

Genotyping and antimicrobial susceptibility of *Salmonella enterica* serotype Panama isolated in Taiwan

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Background and Purpose: Previous studies have indicated that *Salmonella enterica* serotype Panama causes systemic infections in humans. The present study was undertaken to gain more understanding of the molecular epidemiology and antimicrobial resistance of *Salmonella* Panama.

Methods: Antimicrobial susceptibility testing and molecular typing were performed on 9 clinical isolates by pulsed-field gel electrophoresis (PFGE). The presence of resistance genes, *Salmonella* genomic island 1 (SGI1), and integrons was examined by polymerase chain reaction. Plasmid profiles of these isolates were also determined.

Results: Molecular typing showed 3 predominant PFGE types with 6 subtypes among these isolates. High rates of antimicrobial resistance to trimethoprim-sulfamethoxazole (66.7%), tetracycline (66.7%), chloramphenicol (66.7%), ampicillin (55.6%), streptomycin (55.6%), kanamycin (55.6%), and gentamicin (44.4%) were found. All 9 isolates were susceptible to ceftriaxone, cefixime, imipenem, amikacin, and ciprofloxacin. Isolates with PFGE type P1 and subtype P1-1 contained a class 1 integron and resistance genes *sull* and *str* ($p=0.048$). Plasmids of 3 to 20 kb were found in all isolates belonging to PFGE type P1, subtypes P1-1 and P1-2, which were associated with multidrug resistance ($p=0.012$) and the resistant gene *bla*_{TEM} ($p=0.048$). There was no SGI1 found in these 9 isolates.

Conclusions: In view of the high rates of drug resistance to the antimicrobial agents tested, extended-spectrum cephalosporins and fluoroquinolones seem to be a better choice for treatment of systemic infection caused by *Salmonella* Panama. There is a major clone (P1 and its subtypes) among the *Salmonella* Panama isolates. Multi-drug resistance was conferred by integrons or plasmids, rather than SGI1.

Key words: Drug resistance; Genotype; Microbial sensitivity tests; Plasmids; *Salmonella enterica*

Introduction

Non-typhoidal *Salmonella* usually causes diarrheal diseases in humans. However, serious complications of extraintestinal infections, such as bacteremia, meningitis, arthritis or osteomyelitis can occur following gastroenteritis [1-3]. Age younger than 3 months and immunocompromised status have been suggested as risk factors for the development of bacteremia [2-7]. Furthermore,

specific *Salmonella* serotypes with invasive properties can also predispose to extraintestinal *Salmonella* infections [1,8]. In a previous study, *Salmonella* serotype Panama and *Salmonella* serotype Dublin, both of which belong to *Salmonella* group D1, were found to cause bacteremia at a higher rate than other serotypes [9]. In a retrospective study, 320 *Salmonella* isolates from infants and children with non-typhoidal *Salmonella* infection were serotyped, and only *Salmonella* Panama was shown to be strongly associated with bacteremia in children with gastroenteritis [10]. Furthermore, *Salmonella* Panama accounted for 4 of the 6 isolates causing meningitis [10]. This study was conducted to

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extend the understanding of the genotypes, antimicrobial susceptibility, and resistance mechanism of clinical *Salmonella* Panama isolates in Taiwan.

Methods

Bacterial strains

Nine clinical *Salmonella* Panama isolates from 9 patients were obtained from the Center for Disease Control, Taipei, Taiwan, and National Cheng Kung University Hospital, Tainan, Taiwan, including 5 from stools and 4 from blood. The *Salmonella* Panama isolates were cultured on blood agar or Luria-Bertani (LB) agar at 37°C for 24 h and designated as SAPA01 to SAPA09.

Disk diffusion test

Resistance to antimicrobial agents was determined by the agar diffusion method on Mueller-Hinton agar plates. Ampicillin, cefixime, ceftriaxone, chloramphenicol, ciprofloxacin, imipenem, trimethoprim-sulfamethoxazole, streptomycin, tetracycline, gentamicin, amikacin, and kanamycin were the 12 antibiotics tested for activity against the 9 *Salmonella* Panama isolates. Susceptible and resistant isolates were defined according to the criteria suggested by the Clinical and Laboratory Standards Institute [11]. Intermittent resistance was regarded as resistance in statistical analysis.

Genomic DNA analysis by pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) was performed as described previously by Gautom [12]. The bands of about 150 to 400 kb were tight and difficult

to discriminate (Fig. 1A). Another autoprogram was added under the following conditions: angle, 120°; gradient, 6 V/cm; pulse times, 18.27 to 35.38 sec; running time, 33 h (Fig. 1B).

The fragment patterns obtained were interpreted as described by Tenover et al [13]. Briefly, isolates with more than a 7-band difference were considered to be different genotypes, which were designated arbitrarily in alphabetical order. Isolates with identical fingerprints were considered to be the same genotype, while those with less than a 4-band difference were considered to be subtypes of an existing genotype.

Detection of resistant genes, *Salmonella* genomic island 1 and class 1 integron by polymerase chain reaction

AmpC, *sulI*, *str*, *bla*_{TEM} are resistant genes usually located on plasmids. Integrons that harbor a multidrug resistance gene cassette are a mechanism for transferring drug resistance genes in bacteria. *Salmonella* genomic island 1 (SGI1), which was first found in *Salmonella* serotype Typhimurium definitive type 104 (DT104) [14], is a complex class 1 integron conferring DT104 pentaresistance (resistance to ampicillin, chloramphenicol, sulfonamide, streptomycin, and tetracycline). The existence of SGI1 can be detected by the presence of a conserved left junction flanking the SGI1. *Salmonella* Typhimurium BN 9181 that contained SGI1 was used as a positive control.

A multiplex polymerase chain reaction was designed to characterize the resistance genes, including *ampC*, *sulI*, *str* and *bla*_{TEM}, associated with SGI1 and integron [14]. Polymerase chain reaction products

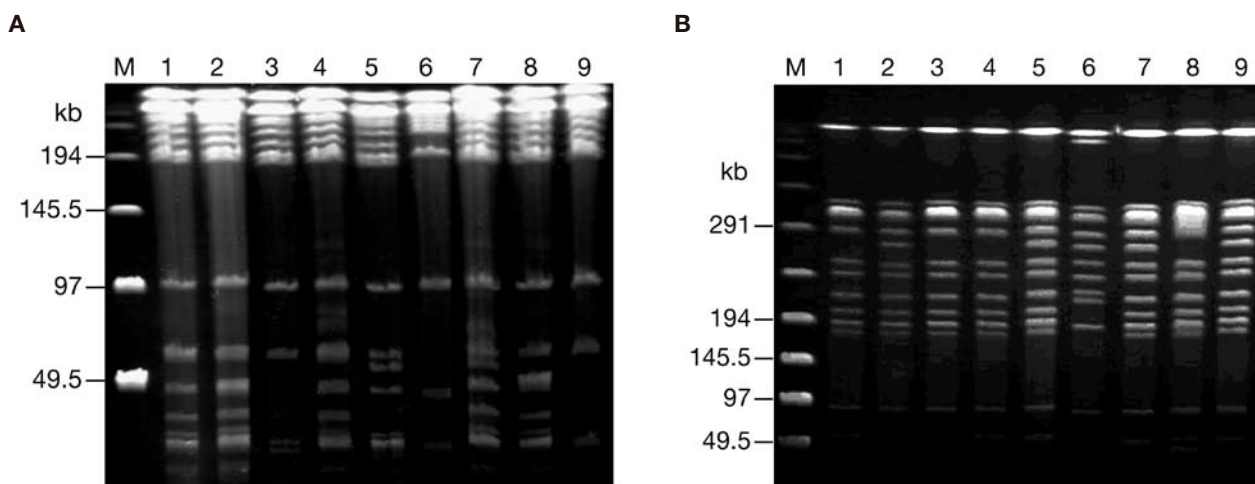


Fig. 1. Pulsed-field gel electrophoresis of the 9 *Salmonella* Panama isolates showed DNA bands of 20~400 kb (A) and 150~400 kb (B) under 2 different electrophoretic conditions. M, lambda DNA marker; lanes 1-9, SAPA01-09.

were purified and sequenced as described previously [15]. The nucleotide sequences obtained were compiled and analyzed using Lasergene software (DNASTAR Inc., Madison, WI, USA).

Plasmid analysis

Plasmid profiles of the isolates were determined by the Kado-Liu method [16]. Bacterial artificial chromosome (BAC)-Tracker™ supercoiled DNA ladder (Epicentre, Madison, WI, USA) and 1-kb DNA ladder (Fermentus, Hanover, MD, USA) were used as size markers.

Statistical analysis

Results of antibiotic susceptibility testing and the presence of resistant genes (*ampC*, *sull*, *str* and *bla*_{TEM}), SGI1, integrons and plasmids in different PFGE types were compared by means of Fisher's exact test using the Statistical Package for the Social Sciences (SPSS)

for Windows (Version 11.5; SPSS, Chicago, IL, USA). Statistically significant differences were defined when $p < 0.05$.

Results

Disk diffusion test

The results of susceptibility testing are summarized in Table 1. SAPA01, SAPA02, SAPA04, SAPA05, SAPA07 and SAPA08 showed resistance to trimethoprim-sulfamethoxazole, chloramphenicol, and tetracycline. SAPA03, SAPA06, and SAPA09 were susceptible to all 12 antibiotics. All 9 isolates were susceptible to ceftriaxone, cefixime, imipenem, amikacin, and ciprofloxacin.

Pulsed-field gel electrophoresis

PFGE analysis of genomic DNA from the 9 *Salmonella* Panama isolates showed banding patterns of

Table 1. Pulsed-field gel electrophoresis (PFGE) type, antimicrobial susceptibility, and presence of resistant genes, integrons, or plasmids among 9 *Salmonella* Panama isolates

	SAPA01	SAPA02	SAPA03	SAPA04	SAPA05	SAPA06	SAPA07	SAPA08	SAPA09
PFGE type	P1	P1-1	P1-3	P1	P1-2	P3	P1-1	P1	P2
Antimicrobial susceptibility									
Trimethoprim-sulfamethoxazole	R	R	S	R	R	S	R	R	S
Chloramphenicol	R	R	S	R	R	S	R	R	S
Tetracycline	R	R	S	R	R	S	R	R	S
Ampicillin	R	R	S	R	R	S	R	S	S
Streptomycin	R	R	S	R	R	S	R	S	S
Kanamycin	R	R	S	R	R	S	R	S	S
Gentamicin	I	I	S	R	S	S	S	I	S
Cefixime	S	S	S	S	S	S	S	S	S
Ciprofloxacin	S	S	S	S	S	S	S	S	S
Ceftriaxone	S	S	S	S	S	S	S	S	S
Imipenem	S	S	S	S	S	S	S	S	S
Amikacin	S	S	S	S	S	S	S	S	S
Resistant genes, integrons, or plasmids									
<i>ampC</i>	-	-	-	-	-	-	-	-	-
<i>sull</i>	+	+	-	+	-	-	-	+	-
<i>str</i>	+	+	-	+	-	-	-	+	-
<i>bla</i> _{TEM}	+	+	-	+	+	-	+	-	-
SGI1	-	-	-	-	-	-	-	-	-
Integron	+	+	-	+	-	-	-	+	-
20-kb plasmid	-	+	-	+	-	-	-	-	-
9-kb plasmid	+	-	-	+	-	-	-	-	-
8-kb plasmid	+	-	-	+	-	-	-	+	-
7-kb plasmid	+	-	-	+	-	-	-	-	-
4-kb plasmid	+	+	-	+	+	-	+	+	+
3-kb plasmid	+	+	-	+	+	-	+	+	+
1-kb plasmid	-	-	-	-	-	-	-	-	+

Abbreviations: SGI1 = *Salmonella* genomic island 1; R = resistant; S = susceptible; I = intermediately resistant; + = presence; - = absence

20~400 kb and 150~400 kb in size (Fig. 1). Although few differences were detected in banding patterns, it was possible to discriminate the 9 isolates into 3 different types: P1, P2, and P3 types. There were 3 isolates belonging to P1 type (SAPA01, SAPA04 and SAPA08), 2 to P1-1 subtype (SAPA02 and SAPA07), 1 to P1-2 subtype (SAPA05), and 1 to P1-3 subtype (SAPA03). SAPA09 was classified as P2 type and SAPA06 as P3 type.

Detection of resistant genes

As shown in Table 1, resistance gene *ampC* was not found in any of the 9 *Salmonella* Panama isolates. Resistance genes *sulI* and *str* were found in SAPA01, SAPA02, SAPA04, and SAPA08. Resistance gene *bla*_{TEM} was found in SAPA01, SAPA02, SAPA04, SAPA05, and SAPA07. None of the 9 isolates contained SGII.

Detection of class 1 integron

The results showed that 1 integron existed in SAPA01, SAPA02, SAPA04, and SAPA08. The resistance cassette was sequenced and 1861 bp were found. The class 1 integron harbored only an *aadA2* gene. The *aadA2* gene is an aminoglycoside (such as streptomycin and spectinomycin) resistance gene from which aminoglycoside adenyltransferase is encoded.

Genotyping

Compared to the non-P1 type, isolates belonging to PFGE type P1 and its subtypes (P1-1 and P1-2) had fewer than 3 different bands and were multidrug-resistant ($p=0.012$). They contained resistant gene *bla*_{TEM} ($p=0.048$) and were resistant to trimethoprim-sulfamethoxazole ($p=0.012$), chloramphenicol ($p=0.012$), ampicillin ($p=0.048$), streptomycin ($p=0.048$), and kanamycin ($p=0.048$). Compared with other genotypes, isolates belonging to PFGE types P1 and P1-1 usually contained integron ($p=0.048$) and resistance genes *sulI* ($p=0.048$) and *str* ($p=0.048$).

Plasmid analysis

Plasmid profiles of all isolates were analyzed by the Kado-Liu method (data not shown). A 20-kb plasmid was found in SAPA02 and SAPA04. An 8-kb plasmid was found in SAPA01, SAPA04, and SAPA08. Two plasmids, 7 kb and 9 kb, were found in SAPA01 and SAPA04. Two smaller plasmids (3 kb and 4 kb) were found in SAPA01, SAPA02, SAPA04, SAPA05, SAPA07, SAPA08, and SAPA09. An additional 1-kb plasmid was found in SAPA09.

The 8-kb plasmid was found in all isolates belonging to PFGE type P1 ($p=0.012$). The 3-kb and 4-kb plasmids were found in 77.8% of all isolates belonging to P1, P1-1, and P2 types ($p=0.012$). The 1-kb plasmid was found only in an isolate belonging to the P2 type.

Discussion

Salmonella Panama has been reported as one of the most virulent non-typhoidal *Salmonella* serotypes, and usually causes bacteremia and meningitis in humans [10]. It used to be common in Taiwan, but after the outbreak of swine hand-foot-mouth disease in 1997, *Salmonella* Panama has been diminishing and has been replaced by *Salmonella* serotype Enteritidis, now the most common serotype among serogroup D *Salmonella* isolated from human sources [17].

In Taiwan, since the 1990s, a high portion of *Salmonella* isolates have been resistant to ampicillin, chloramphenicol, tetracycline, and trimethoprim-sulfamethoxazole [18-20]. The resistance rates to these drugs among *Salmonella* Panama were also high (all above 50%) in this study. An increase in resistance to gentamicin and trimethoprim-sulfamethoxazole from 1989-1992 to 1993-1996 was found in clinical *Salmonella* Panama isolates derived from cerebrospinal fluid (4 isolates), blood (12) and stool (3) [18]. Compared with the previous study [17], these data showed higher resistance rates to trimethoprim-sulfamethoxazole (66.7% vs 0.0%) and chloramphenicol (66.7% vs 42.9%). In addition, high resistance rates to streptomycin (55.6%), kanamycin (55.6%), and gentamicin (44.4%) were found in this study. Fortunately, resistance to tetracycline (66.7% vs 85.7%) and ampicillin (55.6% vs 85.7%) has been declining. Interestingly, chloramphenicol, trimethoprim-sulfamethoxazole, tetracycline, ampicillin, streptomycin, kanamycin, and gentamicin are no longer or infrequently used in the clinical setting for the treatment of *Salmonella* infection. It is thus speculated that the higher resistance rates of *Salmonella* to these agents may be aggravated by use of these antibiotics in domestic animals either therapeutically or for the purpose of growth promotion [21,22].

The most common resistance pattern in *Salmonella* Panama (resistance to ampicillin, chloramphenicol, streptomycin, trimethoprim-sulfamethoxazole, and tetracycline) is the same as that of the multidrug-resistant ACSSuT-type of *Salmonella* Typhimurium DT104,

which has increased in Europe and North America since the 1990s [23] and has also been reported in Asia [14]. In DT104, all resistance genes for ACSSuT phenotype were clustered in SG11. However, SG11 was not found in our 9 isolates of *Salmonella* Panama.

In this study, SAPA01, SAPA02, SAPA04, and SAPA08 were detected to harbor a class 1 integron containing the *aadA2* gene, which has not been reported previously in *Salmonella* Panama. The presence of *aadA1a*, another gene found in class 1 integron cassette encoding streptomycin-spectinomycin resistance, was found in *Salmonella* Panama in the 1990s [24].

Attempts were made to genotype *Salmonella* Panama in Europe in 1995, but failed because of DNA degradation on the addition of endonuclease buffer with the standard methods of DNA preparation of PFGE [25]. The genotypes of *Salmonella* Panama in Taiwan have been surveyed by PFGE recently and a diverse result was found [17]. In this study, multi-stage electrophoresis allowed us to genotype our 9 isolates into 3 PFGE types, which correlated well with the resistance patterns of the isolates. Multidrug resistance was found in 6 isolates, all matching PFGE type P1 and its subtypes (P1-1 and P1-2). Clearly, there is one major PFGE type (P1), which is resistant to multiple antibiotics, circulating in Taiwan. In contrast, isolates belonging to the other 2 PFGE types (P2 and P3) were sensitive to all antibiotics tested.

Bacterial plasmids, which could be transferred by conjugation, may contribute numerous of phenotypes to their bacterial host, such as antibiotic resistance and virulence properties [26]. Plasmid DNA profiles of 337 *Salmonella* isolates belonging to 7 common serotypes and originating from 29 different countries worldwide were investigated in 1985 [27]. A 21-MD (32 kb) plasmid called pRQ32 has been found in 22% of strains of *Salmonella* Panama [27]. In addition, these strains carried a 3-MD cryptic plasmid called pRQ34 [27]. In our study, the 4-kb (3-MD) plasmid was found in 77.8% of all isolates, but no 21-MD plasmid was found. In Europe, 15 of 30 *Salmonella* Panama strains contained plasmids; plasmids of 24, 4, 2.4, and 1.4 MD were common to most of the strains [25]. In this study, no 24-MD plasmid was found. However, the 3-kb and 4-kb (2-MD and 3-MD, respectively) plasmids detected might be similar to the smaller plasmids (4, 2.4, and 1.4 MD) identified in the previous study [25]. From 1982 to 1993, 11 isolates of *Salmonella* Panama in Chile acquired additional self-transferable plasmids (from 61 kb to 120 kb) coding

for multidrug resistance [28]; these larger plasmids found in Chile were not detected in our isolates.

Taken together, class 1 integron and resistance genes *sulI*, *str* and *bla*_{TEM} conferred the multiresistance phenotype to *Salmonella* Panama isolates in Taiwan. These resistance genes as well as the class 1 integron might be located on the plasmids that can spread between bacteria. Extended-spectrum cephalosporins such as ceftriaxone, and fluoroquinolones remain the main therapeutic choices for treatment of systemic infections caused by *Salmonella* Panama in Taiwan.

References

- Huang FY, Huang SH, Chen SH, Hsu YC, Lin CH. Bacteremia in infants with *Salmonella* enterocolitis. *Zhonghua Min Guo Xiao Er Ke Yi Xue Hui Za Zhi*. 1991;32:358-64.
- Nelson SJ, Granoff D. *Salmonella* gastroenteritis in the first three months of life. A review of management and complications. *Clin Pediatr (Phila)*. 1982;21:709-12.
- Zaidi E, Bachur R, Harper M. Non-typhi *Salmonella* bacteremia in children. *Pediatr Infect Dis J*. 1999;18:1073-7.
- Torrey S, Fleisher G, Jaffe D. Incidence of *Salmonella* bacteremia in infants with *Salmonella* gastroenteritis. *J Pediatr*. 1986;108(5 Pt 1):718-21.
- Sirinavin S, Jayanetra P, Lolekha S, Layangkul T. Predictors for extraintestinal infection in *Salmonella* enteritis in Thailand. *Pediatr Infect Dis J*. 1988;7:44-8.
- Schutze GE, Schutze SE, Kirby RS. Extraintestinal salmonellosis in a children's hospital. *Pediatr Infect Dis J*. 1997;16:482-5.
- Davis RC. *Salmonella* sepsis in infancy. *Am J Dis Child*. 1981;135:1096-9.
- Wittler RR, Bass JW. Nontyphoidal *Salmonella* enteric infections and bacteremia. *Pediatr Infect Dis J*. 1989;8:364-7.
- Li WC, Chiang CS, Chiu NC, Weng LC, Yang DI, Cheng CP, et al. Characterization of group D1 non-typhoid *Salmonella* isolates by serotyping and pulsed field gel electrophoresis. *Acta Paediatr Taiwan*. 1999;40:430-3.
- Yang YJ, Huang MC, Wang SM, Wu JJ, Cheng CP, Liu CC. Analysis of risk factors for bacteremia in children with nontyphoidal *Salmonella* gastroenteritis. *Eur J Clin Microbiol Infect Dis*. 2002;21:290-3.
- Performance standards for antimicrobial disk susceptibility tests. 16th informational supplement. CLSI document M2-A9. Wayne: Clinical Laboratory Standards Institute; 2006.
- Gautom RK. Rapid pulsed-field gel electrophoresis protocol for typing of *Escherichia coli* O157:H7 and other Gram-negative organisms in 1 day. *J Clin Microbiol*. 1997;35:2977-80.

13. Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, Persing DH, et al. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol.* 1995;33:2233-9.
14. Chiu CH, Su LH, Chu C, Wang MH, Yeh CM, Weill FX, et al. Detection of multidrug-resistant *Salmonella enterica* serotype Typhimurium phage types DT102, DT104 and U302 by multiplex PCR. *J Clin Microbiol.* 2006;44:2354-8.
15. Su LH, Chen HL, Chia JH, Liu SY, Chu C, Wu TL, et al. Distribution of a transposon-like element carrying *bla*_{CMY-2} among *Salmonella* and other *Enterobacteriaceae*. *J Antimicrob Chemother.* 2006;57:424-9.
16. Kado CI, Liu ST. Rapid procedure for detection and isolation of large and small plasmids. *J Bacteriol.* 1981;145:1365-73.
17. Tsai KS, Yang YJ, Wang SM, Chiou CS, Liu CC. Change of serotype pattern of Group D non-typhoidal *Salmonella* isolated from pediatric patients in southern Taiwan. *J Microbiol Immunol Infect.* 2007;40:234-9.
18. Yang YJ, Liu CC, Wang SM, Wu JJ, Huang AH, Cheng CP. High rates of antimicrobial resistance among clinical isolates of nontyphoidal *Salmonella* in Taiwan. *Eur J Clin Microbiol Infect Dis.* 1998;17:880-3.
19. Ho M, McDonald LC, Lauderdale TL, Yeh LL, Chen PC, Shiau YR. Surveillance of antibiotic resistance in Taiwan, 1998. *J Microbiol Immunol Infect.* 1999;32:239-49.
20. Lee CY, Chiu CH, Chuang YY, Su LH, Wu TL, Chang LY, et al. Multidrug-resistant non-typhoid *Salmonella* infections in a medical center. *J Microbiol Immunol Infect.* 2002;35:78-84.
21. McDonald LC, Chen MT, Lauderdale TL, Ho M. The use of antibiotics critical to human medicine in food-producing animals in Taiwan. *J Microbiol Immunol Infect.* 2001;34:97-102.
22. Chiu CH, Wu TL, Su LH, Chu C, Chia JH, Kuo AJ, et al. The emergence in Taiwan of fluoroquinolone resistance in *Salmonella enterica* serotype Choleraesuis. *N Engl J Med.* 2002;346:413-9.
23. Helms M, Ethelberg S, Mølbak K; DT104 Study Group. International *Salmonella* Typhimurium DT104 infections, 1992-2001. *Emerg Infect Dis.* 2005;11:859-67.
24. Guerra B, Soto S, Cal S, Mendoza MC. Antimicrobial resistance and spread of class 1 integrons among *Salmonella* serotypes. *Antimicrob Agents Chemother.* 2000;44:2166-9.
25. Stanley J, Baquar N, Burnens A. Molecular subtyping scheme for *Salmonella* Panama. *J Clin Microbiol.* 1995;33:1206-11.
26. AitMhand R, Soukri A, Moustaooui N, Amarouch H, ElMdaghri N, Sirot D, et al. Plasmid-mediated TEM-3 extended-spectrum beta-lactamase production in *Salmonella* Typhimurium in Casablanca. *J Antimicrob Chemother.* 2002;49:169-72.
27. Helmuth R, Stephan R, Bunge C, Hoog B, Steinbeck A, Bulling E. Epidemiology of virulence-associated plasmids and outer membrane protein patterns within seven common *Salmonella* serotypes. *Infect Immun.* 1985;48:175-82.
28. Cordano AM, Virgilio R. Evolution of drug resistance in *Salmonella* Panama isolates in Chile. *Antimicrob Agents Chemother.* 1996;40:336-41.