

## Emergence of plasmid-mediated multidrug resistance in epidemic and non-epidemic strains of *Salmonella enterica* serotype Typhi from Jordan

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### Abstract

**Background:** Enteric fever caused by *Salmonella enterica* serovar Typhi has not been adequately explored in Jordan.

**Methodology:** In this study we investigated antibiotic resistance patterns and resistance determinants coupled with fingerprint methods of forty-eight isolates of *S. Typhi* obtained from 113 patients with suspected enteric fever admitted at six governmental hospitals in different directorates in Jordan. Twenty-four isolates were from an outbreak of typhoid fever that occurred between October 2004 and January 2005, and another twenty-four were from sporadic cases from 2005.

**Results:** All isolates of *S. Typhi* were resistant to streptomycin. A multidrug resistant (MDR) pattern of ampicillin, chloramphenicol, co-trimoxazole with tetracycline and streptomycin (R-type ACCoTS) was found in 58% of the epidemic strains causing the outbreak and in 98% of the strains from sporadic cases. MDR isolates harbored a single IncHI1 plasmid containing a class 1 integron (*dfrA7*). Plasmid conjugation studies demonstrated a genetic transfer of resistance (ACCoT). *S. Typhi* isolates were all sensitive to fluoroquinolones and cefotaxime, the alternative drugs recommended for treatment of typhoid fever. The genomic analysis using PFGE showed: a) the outbreak was caused by an introduced circulating clone with/without an MDR plasmid, and b) isolates from the sporadic cases from 2005 are the same MDR clone that persisted and spread in the country.

**Conclusion:** The emergence of MDR *S. Typhi* strains is a major important public health issue in Jordan. This study should guide selection of effective antibiotic therapy for the treatment of typhoid and monitoring of the spread of MDR of *S. Typhi*.

**Key Words:** *Salmonella enterica* serovar Typhi, multi-drug resistance plasmid, integrons, Jordan.

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### Introduction

Typhoid fever caused by *Salmonella enterica* serotype Typhi (*S. Typhi*) is currently a significant cause of morbidity and mortality among children and adults in developing countries. In 2004, the World Health Organization (WHO) reported an annual incidence of 22 million cases with more than 216 thousand deaths per year [1]. Transmission of the infection occurs mainly through consumption of contaminated food or water. For this reason, public health measures for the prevention of typhoid fever in developing countries include access to safe chlorinated water, the improvement of food handling practices, and provision of hygiene education to the population.

Chloramphenicol, amoxicillin and trimethoprim-sulfamethoxazole represent the first-line drugs for

the treatment of uncomplicated typhoid fever, followed by the fluoroquinolones which have been widely introduced for their excellent tissue diffusion [2]. However, multiple resistance to these antibiotics, and more recently to fluoroquinolones, has developed among *S. Typhi* strains [3-6]. Multi-drug resistant (MDR) isolates have been reported as causative agents of numerous outbreaks, dramatically reducing the choice of therapy in many areas of Africa, Southeast Asia and the Indian subcontinent [7-10] where typhoid fever is endemic.

Data, epidemiological studies or reports from the Ministry of Health in Jordan concerning typhoid fever and other enteric diseases in the country are limited. The incidence of infection and outbreaks is not well known and probably underestimated as

many outbreaks go unnoticed. This is due to the difficulty of monitoring and tracing the spread of pathogens, as there is often a lack of laboratory identification and characterization of the causative agents and no well-organized surveillance system in place.

However, despite improving sanitation levels of drinking water and sewage disposal, Jordan experiences several outbreaks of enteric pathogens every year, implicating contaminated water or food as potential sources of infections. Furthermore, few published reports describing the outbreaks of food-borne and water-borne diseases in the country are available [11,12] and information on antimicrobial resistance determinants and profiles of *Salmonella* isolates are lacking.

In view of the emergence of MDR strains and their increased involvement in infections and outbreaks worldwide, combining antimicrobial susceptibility testing and molecular fingerprinting of *S. Typhi* has become an important function in public health laboratories (PHLs).

Molecular tools, such as plasmid analysis and Pulsed-Field Gel Electrophoresis (PFGE), extensively used in the laboratories of developed countries for characterizing and subtyping pathogens, are conversely quite limitedly used in laboratories in developing countries, either due to the lack of infrastructure or well-trained microbiologists.

For these reasons, collaboration with institutions from developed countries represents an effective way of improving diagnostic capacity of laboratories and facilitates acquisition of well-established molecular typing methods for recognizing and investigating outbreaks of communicable disease in developing countries.

In this context, the WHO Office for Epidemic and Pandemic Alert and Response in Lyon has launched training programmes for strengthening capacity of the national PHLs in developing countries such as Jordan, and to facilitate collaborative research activities and partnership between laboratories from less developed and developed countries.

In the present study we have demonstrated a successful example of a joint research activity involving the University of Sassari in Italy and the Central Laboratory, Ministry of Health, in Jordan, in the investigation of a typhoid outbreak that occurred between October 2004 and January 2005

in the Jordan valley and subsequently in the study of sporadic cases of typhoid fever that occurred in 2005. In this report, *Salmonella* isolates of serovar Typhi have been studied for susceptibility to antimicrobial agents, molecular determinants of resistance, and their localization on plasmid or integrons. Furthermore, isolates were investigated for their clonal relationship by ribotyping and PFGE techniques.

## Materials and Methods

### Patients

From October 2004 to January 2006, suspected cases (113 in total) of typhoid fever were admitted at six Government Hospitals (Deir Alla, Al-shoneeh, Ghor-Alsafi, Alsat, Madaba and Al-Karak) located in different directorates in Jordan (Table 1). Patients resided in different rural and periurban areas mostly from Deir Alla in Jordan Valley, about 50 km west of the capital Amman. Eighty-three cases of typhoid fever were recovered during an outbreak from October 2004 to January 2005. Thirty more sporadic cases were recorded after the outbreak in 2005. All patients exhibited fever ( $\geq 39^{\circ}\text{C}$ ), abdominal pain and diarrhoea, and were treated with ceftriaxone intravenous therapy with favourable outcome.

**Table 1.** Characteristics of *Salmonella enterica* serovar Typhi strains isolated during an outbreak and from sporadic cases that occurred in Jordan between November 2004 and January 2006.

	Location (no)	Resistance pattern	Plasmid profile (MDa)	Integron cassette	PFGE pattern (XbaI)
Epidemic isolates	Deir-Alla (21)	S (10)	plasmid free	Nil	A
		ACC <sub>o</sub> TS (11)	98	<i>dfra7</i> *	A1
	Al-shoneeh (2)	ACC <sub>o</sub> TS (2)	98	<i>dfra7</i>	A1
	GhorAl-Safi(1)	ACC <sub>o</sub> TS (1)	98	<i>dfra7</i>	A1
Sporadic isolates	Alsalt (8)	ACC <sub>o</sub> TS (8)	98	<i>dfra7</i>	A1
	Alkarak (1)	ACC <sub>o</sub> TS (1)	98	<i>dfra7</i>	A1
	Deir-Alla (4)	ACC <sub>o</sub> TS (2)	98	<i>dfra7</i>	A1
		S (2)	plasmid free	Nil	A
	Al-shoneeh (7)	ACC <sub>o</sub> TS (7)	98	<i>dfra7</i>	A1

Madaba (4)	ACCoTS (4)	98	<i>dfrA7</i>	A1
A ampicillin, C chloramphenicol, Co cotrimoxazole, T tetracycline, S streptomycin; * the variable region of two isolates was sequenced and indicated the presence of the <i>dfrA7</i> gene cassette.				

### Bacterial isolation

Forty-eight cases of typhoid fever, 24 epidemic (29%) and 24 sporadic (80%) were bacteriologically confirmed at the Central Laboratory of Ministry of Health in Amman, Jordan. Among the epidemic cases, 20 samples were positive by blood culture and 5 samples by faecal culture (one patient was positive for both faeces and blood; only one isolate was used for further study). All sporadic samples were blood culture-positive. Blood samples were cultured in GCC mono-phase blood culture bottles at 37°C, examined daily up to 7 days, and subcultured when turbid on blood, MacConkey and Hektoen enteric agar plates (Oxoid, Basingstoke, UK). Stool samples were collected in Cary-Blair transport medium and subsequently plated onto Selenite broth (Oxoid) and cultured on MacConkey and Hektoen enteric agar plates. Definitive identification of the organism was assessed by use of standard biochemical tests followed by serotyping with commercial antisera (Biorad, Segrate, Italy).

### Antimicrobial susceptibility testing

Sensitivity tests of *S. Typhi* isolates were conducted by the standard disc diffusion method and interpreted according to the guidelines provided by the National Committee for Clinical Laboratory Standards (NCCLS, more recently CLSI) [13]. The antibiotic discs (Oxoid) contained ampicillin (10 µg), tetracycline (30µg), cotrimoxazole (25µg), chloramphenicol (30µg), streptomycin (10µg), gentamicin (10µg), amoxicillin (20µg)-clavulanic acid (10 µg), ciprofloxacin (5 µg), nalidixic acid (10µg) and cefotaxime (30µg). *Escherichia coli* ATCC 25922 was used as a control for the effectiveness of the antibiotic discs.

### Plasmid profile analysis and PCR for *IncHI1*

Plasmid DNA was extracted from *S. Typhi* isolates by the alkaline lysis procedure as previously described [14]. Plasmid size was determined by comparing to the standard plasmids of the V517 and 39R861 *E. coli* reference strains.

To determine the plasmid incompatibility group, PCR amplification was conducted with specific primers for the *IncHI1* plasmid as previously described [15].

### Integrations detection

The presence of class 1 and class 2 integrations in genome DNA was detected by PCR with specific primers for integrase gene *intI1* [16]. Cassette insertions were detected by sequencing the variable region of the class 1 integron amplified with 5'CS and 3'CS primers and by PCR with specific primers for *dhfrA7* [15,16].

### Conjugational transfer of drug resistance

Conjugational transfers were carried out using MDR *S. Typhi* strains as the donor and *E. coli* K12 resistant to kanamycin as the recipient. All mating procedures were performed on filters for 18 hours at 37°C. Transconjugants were screened on LB agar plates containing ampicillin (100 µg/ml), kanamycin (50 µg/ml) or tetracycline (10 µg/ml). *E. coli* transconjugants were confirmed by lactose fermentation and/or failure to agglutinate Omni-O and Vi antisera, and then tested for antibiotic susceptibility and analysed for the presence of both the plasmid and the *intI1* gene.

### Ribotyping

Genomic DNA was extracted according to Ausubel *et al.* [17]. PvuII digested DNA was transferred to a nylon membrane (Hybond N+, Amersham) using an VacuGene™ XL vacuum system (Pharmacia Biotech) and hybridized with a PCR-generated non-radioactive labelled probe for 16S RNA under stringent conditions as described by Stanley *et al.* [18].

### PFGE genotyping

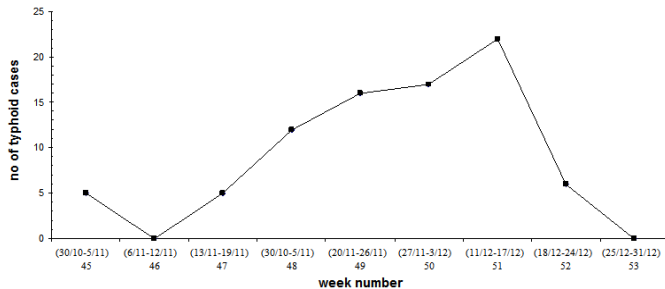
A representative set of epidemic and sporadic strains of *S. Typhi* were typed by PFGE according to a European standardised protocol described by Peters *et al.* in the Salm-gene project [19]. Genomic DNA plugs were digested with 20 units of *Xba*I or *Spe*I (Promega) and subjected to electrophoresis (Pharmacia system) at 14°C for 22 hours at 180V, interpolating, with a pulse time of 2s-64s. Analysis of macrorestriction patterns was carried out according to Tenover *et al.* [20].

## Results

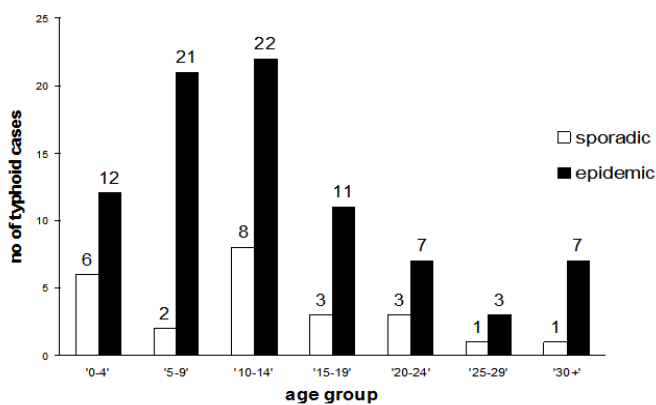
**The outbreak**

The weekly curve of the outbreak (Figure 1) shows a constant increase of cases per week until 17 December 2004, when the incidence of cases started to decline. The age group of patients was distributed between 2 and 38 years, with a predominance in children 5 to 14 years old, as shown in Figure 2.

**Figure 1.** Epidemic curve of the outbreak by week number (October 2004-January 2005).



**Figure 2.** Distribution of typhoid cases by age.

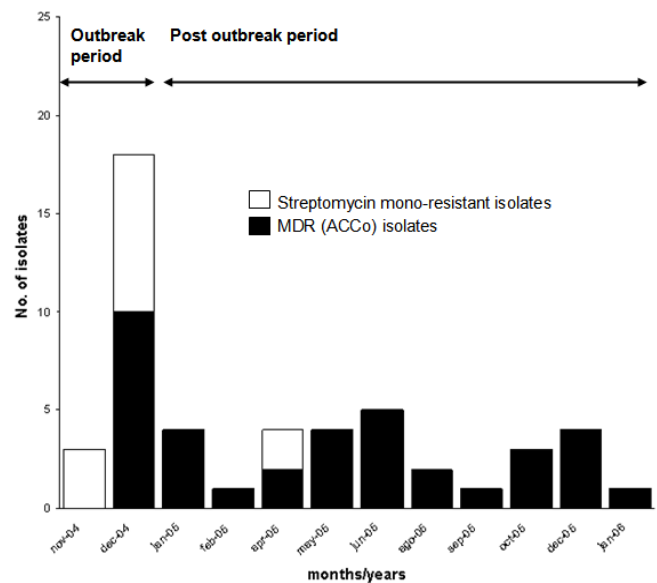


**Bacterial isolates and antimicrobial sensitivity testing**

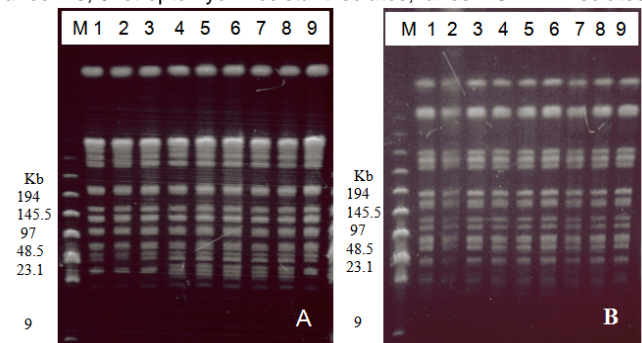
A total of forty-eight *S. Typhi* strains, 24 from the outbreak and 24 sporadic, were isolated from bloodstream or stool samples. The table provides the details of antibiotic resistance patterns, plasmid profiles, integrons, and PFGE patterns among the isolates. There was no consistent change in antimicrobial resistance patterns. All *S. Typhi* isolates were resistant to streptomycin, 75% of which showed additional multidrug resistance to ampicillin, chloramphenicol, co-trimoxazole and tetracycline (R-type ACCoTS), which represents the first-line of drugs for treating typhoid fever in Jordan. The MDR strains persisted after the outbreak (October 2004-January 2005), whereas

streptomycin mono-resistant strains seem to disappear (Figure 3). None of the isolates collected from 2004 to 2006 were resistant to nalidixic acid, ciprofloxacin, amoxicillin, gentamicin or cefotaxime.

**Figure 3.** Trends of Multiple drug resistant (MDR) *Salmonella enterica* serovar Typhi strains from epidemic and non-epidemic cases of typhoid fever from Jordan (November 2004-January 2006). A, ampicillin; C, chloramphenicol; Co, Co-trimoxazole.



**Figure 4.** Pulsed-field gel electrophoresis (PFGE) patterns of *S. Typhi* isolates from Jordan. A. XbaI patterns B. SpeI patterns. M 0.1-200 kb molecular weight marker (Sigma). Lanes 1-3, 9: streptomycin resistant isolates; lanes 4-8: MDR isolates.



**Antibiotic resistance determinants**

We investigated the possible relationship between antibiotic resistance and genetic determinants (plasmids and integrons). Since MDR in *S. Typhi* is mainly due to large conjugative plasmids, strains were subjected to alkali lysis to isolate high molecular weight plasmids. A plasmid of incompatibility group IncHI1 of about 98 MDa

was found in all MDR Typhi isolates (R-type ACCoTS); on the contrary, streptomycin resistant isolates were plasmid-free (Table 1). R-type ACCoTS Typhi also harbored a class 1 integron of 800bp. Class 2 integrons were not detected. Sequencing of class 1 integron variable regions revealed the presence of a single gene cassette *dhfrA7* which confers resistance to trimethoprim. A representative number of MDR Typhi isolates were tested for the transferability of their resistances to *E. coli* Km<sup>R</sup> by conjugation experiments. Interestingly, resistance to ACCoT, but not streptomycin, was co-transferred with the R-plasmid and the *dhfrA7*-integron, which was found to be plasmid-borne as demonstrated by *int1* gene PCR-detection of *E. coli* transconjugants.

#### *Molecular fingerprint methods*

In order to study the clonal relationship among *S. Typhi* isolates responsible for the outbreak and the relationship between these isolates and those from sporadic cases isolated after the outbreak, molecular typing was performed by ribotyping and PFGE methods on 30 randomly selected strains (Table). A remarkable homogeneity of PFGE patterns with two to three fragments difference in the range 48.5-23.1 kb was identified after analysis of DNA restricted with *Xba*I or *Spe*I endonucleases (Figure 4). Thus, only the results of *Xba*I digestion were used for analysis. In particular, plasmid-free isolates lacked the fragments present in MDR strains after restriction, relating these fragments to plasmid carriage. This association was also confirmed by hybridization experiments with a specific probe for the integrase 1 gene (data not shown). When plasmid-associated bands were removed from the analysis, all isolates had an identical PFGE pattern. Results also showed complete correlation between resistance typing and PFGE. The PFGE patterns of *S. Typhi* isolates were compared with the patterns obtained previously with an rRNA gene probe. Ribotyping identified the clonality by showing a unique pattern (data not shown), while the PFGE approach was able to discriminate between MDR-plasmid-bearing and plasmid-free isolates.

#### **Discussion**

Typhoid fever is becoming a serious problem in several countries of the Middle East such as Oman, Qatar, Kuwait, Saudi Arabia, Turkey and

Iran [21-25], possibly due to the introduction of MDR isolates of *S. Typhi* by expatriates from the Indian sub-continent employed in these countries, as well as from nationals visiting the Indian sub-continent. Although typhoid caused by MDR serovar Typhi in most endemic parts of Asia and the Indian subcontinent [26,27] have been well characterized, to date data on antimicrobial resistance among *S. Typhi* from Jordan are limited.

This is the first report of *S. Typhi* from Jordan since 1980, which arguably is tightly linked to the movement of migrant workers continuously employed in the national agricultural field, particularly in the Jordan Valley region, the area under study in the present investigation. We report in this study the characterization of 48 clinical isolates of *S. Typhi* geographically distributed in Jordan by combining phenotypic and molecular typing methods. The data presented highlight the emergence of a high rate of *S. Typhi* showing multiple resistance to the common antimicrobial agents used in the treatment of typhoid fever in developing countries. The prevalence of MDR resistant to ampicillin, chloramphenicol, cotrimoxazole, streptomycin and tetracycline was high in epidemic isolates (58%), but was more pronounced (92%) in isolates from sporadic cases that occurred in different locations during 2005, suggesting a spread of this MDR clone through the country. This finding contradicts recent reports from some regions where the incidence of MDR Typhi isolates appears to have decreased [28,29].

The high frequency of MDR *S. Typhi* isolated is remarkable, since resistance to first-line drugs will require more expensive drugs for effective treatment of typhoid fever and may pose a major challenge to the health care system.

However, the susceptibility exhibited to fluoroquinolone drugs (nalidixic acid and ciprofloxacin) and cefotaxime leaves valuable therapeutic options in the treatment of MDR typhoid fever. This again contradicts what was recently reported from different studies that showed an increased incidence of high resistance to fluoroquinolone which was also associated with treatment failures [30].

Interestingly, the emergence of MDR in *S. Typhi* isolates from Jordan is linked to an IncHI1 [15] conjugative plasmid which mediates multiple resistance, demonstrated by conjugation

experiments. It has been reported that similar plasmids have been isolated in *S. Typhi* strains from different countries in the Middle East and Asia that are able to transfer full MDR or partial resistance [31], which is similar to our findings. The presence of strains harboring the IncHI1 plasmid after the outbreak is of concern and poses the problem of it continuing to spread [32] and transfer resistance.

Besides plasmids, mobile elements contribute to the dissemination of antibiotic resistance and integrons represent the major vehicle for spread [33]. In this study, all MDR *S. Typhi* strains harbored a class 1 integron carrying the *dfrA7* gene cassette, conferring resistance to trimethoprim, which is similar to the results of earlier studies on other *S. Typhi* isolates from different countries of South East Asia [8,15]. The presence of the integron must not be undervalued since two reports have already described multidrug-resistant *S. Typhi* strains harboring integrons with up to six drug resistance genes [34,35]. Furthermore, integrons can acquire additional resistance gene cassettes such as *veb-1* or *blaVIM*, leading to serious problems in the management of these infections.

PFGE of macrorestricted DNA produced two major patterns when *XbaI* and *SpeI* were used. *S. Typhi* strains circulating in Jordan and responsible for the outbreak were most probably derived from an introduced clone possessing a plasmid or not, based on the analysis of resistance patterns and PFGE. Our results indicate that these MDR *S. Typhi* strains are spreading from the outbreak-affected areas to other regions. This situation is of concern and should be carefully monitored in Jordan.

The PFGE pattern of MDR *S. Typhi* strains investigated was apparently identical to one of the five *XbaI*-PFGE-patterns identified as circulating MDR *S. Typhi* strains in Asia and Middle Eastern countries in previous studies [8,26,27,36]. These findings confirm that MDR strains from Jordan are related to those in other countries and could have been introduced possibly about November 2004 or even before. Unfortunately, no samples of *S. Typhi* strains are available from previous years to investigate that retrospectively.

Phenotypic and genotypic typing methods have been demonstrated to be useful to investigate outbreaks and track the spread of MDR

of *S. Typhi* in Jordan. Thus, for the implementation of national prevention measures, it is important to identify the source of an outbreak and to understand the mode of transmission of MDR *S. Typhi*. Further studies based on molecular typing methods are also needed in Jordan to characterize *S. Typhi* isolates associated with sporadic cases in order to compare their genotypic profile to those characterized in the current study. Such an effort would will provide background information on the distribution of MDR *S. Typhi* strains and enable the national health authorities to forecast potential sources of outbreak infections.

In conclusion, this study describes the molecular characterization of clinical isolates of *S. Typhi* for the first time in Jordan, and the importance of the use of molecular typing techniques from a public health perspective.

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**Conflict of interest:** No conflict of interest is declared.