Epidemiol. Infect. (2002), **129**, 155–161. © 2002 Cambridge University Press
DOI: 10.1017/S0950268802007239 Printed in the United Kingdom

Drug resistances in salmonella isolates from animal foods, Italy 1998–2000

C. MAMMINA[1]*, L. CANNOVA[1], S. MASSA[2], E. GOFFREDO[3] AND A. NASTASI[4]

[1] *Centre for Enteric Pathogens of Southern Italy* (*CEPIM*), *Dipartimento di Igiene e Microbiologia 'G. D'Alessandro', University of Palermo, Italy*
[2] *Istituto di Produzioni e Preparazioni Alimentari, University of Foggia, Italy*
[3] *Istituto Zooprofilattico Sperimentale della Puglia e Basilicata, Foggia, Italy*
[4] *Dipartimento di Sanità Pubblica, University of Florence, Italy*

(*Accepted 20 March 2002*)

SUMMARY

We investigated the distribution of serotypes and patterns of drug resistance of 206 strains of salmonella isolated in southern Italy in the years 1998–2000 from raw food of animal origin, faeces of food animals and animal feed. To improve knowledge of mobile genetic elements carrying the resistance genes, some molecular features were also investigated within isolates resistant to three or more antibiotics. A high proportion of isolates, 52·2% and 37·7%, respectively, belonging to both Typhimurium and other serotypes of animal origin, proved to be multidrug resistant. The DT104 complex specific multidrug pattern of resistance was quite infrequent among isolates other than Typhimurium, but resistances to nalidixic acid and kanamycin were more frequent within these last ones (36·9% *vs.* 11·4% and 56·5% *vs.* 2·2%, respectively). Class I integrons were detected in isolates of Typhimurium and seven different serotypes. The relevance of food animal environment as a drug resistance reservoir and animal food as a potential resistance gene vehicle between the farm and human ecological niches is confirmed by our findings.

INTRODUCTION

Food animals represent a large reservoir of resistant bacteria and resistance genes. Widespread use of antibiotics in animal husbandry has a major role in determining the ecological impact of resistant organisms and the increasing worldwide evidence of drug resistant zoonotic pathogens [1, 2]. Food may be also the vector of resistance determinants between the animal ecological niche and the human beings [3]. Intensive rearing, changing consumer's demand, choice reasons and life style modifications are amplifying these problems in an epidemiological vicious circle [4].

Surveillance on drug resistant pathogens in the feed-food chain is essential. Moreover, need of local monitoring of resistances has been previously stressed, because of substantial differences between countries in practice and policy about drug resistance [5].

In this respect, we have examined distribution of serotypes and pattern of resistances of 206 strains of salmonella isolated in southern Italy during the years 1998–2000 from 172 samples of raw food of animal origin, 22 faecal samples from food animals and 12 samples of animal feed. Some molecular features of drug resistance, such as plasmid content and presence of class I integrons, were also investigated in multidrug resistant isolates.

* Author for correspondence: Dipartimento di Igiene e Microbiologia, Via del Vespro 133, I-90127 Palermo, Italy.

MATERIALS AND METHODS

Bacterial strains

Two-hundred-and-six strains of salmonella had been isolated in the years 1998–2000 at the Istituto Zooprofilattico Sperimentale of Foggia (Italy) from 172 samples of raw food of animal origin, 22 faecal samples from farm animals and 12 feed samples. Raw food was swine meat in 65 cases, poultry meat in 26, bovine meat in 17, horse meat in 7, ovine meat in 6, rabbit meat in 4, mixed preparations in 26 and eggs or egg-based preparations in 21 cases. Ten faecal samples were from poultry, 8 from bovine, 3 from rabbit and 1 from ovine. All the above samples had been submitted for microbiological analysis by Public Health Services during their routine surveillance activity at the rearing plants, slaughterhouses, food processing plants, catering services and grocery stores. During the period 1998–2000, approximately 1·5% of the food samples of animal source, which had been processed at the Istituto Zooprofilattico Sperimentale, tested positive by culture for salmonella.

Salmonella isolates had been sent for confirming serological identification and performing phenogenotypic analysis to the Centre for Enteric Pathogens of Southern Italy (CEPIM), Palermo, Italy.

All isolates were serotyped by agglutination tests with specific O and H antisera (Pasteur) and serotypes classified according to the Kauffman–White scheme.

Antibiotic susceptibility testing

Antibiotic susceptibilities were evaluated by the disk diffusion method on Mueller-Hinton agar according to the standards of the National Committee for Clinical Laboratory Standards [6]. The antimicrobial drugs and concentrations in μg were: ampicillin 10 (Ap), cephalotin 30 (Cf), cefotaxime 30 (Ctx), chloramphenicol 30 (Cm), ciprofloxacin 5 (Cip), gentamicin 10 (Gm), kanamycin 30 (Km), streptomycin 10 (Sm), nalidixic acid 30 (Na), tetracycline 30 (Tc), sulphonamides 300 (Su), trimethoprim 5 (Tp). *Escherichia coli* ATCC 25922 was used as reference strain.

Resistance transfer

Salmonella strains resistant to at least three antibacterial drugs were tested for their ability to transfer resistance determinants to the recipient *E. coli* strain K12 J53 rif^{R}. Overnight broth cultures of donor and recipient were mixed in a ratio of 1:1 and incubated for 18 h at 37 °C. Transconjugants were cultured by plating broth cultures on McConkey agar plates containing 250 μg/ml of rifampicin and 20 μg/ml of the appropriate antibiotic. Transconjugant colonies were further purified by plating on selective agar plates and submitted to susceptibility testing.

Plasmid analysis

Plasmids were extracted by the alkaline lysis method [7], resolved on 0·7% agarose gels and stained with ethidium bromide simultaneously with reference size plasmids (39R861, MIP233, R27 and R477). For Typhimurium serotype, extrachromosomal DNA consistent by molecular size and unable to transfer any resistance determinant was considered as the serotype specific virulence plasmid.

Polymerase chain reaction (PCR) based procedures

Screening of isolates for presence of class I integrons was performed by a high-stringency protocol with oligonucleotide primers specific for the sequence of the published 5′-CS and 3′-CS regions adjacent to the site-specific recombinational insertion sequence [8]. Primer sequences were: 5′-CS, GGCATCCAAGCAGCAAG and 3′-CS, AAGCAGACTTGACCTGA [8].

Further PCR analysis was performed on the isolates harbouring class I integrons to better characterize the antibiotic resistance genes associated with the integron structure. This was done by using primers located at the beginning extremities of the inserted resistance genes in combination with the primer specific for the 5′-CS conserved segment. The following sequences were tested: sulfonamide resistance gene *sulI*; beta-lactam resistance genes *oxa2*, *pse1* and *tem*; aminoglycoside resistance genes *aac*(*3*)-*Ia*, *aac*(*3*)-*IIa*, *aac*(*6′*)-*Ib*, *ant*(*3″*)-*Ia*, *aadA2* (also named *ant*(*3″*)-*Ib*); trimethoprim resistance gene *dhfrI* [8, 9]. The presence of the *pasppflo*-like (*floR*) and *tetG* genes, conferring resistance to chloramphenicol, florfenicol and tetracycline in multidrug resistant DT104, was also investigated by using PCR primers specific for these sequences [9].

RESULTS

Serotyping

Serotyping of the 206 isolates gave 47 different serotypes, the most frequent being Typhimurium

Table 1. *Proportion of MDR isolates within the salmonella serotypes*

Serotype	Number (%) of MDR isolates
Typhimurium	35 (52·2)
Blockley	10 (83·3)
Derby	7 (50·0)
Anatum	5 (41·7)
Agona	3 (75·0)
Bredeney	3 (100)
Enteritidis	3 (15·0)
Isangi	3 (100)
Lindenburg	3 (100)
Saintpaul	3 (50·0)
Hadar	2 (66·7)
Livingstone	2 (66·7)
Heidelberg	1 (100)
Infantis	1 (20·0)
Panama	1 (100)
Sandow	1 (50·0)

(32·5%), Enteritidis (9·7%), Derby (7·3%), Anatum (5·8%) and Blockley (5·8%).

Antimicrobial drug resistance

Strains resistant to three or more antibacterial drugs were defined multiresistant (MDR). Distribution by serotype of MDR isolates is summarized in Table 1. Analysis of patterns of antibiotic resistance was further performed by separating serotype Typhimurium and other serotypes because of peculiar features due to the prominent role of the 'DT104 complex' and its resistance pheno-genotype in the epidemiological environment of such a serotype.

Typhimurium isolates

Distribution of resistance patterns of Typhimurium isolates is illustrated in Table 2. Thirty-five of 67 (52·2%) isolates were multidrug resistant. The characteristic ACSSuT profile was identified in 17 strains; in three additional isolates such a pattern was completed by resistance to trimethoprim or nalidixic acid or both. Comparison between MDR and no MDR isolates (Fig. 1a) shows that resistances to ampicillin, streptomycin, sulfonamides and tetracycline are largely prevalent, but at a lower percentage, among Typhimurium isolates not bearing a multiresistance phenotype.

Other serotype isolates

Forty-six of 122 (37·7%) strains were categorized as MDR. Yet, such heterogeneous group includes Enteritidis, a serotype very infrequently linked to drug resistance. Table 2 lists the pattern of antimicrobial resistance of the MDR isolates. Twenty-two different resistant phenotypes were recognized, but a DT104-like pattern was shown by one strain only of Anatum serotype. Comparison between MDR and no MDR groups (Fig. 1b) enlightens a similar trend to that of Typhimurium serotype, but at lower values. Two notable exceptions are the proportions of isolates resistant to nalidixic acid and kanamycin, both at higher level than Typhimurium (36·9% *vs.* 11·4% and 56·5% *vs.* 2·2%, respectively).

Plasmid and class I integron content of MDR strains

Plasmids in the approximate range 30–98 MDal were identified, alone or in association with those presumably identified as the serotype-specific virulence plasmids on the basis of their molecular size. They were detected in 15 of 46 isolates belonging to serotypes other than Typhimurium and in 3 only of 35 Typhimurium isolates. In three strains of Anatum, Enteritidis and Panama, respectively, the plasmids proved to be self-transferable and conferred to the recipient *E. coli* strain the whole pattern of resistance. For the remaining cases, no further attempts were made to correlate resistances with extrachromosomal DNA structures.

Class I integrons were detected in 22 Typhimurium and in 13 other than Typhimurium isolates. Among the Typhimurium isolates, 17 had the characteristic pattern 1·2–1·0 kb. The 13 other than Typhimurium isolates were identified as belonging to 7 different serotypes.

Distribution of integrons and integron-associated resistance genes is summarized in Table 3. Serotypes Panama and Saintpaul carried the *ant3″-Ia* and *pseI* gene cassettes previously described in two separate integrons in MDR-DT104 [10], but located on single integronic structures of approximately 1·6 kb molecular size. Within MDR Typhimurium isolates, a *tem* type gene was found in two (5·3%) and an *oxa2* gene was detected in four (10·5%) isolates with an otherwise typical DT104 pattern. Three isolates from different animal sources contained two copies of the *ant3″-Ia* gene cassette.

*Tet*G gene determinants were identified in two strains of Agona serotype from poultry feed and from swine liver, respectively.

Table 2. *Distribution of resistance patterns among MDR Typhimurium and other than Typhimurium isolates*

R-type	Antibacterial drugs*									Number of isolates
MDR Typhimurium										
R10	Ap					Su		Sm	Tc	3
R30	Ap					Su	Tp		Tc	1
R24	Ap		Na	Cm		Su	Tp	Sm	Tc	1
R25	Ap		Na	Cm		Su		Sm	Tc	2
R26	Ap			Cm		Su	Tp	Sm	Tc	1
R27	Ap			Cm		Su		Sm	Tc	17
R28	Ap					Su	Tp	Sm	Tc	2
R29				Cm		Su	Tp	Sm	Tc	3
R19		Km						Sm	Tc	1
R20			Na					Sm	Tc	1
R22						Su	Tp		Tc	1
R23						Su		Sm	Tc	2
MDR other than Typhimurium										
R1	Ap	Km							Tc	1
R2	Ap	Km		Cm		Su			Tc	1
R3	Ap	Km		Cm		Su		Sm	Tc	1
R4	Ap	Km		Cm		Su	Tp	Sm	Tc	1
R5	Ap	Km						Sm		1
R6	Ap		Na					Sm	Tc	2
R7	Ap			Cm		Su	Tp	Sm	Tc	3
R8	Ap					Su	Tp			2
R28	Ap					Su	Tp	Sm	Tc	1
R9	Ap					Su		Sm		4
R11		Km	Na	Cm	Gm	Su	Tp	Sm	Tc	2
R12		Km	Na	Cm		Su	Tp	Sm	Tc	2
R13		Km	Na	Cm				Sm	Tc	5
R14		Km	Na	Cm					Tc	1
R15		Km	Na					Sm	Tc	2
R16		Km	Na						Tc	1
R17		Km		Cm				Sm	Tc	2
R18		Km				Su		Sm	Tc	1
R19		Km						Sm	Tc	5
R20			Na					Sm	Tc	2
R22						Su	Tp		Tc	3
R23						Su		Sm	Tc	3

* Abbreviations are explained in the Materials and Methods section.

DISCUSSION

Resistant organisms are common in the environment. Human beings, food-producing animals and household pets frequently harbour organisms resistant to two or more antibiotics [11]. Resistant strains have been also isolated from food, which plays undoubtedly a role in the 'trading' of drug resistance genes within pathogen, commensal and opportunistic bacteria [11].

Our study confirms the relevance of food from animal origin as a carrier of resistant bacterial strains and resistance gene determinants. Indeed, a high proportion of isolates belonging to both Typhimurium and other serotypes with a farm ecology have shown a multidrug resistance pattern. Drug resistance has further proved to be in some cases vehicled by mobile elements, such as plasmids and integrons. These last elements represent a very effective strategy of dissemination of resistance among bacterial pathogens in both community and hospital environments [12–14]. Moreover, resistances to ampicillin, streptomycin, sulphonamides and tetracycline have been proved to be widely diffuse among no MDR isolates of many serotypes.

MDR Typhimurium appeared to some extent heterogeneous with different genetic make-up cor-

Table 3. *Distribution of integrons and integron-associated resistance genes in MDR isolates of salmonella*

Serotype	Number of isolates	R-type	Conjugative R-plasmids	Integron molecular weight (kb)	*pseI*	*tem*	*oxa2*	*dhfrI*	*ant 3″-Ia*
Agona	1	R22							
Agona	1	R23		1·0					
Agona	1	R22		1·2, 1·0					
Anatum	1	R28		1·8		pos			
Anatum	1	R8		1·8		pos			
Anatum	1	R8							
Anatum	1	R12	pos	1·6				pos	pos
Anatum	1	R12		1·6				pos	pos
Blockey	1	R13							
Blockey	1	R15							
Blockey	3	R19							
Blockey	2	R17							
Blockey	1	R14							
Blockey	1	R13							
Blockey	1	R15							
Bredeney	1	R18							
Bredeney	2	R11		1·6				pos	pos
Derby	1	R1							
Derby	1	R2							
Derby	1	R3							
Derby	1	R23		1·0					
Derby	2	R19							
Derby	1	R23							
Enteritidis	1	R16							
Enteritidis	1	R9							
Enteritidis	1	R22	pos	2·0					
Hadar	2	R20							
Heidelberg	1	R6							
Infantis	1	R5							
Isangi	3	R9							
Lindenburg	3	R13							
Panama	1	R4	pos	1·6	pos			pos	pos
Saintpaul	2	R7		1·6	pos			pos	pos
Saintpaul	1	R7							
Sandow	1	R6							
Typhimurium	9	R27		1·2, 1·0	pos				pos
Typhimurium	1	R22							
Typhimurium	2	R25							
Typhimurium	1	R10		1·5	pos				pos
Typhimurium	1	R23		1·6	pos	pos			pos
Typhimurium	1	R23							
Typhimurium	1	R19							
Typhimurium	1	R30		1·5	pos	pos			
Typhimurium	1	R20							
Typhimurium	1	R10							
Typhimurium	1	R24		0·5	pos				
Typhimurium	3	R29							
Typhimurium	1	R26		0·5	pos				
Typhimurium	2	R28							
Typhimurium	1	R10		1·2, 1·0	pos				pos
Typhimurium	4	R27		1·2, 1·0			pos		pos
Typhimurium	3	R27		1·2, 1·0	pos				pos (two copies)

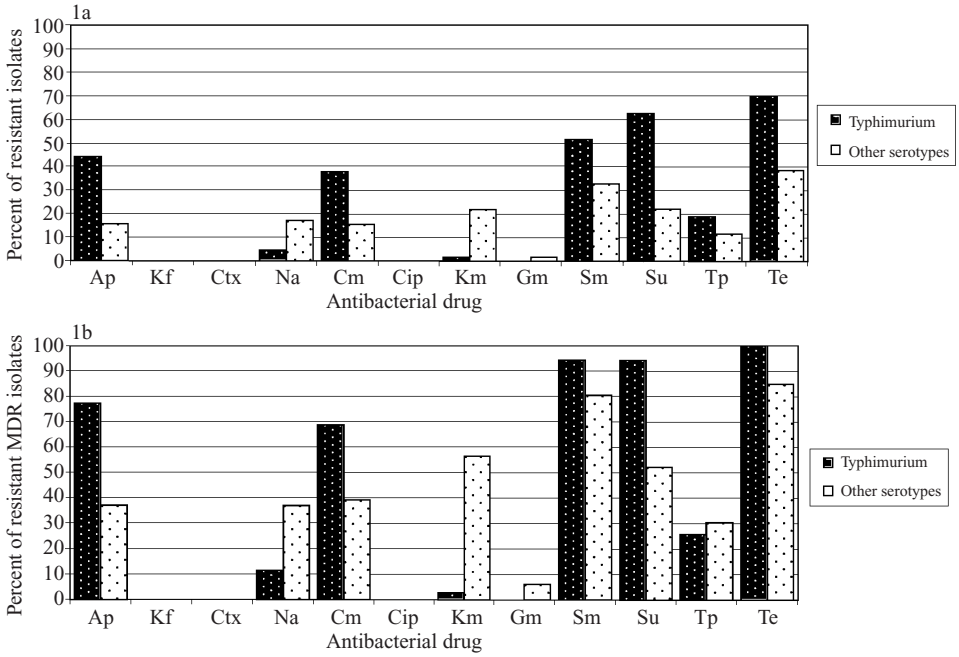

Fig. 1. Percentage of resistant isolates of salmonella from food animals or from food/feed sources, Italy 1998–2000. (a) Isolates resistant to two or less antibacterial drugs; (b) isolates resistant to three or more. Abbreviations for antibacterial substances are explained in the Materials and Methods section.

responding to an apparently monotonous phenotype. This result further confirms the conclusions of a previous study on phage types and ribotypes of Typhimurium in southern Italy, where a policlonal circulation of MDR Typhimurium was evident as well as the predominance of PT 193 [15]. Similar findings have also been described by Markogiannakis *et al.* [16] about Greek MDR Typhimurium isolates based upon PFGE and class I integron size and structure. The world-wide success of MDR Typhimurium strains is indeed now thought better attributable to the dissemination of a cluster of strains with related phage type patterns than to a monoclonal spread [17, 18].

Drug resistance patterns of the other salmonella serotypes were at first sight similar to those of Typhimurium with regard to the involved families of antimicrobial drugs, but some remarkable divergences could be observed. The standard DT104-like pattern has been very infrequent, thus confirming the peculiar relationship between the gene cluster SGI1 and Typhimurium serotype. Evidence for horizontal transfer of this pentaresistance genomic island towards other serotypes, namely Agona [19] and Paratyphi B [20] is concerning, but appears so far a quite limited phenomenon in our geographic area. Nevertheless, the great selective advantage provided by the acquisition of these genetic elements further strengthens the need of a prudent use and an overall limitation of antibiotics on a broad scale.

Moreover, the high proportion of isolates resistant to kanamycin and nalidixic acid is striking. Of particular interest, this last one, because nalidixic acid resistance is a reliable marker of low-level resistance to fluoroquinolones [21] and has proved to be associated with treatment failure in invasive salmonellosis [22]. Role of employ of fluoroquinolones in veterinary medicine towards selection of these first-step mutants within zoonotic serotypes of salmonella has been thoroughly investigated [23]. Choice of antibiotics depending on different food-producing animal species and rearing and production type could reasonably explain the very different distribution of resistant strains to these two antimicrobial drugs among Typhimurium and no Typhimurium isolates in our geographic area.

Evolution of bacterial resistance mechanisms by acquisition and dissemination of new and possibly multiple resistance traits is worrying. Surveillance and monitoring of drug resistance, including use of molecular methods for tracing resistant clones and screening of epidemic mobile elements, with special attention to integrons, appear more and more critical steps for researchers and policy makers in the field of both human and veterinary health.

REFERENCES

1. Chaslus-Dancla E, Lafont JP, Martel JL. Spread of resistance from food animals to man: the French experience. Proceedings of the Symposium on antibiotic resistance with emphasis on animal-human transfer. Falkenberg, Sweden, 13–14 September 1999. Acta Vet Scand 2000; **93** (Suppl): 53–61.
2. Shryock TR. Relationship between usage of antibiotics in food-producing animals and the appearance of antibiotic-resistant bacteria. Int J Antimicrob Agents 1999; **12**: 275–8.
3. Holmberg SD, Wells JG, Cohen ML. Animal to man transmission of antimicrobial resistant *Salmonella*: investigations of U.S. outbreaks 1971–1983. Science 1984; **225**: 833–5.
4. Collins JE. Impact of changing consumer lifestyles on the emergence/reemergence of foodborne pathogens. Emerg Infect Dis 1997; **3**: 471–9.
5. O'Brien TF. The global epidemic nature of antimicrobial resistance and the need to monitor and manage it locally. Clin Infect Dis 1997; **24**: S2–8.
6. National Committee for Clinical Laboratory Standards. Performance standards for antimicrobial disk susceptibility tests for bacteria that grow aerobically. Approved standard M7-A4. Villanova (PA): The Committee, 1997.
7. Birnboim HC, Doly J. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucl Acids Res 1979; **7**: 1513–23.
8. Lévesque C, Piché L, Larose C, Roy PH. PCR mapping of integrons reveals several novel combinations of resistance genes. Antimicrob Agents Chemother 1995; **39**: 185–91.
9. Ng L, Mulvey MR, Martin I, Peters GA, Johnson W. Genetic characterization of antimicrobial resistance in Canadian isolates of *Salmonella* serovar Typhimurium DT104. Antimicrob Agents Chemother 1999; **43**: 3018–21.
10. Arcangioli MA, Leroy-Sétrin S, Martel JL, Chaslus-Dancla E. A new chloramphenicol and florfenicol resistance gene flanked by two integron structures in *Salmonella typhimurium* DT104. FEMS Microbiol Lett 1999; **174**: 327–32.
11. Sørum H, Sunde M. Resistance to antibiotics in the normal flora of animals. Vet Res 2001; **32**: 227–41.
12. Jones ME, Peters E, Weersink AM, Fluit A, Verhoef J. Widespread occurrence of integrons causing multiple antibiotic resistance in bacteria. Lancet 1997; **349**: 1742–3.
13. Carattoli A. Importance of integrons in the diffusion of resistance. Vet Res 2001; **32**: 243–59.
14. Nastasi A, Mammina C. Presence of class I integrons in multidrug-resistant, low-prevalence *Salmonella* serotypes, Italy. Emerg Infect Dis 2001; **7**: 455–8.
15. Nastasi A, Mammina C. Surveillance of multidrug-resistant strains of *Salmonella enterica* serotype Typhimurium in southern Italy in the years 1992–1997. Eur J Epidemiol 2000; **16**: 135–9.
16. Markogiannakis A, Tassios PT, Lambiri M, et al. Multiple clones within multidrug-resistant *Salmonella enterica* serotype Typhimurium phage type DT104. J Clin Microbiol 2000; **38**: 1269–71.
17. Glynn MK, Ribot EM, Barrett TJ. Multidrug-resistant *Salmonella enterica* serotype Typhimurium infections. New Engl J Med 1998; **339**: 922.
18. Tauxe RV. *Salmonella* Enteritidis and *Salmonella* Typhimurium DT104: successful subtypes in the modern world. In: Scheld WM, Craig WA, Hyghes JM, eds. Emerging infections 3, Washington DC: ASM Press, 1999: 37–53.
19. Cloeckaert A, Sidi Boumedine K, Flaujac G, Imberechts H, D'Hooghe I, Chaslus-Dancla E. Occurrence of a *Salmonella enterica* serotype Typhimurium DT104-like antibiotic resistance gene cluster including the *flo*R gene in *S. enterica* serotype Agona. Antimicrob Agents Chemother 2000; **44**: 1359–61.
20. Meunier D, Boyd D, Mulvey MR, et al. *Salmonella enterica* Serotype Typhimurium DT 104 antibiotic resistance genomic island I in serotype paratyphi B. Emerg Infect Dis 2002; **8** (Available from: URL: http://www.cdc.gov/ncidod/EID/eid.htm).
21. Hakanen A, Kotilainen P, Jalava J, Siitonen A, Huovinen P. Detection of decreased fluroquinolones susceptibility in salmonellas and validation of nalidixic acid screening test. J Clin Microbiol 1999; **37**: 3572–7.
22. Vasallo FJ, Martin-Rabadan P, Alcala L, Garcia-Lechuz JM, Rodriguez-Creixems M, Bouza E. Failure of ciprofloxacin therapy for invasive nontyphoidal salmonellosis. Clin Infect Dis 1998; **26**: 535–6.
23. Piddock LJ, Ricci V, McLaren I, Griggs DJ. Role of mutation in the *gyr*A and *par*C genes of nalidixic-acid resistant salmonella serotypes isolated from animals in the United Kingdom. J Antimicrob Chemother 1998; **41**: 635–41.