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Plasmid Encoding Antimicrobial Resistance among Environmental *Salmonella* Species and Molecular Characterization Using Random Amplified Polymorphic DNA Analysis

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Abstract: The increase in antibiotic resistance among Gram-negative bacteria is a notable example of how bacteria can procure, maintain and express new genetic information that can confer resistance to one or several antibiotics. The emergence of multidrug resistant (MDR) Salmonella has become a serious problem. Our study was done to investigate the role of plasmid as a factor mediating such resistance pattern. This study was done on 40 *Salmonella* isolates from different food products. Antibiogram was done by Kirby Bauer disc diffusion method and the minimum inhibitory concentration (MIC) was determined using the microtiter broth dilution method. MDR isolates (23/40) were confirmed and identified to species level using API 20E. Plasmid extraction was done for MDR isolates by alkaline lysis. It was revealed that 60.86% (14/23) of MDR isolates were found to harbour plasmids. To demonstrate the role of plasmid in resistance, *Salmonella* isolates were subjected to plasmid curing using sodium dodecyl sulfate (SDS). The present study has investigated the degree of similarity between five MDR isolates on molecular basis using random amplified polymorphic DNA (RAPD) technique, in which a total of 5 primers were used showing good discriminatory power for all five isolates. The dendrogram showed a common lineage among all five isolates. Finally 16S ribosomal DNA sequencing has been performed for one *Salmonella* isolate.

Key words: *Salmonella* · Plasmid · RAPD-PCR · 16S ribosomal DNA sequencing

INTRODUCTION

The genus *Salmonella* belongs to the family *Enterobacteriaceae*. They are facultatively anaerobic rods [1]. *Salmonella* is mostly motile, non spore forming Gram-negative bacterium [2]. *Salmonella* infection of humans and animals continues to be a distressing health problem worldwide [3]. *Salmonella* spp. are mainly transmitted by the fecal-oral route [4].

People are often infected when they eat contaminated food of animal origin such as meat or eggs. They can also be infected by ingesting organisms in animal feces, either directly or in contaminated food or water [5].

Infection with organisms of the genus *Salmonella* results in three main syndromes which are infection localized to the intestine, often known as salmonellosis, invasive or bacteraemic disease and enteric fever [1].

Salmonellosis is the major cause of food borne infections and the second most common food borne illness after *Campylobacter* infection [6].

Salmonella enterica serovar *arizona* is naturally found in reptiles but also causes outbreaks of salmonellosis in turkeys and sheep and can produce both enteritis and serious disseminated disease in humans [7].

Many isolates are resistant to one or more antibiotics and the choice of drugs should, if possible, be based on susceptibility testing [8]. Some strains may display resistance to gentamicin, kanamycin and trimethoprim/sulfamethoxazole.

Resistance to third generation of cephalosporins in *Salmonella* is of interest because these are drugs of choice for treating salmonellosis in children, where fluoroquinolones are contraindicated [7]. Cephalosporins

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are not active against many MDR *Salmonella* strains and this limits their use in empirical treatment when resistant typhoid is likely [1].

Resistance genes are often located on extrachromosomal genetic elements or in segments inserted within the chromosome that originates from other genomes. The acquisition of a new gene may occur by genetic transformation, but when resistance genes are located on plasmids-self-replicating double-stranded circles of DNA, they can be mobilised by conjugative transfer. The latter may occur at high frequency and efficiency and several resistance genes can be acquired simultaneously [9].

Resistance to chloramphenicol, ampicillin and trimethoprim is generally plasmid encoded, although additional chromosomal resistance to ciprofloxacin has been reported [10]. Methods for the detection of *Salmonella* include the conventional culture method, rapid screening methods, immunological methods and DNA-based methods. The traditional techniques used for the detection of *Salmonella* mostly suffer from being either time-consuming, labor intensive, or expensive [11].

The application of the PCR is one approach for the rapid and effective detection and identification of *Salmonellae*, so the application of RAPD analysis based on the random amplification of genomic DNA fragments through short arbitrarily designed primers is an attractive alternative for the detection and identification of microorganisms, especially where previous sequence information is not available [11].

This study was undertaken to investigate the role of plasmid in mediating antimicrobial resistance among selected *Salmonella* isolates and to detect the degree of similarity between five of studied MDR isolates based on RAPD-PCR analysis.

MATERIALS AND METHODS

Microorganisms: This study was performed on 40 *Salmonella* isolates recovered from various food products such as raw meat, raw vegetables, mayonnaise and raw chickens which were obtained from hotels and industrial locations in Cairo, Alexandria and Sharm el sheikh in Egypt.

Identification of Bacterial Isolates: All the isolates were previously confirmed and identified by the standard bacteriological methods and were further confirmed and identified to species level using API 20E.

Plasmid Analysis: Plasmid DNA was extracted from cultured cells following alkaline lysis method of plasmid preparation according to Maniatis *et al*. [12]. The samples were processed using gel electrophoresis to identify the number of plasmid copies present in different isolates. For this purpose, an agarose gel of 0.8% was used according to Sambrook and Russel [13]. Staining of DNA fragments was carried out using ethidium bromide and they were visualized by UV-Trans illumination. Standard DNA molecular weight marker was used to estimate the plasmids sizes. The standard DNA molecular weight marker used in the present study was lambda DNA / Hind III Digest ladder.

Plasmid Curing: Plasmid curing was carried out using sodium dodecyl sulfate (SDS) as described by Mirmomeni *et al*. [14]. Sodium dodecyl sulfate solution (10% w/v, pH 7.4) was added to lauria-bertani (LB) broth (double strength) and the final volume was made with sterile distilled water to give 5, 4, 3, 2, 1 and 0.5% SDS in the LB (normal strength). An inoculum of 100 µl of the wild-type was then used to seed the SDS containing LB broth and the cultures were incubated overnight with shaking at 45°C and sub cultured for 6 days.

After which a dilution from each culture was cultivated on to selective media (S.S agar). To select the derivatives that had lost antibiotic resistance the colonies were transformed by tooth picking on to Muller Hinton agar (MHA) media or onto LB agar plates containing minimal inhibitory concentration for each isolate to the selected antimicrobial agent. Colonies that were unable to grow in the presence of any or the entire used antimicrobial agents were further tested for the presence of their plasmids.

DNA Extraction for RAPD PCR: Isolation of genomic DNA was performed according to Maloy [15] and Gu¨ rakan *et al*. [11] as follow; bacterial cells from an overnight culture previously grown at 37°C in 10 mL of L.B. broth medium were transferred to 1.5-mL eppendorf tubes and spun at 18,000 × *g* for 2 min.

After supernatant was removed, a new 1.5-mL cell culture was added onto the pellet and again spun at 18,000 × *g* for 2 min. Then, the pellet was resuspended in 467 µl of Tris-EDTA buffer; 30 µl of 10% SDS and 3 µl of 20 mg/mL of proteinase K were added and this mixture was incubated at 37°C for 1 hr.

An equal volume (500 µl) of phenol-chloroform-isoamyl alcohol was added and mixed well by inverting the tube until the phases were completely mixed and then

the tube was spun at 18,000 × *g* for 2 min. The upper aqueous phase containing nucleic acids was carefully transferred to a new tube and phenol-chloroform-isoamyl alcohol extraction was repeated until the interphase was clear.

The aqueous phase obtained after the last in the series of deproteinization was mixed with 0.1× volume of 3 M sodium acetate and ethanol precipitation was performed by adding 2× volume of ice cold 95% ethanol.

The DNA was precipitated overnight at-20°C and collected by centrifugation at 25,000 × *g* for 15 min. Finally, the supernatant was discarded; the pellet was washed with 1 ml of ice-cold 70% ethanol to eliminate salt and centrifuged for another 30 s at 25,000 × *g*. The tube was allowed to air dry until all the ethanol was evaporated.

The DNA was dissolved in 30 to 50 µl of Tris-EDTA buffer and stored at-20°C. The resulting DNA concentration was determined spectrophotometrically and also by comparing the intensity of the band with that of the bands of known concentration on agarose gel.

Random Amplified Polymorphic DNA-PCR (RAPD-PCR): PCR amplification was carried out according to Yaqoob *et al.* [16] and Smith *et al*. [17]; in a 25 µl volume with the gene amplification PCR-system using DNA thermal cycler (Whatmann Biometra® UNO²², Gottingen, Germany). Reaction mixture contained 2 µl DNA of the selected isolates, 0.5 µl dNTPs, 3 µl $MgcL_{2,}$ 0.1 µl of Taq DNA polymerase (promega) and 1.25 µl of each of the following primers; OPD2, OPD3, OPD4, OPD5 and OPD8 (Table 1). The primers sequences used in this technique were shown in table (1).

PCR program consisted of initial denaturation step at 95 °C for 5 min, followed by thirty seven cycles of DNA denaturation at 93 °C for 40 sec., primer annealing at 36 °C for 40 sec., an elongation step at 72 °C for 2 min and a final elongation step at 72 °C for 10 min, after the last cycle, the product was stored at 4 ºC. After PCR, 10-20 µl aliquots of products were subjected to electrophoresis in 1.5 % agarose gel, followed by ethidium bromide staining and photography as previously mentioned.

16S Ribosomal DNA Sequencing: Sequencing was done according to Amit-Romach *et al*. [18]. Bacterial DNA extraction was performed by PrepMan® Ultra Sample Preparation Reagent Protocol (Part Number 4367554) (Applied Biosystems) and DNA concentration was measured by Nanodrop Spectrophotometer.

Table 1: Primers sequences of *Salmonella*

Primer	Sequence
OPD2	5'GGACCCAACC'3
OPD3	5'GTCGCCGTCA'3
OPD4	5'TCTGGTGAGG'3
OPD5	5'TGAGCGGACA'3
OPD8	5' GTGTGCCCCA'3
Sal201-F	CGGGCCTCTTGCCATCAGGTG
Sal597-R	CACATCCGACTTGACAGACCG

PCR Amplification for Sequencing: PCR amplification was carried out in a 25 µl volume with the gene amplification PCR-system using DNA thermal cycler (Whatmann Biometra® UNO²², Gottingen, Germany). The used Taq PCR master mix provided QIAGEN Taq DNA polymerase in a premixed format.

This ready to-use solution included Taq DNA polymerase, PCR buffer, MgCl2 and ultrapure dNTPs at optimized concentrations. Only primers and template DNA were added to prepare the final PCR. Taq PCR master mix contributes to highly reproducible PCR by reducing pipetting errors and miscalculation.

Reaction mixture containing 20ng DNA template, 0.4 µl of Sal201-F as forward primer, 0.4 µl of Sal597-R as reverse primer (Table 1), 200 µM each dNTPs, 1.5 mM MgCl2 and 12.5 µl of Taq DNA polymerase. PCR program consisted of initial denaturation step at 94 °C for 3 min, followed by thirty five cycles of DNA denaturation at 94 °C for 30 sec., primer annealing at 60 °C for 1 min, 68 °C for 2 min and a final elongation step at 68 °C for 7 min, after the last cycle the product was stored at 4 ºC. After PCR, 10-20 µl aliquots of products were subjected to electrophoresis in 2 % agarose gel, followed by ethidium bromide staining and photography as previously mentioned.

RESULTS

Identification of Bacterial Isolates: In this study, a total of 40 bacterial strains were isolated from food specimens. Investigations using different biochemical tests revealed that 100% (40/40) of isolates belonged to *Salmonella* spp., regarding to species identification carried out on MDR isolates using API 20E, 56.52% (13/23) of isolates were identified as *Salmonella arizonae* and 43.48% (10/23) were identified as *Salmonella species*.

Table 2: Molecular size (kb) of plasmid DNA molecules extracted from tested MDR Salmonella isolates

Lane Number	Isolate	Plasmid Number	Plasmid M.WT(Kb)
1	HA029	7	7.897, 4.361, 2.821, 1.765, 1.553, 1.153, 0.880
2	ISO100	0	-
3	HA042	3	4.361, 1.503, 1.197
4	HA013	2	4.361, 1.197
5	HA019	3	4.361, 2.122, 0.942
6	HA009	0	-
7	HA006	2	4.361, 1.265
8	HA039	2	4.361, 1.265
9	HA037	0	-
10	HA003	0	-
11	HA008	0	-
12	HA051	0	-
13	HA012B	1	1.405
14	HA034	2	6.973, 2.977
15	Salm.	1	6.973
16	HA023I	0	-
17	HA023II	1	1.453
18	HA025	4	7.422, 3.682, 2.194, 1.220
19	HA005	0	-
20	ATCC	1	7.422
21	HA012A	3	18.592, 8.641, 1.757
22	HA017	2	10.978, 2.131
23	HA016	0	-

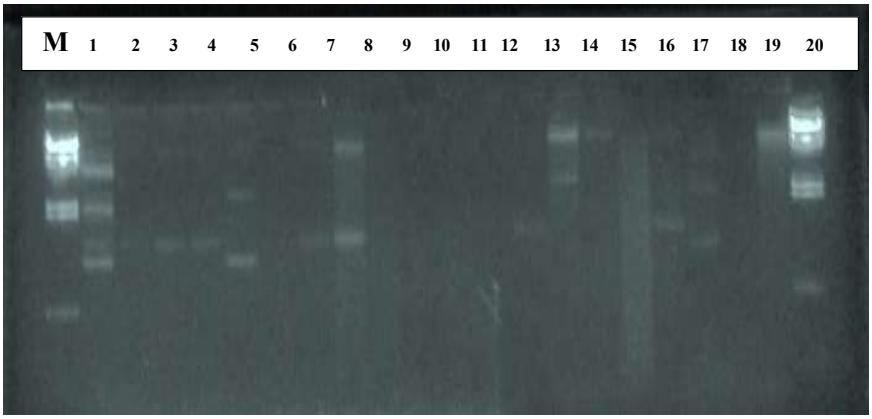

Fig. 1: Plasmid profile of *Salmonella* isolates; Lane (1): lambda DNA/ Hind III Digest; Lane (2)-Lane (20): S_1 to S_{20} *Salmonella* isolates; Lane (21): lambda DNA/ Hind III Digest

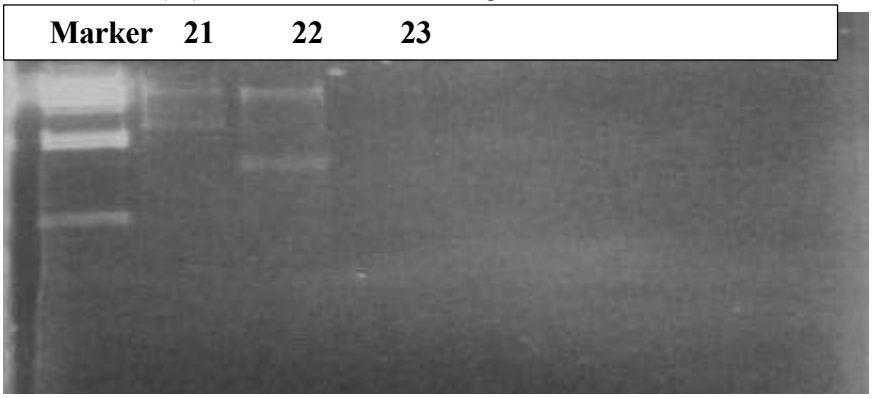

Fig. 2: Plasmid profile of *Salmonella* isolates; Lane (1st): lambda DNA/ Hind III Digest; Lane (21)-Lane (23): S_{21} to S_{23} *Salmonella* isolates

Table 3: Molecular size (kb) of plasmid DNA molecules extracted from wild type and SDS treated of the tested bacterial strains

Lane Number	Isolate	Wild isolate Plasmid M.WT(Kb)	Cured isolate Plasmid M.WT(Kb)
1	HA025	7.317, 3.576, 2.783, 1.209	3.576, 1.209
2	HA012A	18.778, 5.814, 2.976	18.778
3	HA019	4.361, 2.197, 1.759	1.759
4	HA039	4.361, 1.672	1.672
5	HA034	6.557, 2.778	6.557
6	HA023II	1.619	Zero

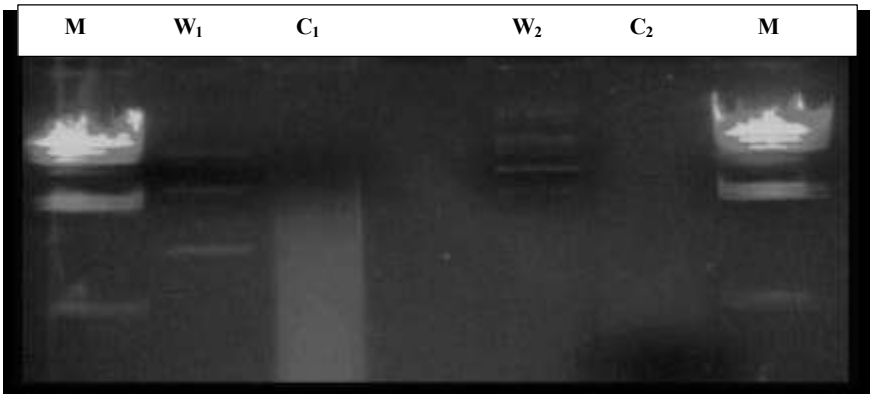

Fig. 3: Plasmid profile of cured *Salmonella* isolates; Lane (M): lambda DNA/ Hind III Digest; Lane (W_1):HA025; Lane (C_1): cured HA025; Lane (W_2): HA012A; Lane (C_2): cured HA012A; Lane (last): lambda DNA/ Hind III Digest.

Plasmid Extraction: The plasmid extraction that was performed on MDR isolates (23/40) revealed that most of MDR isolates harbour at least one plasmid. When all the 23 MDR *Salmonella* isolates were screened for plasmids, fourteen isolates (60.86%) were found to harbour plasmids. Some isolates possessed single sized plasmid while others had multiple plasmids (Table 2 and Fig. 1 and 2). So, plasmids occurred in more than half of MDR isolates of *Salmonella.*

Plasmid Curing: A cell lysate of each of the wild type MDR isolates and the SDS treated ones were screened for the presence of plasmid DNA using agarose gel electrophoresis (Table 3 and Fig. 3 and 4).

The change in the plasmid profile of the wild type isolates and the SDS treated one was found to be associated with antimicrobial susceptibility changes; where the SDS treated *Salmonella* HA 025, *Salmonella* HA 012A, *Salmonella* HA019, *Salmonella* HA039, *Salmonella* HA034 and *Salmonella* HA023II become more susceptible to chloramphenicol, cefepime and cefoperazone rather than the wild type (Table 4).

These data proved that chloramphenicol, cefepime and cefoperazone resistance are more probably associated with plasmid.

Random Amplified Polymorphic DNA-PCR (RAPD-PCR): Molecular typing of the MDR-isolates was done using a DNA-based typing method (random amplified

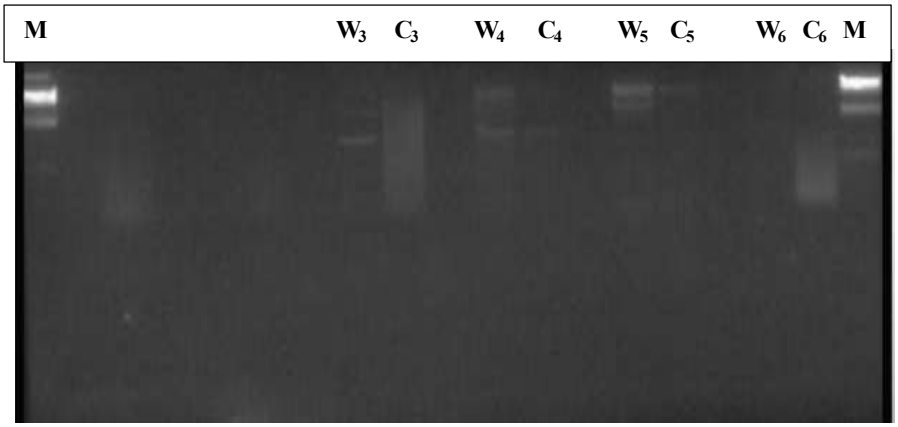

Fig. 4: Plasmid profile of cured *Salmonella* isolates; Lane (M): lambda DNA/ Hind III Digest; Lane (W_3): HA019; Lane (C_3): cured HA019; Lane (W_4): HA039; Lane (C_4): cured HA039; Lane (W_5): HA034; Lane (C_5): cured HA034; Lane (W_6): HA023II; Lane (C_6): cured HA023II; Lane (last): lambda DNA/ Hind III Digest.

Table 4: Antibiogram for wild and cured *Salmonella* isolates

Antibacterial agent* (Conc./disc)	*Salmonella* isolates											
	HA 025		HA 012A		HA019		HA039		HA034		HA023II	
	Wild isolate	Cured isolate	Wild isolate	Cured isolate	Wild isolate	Cured isolate	Wild isolate	Cured isolate	Wild isolate	Cured isolate	Wild isolate	Cured isolate
CPM* (30µg)	R	S	R	S	R	S	R	S	S	S	R	S
CS* (75µg)	S	S	I	S	R	R	I	S	R	S	R	R
CL* (30µg)	S	S	I	I	R	R	I	I	S	S	R	R
C* (30µg)	R	S	S	S	R	S	S	S	R	S	S	S

CPM*; Cefepim, CS*; Cefoperazone, CL*; Ceftriaxone, C*; Chloramphenicol

polymorphic PCR). RAPD PCR was done for five isolates out of 23 MDR *Salmonella* isolates for molecular typing of the five isolates. The analysis of RAPD assay results was performed using uvitech super cool documentation equipment and software, version 11.9. and phylogenetic tree, as dendrogram analysis. Data obtained from dendrogram tree proved that there was a correlation between our MDR *Salmonella* isolates as shown in the figures 5,6.

Sequencing: 16S ribosomal DNA sequencing has been for one *Salmonella* isolate which confirmed our previous identification for *Salmonella* isolates by using gene bank program blast to ensure that the proposed primers were complementary with the target *Salmonella* species but not with other bacterial groups by comparing the complete 16S rDNA sequence of *Salmonella* HA009 using the program NCBI blast.

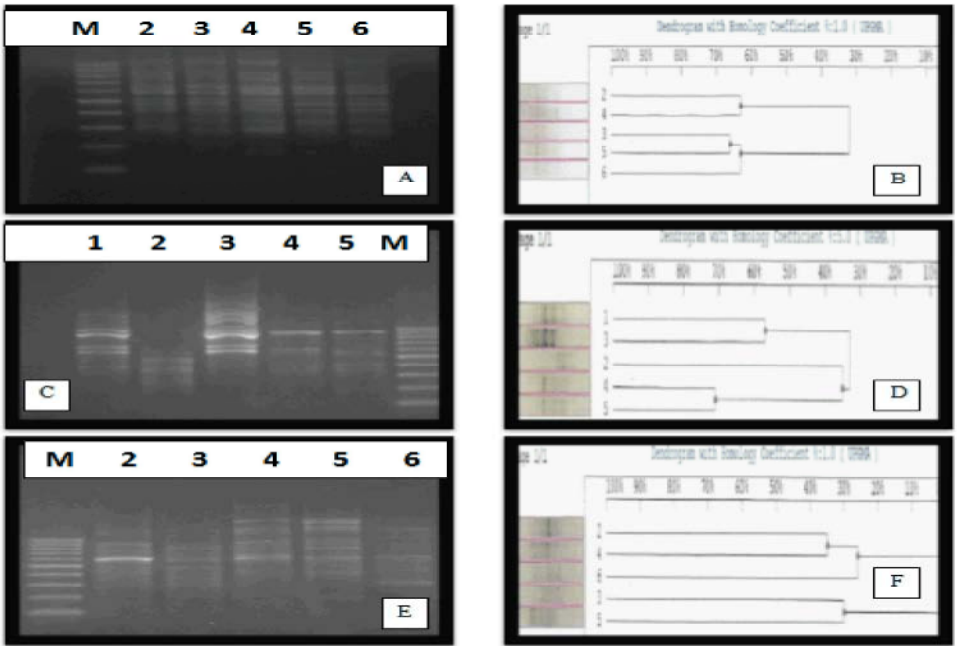

Fig. 5: (A): Random amplified polymorphic-PCR typing of the MDR isolates using OPD3 primer, Lane (1):100bp DNA ladder; Lane (2): HA029; Lane (3): HA042; Lane (4): HA019; Lane (5): HA009; Lane (6): HA039; (B): Dendrogram illustrates the correlation between the analyzed data and PCR result of the MDR-*Salmonella* isolates using OPD3 primer; (C): Random amplified polymorphic-PCR typing of the MDR isolates using OPD2 primer, Lane (1): HA029; Lane (2): HA042; Lane (3): HA019; Lane (4): HA009; Lane (5): HA039; Lane (6): 100bp DNA ladder; (D): Dendrogram illustrates the correlation between the analyzed data and PCR result of the MDR-*Salmonella* isolates using OPD2 primer; (E): Random amplified polymorphic-PCR typing of the MDR isolates using OPD4 primer, Lane (1):100bp DNA ladder; Lane (2): HA029; Lane (3): HA042; Lane (4): HA019; Lane (5): HA009; Lane (6): HA039; (F): Dendrogram illustrates the correlation between the analyzed data and PCR result of the MDR-*Salmonella* isolates using OPD4 primer

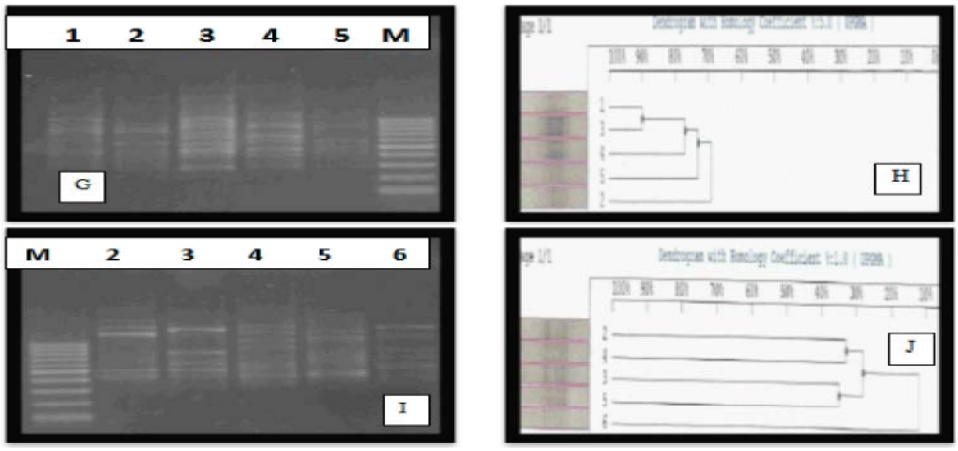

Fig. 6: (G): Random amplified polymorphic-PCR typing of the MDR isolates using OPD5 primer, Lane (1): HA029; Lane (2): HA042; Lane (3): HA019; Lane (4): HA009; Lane (5): HA039; Lane (6): 100bp DNA ladder; (H): Dendrogram illustrates the correlation between the analyzed data and PCR result of the MDR-*Salmonella* isolates using OPD5 primer; (I): Random amplified polymorphic-PCR typing of the MDR isolates using OPD8 primer, Lane (1):100bp DNA ladder; Lane (2): HA029; Lane (3): HA042; Lane (4): HA019; Lane (5): HA009; Lane (6): HA039; (J): Dendrogram illustrates the correlation between the analyzed data and PCR result of the MDR-*Salmonella* isolates using OPD8 primer

DISCUSSION

Gram-negative bacterial resistance possibly now equals or usurps that of Gram-positive bacterial resistance. Many isolates are resistant to one or more antibiotics and the choice of drugs should be based on susceptibility testing [7]. During this study antimicrobial resistance to more than three unrelated classes of antimicrobial agents was observed for many isolates so we try to detect if this resistance pattern was plasmid mediated.

Plasmids represent an altogether diverse category of extra-chromosomal genetic elements. They are circular double-stranded DNA molecules critically present intracellularly and symbiotically in most microorganisms [19].

In our study, plasmid profile analysis of *Salmonella* HA 025 showed that it has four DNA plasmid molecules of molecular weights 7.317, 3.576, 2.783 and 1.209 kb. While the SDS treated one lost the DNA plasmid molecules of the molecular sizes 7.317 and 2.783 kb.

Also, plasmid profile analysis of *Salmonella* HA 012A showed that it has three DNA plasmid molecules of molecular weights 18.778, 5.814 and 2.976 kb While the SDS treated one lost the DNA plasmid molecules of the molecular sizes 5.814 and 2.976 kb., while *Salmonella* HA019 showed that it has three DNA plasmid molecules of molecular weights 4.361, 2.197 kb and 1.759 While the cured derivative of this strain lost the DNA plasmid molecules of the molecular sizes 4.361 and 2.197 kb.

For *Salmonella* HA039, the plasmid profile showed that it has two DNA plasmid molecules of molecular weights 4.361 and 1.62 kb while the SDS treated one lost the DNA plasmid molecule of the molecular size 4.361 kb.

Salmonella HA034 showed that it has two DNA plasmid molecules of molecular weights 6.557 and 2.778 kb, while the cured derivative of this strain lost the DNA plasmid molecule of the molecular size 2.778 kb. So these data from the plasmid profile analysis demonstrated that the resistant genes might be carried on one of the lost plasmids.

Over the last few years several genetic typing methods for *Salmonella* spp. have been evaluated and these methods appear to be useful tools for epidemiological and phylogenetic purposes. A rapid method which is used universally, randomly amplified polymorphic DNA (RAPD) analysis performed with different primers, has been used [20].

The present study aimed to determine to what degree RAPD analysis can be used to give concordant results using *Salmonella* isolates.

The study showed that RAPD provides an excellent screen for typing of *Salmonella* isolates and this is also supported by data obtained by other studies, in which Gu¨ rakan *et al*. [11] revealed that random amplified polymorphic DNA analysis has the potential to detect polymorphism throughout the entire genome as compared with other techniques, also Smith *et al*. [17] showed that the RAPD-PCR would be useful for epidemiological typing of the *Salmonella* spp in Nigeria and Yaqoob *et al*. [16] reported that RAPD analysis was applied for molecular characterization of *Salmonella enteritidis* strains.

In the present study the phylogenetic tree constructed from RAPD data showed a single lineage pattern, which indicated that all isolates are descendents of the same microorganism and are related to each others.

It can be concluded that antimicrobial resistance among most of our *Salmonella* isolates was more probably due to resistant genes carried on plasmids. RAPD-PCR provided an excellent screen for typing of *Salmonella* isolates.

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