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Use of Different Antibiotic Combinations against Fluoroquinolone-Resistant *Salmonella enterica* from Humans in Egypt

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EMERGENCE of antibiotic resistance by pathogenic *Salmonella* spp. is a worldwide problem. Antibiotic combination was used as a clinical cure to solve the problem. Over one and a half years , one hundred gastroenteritis bacterial pathogens were collected from three Egyptian hospitals from fecal and blood sources. 58% *Salmonella* isolates were purified and identified using phenotyping and serotyping techniques. Out of the 58% *Salmonella* spp., 41.4% represented *S.* Typhimurium, 27.6% *S.* Enteritidis, 5.2% *S*. Typhi, 3.4 % *S*. Paratyphi A, 3.4 % *S*. Paratyphi B and 19.0% other *Salmonella*. A total of 36 (62%) out of 58 *Salmonella* spp. were fluroquinolone resistant by disk diffusion method. Resistance of five *Salmonella* strains to the fluroquinolone group was confirmed by MICs values. The level of these values was from 32 - >512 µg/ml, which is higher than those recommended by CLSI. Seventy-five combined microtitre checkerboards were performed on the five fluroquinolone multi-resistant *Salmonella* strains to assess the potential for combination therapy. No antagonism was observed with any combination. Synergy and additivity were achieved with 41.4% and 58.6%, respectively. Time-kill synergy was more often seen at 24hr. There is 100%, 50% and 40% agreement between time-kill and checkerboard results for three *Salmonella* strains. Resistant *Salmonella* has increased in Egypt. Fluroquinolone combination with β-lactams (gentamycin, amikacin) and aminoglycosides (cefotaxime) were effective in the treatment of resistant *Salmonella* Typhimurium, Enteritidis and Typhi.

Keywords: *Salmonella enterica*, Antibiotic combination, Antibiotic resistance, Fluoroquinolone.

Salmonella spp. are important clinical pathogens, causing *Salmonella* infections among humans and animals. Infections due to *Salmonella enterica* include enteric or typhoid fevers, caused by *S. enterica* serovar Typhi and *S. enterica* serovar Paratyphi, and salmonellosis caused by a large number of non-typhodial *Salmonella* (NTS) such as *Salmonella* Typhimurium, and *Salmonella enterica* serovar Enteritidis. *S. enterica* serovar Typhi and *S. enterica* serovar Paratyphi are human-restricted in their epidemiology and highly adapted in their

pathogenesis (Gordon, 2008). Animals are the main reservoirs for NTS. The transmission of this microorganism occurs by the consumption of inadequately cooked or pasteurized foods of animal origin, including poultry, beef, fish, eggs, and dairy products. The incidence of human salmonellosis varies with geographic, socioeconomic and environmental factors (AL-Dawodi *et al*., 2012).

Salmonellosis and typhoid fever remain important public health problems worldwide (Ayana & Surekha, 2008). Although antibiotics are not usually recommended for *Salmonella* gastroenteritis, they are recommended for invasive *Salmonella* infections, such as septicemia and meningitis that are common in infants, elderly and immunocompromised patients. Up to a decade ago, in many countries, conventional 1st-line antimicrobial agents, such as ampicillin, chloramphenicol, and sulfamethoxazole-trimethoprim, were the drugs of choice for the treatment of life-threatening *Salmonella* infections and still remain the main therapeutic drugs of choice in most African countries with poor resources. However, in the past two decades, isolation of multidrug-resistant *Salmonella* spp. has been reported from many parts of the world (Wedel *et al*., 2005). So, fluoroquinolones have become the 1st-line drugs for the treatment of life-threatening salmonellosis and typhoid fever, but with treatment failures due to multidrug-resistant strains (Hakanen *et al*., 2001; Hirose *et al*., 2002; Baucheron *et al*., 2004 and Lunguya *et al*., 2013). Treatment of the latter with combination therapy, using two or more antibacterial agents, has become commonplace (Rybak & McGrath, 1996).

Two of the most widely used *in vitro* methodologies to assess drug–drug interactions are the checkerboard minimum inhibitory concentration (MIC) technique, yielding the fractional inhibitory concentration index (FICI) and time-kill kinetics (Rybak & McGrath, 1996 and White *et al*., 1996). The checkerboard MIC method is prone to error and by necessity, its results are often confirmed with the more dynamic interaction provided by the time-kill kinetic study format (Cappelletty & Rybak, 1996 and Jacqueline *et al*., 2005). The present study evaluates the effect of two combinations; fluoroquinolone with β-lactams and fluoroquinolone with aminoglycosides, against antibiotic resistant typhodial and non-typhodial clinical *Salmonella* isolates in Egypt.

Materials and Methods

Clinical sitting

Samples for the present study were obtained from July 2008 through December 2009 from microbiological laboratories of three government hospitals in Cairo. A total of one-hundred bacterial isolates; sixty from Abo El-Reesh Hospital for children, twenty-five from El-Hommiate Hospital and fifteen from El-Demerdash Hospital, were used in this study. Clinical isolates were collected from fecal and blood samples of patients with proven acute gastroenteritis, enteric fever, septicemia and bacteremia.

Purification and Identification of Salmonella isolates

The morphology of colonies was observed by optical microscope, a single colony of expected bacteria (according to cell morphology) was picked and inoculated to the fresh agar plates. The purified colonies were obtained by repeated streaking of the single colony on fresh agar plates and their morphology was recorded as the basis for classification.

Pure bacterial isolates were identified using microbiological standard methods; selective and differential media (Brenner & Farmer, 2004 and Thompson & Miller, 2003). Confirmation of putative *Salmonella* isolates was performed by biochemical reaction and serological method using *Salmonella* O antisera polyvalent slide agglutination tests (Demka, Sejken, Co., LTD, Tokyo, Japan) (Table 1). The pure isolates were also further grouped into serogroup A, serogroup B or serogroup D *Salmonella* by the use of slide agglutination tests for antiserum factors (O) then tested to flagella (H) antiserum phase I & II according to Kauffman-White serotyping scheme (Popoff & LeMinor, 1997 and Popoff, 2001).

TABLE 1. Method of *Salmonella* identification.

Method	**Control**	***Salmonella* reaction**		
1. Selective media				
MacConky agar	Reddish orange	Coloreless		
Salmonella /Shigella agar	Red-orange	Transparent with black center		
Brilliant green agar	Orange-brown	Red		
Bismuth sulfite agar	Light grey-green to medium green Opaque	black w/metallic sheen (*S.* Typhi)	black or greenish-grey, may have sheen (*S.* Typhimurium)	
Xylose Lysine Deoxycholate agar	Red	Red with black center		
2. Biochemical reaction				
Motility Indole Ornithine	Purple, semi-solid.	**Motility**	**Indol**	**Orithinine decarboxylation**
		+ve	-ve	+ve
Lysine iron agar	Purple	Lysine decarboxylation BUTT	Lysine deamination Slant	H_2S Apex of slant
		+ve Purple	-ve Purple	+ve black
Simmons Citrate agar	Forest green	Blue		
Methyl Red-Voges-Proskauer	Light amber, clear	+ve Bright red color (MR)		
		-ve reaction (VP)		
Triple Sugar Iron agar	Red	**Slant/Butt**	**Gas**	**H_2S(black)**
		Red/Yellow	+ ve for *S.* Entritidies	+ve
		Red/Yellow	- ve for *S.*Typhimurium	+ve
Urease	Yellow	Yellow (-ve reaction)		
3 . Serology (agglutination test)				
Somatic (O antisera)		Polyvalent O I,II,III Serogroup A,B,C and factors		
Flagella (H antisera)		Phase I &II		
Virulance (Vi antisera)				

Antimicrobial susceptibility testing

Fifty-eight *Salmonella* spp. were obtained from 100 isolates and were screened for resistance to antimicrobial agents by disc diffusion method on Mueller-Hinton agar using commercial antibiotic disks including the aminoglycosides group [gentamycin (GN) (10 µg) and amikacin (AK) (30 µg)], β-lactams group [ampicillin (AMP) (10 µg), ampicillin/sulbctam (SAM) (20 µg), amoxicillin/clavulinic acid (AMC) (30 µg), piperacillin (PRL) (100 µg), piperacillin/tazbactam (TZP) (110 µg), cefotaxime (CTX) (30 µg), ceftriaxone (CRO) (30 µg), ceftazidime (CAZ) (30 µg), cefepime (FEP) (30 µg), impenem (IMP) (10 µg), aztroname (ATM) (30 µg)], phenicols [chloramphenicol (C) (30 µg)], sulfadrugs group [sulfamethoxazole-trimethoprim (SXT) (25 µg)] and fluoroquinolones group [nalidixic acid (NA) (30 µg), ciprofloxacin(CIP) (5 µg), levofloxacin (LEV) (5 µg), ofloxacin (OFX) (5 µg) & norfloxacin (NOR) (30 µg)] (Oxoid, UK). The break points used were according to the interpretative criteria recommended by Clinical and Laboratory Standards Institute (CLSI, 2008).

Determination of minimum inhibitory concentrations (MICs)

Standard powder forms of ciprofloxacin, levofloxacin, ofloxacin (Eipico), cefotaxime (Aventis Pharma), ceftazidime (GlaxoSmithKline), cefepime (Bristol-Myers Squibb), amikacin (Bristol-Myers Squibb) and gentamycin (Schering-Plough) were used. The MICs of these antibiotics were determined by the broth microdilution method according to CLSI (2008) at concentration range 0.1-512 µg/ml for each drug immediately prior to testing. Mueller-Hinton broth (LAB M, USA) was used as the culture medium. *Escherichia coli* ATCC 25922 and *Salmonella* Typhimurium ATCC 14028 were used as control strains for evaluation of antibiotic potency.

Evaluation of antibiotics synergy

Two different methods were compared for the determination of synergy: checkerboard and time–kill method.

Checkerboard method

The dynamic checkerboard method was performed to evaluate the interaction of fluoroquinolones in combination with β-lactams and aminoglycosides, each at a time, against *Salmonella* isolates. The concentration range of each antibiotic combination ranged from 1/4 to 1/5 concentrations of MIC up to 2X MIC dilution. Organisms and antibiotic concentrations were prepared as described for the MIC determination using microtitre plates. To evaluate the effect of the combinations, the fractional inhibitory concentration (FIC) index was calculated for each antibiotic in each combination using the following formula:

$$\text{FIC index} = FIC_A + FIC_B$$

where: FIC_A = MIC of drug A in combination/MIC of drug A alone

FIC_B = MIC of drug B in combination/MIC of drug B alone

Synergy was defined as an FIC index of $\leq$ 0.5. Additive or indifferent was defined as an FIC index of $>$ 0.5 but of $\leq$ 4.0. Antagonism was defined as an FIC index of $>$ 4.0 (White *et al.*, 1996 and Satish *et al.*, 2005).

Time-kill curve

Drug concentrations used for time-kill assays were based on three criteria: (i) Concentrations likely to produce synergy as seen in checkerboard testing; (ii) Concentrations those were not more than twice the MIC of each drug; (iii) Concentrations that were within clinically achievable serum levels for each drug Time-kill assays were performed in 10ml Mueller-Hinton broth (LAB M, USA). Each assay included a growth control tube with no antibiotic. The inoculum and antibiotic dilution were prepared as in MIC determination. For determination of viable counts, the surviving bacteria were counted after 0, 3, 6 and 24 hr post-inoculation at 37°C by sub-culturing 100 µl after serially dilution in saline solution (0.9%) onto Mueller-Hinton agar plates in duplicate. Total bacterial count (log_{10} cfu/ml) was determined after 24 h of incubation at 37°C. A bactericidal effect was defined as $\geq$ 3 log_{10} cfu/ml decrease after 24 h of incubation compared to the size of the initial inoculum. Synergy was defined as a decrease in colony count of ≥ 2 log_{10} cfu/ml with the combination compared to the count obtained with the most active single drug. Antagonism was defined as an increase in colony count of ≥ 2 log_{10} cfu/ml with the combination compared to the count obtained with the most active single drug. Additive was defined as a change in colony count of < 10 fold decrease in viable count at 24 h with the combination compared to the count obtained with the most active single drug (Eliopoulos & Moellering, 1991; White *et al*., 1996 and Satish *et al*., 2005).

Results

Distribution of Salmonella isolates

During this study a total of 100 bacterial samples from enteric infections, during 18 months, were purified and identified. Out of the 100 isolates, 58 % proved to be *Salmonella* by microscopy, selective and differential media and biochemical examination. Distribution of the 58 *Salmonella* isolates, were: 40 from Abo-El-Reesh Children Hospital, 15 from El-Hommiate Hospital and 3 from El-Demerdash Hospital. Out of the 58 *Salmonella* spp., 64% were from males and 36% were from females, so incidence of infection was higher in male than in female, also the incidence of *Salmonella* infection was higher in children (69%) than in adults (31%) and stool was a higher source of infection (81.1%) than blood (18.9%).

Identification of pure Salmonella isolates

Out of the 58 *Salmonella* isolates, 24 (41%) were *Salmonella enterica* serovar Typhimurium, 16 (28 %) were *Salmonella enterica* serovar Enteritidis, 3 (5 %) were *Salmonella enterica* serovar Typhi, 2 (3 %) were *Salmonella enterica* serovar Paratyphi A, 2 (3 %) were *Salmonella enterica* serovar Paratyphi B and 11 (19%) were other serovar. The most common serotypes among *Salmonella* strains were the two non-typhodial, *S.* Typhimurium followed by *S.* Enteritidis.

Antimicrobial susceptibility testing

The antibiotic resistance incidence rate in *Salmonella* Typhimurium was higher than in *S.* Enteritidis followed by *S.* Typhi and *S.* Paratyphi A & B.

Out of the 58 *Salmonella* isolates, five multi-resistant *Salmonella* isolates were selected (Fig.1), these strains showed resistance to most tested antibiotics especially the fluoroquinolone group where *Salmonella enterica* serovar Typhimurium (*S.* Tm) (no. 7) was resistant to all tested 20 antibiotics; *S. enterica* serovar Enteritidis (*S.* En) (no. 22), resistant to 19 antibiotics (except SXT); *S. enterica* serovar Typhimurium (*S.* Tm) (no. 54), resistant to 17 antibiotics (except PRL, TZP & IMP from the β-lactams group) and *S. enterica* serovar Typhi (*S.* Ty) (no. 49), resistant to 17 antibiotics (except CTX,CAZ & IMP from the β-lactams group); while *S. enterica* serovar Typhimurium (*S.* Tm) (no. 57) was resistant to 14 antibiotics (except AK from the aminoglycosides group, PRL, TZP, CRO, CAZ from the β-lactams group & NOR from the fluoroquinolones group).

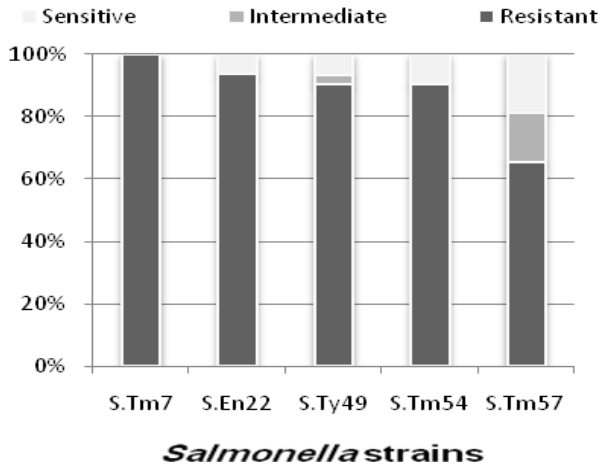

Fig. 1. Antibiotic resistance of five multi-resistant *Salmonella* strains against 20 different antibiotics by disk diffusion method.

As presented in Table 2, the MIC for the five multi-resistant *Salmonella* isolates was tested and the data showed that *S.* Typhimurium (54) was the most resistan, t with MICs ranging from 128-512 µg/ml; while *S.* Typhi (49) had the lowest MIC ranging from 16-128 µg/ml for tested antibiotics. The MIC of ciprofloxacin for *S.* Typhimurium (57 and 54) was 512 µg/ml followed by *S.*Enteritidis (22) with 256µg/ml then *S.* Typhimurium (7) with 64 µg/ml while *S.* Typhi (49) had the lowest MIC of 16 µg/ml.

TABLE 2. MIC of five multi-resistant *Salmonella* strains using microdilution method.

***Salmonella* sp.** / **Antibiotics**		**MIC[a] (µg/ml)**				
		***S.* Tm 7[b]**	***S.* En 22[c]**	***S.* Ty 49[d]**	***S.* Tm 54[b]**	***S.* Tm 57[b]**
Fluroquinolone	Ciprofloxacin	64	256	16	>512	512
	Levofloxacin	64	128	32	>512	64
	Ofloxacin	128	128	32	>512	128
β-lactam	Cefotaxime	512	512	128	>512	>512
	Ceftazidime	>512	>512	64	>512	>512
	Cefipime	>512	>512	64	>512	512
Aminoglycoside	Amikacin	128	64	16	128	32
	Gentamycin	512	>512	32	256	16

[a] MIC, minimum inhibitory concentration.
[b] *Salmonella enterica* serovar Typhimurium.
[c] *Salmonella enterica* serovar Enteritidis.
[d] *Salmonella enterica* serovar Typhi

For the β-lactams group, the MICs were similarly high for all strains except *S.* Typhi (49) which had lower MICs than others. For the Aminoglycosides group, gentamycin had higher MICs than amikacin, except for *S.* Tm (57).

Combination of antibiotics

Table 3 summarizes the results of the checkerboards. of the 75 checkerboards, 41.3% were found to be synergistic according to FIC index and 58.6% additive for the test strains. There was no antagonism reported for any combination (Fig. 2).

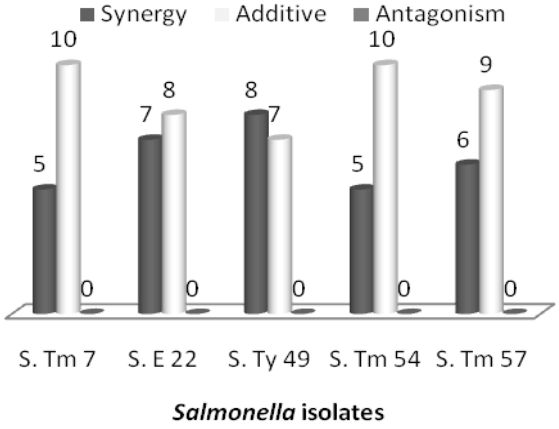

Fig. 2. Checkerboard activity of five multidrug resistant *Salmonella* strains according to fractional inhibitory concentration (FIC) index.

TABLE 3. Checkerboard of five multi-resistant *Salmonella* strains.

Antibiotic Combination	S. Tm 7			S. En 22			S. Ty 49			S. Tm 54			S. Tm 57		
	Conc.	FIC	Activity	Conc.	FIC	Activity	Conc.	FIC	Activity	Conc.	FIC	Activity	Conc.	FIC	Activity
CIP/AK	64/64	1.50	A	32/16	0.75	A	4/4	0.50	S	256/64	1.50	A	64/16	0.5	S
CIP/GN	64/256	1.50	A	16/128	0.50	S	4/16	0.50	S	256/256	2.50	A	256/16	1.5	A
CIP/CTX	16/128	0.25	S	8/128	0.37	S	4/32	0.50	S	256/64	1.00	A	128/64	0.37	S
CIP/CAZ	128/256	2.50	A	32/128	1.50	A	4/8	0.37	S	256/512	1.50	A	128/64	0.37	S
CIP/FEP	128/256	2.50	A	8/128	0.37	S	4/8	0.37	S	256/256	2.50	A	64/256	0.62	A
OFX/AK	16/32	0.25	S	64/32	1.50	A	8/4	0.50	S	128/32	0.50	S	64/32	1.5	A
OFX/GN	64/256	1.00	A	64/128	1.50	A	8/16	0.75	A	128/64	0.50	S	32/4	0.5	S
OFX/CTX	32/128	0.25	S	128/128	1.25	A	16/64	1.00	A	256/512	1.50	A	64/128	0.75	A
OFX/CAZ	64/256	1.00	A	64/64	1.00	A	16/16	0.75	A	256/256	1.00	A	32/256	0.75	A
OFX/FEP	32/256	0.75	A	64/128	0.75	A	16/16	0.75	A	256/256	1.50	A	32/16	1.0	A
LEV/AK	32/32	0.75	A	32/16	0.50	S	16/8	1.00	A	32/64	0.50	S	8/16	1.0	A
LEV/GN	16/128	0.50	S	16/64	0.25	S	8/16	0.75	A	64/64	0.25	S	8/128	0.92	A
LEV/CTX	16/128	0.50	S	16/128	0.50	S	16/16	0.37	S	64/128	0.37	S	64/256	0.37	S
LEV/CAZ	64/512	2.00	A	16/128	0.37	S	16/8	0.37	S	128/256	0.75	A	32/128	0.75	A
LEV/FEP	32/256	1.00	A	64/128	0.75	A	8/8	0.75	A	128/256	0.75	A	8/128	0.37	S

Checkerboard combination using microtitre method at 24 h incubation time. Conc, concentration. FIC, fractional inhibitory concentration. S, Synergy. A, Additive Aminoglycosides group [gentamycin (GN) and amikacin (AK)]

Six of 18 checkerboards were found synergic by $\geq$ 2 $\log_{10}$ decrease in killing curve as compared to the most active drug in combination at 24 h. One of the boards was defined as synergy by these criteria at 3 h, also one combination at 6h. One of the boards at 3 and 6h was defined as synergy and also one at 3 and 24 h and 6 and 24 h were defined as synergy by these criteria against three resistant *Salmonella* isolates. Out of 18 checkerboards, twelve recorded synergic action as compared with the most active drug. Killing-curve for *Salmonella*

isolates (no.7, 49 & 54) showed 100% (5/5), 50% (4/8) and 40% (2/5) agreement between the checkerboard and time-kill, respectively.

Time-kill results showed that ciprofloxacin combined with gentamycin for *S.*Typhi (49) and with cefotaxime for *S.* Typhimurium (7) achieved synergy action as represented in Fig. 5c and 3a .

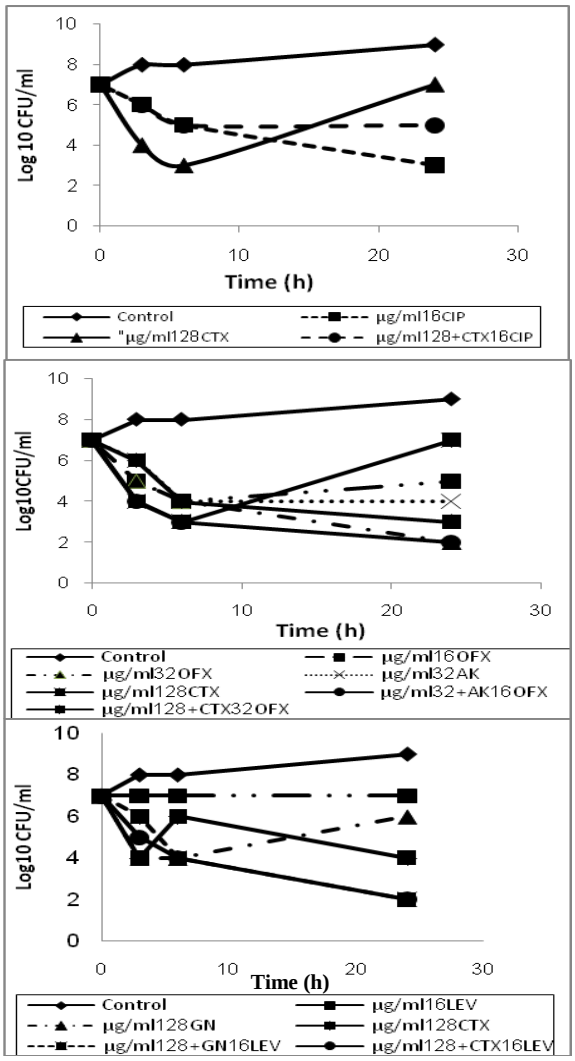

Fig. 3. Killing assay of *Salmonella* Typhimurium (*S*. Tm no. 7) at 0, 3, 6, 24 h incubation time. a) Time kill assay of ciprofloxacin at 16µg/ml + cefotaxime at 128µg/ml. b) Time kill assay of ofloxacin at 16µg/ml + amikacin 32µg/ml and ofloxacin at 32µg/ml +cefotaxime at 128µg/ml. c) Time kill assay of levofloxacin at 16µg/ml + gentamycin at 128µg/ml and levofloxacin 16µg/ml + cefotaxime 128µg/ml.

Salmonella Typhimurium (7) killing-curve combination of ofloxacin with amikacin and cefotaxime also levofloxacin with gentamycin and cefotaxime showed synergistic action (Fig. 3b & 3c). Killing-curve for *S.* Typhimurium (54) (Fig. 4a,b,c) represented antagonism when levofloxacin was combined with amikacin, gentamycin, and cefotaxime while when ofloxacin was combined with amikacin and gentamycin they had synergy action with 40% agreement with checkerboard .

Figure 5a to 5e show that synergistic action was achieved in combination of ciprofloxacin with amikacin and gentamycin, ofloxacin with amikacin and levofloxacin with cefotaxime for killing curve of *S.* Ty (49), while three time-kill showed additive reaction for ciprofloxacin with cefotaxime and ceftazidime and ciprofloxacin combined with cefepime had antagonism action.

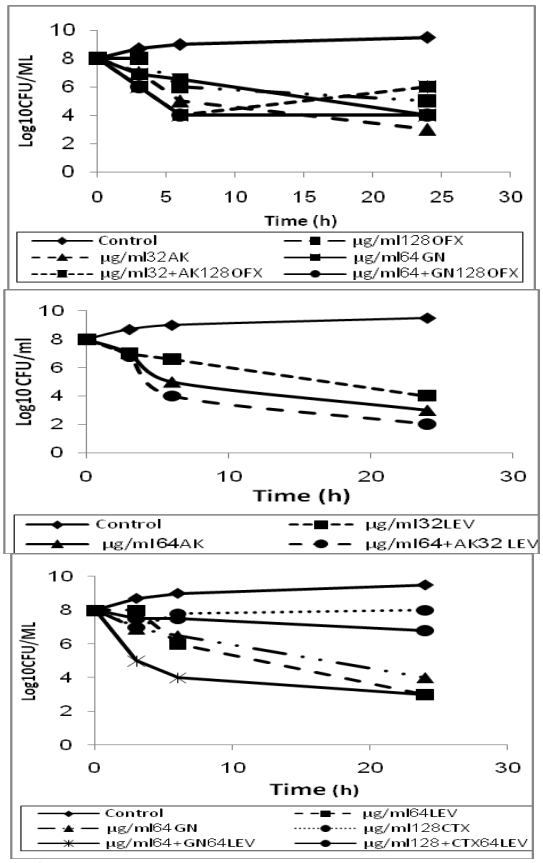

Fig. 4. Killing assay of *Salmonella* Typhimurium (S.Tm no. 54) at 0, 3, 6, 24 h incubation time. a) Time kill assay of ofloxacin at 128µg/ml + amikacin 32µg/ml and ofloxacin at 128µg/ml + gentamycin at 64µg/ml. b) Time kill assay of levofloxacin at 32µg/ml +amikacin at 64µg/ml. c) Time kill assay of levofloxacin at 64µg/ml + gentamycin at 64µg/ml and levofloxacin at 64µg/ml + cefotaxime at 128µg/ml.

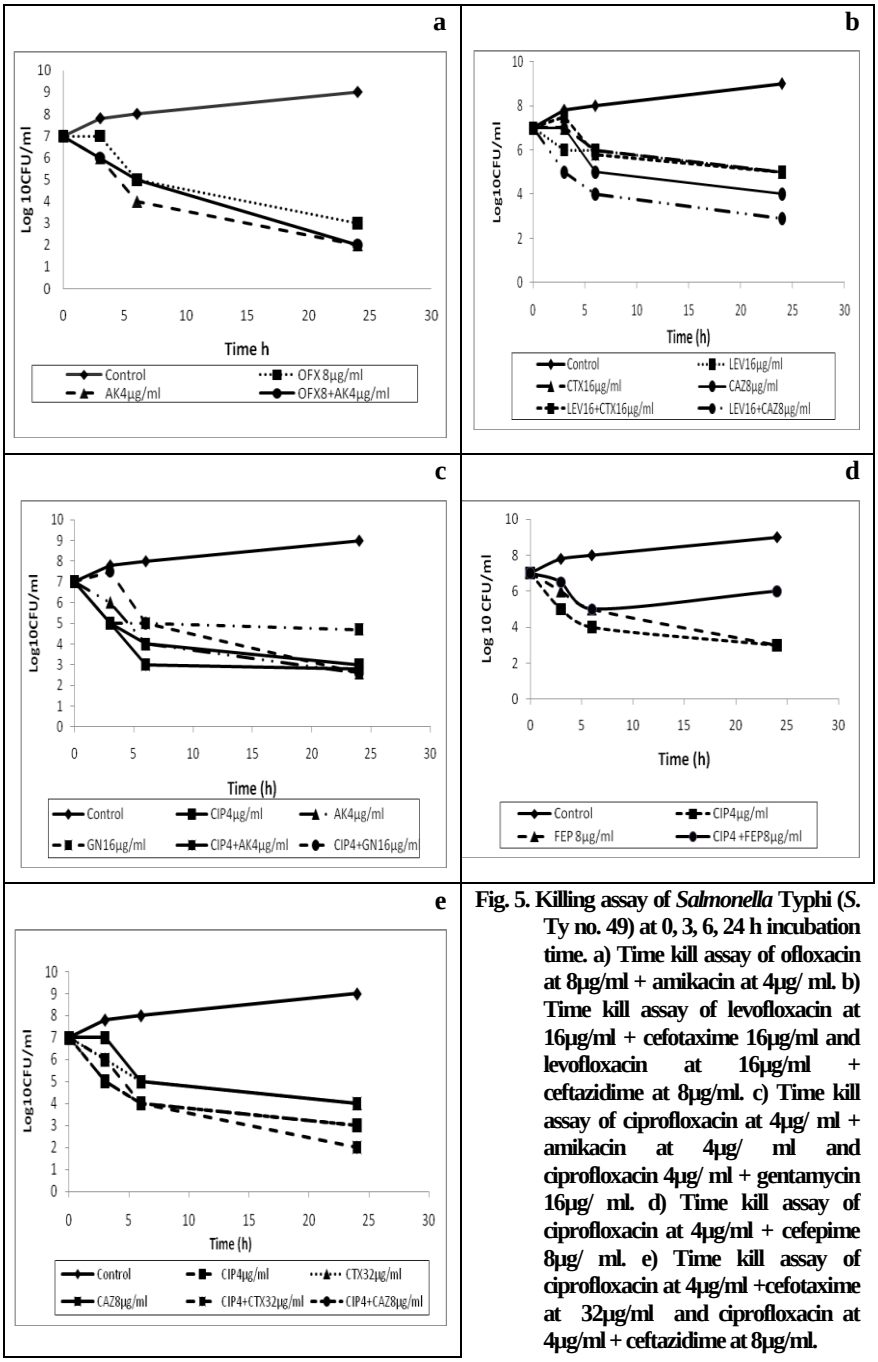

Fig. 5. Killing assay of *Salmonella* Typhi (*S.* Ty no. 49) at 0, 3, 6, 24 h incubation time. a) Time kill assay of ofloxacin at 8µg/ml + amikacin at 4µg/ ml. b) Time kill assay of levofloxacin at 16µg/ml + cefotaxime 16µg/ml and levofloxacin at 16µg/ml + ceftazidime at 8µg/ml. c) Time kill assay of ciprofloxacin at 4µg/ ml + amikacin at 4µg/ ml and ciprofloxacin 4µg/ ml + gentamycin 16µg/ ml. d) Time kill assay of ciprofloxacin at 4µg/ml + cefepime 8µg/ ml. e) Time kill assay of ciprofloxacin at 4µg/ml +cefotaxime at 32µg/ml and ciprofloxacin at 4µg/ml + ceftazidime at 8µg/ml.

Discussion

Bacterial enteropathogens account for gastroenteritis infections observed worldwide with *Salmonella* being the principal cause. The majority of *Salmonella* isolates from Egyptian hospitals are of *S*. Typhimurium and *S*. Enteritidis as non-typhoidal *Salmonella* and *S.* Typhi and, *S.* Paratyphi A, B as typhodial. Hakanen *et al*. (2006) reported that the countries of origin of non-typhodial *Salmonella* infections are most commonly Thailand with 34% of isolates, Egypt with 31% of isolates and Spain with 25% of isolates. The present study revealed that incidence of *Salmonella* in Egypt from July 2008 through December 2009, increased to 58% due to absence of sanitary measures regarding food habits. Aktas *et al*. (2007) stated that in Istanbul, Turkey 63% of *Salmonella* spp. were identified as *S*. Enteritidis and 36% as *S*. Typhimurium.

Salmonella incidence in children is higher than in adults and stool was recorded as the main source of infection compared to blood source. Marimón *et al.* (2004) reported that 53% of *Salmonella* isolates come from children compared to 47% from adults and stool represents 81.9% of isolates.

Within this context, the increasing incidence of fluoroquinolone resistance in *Salmonella* spp. is of great concern. The resistance rate is higher among *S.* Typhimurium isolates (41.3%) compared with *S.* Enteritidis (27.5%). This result is in good agreement with previous data reported by Ricci & Piddock (2009), who found that *S.* Typhimurium are generally more often resistant than *S.*Enteritidis but contrasts with data obtained by Marimón *et al*. (2004), who stated that nalidixic acid resistance in *S.* Enteritidis was more frequent than in *S.* Typhimurium (18.1 versus 3.0%).

The MIC breakpoints for the fluoroquinolone used in the study are much higher than those recommended by CLSI (2008) and strains resistant to one fluoroquinolone prove to be resistant to all other fluoroquinolones. The present results are in agreement in resistance effect but higher in percentage with the results obtained by Rotimi *et al*. (2008), who showed that the resistance rates in Kuwait and United Arab Emirates for ciprofloxacin are 1.2% and 0.8%. Yang *et al*. (2011) studied the resistance of 30 *Salmonella* isolates to seven antibiotics and found that all (*i.e.* 100%) are resistant to nalidixic acid while 96.7% were resistant to difloxacin, 93.3% were resistant to sarafloxacin, 73.3% were resistant to enrofloxacin, 66.7% were resistant to ciprofloxacin and gatifloxacin and 13.3% were resistant to levofloxacin.

The combination of fluoroquinolones with β-lactams is an attractive alternative to the classic combination of aminoglycosides and β-lactams antibiotics in the empiric treatment of serious infections (Maiche & Teerenhovi, 1991). The combination of ciprofloxacin with β– lactams antibiotic can be synergistic (Gould *et al*., 1997).

Time-kill curve experiments are frequently used to assess the activity of antimicrobial combination *in vitro*; however, the numbers of antimicrobial combinations that can be tested are limited. Therefore, we used the dynamic checkerboard method as a screen to determine the pertinent antibiotic concentration to be tested by the time-kill curve (Jacqueline *et al*., 2005).

In the treatment of enteric fever, gentamycin alone is avoided considering its low intracellular concentration and the survival of *Salmonella* inside macrophages, so gentamycin is used with fluoroquinolones derivatives. In this study gentamycin achieved synergistic action in combination with fluoroquinolones, these results are in line with Mandal *et al*. (2003) who show that ciprofloxacin in combination with gentamycin using the time-kill method has a 2.64 $\log_{10}$ decrease in cfu/ml against one isolate.

From the β-lactams group, the third-generation cephalosporin (cefotaxime) retained high activity and continues to be an important agent treating enteric *Salmonella* species; therefore, cefotaxime is chosen for time-kill synergy. Kim *et al*. (2010) stated that in combination therapy, ciprofloxacin with cefotaxime might be the treatment of choice for patients with typhoid fever; they confirm this by the time-kill technique. The combination of ciprofloxacin and cefotaxime against all three nalidixic acid resistant *S.* Typhi strains and one nalidixic acid-susceptible *S.* Typhi ATCC 9992 strain is significantly more effective *in vitro* in reducing bacterial counts by ≥ 3 $\log_{10}$ cfu/ml at 24 h and shows synergistic effects (Kim *et al*., 2010). Cefotaxime and ciprofloxacin in combination may be considered as an option for difficult-to-treat salmonellosis (Chang *et al*., 2006).

The antagonism action achieved in the time-kill combination may be explained as follows: A large proportion of the bacterial population may be affected by antibiotic, with resultant death, but some cells may remain viable because: (i) They are in a different growth stage and less susceptible; (ii) The concentration of antibiotics is not sufficient to reach the intracellular levels required to effect the death of all the cells in the culture; or (iii) Resistance emerges. The same discrepancy has been encountered with *P. aeruginosa* as reported by Cappelletty & Rybak (1996).

Conclusion

The fluoroquinolone resistant human *Salmonella* infections have markedly increased and to overcome this problem we used a combination therapy. Fluroquinolone combination with β-lactams (gentamycin, amikacin) and aminoglycosides (cefotaxime) were effective in the treatment of resistant *Salmonella* Typhimurium, *S.* Enteritidis and Typhi.

References

Aktas, Z., Day, M., Kayacan, C.B., Diren, S. and Threlfall, E.J. (2007) Molecular characterization of *S.* Typhimurium and *S.* Enteritidis by plasmid analysis and pulsed-field gel electrophoresis. *International Journal of Antimicrobial Agents*, **30,** 541–545.

AL-Dawodi, R., Farraj, M.A. and Essawi, T. (2012) Antimicrobial resistance in non-typhi *Salmonella enterica* isolated from humans and poultry in Palestine. *The Journal of Infection in Developing Countries*. **6**(2), 132-136.

Ayana, N. and Surekha, K. (2008) Antimicrobial susceptibility pattern and characterization of ciprofloxacin resistant *S.* Typhi. *Research Journal Microbiology*, **3**(11), 654-660.

Baucheron, S., Chaslus-Dancla, E. and Cloeckaert, A. (2004) Role of TolC and *parC* mutation in high-level fluoroquinolone resistance in *Salmonella enterica* serotype Typhimurium DT204. *Journal of Antimicrobial Chemotherapy*, **53,** 657-659.

Brenner, D.J. and Farmer, J.J. III. (2004) Family I. Enterobacteriaceae. In: "*Bergey's Manual of Systemic Bacteriology"*, Brenner D.J., Krieg, N.R., Staley, J.T., Garrity, G.M., Boone, D.V., De Vos, P., Goodfellow, M., Rainey, F.A. and Schleifer, K.H. (Ed.), 2*nd*. Springer, USA.

Cappelletty, D.M. and Rybak, M.J. (1996) Comparison of methodologies for synergism testing of drug combination against resistant strains of *P. aeruginosa*. *Antimicrobial Agents and Chemotherapy*, **40,** 677-683.

Chang, C.M., Lee, H.C., Lee, N.Y., Huang, G.C., Lee, **I.W. and Ko**., **W.C. (2006)** Cefotaxime-Ciprofloxacin combination therapy for nontyphoidal *Salmonella* bacteremia and paravertebral abscess after failure of monotherapy. *Pharmacotherapy*, **26** (11), 1671-1674.

Clinical and Laboratory Standards Institute (CLSI) (2008) Performance standard for antimicrobial susceptibility testing; Eighteen Information Supplement Document M100-S18.8, (1) Wayne, PA.

Eliopoulos, G.M. and Moellering, R.C. (1991) Antimicrobial combinations. In: "*Antibiotics in Laboratory Medicine"*, V. Lorian (Ed.), 3*rd* ed. The Williams & Wilkinins Co. Baltimore, Maryland, USA.

Gordon, M.A. (2008) *Salmonella* infections in immunocompromised adults. *Journal of Infection*, **56,** 413-422.

Gould, I.M., Wilson, D., Milne, K., Paterson, A., Golder, D. and Russell, D. (1997) *In vitro* pharmacodynamics studies of piperacillin/tazobactam with gentamycin and ciprofloxacin. *Journal of Antimicrobial Chemotherapy*, **39,** 53-61.

Hakanen, A., Kotilainen, P., Huovinen, P., Helenius, H. and Siitonen, A. (2001) Reduced fluoroquinolone susceptibility in *Salmonella enterica* serotypes in travellers returning from Southeast Asia. *Emerging Infectious Diseases*, **7,** 996-1003.

Hakanen, A., Kotilainen, P., Pitkanen, S., Huikko, S., Anja Siitonen, A. and Huovinen, P. (2006) Reduction in fluoroquinolone susceptibility among non-typhodial strains of *Salmonella enterica* isolated from Finnish patients. *Journal of Antimicrobial Chemotherapy*, **57,** 569-572.

Hirose, K., Hashimoto, A., Tamura, K., Kawamura, Y., Ezaki, T., Sagara, H. and Watanabe, H. (2002) DNA sequence analysis of DNA gyrase and DNA topoisomerase IV quinolone resistance-determining regions of *Salmonella* enterica serovar Typhi and serovar Paratyphi A. *Antimicrobial Agents and Chemotherapy*, **46**(10), 3249-3252.

Jacqueline, C., Navas, D. and Batard, E. (2005) *In vitro* and *in vivo* synergistic activities of linezolid combined with subinhibitory concentrations of imipenem against methicillin-resistant *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy*, **49,** 45-51.

Kim, D.M., Neupane, G.P., . Jang, S.J., Kim, S.H. and Lee, B.K. (2010) *In vitro* efficacy of the combination of ciprofloxacin and cefotaxime against nalidixic acid-resistant *Salmonella enterica* serotype Typhi. *International Journal of Antimicrobial Agents*, **36**(2), 155-158.

Lunguya, O., Lejon, V., Phoba, M.F., Bertrand, S., Vanhoof, R., Glupczynski, Y., Verhaegen, J., Tamfum, J.J.M. and Jacobs, J. (2013) Antimicrobial Resistance in Invasive Non-typhoid Salmonella from the Democratic Republic of the Congo: Emergence of Decreased Fluoroquinolone Susceptibility and Extended-spectrum Beta Lactamases. PLOS Neglected Tropical Diseases | www.plosntds.org, **7** (3), 1-9.

Maiche, A.G. and Teerenhovi, L. (1991) Empiric treatment of serious infections in patients with cancer: randomized comparison of two antibiotics. *Infection,* **19** (Suppl.6), S326-S329.

Mandal, S., Mandal, M.D. and Pal, N.K. (2003) Combination effect of ciprofloxacin and gentamycin against clinical isolates of *Salmonella* Typhi with reduced susceptibility to ciprofloxacin. *Japanese Journal of Infectious Diseases*, **56**(4), 156-157.

Marimón, J.M., Gomáriz, M., Zigorraga, C., Cilla, G. and Pérez-Trallero, E. (2004) Increasing prevalence of quinolone resistance in human nontyphoid *Salmonella enterica* isolates obtained in Spain from 1981 to 2003. *Antimicrobial Agents and Chemotherapy*, **48**(10), 3789-3793.

Popoff, M.Y. (2001) Antigenic Formulas of the *Salmonella* serovars, Report of the WHO Collaborating Center for Reference and Research on *Salmonella*, 8th ed. WHO.

Popoff, M.Y. and Le Minor, L. (1997) Antigenic formula of *Salmonella* serovars. Report of WHO. Collaborating Center for Reference and Research on *Salmonella*, Institute Pasteur, Paris, France.

Ricci, V. and Piddock, L.J.V. (2009) Ciprofloxacin selects for multidrug resistance in *Salmonella enterica* serovar Typhimurium mediated by at least two different pathways. *Journal of Antimicrobial Chemotherapy*, **63,** 909-916.

Rotimi, V.O., Jamala, W., Palb, T., Sonnevendb, A., Dimitrovc, T.S. and Alberta, M.J. (2008) Emergence of multidrug-resistant *Salmonella* spp. and isolates with reduced susceptibility to ciprofloxacin in Kuwait and the United Arab Emirates. *Diagnostic Microbiology and Infectious Disease*, **60,** 71-77.

Rybak, M.J. and McGrath, B.J. (1996) Combination antimicrobial therapy for bacterial infections. Guidelines for the clinician. *Drugs*, **52,** 390-405.

Satish, K.P., Robert, C.M.J. and George, M.E. (2005) Multidrug-resistant urinary tract isolates of *Escherichia coli* from Ribeirao Preto, Sao Paulo, Brazil. *Brazilian Journal of Infectious Diseases*, **11,** 575-578.

Thompson, R.B. and Miller, J.M. (2003) Specimen collection, transport and processing: bacteriology, pp. 286–330. In: "*Manual of Clinical Microbiology"*, Murray P.R., Baron, E.J., Jorgensen, J.H., Pfaller, M.A. and Yolken, R.H. (Ed.), 8th ed. American Society for Microbiology, Washington, D.C.

Wedel, S.D., Bender, J.B., Leano, F.T., Boxrud, D.J., Hedberg, C. and Smith, K. (2005) Antimicrobial drug susceptibility of human and animal *S.* Typhimurium, Minnesota, 1997–2003. *Emerging Infectious Diseases,* **11,** 1899–1906.

White, R., Burgess, D. and Manduru, M. (1996) Comparison of three different *in vitro* methods of detecting synergy: time-kill, checkerboard, and E test. *Antimicrobial Agents and Chemotherapy*, **40,** 1914–1918.

Yang, B., Xi, M., Cui, S., Zhang, X., Shen, J., Sheng, M., Qu, D., Wang, X. and Meng, J. (2011) Mutations in gyrase and topoisomerase genes associated with fluoroquinolone resistance in *Salmonella* serovar from retail meats. *Food Research International*, **45** (2), 935-939.

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توليفة من مضادات حيوية مختلفة ضد *سالمونيلا إنتيريكا* المقاومة للفلوروكينولون و المعزولة من الانسان بمصر

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لقد اصبح نشوء المقاومة للمضادات الحيوية في انواع السالمونيلا الممرضة مشكلة عالمية و قد تم إستخدام توليفة من مضادات الحياة كعلاج سريري (إكلينيكي) لحل هذه المشكلة. تم جمع 100 من البكتريا الممرضة المسببة للنزلات المعوية على مدي عام و نصف العام مصدرها البراز و الدم من ثلاث مستشفيات مصرية و بإستخدام تقنيات النمط الظاهري و السيرولوجية تم تنقية و تعريف 58 من عزلات السالمونيلا تبين منها 41,4% سالمونيلا تايفيموريم ، 27,6% سالمونيلا إنتريتيدس ، 5,2% سالمونيلا تايفي ، 3,4% سالمونيلا باراتايفي أ ، 3,4% سالمونيلا باراتيفي ب و 19% أنواع اخري من السلمونيلا . و بينت طريقة قرص الانتشار أن 36 من ال 58 نوع سالمونيلا (نسبة 62%) كانت مقاومة للفلوركينولون و تم التاكد أن خمس سلالات من السالمونيلا مقاومة لمجموعة فلوركينولون و ذلك من قيم التركيزات للحد الادني المثبط و تراوح مستوي هذه القيم بين 32إلى > 512 مليجرام/ملي و هى أعلى من تلك التي اوصي بها معهد القياسات الطبية و المعملية (CLSI) و قد تم إجراء 75 معايرة دقيقة مشتركة بطريقة الشطرنج (combined microtitre cheackerboard) علي سلالات السالمونيلا الخمس ذات المقاومة المتعددة للفلوركينولون لتقدير إمكانية المعالجة بالتوليفة . هذا و لم يلاحظ أي تضاد في أى توليفة بل تحقق التاثيرالتعاوني و تاثير الإضافي في 41,4% و 58,6% علي الترتيب . كما لوحظ كثيرا ان منحي الابادة التعاوني هو 24 ساعة. و قد وجد ان هناك تطابق بين نتائج منحني الابادة وطريقة الشطرنج يبلغ 100% و 50% و 40% لثلاث سلالات من السالمونيلا. لوحظ ان السالمونيلا المقاومة قد ازدادت في مصر. و قد وجد ان توليفة الفلوروكينولون مع بيتا لامتامز (جنتاميسين و اميكاسين) وامينوجلايكوسايدز (سيفوتاكسايم) ذات تاثير فعال في معالجة سالمونيلا تايفيموريام و انتريتيدس و تايفي .