Plasmid content studies of local isolates of Salmonella

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Summary

A genus of Salmonella was isolation and identification from 40 stool samples of patients at different hospitals in Baghdad, during the period between June 2001 and July 2002. The following serotypes were isolated: - *S.typhimurium* (69%), *S.enteritidis* (10%), *S.typhi* (8%) and (13%) of other *Salmonella* species. All isolates were screened for their resistance to amoxicillin, ampicillin, chloramphenicol, co-trimexazole, cephalexin, cefotaxaime and ciprofloxacin, using disc diffusion method. *Salmonellae typhimurium* isolates (80%) were multidrug resistance (MDR) for six antimicrobial agents. Screening of plasmid content revealed that the presence of single mega plasmid was found in 72.7% of multidrug resistance *Salmonella* isolates and presence of small plasmid in all tested isolates was detected. High frequency molecular weight was observed in the range between 2.1-2.93kb (1.33-1.86Md), in *Salmonella* isolates.

Introduction

Salmonellosis is a global health problem. Most *Salmonella* infections arise from oral ingestion of contaminated food or water (1). It is estimated that from 2 to 4 million cases of salmonellosis occur in the U.S annually (2). Nontyphoidal salmonellosis, a disease caused by *Salmonella* other than *S.typhi,* is an important food-borne infection with a worldwide distribution (3, 4). The two most common causes of nontyphoidal salmonellosis are *S.typhimurium* and *S.enteritidis* (5). Typhoid (enteric) fever is more serious form of salmonellosis (6). The enteric fevers are caused by infection with *S.typhi* and *S.paratyphi* A, B and C (7). Several antibiotics are effective in enteric fever. Ciprofloxacin (500 mg/ 12hr) is the drug of choice. Alternatives include co-trimoxazole (500 mg 6-hourly), amoxycillin (750mg 6-hourly) and chloramphenicol (500mg 6-hourly) (8). Data obtained in 1996 survey CDC of *Salmonella* isolates revealed that 37% were resistant to at least one antimicrobial agent (6, 9). Local study in 1993 reported that all *S.typhimurium*, *S.wien*, *S.muencher*, *S.senftenberg* and S.enek have resistance range from 11%- 66% to chloramphenicol, ampicillin, streptomycin, tetracycline, and gentamycin (10). Other hocal studies revealed some identical antibiotic resistance patterns among *Salmonella*, S*higella,* and

E.coli, with inclusive resistance to ampicillin penicillin G and tetracycline (11, 12).

Epidemiological investigations have traditionally relied on biochemical and serological methods for the primary identification of strains (13). The modern typing methods are based on characterization of the genotype of the organism by analysis of plasmid and chromosomal DNA (14). The LD_{50} values obtained from plasmid-positive strains of *S.typhimurium*, *S.enteritidis* and *S.dublin* were up to 10^6- fold lower than the values obtained for the plasmid free strains of the same serotype (15, 16). Various Iraqi studies investigated plasmids in *Salmonella* spp. There were reported that S.typhi isolates showed one to two large plasmids (17) and only one isolate of *S.typhimurium* have one mega plasmid and three small plasmid in different molecular weight. Other study showed that plasmid contents of *S.typhimurium* isolates revealed the presence of single mega plasmid involved in antibiotic resistance isolates (11).

The present study was planned and designed to reach the following aims:

1- To study the rate of *Salmonella* species among suspected typhoid fever and diarrhea patients.

2- The susceptibility of Salmonella isolates to antimicrobial agents used against Gram-negative bacteria.

3- Determination of the plasmid profile and the relationship between the known number of plasmids and resistance to antimicrobial agent.

Materials and Method

Bacterial isolates

Forty *Salmonella* isolates were obtained from the Central Public Health Laboratory (CPHL), Central Child Hospital and AL-Elwea Child Hospital. Stool specimens were collected in sterile plastic containers, and a loopful was inoculated in tetrathionate broth within 15 minutes. Conventional laboratory procedures for isolation and identification of enteric microorganisms were used according to the Cappuccino & Sherman manual (18). All strains were biotyped by determination the results of 20 different biochemical tests with API 20E strips (Biomerieux) and diagnostic antisera (Fisher) (Salmonella: polyvalent O, Specific O-group: 9.12(D) and VI, Specific O-group: 4.5(B), Specific H: d, i).

Antimicrobial susceptibility testing

All *Salmonella* isolates were tested with disc diffusion method for susceptibility to the following antimicrobial agents: (AL-Razi Center): Ciprofloxacin 5 μg, chloromphenicol 30 μg, cefotaxime 30 μg, amoxicillin 10 μg, ampicillin 10 μg, trimethoprime 1.25 μg + sulfamethoxazole 23.25 μg, tetracycline 30 μg , Cephalexine 30 μg.

Plasmid DNA isolation procedure

All *Salmonella* isolates were screened for plasmid content by the alkaline method of Brinboim and Doly (19), with modifications by Popiech and Neumann (20) and Nasir (21). Bacterial cells were grown overnight in 50ml Luria-Bertain (LB) medium at 35^{o}C, harvested by centrifugation (10 000 rpm/ 20 min.), and washed twice by tris-EDTA-salts (TES) buffer. The pellet was resuspended by 1.5ml Tris-EDTA glucose (TEG), and 2.5mL sodium dodecyl sulfate (SDS) (1%) were added, then incubated at 25^{o}C for 30 minutes. 1.5ml of an ice-cold solution of potassium acetate was added . The tube was vortexes gently in an inverted position three times. It was then stored on ice for 10 minutes. The preparation was centrifuged for 20 minutes at 6000 rpm. 600ml of the supernatant was transferred to an Eppendrof tube. The lysate was extracted once with an equal volume of phenol /chloroform / isoamyl alcohol mixture. The contents were mixed by inverting the Eppendrof tube rapidly (2-3) times. The mixture was centrifuged for 10 minutes at 10 000 rpm. Then supernatant was transferred to a fresh tube. The DNA was precipitated with two volumes of absolute ethanol, and mixed by vortexing. The mixture was stored at -20^{o}C overnight. It was centrifuge for 10 minutes at 10 000 rpm. The supernatant was removed, and the tube placed in an inverted position on a filter paper to allow all of the fluid to drain away. The DNA was redissolved in 20μ l tris-EDTA (TE) buffer and stored at -20^{o}C.

Agarose gel electrophoresis:

Plasmid DNA samples were resolved by horizontal agarose gel LKB electrophoresis (22). Agarose (0.7 & 1%) was made by adding 1gm or 0.7gm of agarose to 100mL of 1x Tris-borate EDTA (TBE) buffer. Power was turned on at 60v. for 2hr. for 1% agarose and 75v. for 2hr. for 0.7% agarose . The ethidium bromide stained bands in gel for 15 minutes. The bands were visualized by using LKB UV transilluminator at 350nm and photographed by UV scanner. λ DNA pstI digest, served as markers during electrophoresis.

Result and discussion

Salmonellosis is an important zoonotic disease and of a worldwide spread (23). The present results showed that most currency of *S.typhimurium* was the commonest among all Salmonellae isolates mainly from children hospital. *Salmonella typhimurium* was found to be the most prevalent organism isolated, which represented 69% of isolates. Whereas *S.enteritidis* represented 10%, *S.typhi* 8% and others Salmonellae 13%. Such results have been noticed. Local study reported that 4.9% of 913 stool sample of infants and children under six-year old were *Salmonella* isolates [*S.emek* (42.2%), S.agona (22.2%), S.typhimurium (20%), *S.typhimurium* var.copenhagen (11.1%), *S.muenchen* (2.2%), *S.anatum* (2.2%)] (24). The sources of infection may be either direct or indirect contact with animal or animal products and / or person-to-person spread

of *S.typhimurium* (25). *Salmonella paratyphi* B and *S.typhimurium* were the most common isolates from local foods, mainly kubba, cakes, meat, unpasteurized raw milk, chicken, and sweat foods in varying percentage in three city of Baghdad (26).

Antimicrobial therapy usually is not given to uncomplicated (noninvasive) gastroenteritis caused by nontyphoidal *Salmonella* species because therapy does not shorten the duration of disease (27). Antibiotics are usually given to: infants, the elderly, and the immunocompromised, as well as those whose infections have moved from the intestines (28).

Table (1) showed the percentage of resistance of *S.typhimurium* isolates to each antimicrobial agents used. *Salmonella typhimurium* isolated of resistant to ampicillin, amoxacyline, chloramphenicol, cefotaxaime and co-trimexazol (ranging from 89.2% - 82.1%). Resistant to cephalexine was found to be 78.5% and to tetracycline 7.1%.

Table (2) revealed the percentage of MDR isolates when compared with total resistance of isolates *S.typhimurium* showed high percentage of resistance to ampicillin, amoxicillin, co-trimexazole, chloramphenicol, cefotaxime and cephalexine reaching up to 80% and only 8% resistance to tetracycline in addition to the previous six antimicrobial agents.

All of the chloramphenicol-resistant *S.typhimurium* isolates (except STM_{21}), were resistant at least to four antibiotics.

The alkaline method was used for the detection of characterization of plasmid types in Salmonella species, the isolates selected according to their resistance to antimicrobial agent. The plasmids types detected were small or large. *Salmonella* plasmids were compared with λ DNA digest under different electrophoresis conditions (Fig. 1; 2; 3). Some *Salmonella* isolates were free from plasmids.

S.typhi (ST_1, ST_3) isolates showed that no DNA bands apparent, in spite of the fact the ST_3 isolates revealed resistance for sulphamethaxazole and trimethoprim, this may be due to either destruction occurred during preparation or such isolates were free from plasmids. In addition, due to the limited number of *S.typhi* isolates, verification of the result could not be achieved. However previous local study revealed one to two mega plasmids present in *S.typhi* isolates (17). *S.typhi* is a serovar in which the majority of strains are plasmid free (29).

In the case of *S.enteritidis* (SE_1, SE_2, SE_4), each antimicrobial agents sensitive isolate was free from plasmids. Only SE_1 isolate was resistant to ampicillin and cephalexine revealed two small plasmid 12.28 kb (7.79 Md), and 4.77kb (3.02 Md). Comparison between such isolates may not be enough to

state that there is specific plasmid for each serotype of different *Salmonella* species.

Plasmid profiling method, in case of *S.typhimurium* isolates, revealed significant result, when compared with other *Salmonella* species isolates. Approximately all *S.typhimurium* (STM) isolate revealed mega plasmid (> 60kb), except STM9 which is sensitive for all antimicrobial agent used in this study, and may be non - virulent strains. Plasmid of 60 Md was found to be present in *S.typhimurium*, its prevalence among veterinary isolates was significantly higher than among human isolates (30, 31).

In this study, STM_{11}, STM_{13}, STM_{19}, STM_{18}, and STM_{17} revealed three plasmid bands in different molecular weight (5.29, 2.75, 2.27 kb) which may be responsible for drug resistance. The similarity in plasmid content of strains isolated is widely separated areas suggests that they have a clonal origin (32).

It was also shown that STM_{15} and STM_{14} showed 4 to 5 plasmid bands ranging between 5.29 kb to 2.1 kb. STM_{21} lose of the mega plasmid in spite of appearance of two small plasmids of M.W 3.31kb (2.1 Md) and 2.56kb (1.62 Md), such isolate was resistance to ampicillin only. STM_{21} isolate may have lost the mega plasmid during preparation or may be less virulent than other isolate with mega plasmid. Previous local study showed *S.typhimurium* isolates contain single mega plasmid (90 kb) and only a small number of isolates showed only one small plasmid (11). In conclusion, our data indicate that multidrug resistant nosocomial infection is becoming an important problem and the possibility of transfer of resistance to other enteric organisms.

Table 1 The percentage of resistance by use disc diffusion method for *S.typhimurium*

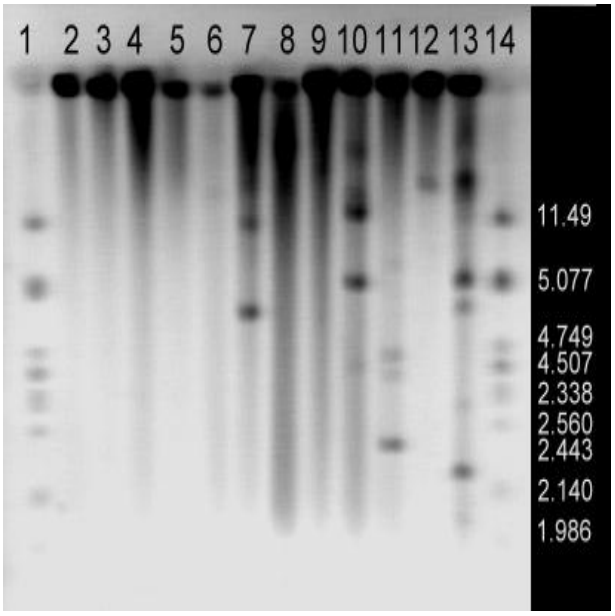
Antimicrobial agent	**No.of resistance isolate**	**%**
Ampicillin	25	89.2
Amoxacyline	25	89.2
Chloramphenicol	24	85.7
Cefotaxime	23	82.1
Co-trimexazol	23	82.1
Cephalexin	22	78.5
Tetracycline	2	7.1
Ciprofloxacin	0	0
Total	28	

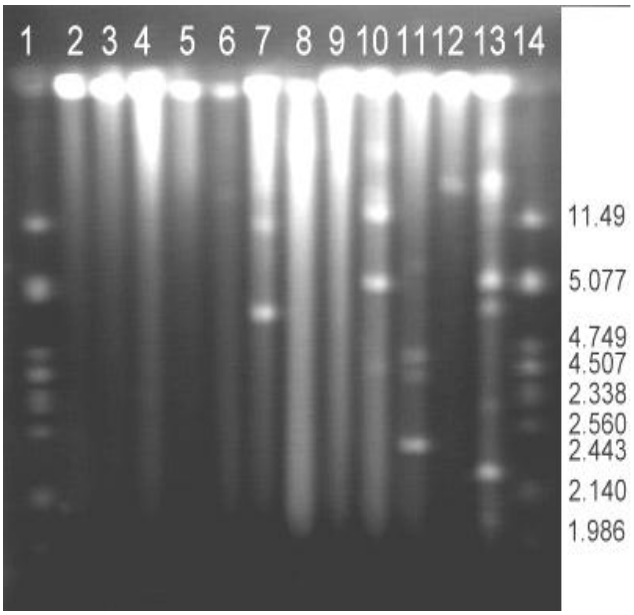
Table 2 The percentage of *MDR isolates in disc diffusion method

Antimicrobial agents	**No.of Anti. Agent**	**No. of MDR isolates**	**%**
Am, Ax, SXT, Te,Cf, CTX,C	7	2	8
Am, Ax, SXT, Cf, CTX, C	6	20	80

Am, Ax, SXT, C Or Am, Ax, CTX, C	4	2	8
Am (STM_{21})	1	1	4
Total	25		

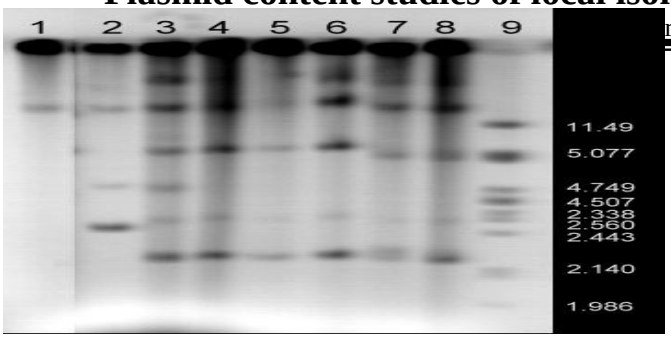
- MDR multidrug resistance





b a

Figure 1 Agarose gel electrophoresis of plasmids extracted from various *Salmonella* species. a. No. 1 & 14 λ DNA (Pst1); 2&3 *S.typhi*; 4 *S.paratyphi* B; 5 *S.paratyphi* A; 6,7,8 *S.enteritidis*; 9 *S.senftenberg*; 10 *S.meterinum*; 11 *S.hadar*; 12,13 *S.typhimurium* (1% agrose, 60 v., 2 hr.). b. Negative picture for a.



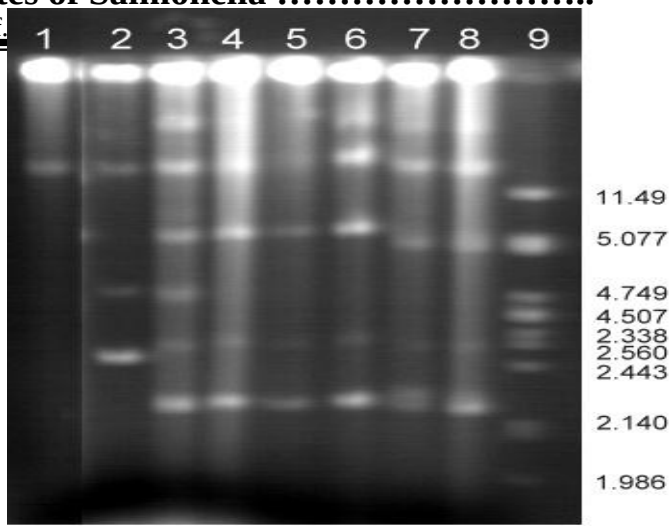


Table 3 Isolates of *Salmonella* spp. tested for antimicrobial resistance and plasmid profile

No.	Isolates	Antimicrobial resistance	No. of plasmid		Mass of plasmids (kilo base) kb 1% agarose
			Mega	Small	
1	ST_1	-	Free		
2	ST_3	Su, Tr	Free		
3	SPB	-	Free		
4	SPA	-	Free		
5	SE_2	-	Free		
6	SE_1	Ce, Am		2	9.73, 4.02
7	SE4	-	Free		
8	SS	Am, Ce, C, Tr,Su, Cf	Free		
9	SM	Tr, Su, Te		2	12.28, 4.77
10	SH	Te		3	3.14, 2.93, 2.40
11	STM_9	-	Free		
12	STM_{15}	C,Cf, Ce, Am, Tr, Su, Te	1	5	5.02, 4.18, 2.65, 2.25, 2.10
13	STM_{21}	Am		2	3.31, 2.56
14	STM_4	C, Cf, Ce, Am, Su, Tr, Te	1	4	5.29, 3.31, 2.7, 2.27
15	ETM_{11}	C, Am, Su, Tr	1	3	5.29, 2.75, 2.27
16	STM_{13}	C, Cf, Ce, Am, Su, Tr	1	3	5.29, 2.75, 2.27
17	STM_{19}	C, Cf, Ce, Am, Su, Tr	1	3	5.6, 2.75, 2.27
18	STM_{18}	C, Cf, Ce, Am, Su, Tr	1	3	4.77, 2.65, 2.3
19	STM_{17}	C, Cf, Ce, Am, Cu, Tr	1	3	5.02, 2.65, 2.27

b a

Figure 2 Agarose gel electrophoresis of plasmids extracted from various *S.typhimurium* isolates. a. No. 1,2,3,4,5,6,7,8 *S.typhimurium* and 9 λ DNA (Pst1)(1% agarose, 60 v., 2hr.). b. Negative picture for a.

b a

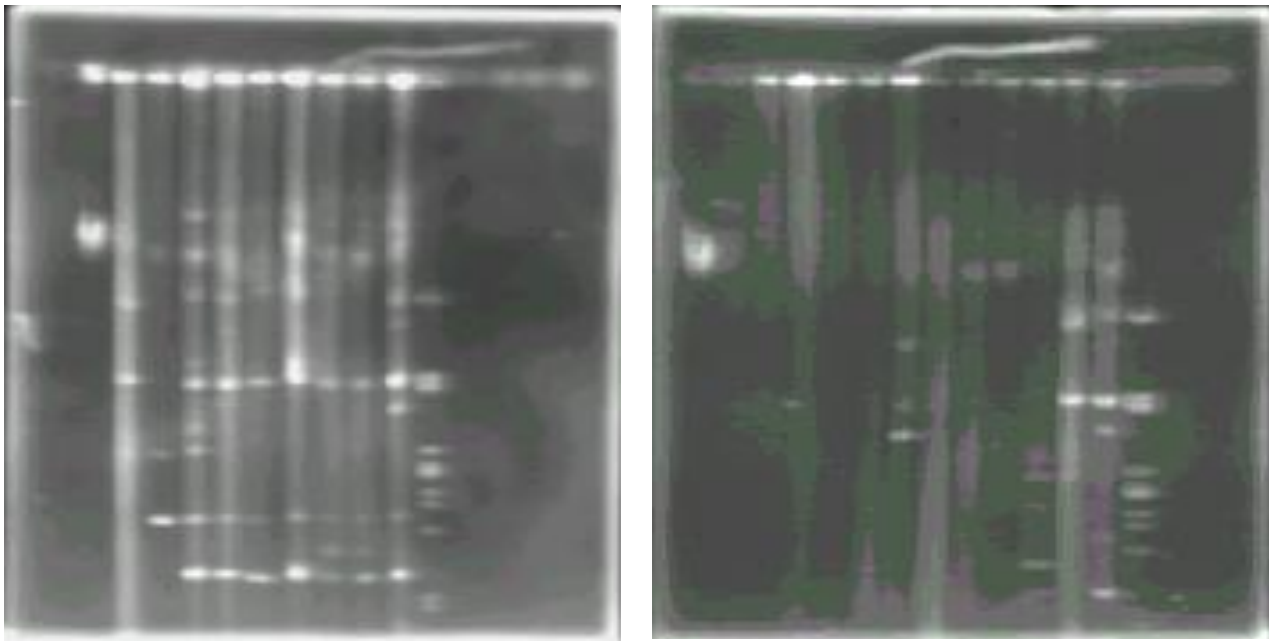


Figure 3 a. Plasmid migration in 0.7% agarose, 75 v., 2 hr. for various *Salmonella* spp. Isolates. b. Plasmid migration in 0.7% agarose, 75 v., 2 hr. for various *S.typhimurium* isolates. Notes: 1st column in the left picture show chromosomal DNA marker in M.W range (60- 100 Kb)

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دراسة المحتوى البلازميدي لعزلات محلية للسلمونيلا

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الخلاصة

تم عزل وتشخيص جنس السلامونيلا من 40 عينة لبراز مرضى في مستشفيات مختلفة من بغداد للفترة من حزيران 2001– تموز 2002. وعزلت الضروب المصلية التالية: S. typhimurium 69% و S. enteritidis 10% و S. typhi 8% وانواع اخرى من السالمونيلا 13%. وتم دراسة حساسيتها للمضادات الحيوية ولكل من ampicillin وِamoxicillin و cephotaxame وciprofloxacin و chloramphenicol و cotrimexazole و cefalexine وذلك باستخدام طريقة الانتشار القرصي، فتبين بان 80% من سلالات S. typhimurium قد ابدت مقاومة لستة منها. وفي مسح لمحتوى البلازميدات تبين وجود ميكا بلازمد واحد في 72.7% من عزلات السالمونيلا المقاومة لعدة عقاقير وعدد من البلازميدات الصغيرة ذات الوزن الجزيئي (1.33-1.86 Md) 2.1-2.93 kb.