from raw meat and poultry products as well as from milk and milk-based products [4]. The detection of *Salmonella* species therefore remains a highly important issue in microbiological analysis for food safety and standards. Most *Salmonella* species produce hydrogen sulfide, which can readily be detected by growing them on media containing ferrous sulfate [5].

Previous studies show that *Salmonella typhimurium* is resistant to at least five antimicrobials; ampicillin, chloramphenicol, streptomycin, sulfamethoxazole and tetracycline [6-9].

Salmonella typhimurium carry about 1160 genes, which are located on the genetic map as following; 1081 are on the circular chromosome, 29 on the 90-kb virulence-associated plasmid and 50 are not yet mapped [10].

Aminoglycosides are compounds that are characterized by the presence of an aminocyclitol ring linked to amino sugars in their structure, such as streptomycin, tobramycin and kanamycin [11]. These compounds irreversibly bind to the bacterial ribosomes and also interact with other cellular structures and metabolic processes [12].

Three mechanisms of resistance have been recognized, namely ribosome alteration, decreased permeability and inactivation of the drugs by aminoglycosides modifying enzymes. Inactivation of aminoglycosides found to be the most clinical importance since the genes encoding the aminoglycosides modifying enzymes can be distributed by bacterial plasmids or genomes [13].

The *aad*A genes, encoding resistance to streptomycin and spectinomycin have been found as gene cassettes in different Gram-negative and

Corresponding Author: Youssry S. El-Sadi ,Scientific Office, Trust Medical Company, Giza, Egypt.
Tel: 00201005465449.

Gram-positive bacterial species [14, 15]. The *aad*A protein confers resistance to streptomycin and spectinomycin, the *aad*A gene has been found in association with several transposons (Tn7, Tn21) and is ubiquitous among Gram-negative bacteria, especially *Salmonella* species [16].

The recorded data has described *aad*A genes of *Salmonella* species as ranging between 780-792 base pair long and encoding a protein of a predicted size ranging between 29-29.35 kDa. The *aad*A gene product inactivates streptomycin and spectinomycin by an adenylation modification [17].

Generally; the *aad*A gene confers its resistance to spectinomycin and streptomycin antibiotics. Spectinomycin is also used as a selection marker in plant's genetic manipulation process. In the early laboratory stage for development of the transgenic plant, the *aad*A gene can be used as a selectable marker gene as it enables the selection of plant cells containing a desired genetic modification [18].

The main aim of this study was molecular cloning of *aad*A coding sequence and full characterization and validation of aminoglycoside 3'-adenyltransferase enzyme activity, to be used as a marker gene in *plastid transformation system* using chloroplast expression cassette.

MATERIALS AND METHODS

Bacterial Culture Conditions: The *Salmonella* isolate used in this study was kindly supplied by *Prof. Dr. Adel Khalil Goher*, Professor of Clinical pathology, Faculty of Veterinary Medicine, Cairo University; Cairo, Egypt. The bacterial strain was isolated from a cattle fecal sample. The strain for this study was first sub-cultured using Hektoen enteric agar [19], to confirm its validity as *Salmonella typhimurium*, the positive culture was inoculated into 25ml sterilized rich LB broth medium; the prepared medium was incubated in a shaker incubator at 36°C with 150rpm for 12- 16 hrs. This Part was carried out in; Microbiology laboratory, Botany department, Faculty of Science, Cairo University.

Antimicrobial Susceptibility Testing (Determination of Phenotypic Resistance): Antimicrobial susceptibility of *Salmonella* isolate towards the antimicrobial agents (streptomycin/ spectinomycin, kanamycin, ampicillin, chloramphenicol and tetracycline) was determined on Mueller Hinton II agar, via the agar diffusion test [20, 21] using Etest® strips (Epsilometer test) (BioMérieux, France).

Amplification of *aad*A gene: The genomic DNA from *Salmonella* isolate was extracted by using ArchivePure™ DNA Yeast and Gram-positive Bacteria Kit (5Prime).

Primers for polymerase chain reaction (PCR) were constructed based on the nucleotide sequence data of *aadA* genes encoding aminoglycoside 3'-adenyltransferase of *Salmonella* species, retrieved from the genomic database GenBank, accession numbers DQ133159; DQ133164; DQ133165.1; NC_003197.1. Primers corresponding to 5' and 3' ends were:

5'-CATATGATGACGCTGTCCATATCGCCCTCGATA-3' and
5'-GCGGCCGCTTATGTGAACTGCGTGGGGATGTA-3', respectively.

The 5'-end primer contains the initiation codon (ATG) in the sequence and the coding sequences for eight amino acid residues of *aadA*. The 3'-end primer a complementary sequences coding for the termination codon (TTA) and for seven amino acid residues. The primers were synthesized by Jena Bioscience, Inc. (Germany).

PCR reactions were performed in a total volume of 50µl with Perfect *Taq* DNA polymerase (5Prime), using the specific primers (10pmols/µl) for amplification of the corresponding *aad*A gene, with 30 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1 min, with a final extension at 72°C for 10 min.

Molecular Cloning of *aad*A Gene: The PCR product with the expected DNA length (800 bp) was further purified using GelElute Extraction Kit (5Prime) and then cloned using Perfect PCR Cloning Kit into pPrime Cloning Vector (5Prime). The resulted construct was designated as pPrime -*aad*A and then was used to transform the *E. coli* strain DH5α (Stratagene) and the positive transformants were selected in Luria Bertani (LB) plates supplemented with ampicillin (50µg/ml) [22].

The *aad*A gene ligated into pPrime Cloning Vector was detected and validated by PCR as a confirmatory test. The promising recombinant clones were validated by PCR amplification using specific *aad*A gene amplification primers.

This Part was carried out in; Private Molecular biology center, Faculty of Veterinary Medicine, Cairo University.

Study the Sequence of Cloned *aad*A Gene: The construct pPrime -*aad*A was purified using Fast Plasmid Mini Kit (5Prime) and then serve as a template for bidirectional DNA sequencing in an automatic sequencer by Jena Bioscience, Inc. (Germany).

Expression of Recombinant Aminoglycoside 3'-adenyltransferase: The amplified DNA sequence (approximately 800bp) was directly ligated into pPP-30UA expression vector (5Prime). The resulted construct was designated as pPP-30UA -*aad*A and then was used to transform the *E. coli* strain XL1-blue (Stratagene). The positive transformants were selected in Luria-Bertani (LB) plates supplemented with ampicillin (50µg/ml).

Miniprep DNA obtained from these transformants was then used to transform the *E. coli* strain BL21 (DE3) -pLysS (Stratagene). The transformed cells were grown in LB liquid medium in the presence of ampicillin at 37°C and the synthesis of recombinant aminoglycoside 3'-adenyltransferase enzyme was induced with 0.1mM isopropyl-b-D-thiogalactopyranoside (IPTG) for 3 hrs [22].

Study the Expression of Recombinant *aadA* Gene: Culture volumes (*E. coli* strain expressing recombinant *aadA*) equivalent to 1ml were collected, cells were harvested by centrifugation (10,000 g, 4°C, 10 min) and then used in extraction of total RNA for RT-PCR assay using *aad*A specific oligonucleotides primers [22].

The total RNA were isolated from the transformed *E. coli*, that grew in IPTG induction condition and used to synthesize a complementary first strand (cDNA) using Reverse-iTTM 1st strand synthesis kit (Fermentas), through a reverse transcription reaction.

Minimum Inhibitory Concentrations (MIC) of Streptomycin/spectinomycin Against *E.coli* Expressing Recombinant *aadA* Aminoglycoside Resistance Protein: Antimicrobial minimum inhibitory concentrations (MIC) of *E. coli* expressing *aad*A aminoglycoside resistance protein toward antimicrobial agents like; streptomycin/ spectinomycin were determined on Mueller Hinton II agar, via the agar diffusion test using Etest® strips (Epsilometer test) (BioMérieux, France).

Purification of Recombinant Aminoglycoside 3'-adenyltransferase: All operations were carried out at 4°C. The resuspended cells were disintegrated by sonic oscillation (90s at 60W) in a Branson Sonifier Cell Disruptor (Danbury, Conn.). The slurry was centrifuged at 14,500 *x*g for 30 min. The supernatant (cell extract) was brought to 20% (wt/vol) sucrose and then poured into Ni-NTA column of PerfectPro Ni-NTA Superflow tagged protein purification system (5Prime).

The induction level of the *aad*A was assessed by the activity of the expressed enzyme measured by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli [23]. To measure *aad*A protein, identical samples (1µg of total soluble protein) were loaded into 8% SDS-PAGE gel [23].

Amino Acid Composition: After the SDS-PAGE analysis, the Coomassie blue-stained protein bands (native and recombinant) were excised and submitted to the Bio-Synthesis, Inc. (Bioanalytical Laboratory, Texas, USA), for analysis of N-terminal sequence and for determination of the amino acid composition.

RESULTS

Bacterial Culture Conditions: The colonial growth pattern displayed by the *Salmonella* isolate on the Hektoen enteric agar was greenish-blue in color with black deposits in the centers.

Antimicrobial Resistance: The *Salmonella* isolate was tested for determination of phenotypic resistance. In general, the incidence of resistance to the tested antibiotics was mainly higher to, streptomycin/spectinomycin (128µg/ml) and kanamycin (64µg/ml); rather than to ampicillin (32µg/ml), chloramphenicol (32µg/ml) and tetracycline (16µg/ml).

Molecular Cloning of Salmonella Isolate *aadA* Gene: The cloning of the *aad*A gene coding aminoglycoside 3'-adenyltransferase was performed via PCR and specific oligonucleotides primers. The PCR assay resulted in a DNA fragment of approximately 800bp in size (Fig. 1).

Fig. 1: Electrophoretic mobility of *Salmonella typhimurium* PCR products amplified from genomic DNA using specific synthetic oligonucleotides primers. Lane (1): 1kb ladder (Fermentas) and Lane (2): *aad*A DNA fragment (800bp).

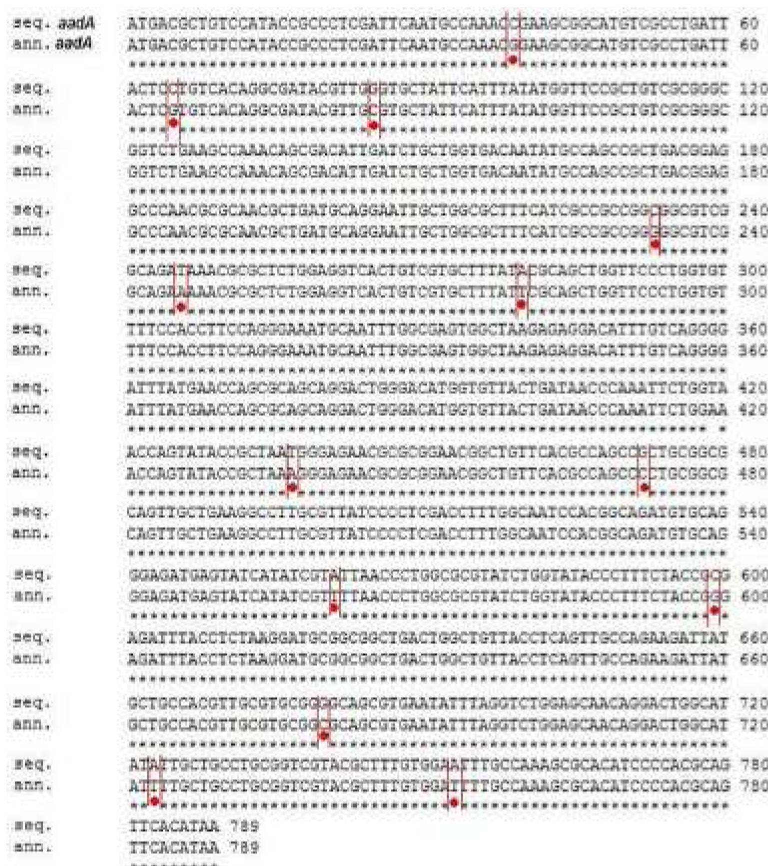

Fig. 2: Shows alignment between the sequenced *aadA* gene (seq. *aadA*) and annotated *aadA* sequence of *Salmonella typhimurium* (ann. *aadA*). The alignment result between the sequenced *aadA* gene and annotated *aadA* sequence of *Salmonella typhimurium* at gene bank, shows that the sequenced *aadA* gene with conservation of 98% identity with the annotated sequence from the database accession number NC_003197.1.

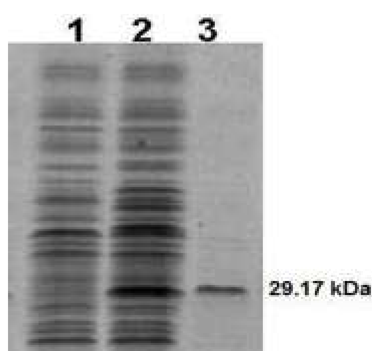

Fig. 3: Over-expression of recombinant *S. typhimurium aadA* in *E. coli*. SDS-PAGE (8% gel) and Coomassie blue staining. Lane (1) whole cell lysate (0.5 µg) of non-induced BL21(DE3) pLysS cells containing the plasmid pPP-30UA -*aad*A; Lane (2) whole cell lysate (0.5 µg) of the same cells obtained at 3 h post-induction with 0.1 mM IPTG; and Lane (3) purified tagged recombinant aminoglycoside 3'-adenyltransferase protein.

The alignment result between the sequenced *aad*A gene from the *Salmonella* isolate and annotated *aad*A genes sequence of *Salmonella* species in the GenBank, showed that the sequenced *aad*A gene has conserved 98% identity to the annotated sequence from the database accession number NC_003197.1 (Fig. 2); along with a conserved 55% identity to the annotated sequence from the database accession number DQ133159 and DQ133164; and 51% identity with the annotated sequence from the database accession number DQ133165.1.

Expression of Recombinant *aadA* Aminoglycoside Resistance Protein: The expression of the recombinant aminoglycoside 3'-adenyltransferase protein at a high level was obtained by the transformation of *E. coli* cells (Fig. 3) with the plasmid pPP-30UA -*aad*A, consisting of the gene coding for *aad*A cloned into the pPP-30UA vector.

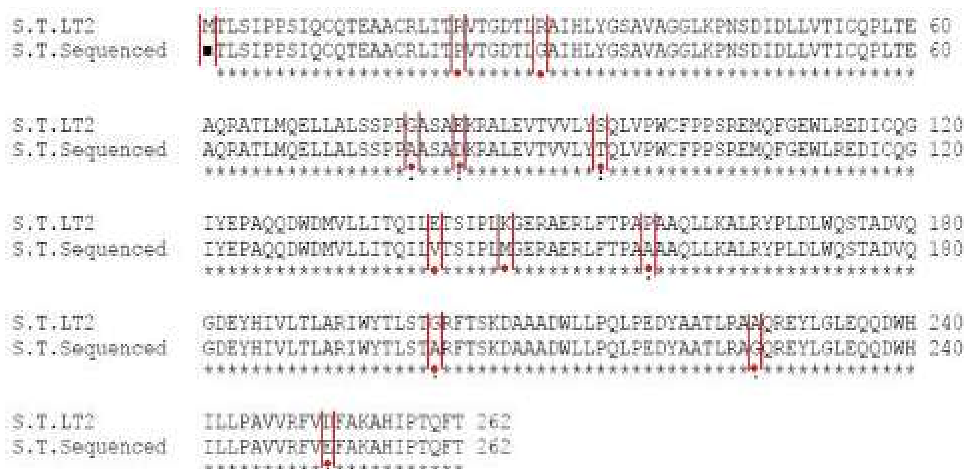

Fig. 4: Shows alignment between the sequenced *aadA* aminoglycoside resistance protein of *Salmonella* isolate (S.T. Sequenced) and annotated *aadA* aminoglycoside resistance protein of *Salmonella typhimurium* from GenBank (S.T.LT2). The alignment shows amino acid variations indicated by red dots.

Antibiotic Resistance Confirmation: The *E. coli* expressing the recombinant *aad*A aminoglycoside resistance protein was used in the determination of phenotypic resistance. In general, the minimum inhibitory concentration (MIC) of streptomycin/spectinomycin used against control strain of *E. coli* (lack *aad*A coding gene), was found to be >25µg/ml, while the incidence of resistance of *E. coli* strain expressing the recombinant *aad*A to the streptomycin/spectinomycin was mainly higher, approximately >96µg/ml.

Amino Acid Composition: The amino acid composition of the recombinant *aad*A protein was estimated on the basis of a 29.17kDa subunit of 262 amino acids. The N-terminal amino acid sequence of the recombinant enzyme matched that of the native *aad*A enzyme purified from *Salmonella* isolate indicating that the initiating methionine was removed post-translationally, as in the natural host (result not shown).

The alignment between recombinant *aad*A protein sequence and *aad*A protein sequence of *S. typhimurium* LT2 indicated variability in several residues (Fig. 4).

DISCUSSION

During the last decade, antibiotic resistance and multi-resistance of *Salmonella* species have increased a great deal. In Egypt, there is a little information on the predominance of *Salmonella typhimurium*, due to the relatively low incidence of infections caused by it (Unpublished data).

The present study characterized the nucleotide sequence and expression of streptomycin and spectinomycin resistance gene (*aad*A) from *Salmonella typhimurium* isolate from Egypt, to be used as a chloroplast transformation marker gene in *plastid transformation system* using plastid expression cassette. During the bacterial isolate study we found that the colonial growth pattern displayed by the isolate on a Hektoen enteric agar was greenish-blue in color because the bacterium did not ferment lactose. The center of the bacterial colonies appeared with black deposits due to production of hydrogen sulfide [24]. From these indicators, we concluded that the isolate under investigation was *S. typhimurium*.

Generally; *Salmonella* species show resistance to ampicillin, streptomycin, kanamycin, chloramphenicol, tetracycline [6-9]. Antimicrobial susceptibility tests for the *Salmonella* isolate in this study showed that the isolate was resistant to streptomycin/spectinomycin, kanamycin, ampicillin, chloramphenicol and tetracycline of minimum inhibitory concentrations (MIC): 128µg/ml, 64µg/ml, 32µg/ml, 32µg/ml and 16µg/ml; respectively. The resistance of the tested organism to the tested antibiotics was mainly higher to, streptomycin/spectinomycin and kanamycin than other antibiotic agents.

Depending on the data collected from GenBank database, the complete gene sequence has been identified in a number of *Salmonella* species as described in many previous studies [8, 10, 17]. The PCR assay performed using the synthesized oligonucleotides based on the identified *aad*A genes of these species and genomic DNA of *Salmonella* isolate as template revealed a DNA fragment approximately 789bp long, being similar to that reported by Benacer *et al*. [5].

Multiple DNA sequence alignments were carried out by using ClustalW (1.83), the multiple alignment program for DNA, (http://www2.ebi.ac.uk/clustalW)

between the sequenced *aad*A gene from the *Salmonella* isolate and the annotated sequence of *aad*A genes (GenBank accession number DQ133159; DQ133164; DQ133165.1; NC_003197.1) from the database, showed that the sequenced *aad*A gene scored maximum identity with *aad*A gene sequence (GenBank accession number NC_003197.1) from *Salmonella typhimurium* LT2 strain with conserved 98% identity, being similar to that reported by Benacer *et al*. [5] and Leung *et al*. [17]. Also the sequenced *aad*A gene from the *Salmonella* isolate possess conservation of 55% and 51% identity with the annotated sequences from the database accession number DQ133159 & DQ133164; and DQ133165.1 respectively, similar to the data reported by Shaw *et al*. [16].

The expression results showed that; the recombinant *aad*A aminoglycoside resistance protein was expressed at a high level, about 15% of soluble *E. coli* protein. We obtained about 25mg of pure protein per liter of culture. The multiple amino acid sequence alignment between the native and recombinant *aad*A aminoglycoside resistance protein revealed that both sequences were matched; also the alignment indicated that the initiating methionine was removed post-translationally, as in the natural host (results not shown). While the alignment between recombinant *aad*A aminoglycoside resistance protein from the *Salmonella* isolate and annotated *aad*A aminoglycoside resistance protein from *Salmonella typhimurium* LT2 showed a number of amino acid variations.

On the other hand; the variability in amino acid residues between the recombinant and annotated *aad*A aminoglycoside resistance protein might lead to change in protein activity against the streptomycin/ spectinomycin antibiotic agents.

During the antimicrobial susceptibility testing; the minimum inhibitory concentrations (MIC) of streptomycin/spectinomycin against the native aminoglycoside 3'-adenyltransferase found to be 128µg/ml, while the MIC value against recombinant aminoglycoside 3'-adenyltransferase was found to be 96µg/ml. The differences in MIC values mainly depend on the *aad*A aminoglycoside resistant protein activity against the antibiotic agents. We regarded the differences in MIC values between the native and the recombinant *aad*A aminoglycoside resistant protein to the differences in the promoters, which might lead to the differences in the expression efficiency and to signal peptides which existed in the native *S. typhimurium* machinery but didn't exist in the *E. coli* expression machinery.

In our study, no plasmid analysis or interference were done; DNA purification was established using a dedicated purification system; in order to eliminate the amplification of plasmid drug-resistant gene and to restrict the study on *Salmonella* genomic island drug resistant genes.

This study indicated that *aad*A coding gene from *Salmonella typhimurium* isolate from Egypt shares identity with the *aad*A gene sequence from GenBank database (*Salmonella typhimurium* LT2 strain) with conserved 98% identity. Also this study indicated that the recombinant *aad*A aminoglycoside resistance protein showed amino acid variability which might affect the protein activity against streptomycin and spectinomycin antibiotics.

The nucleotide sequence of the Egyptian isolate *Salmonella typhimurium aad*A gene has been deposited in the GenBank database under accession no. JF319443.

ACKNOWLEDGMENTS

We are very grateful to *Prof. Dr. Adel Khalil Goher*, Professor of Clinical Pathology, Faculty of Veterinary Medicine, Cairo University; for providing the *Salmonella* isolate for this study.

REFERENCES

1. Gebreyes, W.A., S. Thakur, P.R. Davies, J.A. Funk and C. Altier, 2004. *Salmonella serotypes* from pigs, 1997–2000. J. Antimicrob. Chemother., 53: 997-1003.
2. Threlfall, E.J., J.A. Frost, L.R. Ward and B. Rowe, 1996. *Salmonella typhimurium*. Lancet, 347: 1053-1054.
3. Eman Halawani and Mohamed Shohayeb, 2008. Molecular Characterization of Multiple Antibiotic Resistance in *Salmonella enterica, Serovar typhimurium* and Eenteritidis Isolated in Saudi Arabia. World J. Med. Sci., 3: 65-70.
4. Gorman, R. and C.C. Adley, 2004. Characterization of *Salmonella enterica Serotype typhimurium* isolates from human, food and animal sources in the Republic of Ireland. J. Clin. Microbiol., 42: 2314-2316.
5. Benacer, D., K.L. Thong, H. Watanabe and S.D. Puthucheary, 2010. Characterization of Drug-Resistant *Salmonella enterica, Serotype typhimurium* by Antibiograms, Plasmids, Integrons, Resistance Genes and PFGE. J. Microbiol. Biotechnol., 20: 1042-1052.

6. Cardoso, M.O., A.R. Ribeiro, L.R. Dos Santos, F. Pilotto, H.L.S. De Moraes, C.T.P. Salle, *et al*., 2006. Antibiotic resistance in *Salmonella enteritidis* isolated from broiler carcasses. Braz. J. Microbiol., 37: 368-371.
7. Foley, S.L., D.G. White, P.F. McDermott, R.D. Walker, B. Rhodes, P.J Fedorka-Cray, *et al*., 2006. Comparison of subtyping methods for differentiating *Salmonella enterica* serovar typhimurium isolates obtained from food animal sources. J. Clin. Microbiol., 44: 3569-3577.
8. Rabatsky-Her, T., J.M. Whichard, S. Rossiter, B. Holland, K. Stamey, M.L. Headrick, *et al*., 2004. *Salmonella enterica* typhimurium, United States, 1997-1998. Emerg. Infect. Dis., 10: 795-801.
9. Ribot, E.M., R.K. Wierzba, F.J. Angulo and T.J. Barrett, 2002. *Salmonella enterica* serotype typhimurium DT104 isolated from humans, United States, 1985, 1990 and 1995. Emerg. Infect. Dis., 8:387-391.
10. Sanderson, K.E., A. Hessel, S.L. Liu and K.E. Rudd, 1996. The genetic map of *Salmonella typhimurium*, edition VIII. In: *Escherichia coli* and *Salmonella* cellular and Molecular Biology, Eds., Ingraham, C., L. Lin, M. Reznikoff, R. Schaechter. ASM Press, Washington, DC., 2: 1903-1999.
11. Mingeot-Leclercq, M.P., Y. Glupczynski and P.M. Tulkens, 1999. Aminoglycosides: activity and resistance. Antimicrob. Agents Chemothe., 43: 727-737.
12. Miller, G.H., F.J. Sabatelli, R.S. Hare, Y. Glupczynski, P. Mackey, D. Shlaes, *et al*., 1997. The most frequent aminoglycoside resistance mechanisms--changes with time and geographic area: a reflection of aminoglycoside usage patterns? Aminoglycoside Resistance Study Groups. Clin. Infect. Dis., 24: 46-62.
13. Miller, G.H., F.J. Sabatelli, L. Naples, R.S. Hare and K.J. Shaw, 1995. The changing nature of aminoglycoside resistance mechanisms and the role of isepamicin--a new broad-spectrum aminoglycoside. The Aminoglycoside Resistance Study Groups. J. Chemother., 7: 31-44.
14. Biendo, M., G. Laurans, D. Thomas, B. Canarelli, F. Hamdad-Daoudi, F. Rousseau, *et al*., 2005. Molecular characterisation and mechanisms of resistance of multidrugresistant human *Salmonella enterica* serovar typhimurium isolated in Amiens (France). Int. J. Antimicrob., 26: 219-229.
15. Cardoso, M.O., A.R. Ribeiro, L.R. Dos Santos, F. Pilotto, H.L.S. De Moraes, C.T.P. Salle, *et al.,* 2006. Antibiotic resistance in *Salmonella enteritidis* isolated from broiler carcasses. Braz. J. Microbiol., 37: 368- 371.
16. Shaw, K.J., P.N. Rather, R.S. Hare and G.H. Miller, 1993. Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. Microbiol., 57: 138-163.
17. Leung, K.Y., S.R. Ruschkowski and B.B. Finlay, 1992. Isolation and characterization of the *aad*A aminoglycoside-resistance gene from *Salmonella choleraesuis*. Mol. Microbiol., 6: 2453-2460.
18. Zora Svab and Pal Maliga, 1993. High-frequency plastid transformation in tobacco by selection for a chimeric aadA gene. Proc. Natl. Acad. Sci., 90: 913-917.
19. MacFaddin, J.F., 1985. Media for Isolation, Cultivation, Identification, Maintenance of Medical Bacteria. Baltimore, MD. Williams & Wilkins, 1: 357-359.
20. Maria, I., Maria Garcia-Castillo,Fernando Baquero, Maria Perez-Vazquez, Elena Loza and Rafael Canton, 2005. Breakpoints for Predicting Pseudomonas aeruginosa Susceptibility to Inhaled Tobramycin in Cystic Fibrosis Patients: Use of High-Range Etest Strips. J. Clin. Microbiol., 43: 4480-4485.
21. Manal, M., E. Alaa and S. Sherein, 2011. Assessment of the Immune Status in Nile Tilapia (*Oreochromis niloticus*) Experimentally Challenged with Toxogenic / Septicemic Bacteria During Treatment Trial with Florfenicol and Enrofloxacin. WJFMS., 3: 21-36.
22. Sambrook, J., E.F. Fritsch and T. Maniatis, 1989. Molecular Cloning: a laboratory manual. 2nd ed. N.Y., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, pp: 1659. ISBN 0-87969-309-6
23. Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227: 680-685.
24. James, T., R. David Boone, J. Don Brenner, Paul De Vos, M. George Garrity, Michael Goodfellow R. Noel Krieg, A. Fred Rainey and Karl-Heinnz Schleifer. XXXX. Bergey's Manual of Systematic Bacteriology, USA.