Molecular characterization of antimicrobial resistance in non-typhoidal salmonellae associated with systemic manifestations from India

G. A. Menezes,^{1,2} M. A. Khan,³ B. N. Harish,¹ S. C. Parija,¹ W. Goessens,³ K. Vidyalakshmi,⁴ S. Baliga⁴ and J. P. Hays³

Correspondence

G. A. Menezes

godfredmenezes@gmail.com

¹Department of Microbiology, Institute of National Importance, Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER), Pondicherry, India

²Department of Microbiology, SSR Medical College, Belle Rive, Mauritius

³Department of Medical Microbiology and Infectious Diseases, Erasmus MC, 's Gravendijkwal 230, 3015 CE Rotterdam, The Netherlands

⁴Department of Microbiology, Kasturba Medical College Mangalore, Manipal University, Mangalore, India

Extended-spectrum cephalosporins and fluoroquinolones are essential antimicrobials for treating invasive salmonellosis, although emerging resistance to these antimicrobials is of growing concern, especially in India. Therefore, a study was conducted to characterize the antimicrobial susceptibility phenotypes, types of extended-spectrum β -lactamase (ESBL) gene plasmids and serological relationships of 21 non-typhoidal *Salmonella* isolates from patients who attended three different hospitals in India from 2006 to 2008. The isolates were cultured from stool, blood and cerebrospinal fluid samples obtained from patients presenting with diarrhoea and accompanying systemic manifestations such as fever, vomiting and meningism. Non-typhoidal *Salmonella* isolates were investigated using serotyping and antimicrobial susceptibility testing. PCR screening was also performed to detect the β -lactamase, *qnr* and *aac(6')-Ib-cr* genes and class 1 integrons. Sequencing for quinolone resistance mutations and plasmid replicon typing were also performed. An antimicrobial resistance microarray was used for preliminary screening and identification of *bla*_{TEM} and *bla*_{SHV} genes, and phenotypic testing for the presence of efflux pumps was also performed. Ten out of 21 isolates (48 %) possessed the extended-spectrum cephalosporin resistance phenotype, with PCR amplification and sequencing revealing that isolates possessed TEM-1, SHV-12, DHA-1, OXA-1-like and CTX-M-15 ESBL genes. *FII*_S plasmid replicons were detected in seven isolates (33 %). The involvement of efflux pumps was detected in four isolates (19 %) resistant to ciprofloxacin. It was concluded that SHV-12-carrying *Salmonella* serotype Agona may play an important role in ESBL-mediated resistance in non-typhoidal salmonellae in India. The very high percentage (48 %) of ESBL-producing non-typhoidal salmonellae isolated from these patients represents a real and immediate challenge to the effective antimicrobial therapy of *Salmonella* infections associated with systemic manifestations. Continued surveillance for the presence of ESBL-producing (non-typhoidal) salmonellae in India is essential.

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INTRODUCTION

Non-typhoidal *Salmonella* is one of the principal pathogens implicated in food-borne gastroenteritis worldwide. In severe infections such as meningitis and septicaemia, antibiotic treatment is essential, with extended-spectrum cephalosporins being used preferentially to treat salmonellosis in children (Threlfall, 2002). However, treatment

failures due to the *in vivo* acquisition of extended-spectrum β -lactamase (ESBL) or fluoroquinolone resistance genes in *Salmonella* isolates are now well established (Whichard *et al.*, 2007). *Salmonella* species resistant to extended-spectrum cephalosporins have been known since 1988 (Hammami *et al.*, 1991) and may be isolated from hospitals in many different continents including Europe, North and South America, North Africa and countries such as India and South Korea (Weill *et al.*, 2004). Salmonellae have been found to possess a wide variety of ESBL enzymes,

Abbreviation: ESBL, extended-spectrum β -lactamase.

including TEM, SHV, PER and CTX-M (Gniadkowski, 2001), as well as plasmid-borne AmpC enzymes (e.g. CMY, DHA, ACC-1) (Miriagou *et al.*, 2004). CTX-M enzymes are particularly important in facilitating the ESBL resistance phenotype and have been described in several *Salmonella* serovars. In fact, 12 out of more than 50 currently known CTX-M enzymes (which are divided into five major phylogenetic groups) have been identified in the genus *Salmonella*.

Fluoroquinolone resistance is also a growing problem in non-typhoidal salmonellae, with the first report of ciprofloxacin resistance in *Salmonella enterica* infection (eventually leading to treatment failure) being published in 1990 (Piddock *et al.*, 1990). Since then, there have been reports of ciprofloxacin-resistant isolates being found in many countries, including India, Pakistan, Vietnam and Spain (Nath *et al.*, 2000).

Combined ESBL and fluoroquinolone resistance are major problems in the effective treatment of bacterial infections, both in the community and in the nosocomial setting. This problem is greatly increased when bacterial pathogens such as non-typhoidal salmonellae acquire both ESBL and fluoroquinolone resistance, as the consequences could have important health implications for both livestock and the human community. In order to manage and prevent the spread of antimicrobial resistance, both governing bodies and clinicians require accurate information as to the prevalence and molecular mechanisms of antimicrobial resistance within currently circulating bacterial pathogens. At present, this information tends to be lacking, especially in countries with large populations and unrestricted 'over-the-counter' prescription policies, such as India. Therefore, this study was set up to investigate the extent of antimicrobial resistance, including ESBL and fluoroquinolone resistance, in non-typhoidal salmonellae originating from patients who attended three different hospitals in India. Information was also obtained regarding the circulating serovars of non-typhoidal salmonellae in order to relate these characteristics to specific antimicrobial phenotypes.

METHODS

Bacterial isolates and serotyping. Twenty-one isolates of non-typhoidal salmonellae were collected from patients attending three different hospitals in India (Pondicherry, Bangalore and Mangalore) from 2006 to 2008. These represented isolates that were cultured from patients with diarrhoea associated with the systemic manifestations fever, vomiting and meningism. Non-typhoidal salmonellae were isolated from 17 stool cultures, three blood cultures and one cerebrospinal fluid sample. Isolates were initially identified biochemically, followed by confirmation using specific antisera. Serovar determination was performed at the Rijksinstituut voor Volksgezondheid en Milieu, Bilthoven, The Netherlands.

Antimicrobial susceptibility. Antibiotic susceptibility profiles were obtained against 16 antibiotics using the Vitek (bioMérieux) system. The MICs of ceftriaxone and ciprofloxacin were determined using

Etest (AB Biodisk). Isolates were confirmed as ESBL producers via the combination disc method using cefotaxime (30 µg) and ceftazidime (30 µg) and a disc of cefotaxime + clavulanate (30 µg + 10 µg) and ceftazidime + clavulanate (30 µg + 10 µg). A ≥ 5 mm increase in diameter of the inhibition zone of the cefotaxime + clavulanate and ceftazidime + clavulanate disc, when compared with the cefotaxime and ceftazidime disc alone, was interpreted as phenotypic evidence of ESBL production (NCCLS, 2000). AmpC β -lactamase production was measured using cefoxitin (a zone diameter of <18 mm was considered to be AmpC-positive). The AmpC disc test for plasmid-mediated AmpC β -lactamase detection was carried out according to a procedure described previously (Singhal *et al.*, 2005). Resistant control strains were used for all ESBL and AmpC β -lactamase detection methods. For detection of the involvement of efflux pumps, a two-step dilution series of the antibiotics nalidixic acid and ciprofloxacin was made to detect MIC in the absence and presence of the efflux pump inhibitor Phe-Arg- β -naphthylamide. MIC values were expressed as the highest dilution that inhibited growth, as determined by a lack of opacity in the respective wells of the microtitre plate used. A fourfold or greater reduction in the MIC values after the addition of Phe-Arg- β -naphthylamide was considered significant (Schumacher *et al.*, 2006).

PCR screening, sequence analysis and IEF. Isolates were screened initially for the presence of TEM and SHV β -lactamases using a commercially available antimicrobial resistance gene microarray (Check-Points BV). PCR screening and sequencing was performed to identify the β -lactamase resistance genes *bla*_{TEM}, *bla*_{SHV}, *bla*_{OXA-1} group, *bla*_{CTX-M} and AmpC, as described previously (Karisik *et al.*, 2006; Pérez-Pérez & Hanson, 2002; Taşlı & Bahar, 2005; Woodford *et al.*, 2006). The mechanism associated with any phenotypic quinolone resistance was determined by investigating nucleotide sequence mutations in the DNA gyrase (*gyrA* and *gyrB*) and DNA topoisomerase IV (*parC* or *parE*) genes (Giraud *et al.*, 1999; Komp Lindgren *et al.*, 2003; Renuka *et al.*, 2004). Sequencing was performed using both forward and reverse PCR primers and standard methods on an ABI Prism 3130X1 Genetic Analyzer (Applied Biosystems). The BLASTN program was used for database searching (<http://www.ncbi.nlm.nih.gov/BLAST/>). Additional sequencing primers were required for *bla*_{TEM} PCR product sequencing (lagging strand 7, 5'-TTACT-GTCATGCCATCC-3'; and lagging strand 3, 5'-AGAGAATTATGC-AGTGC-3'). PCR primers corresponding to sequences downstream (ORF1) of the *bla*_{CTX-M} genes (M3 int upp, 5'-TCACCCAGCCT-CAACCTAAG-3'; and ORF1 pol M3, 5'-GCACCGACACCTCA-CACCT-3') were also used (Eckert *et al.*, 2004). Finally, the PCR products of *bla*_{CTX-M} were subjected to sequencing using primers CTX-M-1 fw multi (5'-AAAAATCACTGCGCCAGTTC-3'), CTX-M-1 multi (REV)F seq (5'-AACGTGGCGATGAATAAGCT-3') and ORF1 pol M3. Plasmid-mediated quinolone resistance was detected as described previously (Wang *et al.*, 2004). PCR screening for the *aac(6')-Ib-cr* gene was performed according to Robicsek *et al.* (2006). The presence of individual plasmid types was determined by PCR screening of I1, FIA, FIB, FII, A/C, HI1, Frep, K/B and B/O replicons (Carattoli *et al.*, 2005). The presence or absence of class 1 integrons was determined using 5'- and 3'-conserved segment primers specific for the variable regions of integrons as described by Lévesque *et al.* (1995). IEF of β -lactamase enzymes was performed using standard methods (Paterson *et al.*, 2001).

RESULTS

The 21 non-typhoidal *Salmonella* isolates investigated comprised six serovars, namely *Salmonella* Agona (ten), *Salmonella* Typhimurium (five), *Salmonella* Enteritidis (three), *Salmonella* Senftenberg (one), *Salmonella* Lexington

(one) and *Salmonella* Kirkee (one) (Table 1). Serovars were not linked to a particular hospital. Ten of the 21 isolates were resistant to extended-spectrum cephalosporins (cefotaxime, ceftazidime and ceftriaxone) (Table 2). Of the 10 isolates that exhibited extended-spectrum cephalosporin resistance, three were determined to be resistant to a monobactam (aztreonam), a cephamycin (cefepime) and amoxicillin/clavulanic acid. They exhibited intermediate resistance to piperacillin/tazobactam. Phenotypically, 10 of the 21 isolates (48 %) harboured ESBL enzymes, of which three (30 %) co-produced AmpC enzymes. Seven of the 21 isolates (33 %) were resistant to nalidixic acid and, of these, two ESBL-producing and two non-ESBL-producing isolates were also found to be resistant to ciprofloxacin. At the serovar level, two of the *Salmonella* Agona isolates were resistant to extended-spectrum cephalosporins and ciprofloxacin. No resistance to carbapenems was recorded.

Interestingly, nine of the ten ESBL-positive isolates (90 %) belonged to *Salmonella* serovar Agona (Table 1). ESBL array analysis revealed the presence of ten TEM and nine SHV positives within the 21 isolates, with all TEM-positive isolates being TEM-1-positive, and all SHV isolates being

either SHV-12- or SHV-5-positive. IEF experiments showed that the cell extracts of these ten isolates contained β -lactamases with a corresponding pI of 5.4 (the pI of TEM-1) and nine β -lactamases with a corresponding pI of 8.2 (the pI of SHV-12). Of the ten ESBL-positive isolates, ten (100 %), nine (90 %), two (20 %) and one (10 %) of the isolates were found to be TEM-1-, SHV-12-, OXA-1-like- and CTX-M-15-positive, respectively, by PCR amplification and sequence analysis (Tables 1 and 2). Additionally, three ESBL producers (30 %) were co-producers of DHA-1 plasmid-borne AmpC β -lactamase (Tables 1 and 2). The combinations of β -lactamases found in the isolates are shown in Tables 1 and 2. The four isolates that were ciprofloxacin-resistant showed phenotypic evidence for the involvement of efflux pumps.

The range of mutations found in the DNA gyrase (*gyrA* and *gyrB*) and DNA topoisomerase IV (*parC* or *parE*) genes of the ciprofloxacin (quinolone)-resistant isolates is shown in Table 3. All isolates were negative for *qnr* genes and all four ciprofloxacin-resistant isolates carried the *aac(6')-Ib-cr* gene. Of the isolates carrying the *aac(6')-Ib-cr* gene, two were *Salmonella* Agona and were resistant to extended-

Table 1. Serovar distribution of 21 non-typhoidal salmonellae cultured from patients exhibiting systemic manifestations from Pondicherry, Bangalore and Mangalore, India, from 2006 to 2008

All isolates were cultured from stool specimens apart from isolates B4 (from cerebrospinal fluid) and A19, B5 and B13 (from blood culture).

Isolate	Serovar	Place*	Year	β -Lactamase†		Ep‡	Int§	Plasmids
B3	Typhimurium	P	2008	T-1	C-15	A	Int	FIA and FIB, B/O
B4	Typhimurium	P	2008					FII _s
B5	Typhimurium	P	2008					FII _s
A14	Typhimurium	M	2006					FII _s
B13	Agona	P	2007	T-1	S-12		Int	
A19	Agona	P	2007					FII _s
A16	Enteritidis	M	2006					FII _s
B22	Agona	P	2007	T-1	S-12			FII _s
B23	Enteritidis	P	2008					FII _s
A15	Enteritidis	M	2006			Ep		
B16	Lexington	B	2008					
A13	Senftenberg	M	2006			Ep		
B17	Kirkee	B	2008					
B14	Typhimurium	M	2007					B/O
B21	Agona	B	2006	T-1	S-12			
B7	Agona	B	2006	T-1	S-12		Int	
B8	Agona	P	2008	T-1	S-12	O-1	A	FIA and FIB
B9	Agona	B	2008	T-1	S-12	O-1	Ep	FIA and FIB, B/O
B1	Agona	B	2006	T-1	S-12			
B10	Agona	B	2008	T-1	S-12			
B11	Agona	B	2006	T-1	S-12	A	Int	

*P, Pondicherry; M, Mangalore; B, Bangalore.

†T-1, TEM-1; S-12, SHV-12; C-15, CTX-M-15; O-1, OXA-1 group; A, AmpC (DHA-1).

‡Ep, Efflux pump positive based on antibiotic testing (see Methods).

§Int, Class 1 integrons.

||Major plasmid incompatibility groups FIA, FIB, FII_s and B/O, determined by PCR (Carattoli *et al.*, 2005).

Table 2. Antimicrobial susceptibility profiles for 21 non-typhoidal *Salmonella* isolates cultured from patients exhibiting systemic manifestations from Pondicherry, Bangalore and Mangalore, India

Antimicrobial drugs to which isolates were resistant are listed: Ak, amikacin; Ac, amoxicillin/clavulanic acid; A, ampicillin; Cz, cefazolin; Cpm, cefepime; Cn, ceftazidime; Cep, cefepime; Ce, cefotaxime; Ca, ceftazidime; Ci, ceftriaxone; Cu, cefuroxime; C, chloramphenicol; Cf, ciprofloxacin; M, meropenem; Na, nalidixic acid; Pc, piperacillin; Pc/Tz, piperacillin/tazobactam. R, Resistant; S, sensitive; I, intermediate.

Isolate	Ak	Ac	A	Cz	Cpm	Cn	Cep	Ce	Ca	Ci	Cu	C	Cf	M	Na	Pc	Pc/Tz	β -Lactamase*		
B3	S	R	R	R	S	R	R	R	R	R	R	R	S	S	S	R	I	T-1	C-15	A
B4	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S			
B5	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S			
A14	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S			
B13	S	S	R	R	S	S	R	R	R	R	R	R	S	S	S	R	S	T-1	S-12	
A19	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S			
A16	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S			
B22	R	S	R	R	S	S	R	R	R	R	R	R	S	S	S	R	S	T-1	S-12	
B23	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S			
A15	S	S	S	S	S	S	S	S	S	S	S	I	R	S	R	S	S			
B16	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S			
A13	S	S	S	S	S	S	S	S	S	S	S	S	R	S	R	S	S			
B17	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S			
B14	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S			
B21	I	I	R	R	S	S	R	R	R	R	R	S	S	S	S	R	S	T-1	S-12	
B7	S	S	R	I	S	S	R	R	R	R	R	R	S	S	S	R	S	T-1	S-12	
B8	S	R	R	R	S	R	I	R	R	R	R	R	R	S	R	I	I	T-1	S-12	O-1 A
B9	S	S	R	R	S	S	R	R	R	R	R	S	R	S	R	I	I	T-1	S-12	O-1
B1	I	I	R	R	S	S	R	R	R	R	R	S	S	S	S	R	S	T-1	S-12	
B10	S	S	R	R	S	S	R	R	R	R	R	R	S	S	S	R	S	T-1	S-12	
B11	S	R	R	R	S	R	R	R	R	R	R	R	S	S	S	R	I	T-1	S-12	A

*T-1, TEM-1; S-12, SHV-12; C-15, CTX-M-15; O-1, OXA-1 group; A, AmpC (DHA-1).

spectrum cephalosporins, with one isolate possessing the ESBL phenotype (SHV-12 with TEM-1 and OXA-1 group) and the other a combined ESBL and AmpC co-producing phenotype (SHV-12 and DHA-1 with TEM-1 and OXA-1 group). In this study, FII_s replicon plasmids were found in seven (33 %) and B/O replicons in three (14 %) of the isolates (Table 1). Both FIA and FIB replicons were positive in three (14 %) of the isolates. Plasmids harbouring II, A/C, HI1, Frep and K/B replicons types were not detected. Class 1 integrons were detected in seven (33 %) of the isolates, which were all ESBL-resistant, but were not associated with a particular serovar.

Finally, antibiotic resistance profiles between ESBL producers tended to be similar, with non-ESBL-producing isolates being much more susceptible to a range of common, clinically useful, antibiotics. In contrast, ESBL-producing isolates tended to be resistant to most antibiotics tested, except for amikacin, cefepime, ciprofloxacin, meropenem and piperacillin/tazobactam.

DISCUSSION

In India, non-typhoidal salmonellae constitute approximately 20 % of the *Salmonella* serovars currently circulating,

and dissemination of isolates resistant to ESBLs is frequently observed (Kumar *et al.*, 2009). Indeed, in the present study, 48 % (10/21) of the non-typhoidal salmonellae associated with systemic manifestations analysed harboured ESBL genes and exhibited an ESBL phenotype, representing a very high percentage of isolates. In contrast, in a recent study in Korea, only 1 % of *Salmonella* isolates possessed the ESBL phenotype, with ESBL phenotypes showing a switch from TEM- and SHV- to CTX-M-mediated resistance (Park *et al.*, 2010). Although several different serovars were identified, the majority (90 %) of ESBL producers belonged to the *Salmonella* Agona serovar, which also represented the most abundant serovar isolated in this study. *Salmonella* Agona was reported for the first time in 1961 in Ghana (Guinée *et al.*, 1961), although this particular serovar has since been reported in many countries worldwide in both humans and animals. For example, in Brazil, *Salmonella* Agona has been reported as being the fourth most common *Salmonella* serovar isolated from non-human sources, and is among the top ten serovars associated with human disease (Tavechio *et al.*, 2002). Furthermore, multidrug-resistant *Salmonella* Agona has been responsible for at least two hospital outbreaks in paediatric wards (Asensi *et al.*, 1994). In both cases, the strains were found to harbour large plasmids that conferred

Table 3. Mutations found in the DNA gyrase (*gyrA* and *gyrB*) and DNA topoisomerase IV (*parC* or *parE*) genes of four ciprofloxacin-resistant non-typhoidal salmonellae from India

All four isolates were positive for the *aac(6')-Ib-cr* gene and efflux pumps and negative for mutations in the *gyrB* and *parE* genes. Two nalidixic acid-sensitive control strains (included as negative controls) had no mutations (data not shown).

Isolate no./ <i>Salmonella</i> serovar	Ciprofloxacin MIC ($\mu\text{g ml}^{-1}$)	Mutations in <i>gyrA</i>	Mutations in <i>parC</i>
A15/Enteritidis	>64	Ser83→Tyr, Asp87→Gly	Ser80→Ile
A13/Senftenberg	>64	Ser83→Tyr, Asp87→Gly	Thr57→Ser, Ser80→Ile
B8/Agona	32	Ser83→Leu, Asp87→Asn	Thr57→Ser
B9/Agona	32	Ser83→Leu, Asp87→Asn	Thr57→Ser

resistance to multiple antibiotics. Currently, there are few publications from India that have investigated antibiotic resistance in non-typhoidal salmonellae (Jesudason *et al.*, 1988), especially isolates cultured from patients exhibiting systemic manifestations of disease. From our results, it appears that *Salmonella* Agona may also play an important role in non-typhoidal *Salmonella* ESBL-mediated resistance in India. More importantly, of particular concern is the fact that two isolates of *Salmonella* Agona recovered in this study possessed both ESBLs and mutations in the DNA gyrase and DNA topoisomerase IV genes, seriously limiting antimicrobial treatment options.

Nine of the 21 non-typhoidal *Salmonella* isolates in this study were found to possess the SHV-12-type ESBL gene. SHV-12 is currently one of the most common non-CTX-M ESBLs circulating, and has been identified in many Gram-negative species, including *Salmonella* serovars (Weill *et al.*, 2004). All of the 10 ESBL producers in our study also possessed the TEM-1 (non-ESBL) β -lactamase. TEM-1 is the most commonly encountered β -lactamase in members of the *Enterobacteriaceae*, and is able to hydrolyse narrow-spectrum penicillins and cephalosporins, such as cephalothin and cephaloridine.

Among the isolates studied, three were positive for DHA-1, a plasmid-borne AmpC enzyme first identified in *Salmonella* Enteritidis isolates from Saudi Arabia in 1992, but now reported in several other countries (Verdet *et al.*, 2006). Finally, a single isolate was positive for the CTX-M-15 ESBL enzyme. The ESBL enzymes that tend to be responsible for cephalosporin resistance in the genus *Salmonella* are TEM and SHV β -lactamase derivatives, although CTX-M-type ESBLs have recently (although infrequently) been reported. In a study by Rotimi *et al.* (2008) in Kuwait and the United Arab Emirates, 3.4 % of the *Salmonella* species studied were found to be CTX-M-15-positive.

Replicon-type FII_s plasmids are associated with *Salmonella* virulence (Guerra *et al.*, 2002). Plasmids containing FII_s replicons were the most frequent plasmid types found (33 % isolates), with multiple plasmid types being observed in three isolates only. Ten isolates were negative for all of the replicons tested (Table 1). There was no correlation between carriage of plasmid types and *Salmonella* serovar.

Sorting of plasmids into incompatibility (Inc) groups is advantageous because specific plasmid types have been associated with virulence and/or antimicrobial resistance (Guerra *et al.*, 2002).

Integrins are genetic agents known to harbour and spread antibiotic resistance determinants in pathogenic bacteria (Rotimi *et al.*, 2008). Interestingly, in our set of non-typhoidal salmonellae, all isolates containing class 1 integrins were ESBL-producing isolates, and integrins have previously been associated with the carriage and spread of ESBL enzymes (Novais *et al.*, 2006).

The antimicrobial resistance ESBL array is a DNA microarray system that offers accurate and rapid detection of ESBL genes. The use of such DNA microarrays for rapid genotypic detection of β -lactamases could be an important tool for monitoring the spread of ESBL resistance genes within the hospital setting of the future (Grimm *et al.*, 2004).

Salmonella Agona carrying SHV-12 may play an important role in non-typhoidal *Salmonella* ESBL-mediated resistance in India. Further studies into the current extent of infection with this serovar, as well as its potential to spread into surrounding regions, should be investigated more closely. Continued surveillance of circulating ESBL-producing, as well as fluoroquinolone-resistant, salmonellae is warranted throughout India. Indiscriminate and extended use in animal and human practices has enhanced the emergence and spread of multiresistant bacteria. This resistance can be transferred between animals and humans. Therefore, the cautious use of antibiotics in both human and animals is warranted.

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