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Epidemiological Typing of *Salmonellla enterica* Isolates Causing Acute Food Poisoning in Saudi Arabia Based on Plasmid Profiles and Antibiograms

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Abstract: A total of 41 *Salmonellla* enterica isolates, 23 serotype Typhimurium and 18 serotype Enteritidis isolated in Saudi Arabia were epidemiologically studied. All isolates of both serotypes were resistant to at least one antibiotic of the 15 tested. Multi-drug resistance was prevalent in the majority of isolates of *Salmonellla* serotype Typhimurium and more than 70% of these isolates were resistant to 6-8 antibiotics. It was possible to recognize 10 resistance patterns. *Salmonellla* serotype Enteritidis isolates were mostly resistant to streptomycin, neomycin and Sulfamethoxazole and 3 resistant patterns were dtected. Isolates of both serotypes harbored a sero-specific high molecular weight plasmid; 90 kb in *Salmonellla* serotype Typhimurium and 60 or 65 kb in *Salmonellla* serotype Enteritidis. In addition in *Salmonellla* serotype Typhimurium and serotype Enteritidis, 82.6% and 33.3% of the isolates harbored one or more low molecular weight plasmids. It was possible to recognize 7 and 5 plasmid profiles in serotype Typhimurium and Enteritidis respectively. Dice coefficient analysis of plasmids revealed 4 clusters in serotype Typhimurium and 3 clusters in serotype Enteritidis. Cluster A in serotype Typhimurium was more prominent (69.6% of isolates) and cluster E was more prominent in serotype Enteritidis (61.1% of isolates). Isolates of cluster A were further divided into 4 sub-clones and those of cluster E were divided into 2 sub-clones according to their plasmid profiles. When both plasmid profiles and antibiograms were combined the sub-clones were further divided into several new sub-clones. Data obtained suggest that the misuse of antibiotics resulted in the development and the dissemination of new sub-clones of both serotypes in the community. This fact necessitates the control of antibiotic use both in humans and animals.

Key words: Epidemiology · *Salmonella enterica* · Plasmids · Food poisoning · Typhimurium · Enteritidis

INTRODUCTION

Acute gastroenteritis caused by *Salmonellla enterica* continues to be a worldwide public health problem [1]. The Centers for Disease Control and Prevention has estimated that food-borne *Salmonellla* infections are responsible for 1.3 million illnesses annually worldwide, resulting in 16,000 hospitalizations and 600 deaths [2]. The two predominant agents associated with food-borne nontyphoidal salmonellosis were *S. enterica* serotype Enteritidis and *Salmonella enterica* serotype Typhimurium [3,4]. In addition to the importance of raw and undercooked meat, poultry, eggs and dairy products as potential vehicles of human salmonellosis, there are increasing reports of outbreaks associated with fresh fruits and vegetables [1,5].

Antibiotic therapy is usually not recommended for routine treatment of salmonellosis; however, appropriate antimicrobial therapy can be lifesaving for patients with invasive disease [6,7]. Hence, *Salmonellla* isolates from both humans and the environment or animals should be monitored for antimicrobial resistance. The emergence of antimicrobial-resistant *Salmonellla* strains is of great concern worldwide [8]. During the past few decades, various countries have witnessed a significant increase in human isolates of multiresistant *Salmonella enterica* [3, 9-12], as well as animal isolates [13-16]. Furthermore, the widespread use of antimicrobials in humans and animals is often implicated in the emergence of multidrug-resistant strains of *S. enterica* and there is a link between the use of antibiotics in animals and drug-resistant strains in human infections has been suggested [17-20].

Phage typing is often the initial method used for discrimination of isolates of a serotype outbreaks. However, changes in phage type can occur as the result of plasmid or phage acquisition, changes in

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lipopolysaccharide expression, or spontaneous mutations affecting phage receptor sites [21]. For this reason, molecular subtyping of *Salmonellla* isolates is an invaluable epidemiological tool that can be used to track the source of infection and to determine the epidemiological link between isolates. A number of molecular typing and fingerprinting methods have been investigated including plasmid profiling, pulsed-field gel electrophoresis (PFGE), random amplified polymorphic DNA analysis and ribotyping [4, 22-29].

Plasmid profiling has been proved to be valuable for differentiation of bacteria in epidemiological studies (30-34). Although many of these were performed prior to the widespread implementation of the more advanced method, plasmid profiling technique has significant advantages, like its rapidity and the relative simplicity of the procedure and basic apparatus required [35]. This technique has proved to be invaluable in the discrimination of strains disseminating in a particular community [5, 36, 37].

The aim of the present investigation was to eoidemiologically analyse strains of *Salmonellla enterica* serotypes Typhimurium and serotype Enteritidis isolated from acute cases of food poisoning. For this purpose we analysed the antibiograms and plasmid profiles of some *Salmonellla* isolates belonging to both serotypes.

METHODS

Bacterial Strains: *Salmonellla* strains used in this study were isolated from patients suffering from cases of acute food poisoning admitted to King Abdul aziz and Al-Noor Hospitals in Taif and Makkah respectively.

Antimicrobial Susceptibility Testing: Disk diffusion assay with Muller-Hinton agar in accordance with National Committee for Clinical Laboratory Standards [38]. Antibiotic discs used were, ampicillin (Amp), chefalexin (Cfl),, cefoxitin (Cfx), cefotaxime (Cfxm), pipracillin (Pip), streptomycin(Stm), neomycin (Nem), amikacin (Amk), gentamicin (Gen), tobramycin (Tob), tetracycline (Tet), chloramphenicol(Clm), sulfamethoxazole (Sul), cotrimoxazole (Cot), ciprofloxacin (Cip).

Plasmid Profile Analysis and Plasmid Fingerprinting: Plasmids were extracted from the *S. enterica* serotypes Typhimurium and Enteritidis isolates by the method of alkaline lysis [39]. The resulting DNA preparation was submitted to electrophoresis on horizontal slab gels containing 0.8% agarose (Bio-Rad, USA). Plasmids with known molecular sizes were used for size estimation. Electrophoresis was carried out at 100 V for 3 h. The slab gels were stained in ethidium bromide solution (10 $\mu g\ ml^{-1}$) for 10 min, followed by immersion in distilled water for 45 min. The slab gels were placed under UV light and photographed by a Polaroid camera using 667 instant films (Polaroid incorporation, UK).

Cluster Analysis: Denderograms were generated by NTSYS Ralph 2000 software on a matrix based on the Dice coefficient.

RESULTS

All the 23 isolates of *Salmonellla* serotype Typhimurium and 18 isolates of *Salmonellla* serotype Enteritidis included in this study were resistant to at least one antibiotic of the fifteen antibiotics tested. On the other hand, all isolates were sensitive to Gen, Cfx and Tob. *Salmonellla* serotype Typhimurium was resistant to a wide variety of antibiotics. Resistance to Nem and Stm was most commonly encountered in *Salmonellla* serotype Typhimurium being found in 86.5% and 82.7% respectively (Table 1). Resistance to both Amp and pip was found in 73.9% of isolates (Table 1). One isolate was resistant to Amk and 2 were resistant to Cip Table 1).Multi-resistance was common in *Salmonellla* serotype Typhimurium. A part of two isolates which were resistant to Cip, all other isolates were resistant to 2 to 8 antibiotics. Out of the 23 three isolates of *Salmonellla* serotype Typhimurium, 17 were resistant to 6 or more antibiotics (73.9%) as shown in Table 1. On the other hand *Salmonellla* serotype Enteritidis were resistant to Nem, Sul and/or Stm. No resistance to other antibiotics was detected (Table 2). Eight isolates of *Salmonellla* serotype Enteritidis were resistant to 2 or 3 antibiotics and the majority (55.5%) of isolates were resistant to one antibiotic.

Resistance patterns of *Salmonellla* serotype Typhimurium were categorized into 10 resistance patterns (Table 1). No more than 5 isolates had the same resistance pattern (Table 1). The most common resistance pattern (Amp, Pip, Nem, Stm, Tet, Sul, Cot) was shared by 5 isolates followed by the pattern: Amp, Pip, Nem, Stm, Sul, Cot which was shared by 4 isolates(Table 1). On the other hand, only three resistant patterns were recognized in the 18 tested *Salmonellla* serotype Enteritidis (Table 2). Ten isolates were resistant to only one antibiotic (Nem),

Table 1: Resistance patterns of *Salmonellla enterica* serotype Typhimurium

Serial No.	No. Antibiotaic	Resistance patterns	No. isolates
1	1	Cip	2
2	2	Cfl, Nem	1
3	4	Nm Stm Tet Slx	2
4	4	Cfl Cfx Amk Nem	1
5	6	Amp Pip Stm Tet Sul Clm	2
6	6	Amp Pip Nem Stm Sul Cot	4
7	7	Amp Pip Nem Stm Tet Sul Cot	5
8	7	Amp Pip Nem Stm Tet Sul Clm	3
9	8	Amp Pip Clf Nem Stm Tet Sul Cot	1
10	8	Amp Pip Clf Nem Stm Tet Sul Clm	2

Table: 2 Resistance patterns of *Salmonellla enterica* serotype Enteritidis

Serial No.	Antibiotaic No	Resistance apatterns	Isolates No.
1	1	Nem	10
2	2	Nem Sul	6
3	3	Nem Sul Stm	2

Table 3: Plasmid profiles of *Salmonellla enterica* serotypes Typhimurium and Enteritidies

Salmonellla serotype	Profile group	No. of isolates	Strains	Plasmid profile (kb)
Typhimurium	TP1	2	2, 37	90.0, 20.4, 17.8, 7.9, 6.0
	TP2	3	3, 12, 21	90.0, 20.4, 17.8, 7.9, 6.0, 3.8
	TP3	9	4, 9, 18, 19, 20, 24, 29, 40, 41	90.0, 20.4, 7.9, 6.0, 3.8
	TP4	2	31, 32	90.0, 7.9, 6.0
	TP5	2	35, 38	90.0, 20.4, 17.8, 9.1, 7.9, 6.0, 2.5
	TP6	4	5, 23, 27, 28	90.0
	TP7	1	22	90.0, 20.4
Enteritidis	EP1	8	1,10,14,15,16,26, 34, 39	60.0
	EP2	3	11, 13, 33	60.0, 1.5
	EP3	1	7	65.0, 5.0, 3.4, 2.6, 1.5
	EP4	2	8, 30	65.0, 7.7, 5.0, 3.4
	EP5	4	6, 17, 25, 36	65.0

six were resistant to 2 antibiotics (Nem, Sul) and two isolates were resistant to 3 antibiotics (Nem, Sul, Stm) as shown in Table 2.

Eight different plasmids were detected in *Salmonellla* serotype Typhimurium and 7 different plasmids were detected in serotype Enteritidis (Fig. 1 and 2; Tables 3). The estimated molecular sizes of the plasmids of the eight plasmids of *Salmonellla* serotype Typhimurium were 90.0, 20.4, 17.8, 9.1, 7.9, 6.0, 3.8, 2.5 kb (Fig. 1 and Table 3). The large molecular weight plasmid (90.0kb) was shared by all tested isolates (Fig. 1). Seven different plasmid profiles were identified in *Salmonellla* serotype Typhimurium (Fig. 1 and Table 3).

The most predominant profile (TP3) was shared by 9 isolates and corresponded to five plasmids of molecular weights 90.0, 20.4, 7.9, 6.0, 3.8 kb (Fig. 1 and Table 3). The second predominant profile (TP6) was shared by four isolates and only one plasmid of 90 kb was detected. The third predominant profile, TP2, shared by three isolates, was characterized by six plasmids (90.0, 20.4, 17.8, 7.9, 6.0, 3.8 kb). Plasmid profiles TP1, TP4 and TP5 were each represented by two isolates and were characterized by five (90.0, 20.4, 17.8, 7.9, 6.0 kb), three (90.0, 7.9, 6.0 kb) and seven (90.0, 20.4, 17.8, 9.1, 7.9, 6.0, 2.5 kb) plasmids. Profile TP7, which was found in only one isolate was characterized by the presence of two plasmids (90.0, 20.4 kb) (Fig. 1 and Table 3).

Salmonellla serotype Enteritidis had seven different plasmids which had estimated molecular weights of 65.0, 60.0, 7.7, 5.0, 3.4, 2.6, 1.5 kb (Fig. 2 and Table 3). Isolates

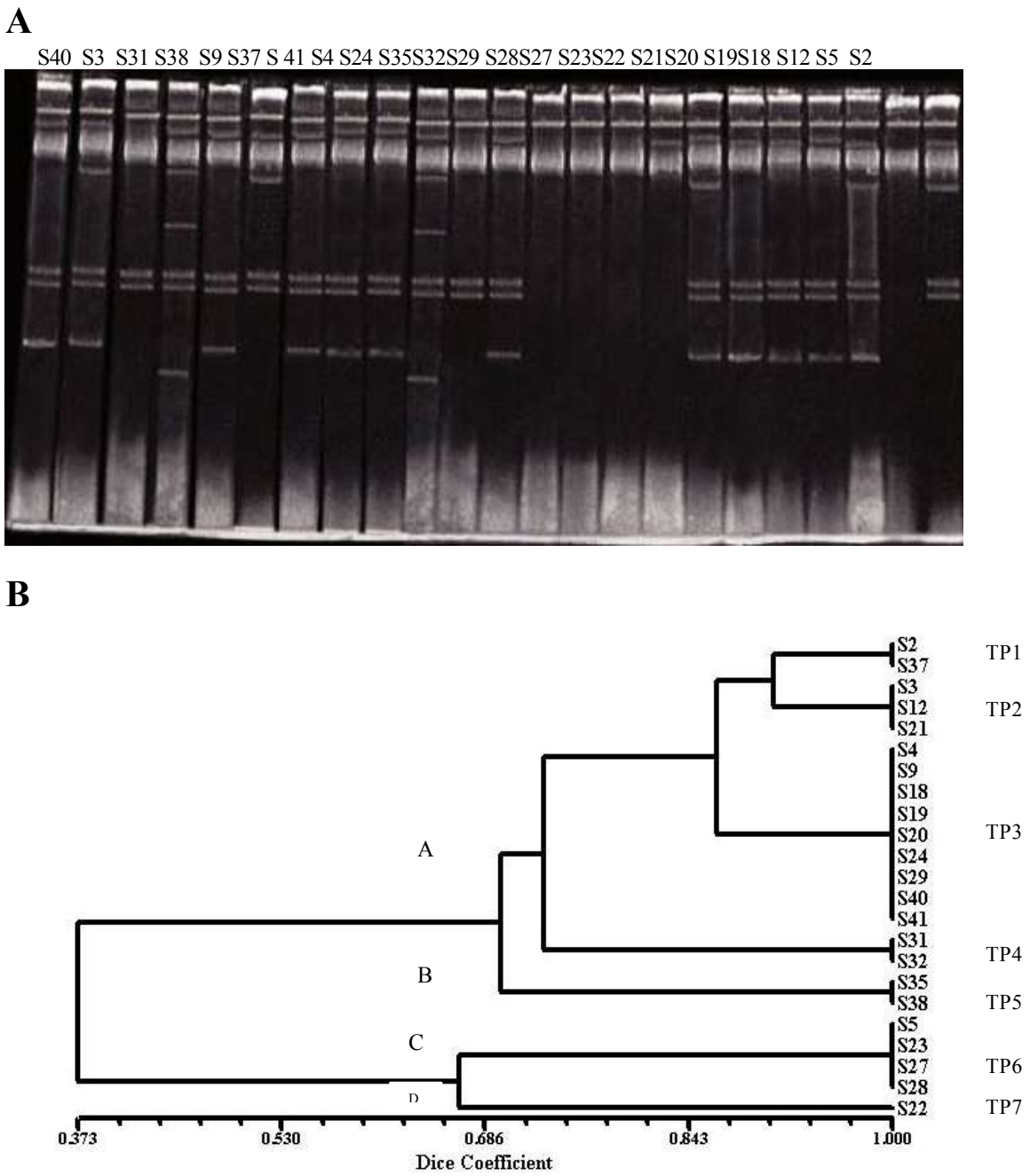

Fig. 1: A: Plasmid profiles of 23 strains of *Salmonellla* serotype Typhimurium.
B: Dendrogram showing the results of cluster analysis based on plasmid profiles of strains

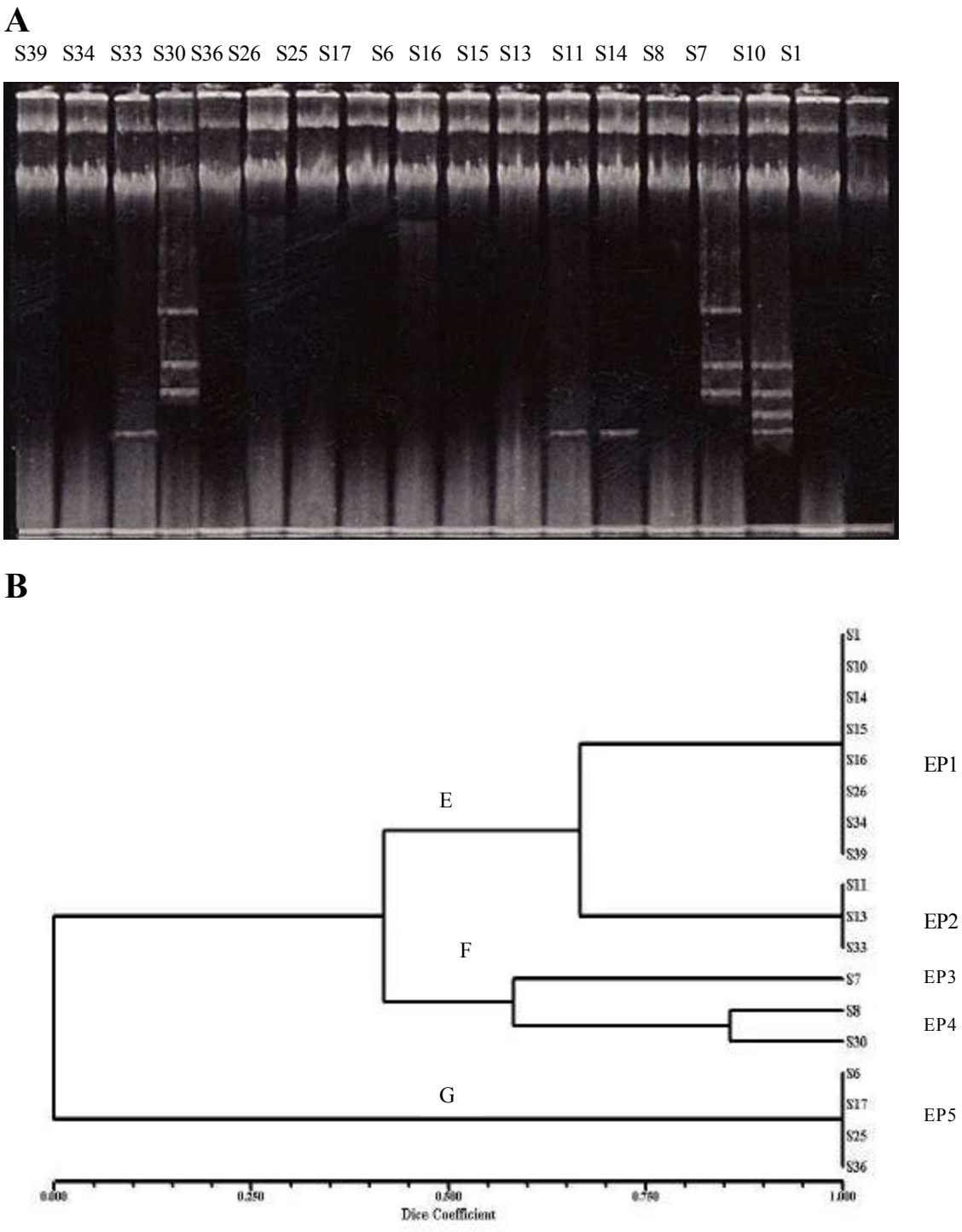

Fig 2: A: Plasmid profiles of 18 strains of *Salmonellla* serotype Enteritidis.
B: Dendrogram showing the results of cluster analysis based on plasmid profiles of strains. The dendrogram was constructed by using NTSYS Ralph 2000 on a matrix based on the Dice coefficient

Table 4: Typing of *Salmonellla enterica* serotype Typhimurium by combination of plasmid profile and biotyping

Serial No	Plasmid profile	No. isolates	Strains	Resistance pattern
1	TP1	2	2, 37	Amp Pip Nem Stm Tet Sul Clm
2	TP2	2	3, 21	Amp Pip Nem Stm Tet Sul Cot
3		1	12	Amp Pip Clf Nem Stm Tet Sul Cot
4	TP3	4	4, 18, 19, 24	Amp Pip Nem Stm Sul Cot
5		2	20, 40	Amp Pip Clf Nem Stm Tet Sul Clm
6		3	9, 29, 41	Amp Pip Nem Stm Tet Sul Cot
7	TP4	2	31, 32	Cip
8	TP5	2	35, 38	Nm Stm Tet Sul
9	TP6	1	5	Cfl, Nem
10		1	23	Amp Pip Nem Stm Tet Sul Clm
11		1	27	Cfl Cfx Amk Nem
12		1	28	Amp Pip Stm Tet Sul Clm
13	TP7	1	22	Amp Pip Stm Tet Sul Clm

Table 5: Typing of *Salmonellla enterica* serotype Enteritidis by combination of plasmid profile and biotyping

Serial No.	Plasmid profile	No. isolates	Strains	Resistance pattern
1	EP1	6	1, 15, 16, 26, 34, 39	Nem
2		2	10, 14	Nem Sul
3	EP2	1	11	Nem
4		2	13, 33	Nem Sul
5	EP3	1	7	Nem
6	EP4	2	8, 30	Nem Sul
7	EP5	2	17, 25	Nem Sul Stm
8		2	6, 36	Nem

of *Salmonellla* serotype Enteritidis harbored only one large molecular weight plasmid (65.0 or 60.0 kb). The large molecular weight plasmid 60.0 kb was found in eleven isolates while the 65 kb plasmid was detected in seven isolates (Fig. 2 and Table 3).

Plasmid contents of *Salmonellla* serotype Enteritidis were categorize into five different profiles (Table 3). The most predominant profile (EP1) and was shared by 8 isolates and was characterized by the presence of one plasmid (60.0 kb). The second predominant profile (EP5) was shared by four isolates and was also characterized by the presence of the other large plasmid (65 kb). Plasmid profiles EP2 (60.0, 1.5 kb), EP4 (65.0, 7.7, 5.0, 3.4 kb) and EP3 (65.0, 5.0, 3.4, 2.6, 1.5 kb), were shared by three, two and one isolates respectively (Fig. 2 and Table 3).

The seven plasmid profiles of *Salmonellla* serotype Typhimurium were examined for cluster analysis at a cut off value of 71.1%. Isolates were differentiated into 4 clusters cluster A (16 isolates), cluster B (2 isolates), cluster C (4 isolates) and cluster D (one isolate). Cluster A was sub-divided into 4 sub-clones on the basis of the four plasmid profiles (TP1, TP2, TP3 and TP4), detected in this cluster. On the other hand one plasmid profile, and hence one clone, was detected in the other three clusters. Nine isolates of the sixteen assigned to cluster A belonged to the sub-clone with plasmid profile PT3 (Fig. 1).

Three different clusters (E, F and G) were recognized at a cut off value of 57.5% in *Salmonellla* serotype Enteritidis (Fig. 2). To cluster E, belonged 11 isolates and to cluster F and G belonged 3 and 4 isolatesrespectively. Cluster E of *Salmonellla* serotype Enteritidis was further subdivided into two subclones, one with 8 isolates and the other with 3 isolates (Fig. 2).

Combination of plasmid profile and antibiograms of *Salmonellla* serotype Typhimurium and *Salmonellla* serotype Enteritidis allowed the differentiation of thirteen and eight clones respectively (Table 4 and 5). In *Salmonellla* serotype Typhimurium, sub-clones corresponding to plasmid profiles, TP2, TP3 and TP6, were split into 2, 3 and 4 sub-clones respectively (Table 4). On the other hand sub-clones corresponding to plasmid profiles, Ep1, EP2 and EP5 were split into 2 clones each (Table 5).

DISCUSSION

The purpose of this study was to investigate clonal diversity of *Salmonellla* serotype Typhimurium and serotype Enteritidis, isolated from cases of acute food poisoning in Taif and Makkah cities. We relied on the patterns of resistance to antibiotics (antibiogram), plasmid profiles and the combination of both of them.

All isolates of both serotypes were tested for their susceptibility to 15 antibiotics. No resistance to Cfm, Tob and Gen was detected. Isolates of both serotypes were resistant to 1-8 antibiotics.

In *Salmonellla* serotype Typhimurium two isolates were resistant to Cip and one isolate was resistant to Cfx and Amk. Fluoroquinolones like Cip are widely used for the treatment of salmonellosis [40]. Therefore, there has been a concern of the appearance of resistant nontyphoid *Salmonellla* isolates to this antibiotic in Saudi Kingdom [41].

Resistance to Amp, Pip, Stm and Nem ranged between 73.9 to 86.5%. More than 70% of the isolates were resistant to 6-8 antibiotics. This prevalence of multi-drug resistance which has been reported in Saudi Arabia [42,43], limits the effective treatment of human *Salmonellla* food poisoning with commonly used antibiotics. In addition this also suggests a misuse of these antibiotics. Miss use of antibiotics as prophylactic and therapeutic drugs and their use as growth promoters in poultry and food animals, leads to the prevalence of resistant strains [44].

Ten resistance patterns were identified in the 23 isolates of *Salmonellla* serotype Typhimurium and no more than 5 isolates of the 23 had the same resistance pattern. This suggests the clonal diversity of Salmonella serotype Typhimurium disseminating in Taif. An increase in the occurrence of antibiotic resistance in *Salmonellla* has been observed in several countries and in some cases the emergence of resistance has been caused by clonal spread of multiresistant strains [2].

Unlike *Salmonellla* serotype Typhimurium, *Salmonellla* serotype Enteritidis isolates were resistant to only Stm, Nem and Sul. No resistance to other antibiotics was detected. This serotype is less common among the Saudi citizens and seems to be introduced to the Saudi Kingdom through foreign labors who came to Saudi mostly from Asia (45). It seems that antibiotics in their countries are not extensively used as the case in Saudi and therefore, these isolates are not equally as resistant as *Salmonellla* serotype Typhimurium. Only three resistance patterns were recognized in *Salmonellla* serotype Enteritidis.

Plasmid profiles represent a reasonably sensitive method for typing *Salmonellla* isolates as long as they are not used for epidemiological studies over a long period of time [35]. It was possible to recognize 7 plasmid profiles. The high molecular weight plasmid (90kb) was present in all isolates either alone or in combination with other plasmids. This plasmid is regarded as a serotype specific plasmid [36]. Plasmid profiles were analyzed by Dice coefficient analysis software. Sixteen isolates were recognized by the dendrogram as cluster A, which suggests that this cluster predominantly circulates in Taif city. The other seven isolates were shared by clusters B, C and D. Cluster A includes 4 plasmid profiles and therefore, can be further split into 4 sub-clones. It seems that the misuse of antibiotics creates selection pressure which keeps the isolates changing their resistance patterns and hence created new sub-clones as was demonstrated when both plasmid profiles and resistance patterns were combined (Table 4).

Five plasmid profiles were recognized in *Salmonellla* serotype Enteritidis. There were two types of serotype specific high molecular weight plasmids (65kb and 60 kb). Either of these plasmids was present in all *Salmonellla* serotype Enteritidis. No plasmids were shared between the two serotypes of *Salmonellla* under investigation. Twelve isolates of the 18 investigated had only one high molecular weight plasmid. The other 6 isolates had 2-5 small plasmids in addition to the serospecific high molecualr weight plasmid. Three clusters were recognized in the dendrogram of *Salmonellla* serotype Enteritidis (E, F and G). Cluster E was more predominant as to it belonged 10 isolates and it included two plasmid profiles (EP1 and EP2). Combination of both antibiogram and plasmid profiles identified 8 clones in *Salmonellla* serotype Enteritidis.

This study demonstrates the clonal diversity of Salmonella serotype Typhimurium and serotype Enteritidis disseminating in Taif and the prevalence of multidrug reistance. Combination of plasmid profiles isolates and their corresponding antibiogram could further demonstrate how the misuse of antibiotics can result in new sub-clones. This study also, implies the need to put and implement a policy for the use of antibiotics both in humans and animals.

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