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Full Length Research Paper

Resistance to β-lactams of human and veterinary *Salmonella* isolates in Egypt and Algeria

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Seventy six non-typhoid *Salmonella* were isolated from both human and poultry in Egypt and Algeria and tested for their antibiotics resistance. The incidence of multiple antibiotics resistance was high. To study β-lactams resistance mechanisms, double disk synergy test (DDST) with and without cloxacilline was used, results revealed the production of extended spectrum β-lactamases (ESBLs) and cephalosporinase in seven and one human Egyptian isolates, respectively. The seven ESBL isolates were identified as *Salmonella enterica* serotype Poona and their molecular typing by ERIC-PCR revealed unrelated genetic patterns, indicating that these isolates are not clonal. The Cephalosporinase-ESBL- producing isolate was identified as *S. enterica* serotype Hadar. Polymerase chain reaction (PCR) with specific primers showed the presence of bla_{TEM} and bla_{SHV} genes, respectively, in all and four ESBL producers, and bla_{AmpC} gene was detected in cephalosporinase-producing isolate. Genetic transfer by conjugation and plasmid profiles analysis showed that these genes and their resistance markers were transferable in association with plasmids of 60 kb for ESBLs and 64 and 3.2 kb for AmpC cephalosporinase.

Key words: Non-typhoid *Salmonella*, extended spectrum β-lactamases, cephalosporinase, Egypt, Algeria.

INTRODUCTION

Salmonella are widely distributed in nature and the most common reservoir of these bacteria is the gut of vertebrates. They are the major cause of food-born bacterial diseases. They cause a wide range of clinical illness: enteric fever, gastroenteritis, and bacteraemia, particularly in infants and in immunocompromised patients (Fluit, 2005). The incidence of salmonellosis is rising in the most of countries, which become one of the public health problems. Along with this incidence, increasing rates of antibiotic resistance have been reported in various regions. Therefore the effectiveness of antimicrobial chemotherapy is being eroded, and multidrug resistance clones were disseminated worldwide; in 2004, *Salmonella* resistant to extended spectrum cephalosporines (ESCs) were identified in 43 countries (Edelstein et al., 2004; Arlet et al., 2006). β-lactams constitute the most important antibiotic family in therapeutic, because of their efficiency and relative low toxicity. ESCs are currently the agents of choice for such chemotherapy especially for infants and neonates, for whom the use of fluoroquinolones is not yet approved (Bouallègue et al., 2005; Wilke et al., 2005). The selective pressure created by the use of ESCs has been described as one of the most important factors in the emergence of plasmid mediated extended spectrum

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β-lactamases (ESBLs) and Amp-C type cephalosporinases (Winokour et al., 2001; Miriago et al., 2004). ESBLs are clavulanate-susceptible enzymes capable of hydrolyzing oxyimino-cephalosporins and monobactams but not cephamycins and carbapenems. They belong to the Ambler class A and functional group 2be of the Bush-Jacoby-Meideros classification (Ambler, 1980; Bush et al., 1995; Bush and Jacoby, 2010). ESBLs are the first cause of resistance of Enterobacteriaceae to extended spectrum β-lactams that hamper infections treatment. They have evolved mainly from the old β-lactamases TEM-1 or TEM-2, and SHV-1 by various amino acid substitutions around active site. Reports described the emergence of ESBLs classes, such as PER, VEB, GES,TLA-1, IBC and CTX-M. The CTX-M β-lactamases are rapidly increasing, they were recognized in outbreaks in many parts of the world (Bonnet, 2004; Pitout et al., 2005; Hawkey and Jones, 2009). AmpC-type cephalosporinases (chromosomal or plasmid encoded), representing class C β-lactamases, are clinically significant as these confer resistance to cephalosporins in the oxyimino group, 7α methoxy cephalosporins, and not inhibited by β-lactams inhibitors (Bush et al., 1995; Bush and Jacoby, 2010). Dissemination of specific clones or/and epidemic resistance plasmids in community and hospitals is the main cause of the widespread of ESBLs and cephalosporinases (Messai et al., 2008; Iabadene et al., 2009).

With the importance of the bacterium *Salmonella* as pathogen of human and animals, and very few resistance data published in Algeria and Egypt, the aim of this study is the determination of susceptibility to 26 antibiotics of clinical and veterinary importance of 76 *Salmonella* and screening for the ESBLs and AmpC type cephalosporinases.

MATERIALS AND METHODS

Bacterial isolates

A total of 76 non-typhoid *Salmonella* were isolated from human and veterinary pathological specimens. Human isolates were collected from microbiology laboratories of two university hospitals in Cairo-Egypt (Demerdash-Ain Shams university and Kasr-Alainy-Cairo university), and two university hospitals in Algiers-Algeria (Mustapha-Bacha and Hadi Flici– Algiers university). Avian isolates were collected from microbiology laboratories of veterinary college-Cairo and Veterinary school- Algiers. The isolates were identified by the API 20E system, (Biomerieux, Marcy l'Etoile, France), PCR using *inv*A primers: *inv*A/F (5'-GTGAAATTATCGCCACGTTCGGGCAA-3') and *inv*A/R (5'-TCATCGCACCGTCAAAGGAACC-3') (Rahn et al., 1992) and serotyping.

Antimicrobial susceptibility and synergy testing

Antibiotic susceptibility was done on Muller-Hinton agar plates with the disk diffusion method and interpreted according to CA-SFM guidelines (2009). Antibiotic disks were purchased from Bio-Rad. *Escherichia coli* ATCC 25922 was used as a control strain. Beta-lactamases production was revealed by iodometric method (Courvalin et al., 1985). Extended-spectrum beta-lactamases and cephalosporinases production was screened by the Double-Disk Synergy Test (DDST) with and without cloxacilline (250 mg/L) (Jarlier et al., 1988).

Plasmid analysis

Plasmid DNA was extracted by alcalin-lysis method as previously described (Kado and Liu, 1981), and analysed by electrophoresis on 0.7% agarose gel in presence of the following reference plasmids : from *E. coli* V517 harboring 8 plasmids: 54.4, 7.3, 5.6, 5.2, 4, 3, 2.7 and 2.1 kb; pBR322: 4.36 kb; RP4: 60 kb.

Characterization of ESBL- and cephalosporinase-encoding genes

DNA was obtained by heating a suspension of colonies in 50 µl of water to 95°C for 10 min. The DNA-containing supernatant was then used as a template in specific PCR for the detection of bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{GES-1}, bla_{PER-1} and bla_{AmpC}. PCR amplification was performed by using the following primers : TEM/F (5'-ATGAGTATTCAACATTTCCG-3') and TEM/R (5'-CCAATGCTTAATCAGTGAGG-3'); SHV/F: (5'-TTATCTCCCTGTTAGCCACC-3') and SHV/R: (5'-GAGCCCGTTTTATGCACCCA-3'); CTX-M/F (5'-GGTTAAAAAATCACTGCGTC-3') and CTX-M/R: (5'-TTGGTGACGATTTTAGCCGC-3'); GES-1/F: (5'-ATCGGCTTCATTCACGCAC-3') and GES-1/R: (5'-CTATTTGTCCGTGCTCAGG-3'); PER-1/F: (5'-AATTTGGGCTTAGGGCAGAA-3') and PER-1/R: (5'-ATGAATGTCATTATAAAAGC-3'); AmpC/F (5'-ATCAAAACTGGCAGCCG-3') and AmpC/R (5'-GAGCCCGTTTTATGCACCCA-3'). Cycling conditions were as following: initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 50°C (SHV), 53°C (TEM), 55°C (CTX-M and GES), 42°C (PER) and 65°C (AmpC) for 1 min, and elongation at 72°C for 1 min. The final elongation step was extended to 10 min at 72°C. The PCR products were separated on 1.5% agarose gels. Bands were visualized under ultraviolet light after being stained with ethidium bromide and photographed. Expected sizes of TEM, SHV and AmpC PCR products were, respectively, 310, 858 and 510 bp (Gebreyes and Altier, 2002; Yates et al., 2003; Kim et al., 2004; Poirel and Nordmann, 2005; Messai et al., 2006; Iabadene et al., 2008).

Conjugation experiments

Mating experiments were performed as previously described (Bakour et al., 1983) with *E. coli* BM21 (Nalidixic acid resistant) as a recipient. Selective agents were used at the following concentrations: 50 µg/ml for nalidixic acid and amoxicillin, and 2 µg/ml for cefotaxime. Transconjugants were subjected to antibiotics susceptibility, DDST and PCR.

Enterobacterial repetitive consensus PCR (ERIC-PCR)

The epidemiological relationships between ESBL-producing *S. enterica* serotype Poona isolates were analysed by ERIC-PCR using primer ERIC-2 (5'-AAGTAAGTGACTGGGGTGACGC-3') (Decré et al., 2004). Cycling conditions were as follows: 3 min at 95°C, 40 cycles of 30 s at 92°C, 1 min at 52°C and 8 min at 72°C, and final extension of 16 min at 72°C. Fingerprints were visually

Table 1. Percentage of resistance to antibiotics according to country and source of isolation.

Antibiotic discs \ Source area	Percentage of resistance (%)			
	Human		Poultry	
	Egypt, n=20	Algeria, n=20	Egypt, n=20	Algeria, n=16
AMX (25 µg)	70	40	0	31.25
AMC (20 +10 µg)	50	0	0	25
ATM (30 µg)	40	0	0	6.25
CRO (30 µg)	40	0	0	0
CAZ (30 µg)	40	0	0	0
FOX (30 µg)	50	0	0	18.75
CXM (30 µg)	40	0	0	12.5
CTX (30 µg)	40	0	0	0
TIC (75 µg)	70	40	0	12.5
TCC (75+10 µg)	55	10	0	0
MEC (10 µg)	70	5	15	6.25
IMP (10 µg)	0	0	0	0
MZ (75 µg)	60	20	0	6.25
CF (30 µg)	60	5	0	25
TZP (75+10 µg)	0	0	0	0
PIP (75 µg)	65	10	0	12.5
TE (30 IU)	85	85	85	100
AN (30 µg)	50	0	0	6.25
S (10)	70	35	55	68.75
GM (15 µg)	70	0	0	0
K (30 µg)	55	5	50	0
CIP (5 µg)	0	0	0	0
NA (30 µg)	35	35	55	18.75
NOR (5 µg)	0	0	10	0
C (30 µg)	20	15	25	0
SXT (1.25+23.75 µg)	5	5	30	18.75

AMX : Amoxicillin, AMC : Amoxicillin/clavulanic acid, ATM : Aztreonam, CRO : Ceftriaxone, CAZ : Ceftazidime, FOX : Cefoxitin, CXM : Cefuroxime, CTX : Cefotaxime, TCC : Ticarcillin/ clavulanic acid, TIC : Ticarcillin, MEC : Mecillinam, IMP : Imipinem, MZ Mezlocilline, CF : Cefalotin, PIP : Piperacillin,TZP : Piperacillin/tazobactam, , TE : Tetracycline, AN : Amikacin, S : Streptomycin, GM : Gentamicin, K : Kanamycin, CIP : Ciprofloxacin, NA : Nalidixic acid, NOR: Norfloxacin, C : Chloramphenicol, SXT : Trimethoprime-sulphamethaxazole.

compared and the patterns differing by at least one amplification band were classified different.

RESULTS

Results showed that isolates were globally multidrug-resistant (MDR); however, the resistance gradually declined or no resistance was observed for third generation cephalosporins, imipinem, piperacillin/ tazobactam, fluoroquinolones, Trimethoprime-sulphamethoxazole, and chloramphenicol (Table 1). Notable findings are the resistance of eight (40%) human Egyptian isolates to all tested third generation cephalosporins (3GCs) and to non-beta-lactams antibiotics as aminoglycosides, chloramphenicol, tetracycline, trimethoprime-sulphamethoxazole and nalidixic acid. The multiple antibiotic resistance patterns includes till fifteen antibiotics (Table 2).

The beta-lactamases production have concerned 70, 40, 31, 5 and 0% of human Egyptian, human Algerian, poultry Algerian and poultry Egyptian isolates, respectively. A synergy between clavulanic acid and third and fourth generation cephalosporins characterizes seven 3GCs resistant human Egyptian isolates, this augurs the ESBL production, and all of these isolates were identified as *S. enterica* serotype Poona. The molecular typing revealed that these isolates have different ERIC-PCR patterns, indicating clearly heterogeneity in genetic profiles. For the remaining 3GCs resistant human Egyptian isolate, the supplementation with cloxacillin restored the activity of cefotaxime and

Table 2. Resistance phenotypes, plasmids and β-lactamases of extended-spectrum cephalosporins resistant isolates

Isolates	Resistance pattern	Plasmids content (kb)	β-lactamases	Transfer (transconjugants)		
				β-lactamase	Transferred resistance	Plasmids
S. poona S3	AMC CRO CAZ FOX CXM CTX AMX TIC AMP MZ TE AN S GN K	60 - 3.4 - 2.5	TEM, SHV	TEM, SHV	AMC CRO CAZ CXM CTX AMX TIC AMP MZ AN S GN K	60
S. Hadar S4	AMC CRO CAZ FOX CXM CTX AMX TIC AMP MZ TE AN S GN K NA	64 - 8 - 4.4 - 3.2	AmpC, TEM,	AmpC, TEM,	AMC CRO CAZ FOX CXM CTX AMX TIC AMP MZ (I) S	64 - 3.2
S. Poona S6	AMC CRO CAZ FOX CXM CTX AMX TIC AMP MZ TE AN S GN K	60 - 3.4 - 2.5	TEM	TEM	AMC CRO CXM CTX AMX TIC AMP MZ AN K	60
S. poona S9	AMC CRO CAZ FOX CXM CTX AMX TIC AMP MZ TE AN S GN K	60 - 3.4 - 2.5	TEM	TEM	AMC CRO CAZ CXM CTX AMX TIC AMP MZ AN K	60
S. poona S11	AMC CRO CAZ FOX CXM CTX AMX TIC AMP MZ TE AN S GN K NA	60 - 3.4 - 2.5	TEM, SHV	TEM, SHV	AMC (I) CRO CAZ CXM CTX (I) AMX TIC AMP MZ AN K	60
S. poona S12	AMC CRO CAZ FOX CXM CTX AMX TIC AMP MZ TE AN S GN K C	60 - 3.4 - 2.5	TEM	TEM	AMC (I) CRO CAZ CXM CTX AMX TIC AMP MZ AN K	60
S. poona S13	AMC CRO CAZ FOX CXM CTX AMX TIC AMP MZ TE AN S GN K	60 - 5.2 - 3.4 - 2.5	TEM, SHV	TEM, SHV	AMC (I) CRO CAZ CXM CTX AMX TIC AMP MZ AN K	60
S. *poona* S15	AMC CRO CAZ FOX CXM CTX AMX TIC AMP MZ TE AN S GN K	60 - 3.4 - 2.5	TEM, SHV	TEM, SHV	AMC (I) CRO CAZ CXM CTX AMX TIC AMP MZ AN K	60

AMC : Amoxicillin-Clavulanic Acid, CRO: Ceftriaxone, CAZ : Ceftazidime, FOX : Cefoxitin, CXM : Cefuroxime, CTX : Cefotaxime, AMX : Amoxicillin, TIC : Ticarcillin, MZ : Mezlocilline, TE : Tetracycline, AN : Amikacin, S : Streptomycin, GN : Gentamicin, K : Kanamycine, C : Chloramphenicol, AMP : Ampicillin, NA : Nalidixic acid, (I) : Intermediate.

increased that of cefepim; this is indicative for cephalosporinase production by this isolate, which was identified as *S. enterica* serotype Hadar.

PCR amplification performed on the 7 ESBL and one cephalosporinase producing Egyptian human isolates revealed the presence of bla_{TEM} and bla_{SHV} in four isolates bla_{TEM} in 3 isolates and bla_{TEM} and bla_{AmpC} in one isolate. The plasmid profile analysis of selected MDR isolates from human and poultry in both countries, revealed the presence of more than two plasmids in most isolates. The number and size of plasmids in MDR human Egyptian isolates were higher than those in other isolates. Six of ESBL Egyptian human isolates have the same plasmid profile (3 plasmids) (Table 2). Mating assays carried out on ESBL and cephalosporinase isolates allowed the transfer of ESBL and cephalosporinase phenotypes to recipient *E. coli* BM21 in association with plasmid of 60, 64 and 3.2 kb respectively (Figure 1A and B).

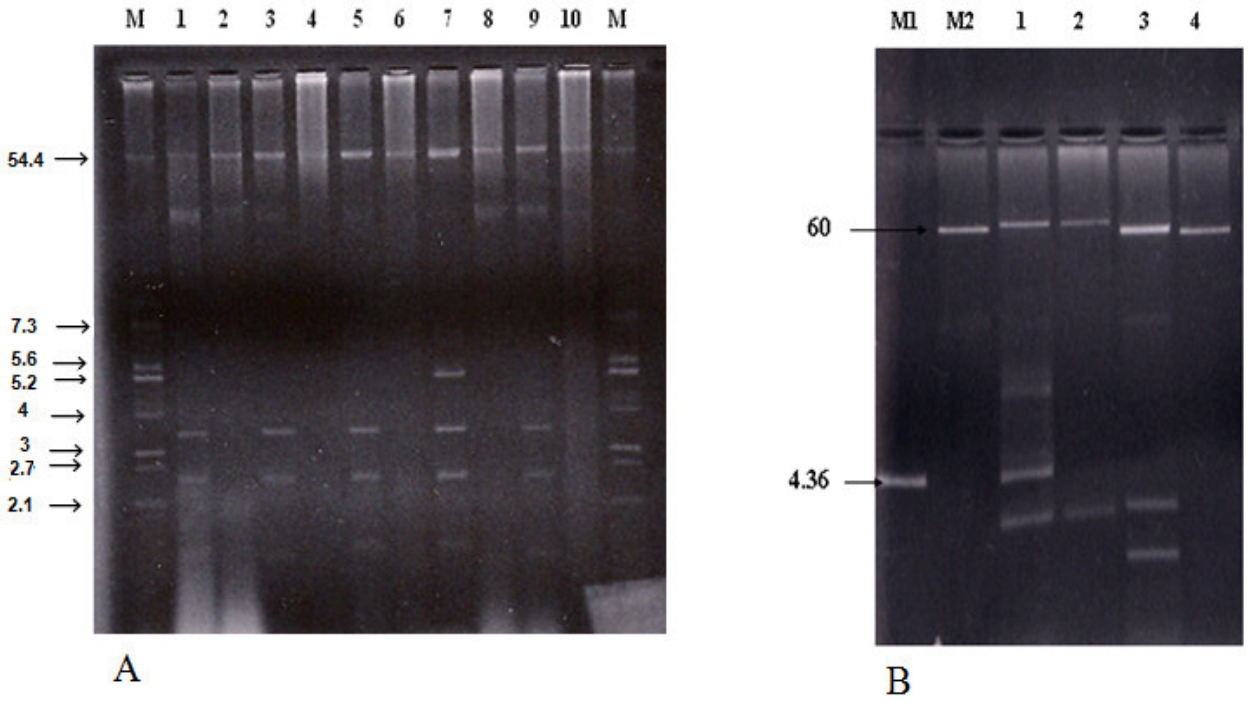

Figure 1. Agarose gel electrophoresis of the plasmidic extract of ESBL and cephalosporinase *Salmonella* from human in Egypt and their transconjugants. (A) Lane 1-2: *Salmonella poona* S9 and its transconjugant; Lane 3-4: *Salmonella poona* S11 and its transconjugant; Lane 5-6: *Salmonella poona* S12 and its transconjugant; Lane 7-8: *Salmonella poona* S13 and its transconjugant; Lane 9-10: *Salmonella poona* S15 and its transconjugant. M: Standard plasmids from *E. coli* V517 harboring 8 plasmids: 54.4, 7.3, 5.6, 5.2, 4, 3, 2.7 and 2.1 kb. (B) Lane 1-2 : *Salmonella* Hadar S4 and its transconjugant; Lane 3-4 *Salmonella poona* S6 and its transconjugant. M1: RP4 (60kb), M2 : pBR322 (4.36 kb).

DISCUSSION

The high rate of resistance to tetracycline, streptomycin and nalidixic acid is mainly due to the early introduction of these antibiotics in veterinary and human medicine. The high rate of resistance to all tested ESCs of human Egyptian isolates constitutes a serious public health problem, especially for neonates and childrens under 7 years, since ESCs are the antibiotics of choice for invasive *Salmonella* infections in children (Hohmann, 2001; Bouallègue et al., 2005). The emergence of ESCs-resistant *Salmonella* in Egypt has been reported many times (Bouchillon et al., 2004; AbdelGhani et al., 2010). The sensitivity of human Algerian isolates to 3GCs is probably due to the fact that these antibiotics are not available in the community and not used extensively in hospital practice. The most active drugs against our isolates were imipinem, piperacillin/tazobactam (TZP) and fluoroquinolones. The resistance phenotypes of non-typhoid *Salmonella* noted in this study may be considered alarming, because this bacterium was reported as sensitive. This result is consistent with the emergence of multidrug-resistance and ESBL production within non-typhoid *Salmonella* (Miriago et al., 2002, 2004; Chen et al., 2010). This high frequency of resistance might be due to the easily acquisition of resistance, or to the exposition of natural reservoirs (human and animal gastrointestinal tracts) to large amounts of antibiotics. The second hypothesis is more probable for this genus when we see that most multi-drug resistant isolates in our study were recovered from neonates, and the evolution of resistance in *Salmonella* was initially more moderate compared to those of other species of Enterobacteriaceae such as *E. coli* and *Klebsiella (*Bradford, 2001a; Yates and Amyes, 2005).

It has been reported worldwide that most non-typhoid *Salmonella* that resist to ESCs had been resulted from human (Gaillot et al., 1997; Banajah et al., 2001; Chande et al., 2002; Miriago et al., 2004; Chen et al., 2010). However, in certain countries, such as USA and Canada, resistance to ESCs is derived from both human and animal (Winokour et al., 2001).

Despite their genetic differences, six ESBL isolates have the same plasmid profile, this suggest that they emerged under the same selective pressure which promotes genetic exchanges between bacteria in hospital environments. The exchange of resistance plasmids between members of Enterobacteriaceae will severely limit the treatment options of infections caused by these microorganisms, which are responsible for nearly half of all infections (Fluit, 2005). Many ESBLs-mediated plasmids also contain virulence genes or regulate their

expression; this confers survival advantage in an unfavorable drug environment and constitutes a new tool in the bacterial evolution (Guerra et al., 2002; Martinez and Baquero, 2002; Chu and Chiu, 2006).

Amplification by PCR showed that ESBLs were bla_{TEM} or bla_{SHV} types, these enzymes have been recorded widely in Enterobacteriaceae worldwide, often in association with resistance to other antimicrobial classes like aminoglycosides. ESBLs TEM and SHV types have been described in *Salmonella* in Egypt and worldwide, but currently trend is the emergence of ESBL CTX-M type in this genus.

(Livermore, 2004 ; Veldman et al., 2009; AbdelGhani et al., 2010). In *S. enterica* serotype Hadar, bla_{TEM} was found with bla_{AmpC}, this come in agreement with results obtained by disk diffusion at the presence of cloxacillin. In recent years, there have been increasing reports of *Salmonella* isolates that produce either an ESBL or a plasmid-mediated AmpC β-lactamase (Dunne et al., 2000; Bradford, 2001b; Menezes et al., 2010), while the coexistence of cephalosporinase and ESBL mechanisms in the same *Salmonella* strain has been rarely documented (Hanson et al., 2002); the acquisition of both an ESBL and an AmpC β-lactamase in our isolate is a significant concern. Some ESBLs and cephalosporinases producers clones have spread, causing major outbreaks, and a few have disseminated across regions or countries (Livermore, 2004; Bouallègue et al., 2005; Rankin et al., 2005).

Mating assays conducted on ESBL Egyptian human isolates allowed the transfer of ESBL phenotype to recipient *E. coli* BM21 in association with bla_{TEM} and/or bla_{SHV} genes and plasmids of 60 kb. Transfer of cephalosporinase phenotype was observed in only one isolate in association with bla_{TEM} and bla_{AmpC} genes, and with plasmids of 64 and 3.2 kb. The first plasmidic AmpC in non-typhoid *Salmonella* was discovered in Saudi Arabia in *S. enteritidis* on mega transferable plasmid (Gaillot et al., 1997) and since it has reported in many other studies (Villa et al., 2002; Kim et al., 2004). Resistance to ESCs was mediated by plasmids as proved by gene transfer. It was reported that plasmid mediated mechanisms have led to resistance to almost every class of clinically important antibiotics (Philippon et al., 2002; Li et al., 2007). bla_{TEM} are so far the most widespread plasmid borne β-lactmases genes as demonstrated in human ESBL-producing *Salmonella* in Egypt. Transfer of resistance to cefoxitin was observed in only one isolate. Resistance to cefoxitin in isolates that do not produce AmpC cephalosporinases may be due to non-enzymatic mechanisms. In Gram negative bacteria, outer membrane permeability barrier and multidrug efflux pumps play synergistically an important role in intrinsic resistance of these bacteria (Li and Nikaido, 2004). We have described ESBL and AmpC cephalosporinase in human isolates of non-typhoid *Salmonella* from Egypt. Their presence may be significant factor of therapeutic failures; therefore, a careful monitoring of their evolution is recommended.

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