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Phenotypic Detection of Potential Antimicrobial Resistance among Environmental *Salmonella* Species

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Abstract: Emergence of multidrug resistance (MDR) in *Salmonella* strains is a serious development. Our study was done to investigate such resistance mechanisms which may play a role in partial or total resistance to these antimicrobial agents. This study was done on 40 *Salmonella* isolates from different food products. All isolates were plated primarily on MacConkey's agar then on bismuth sulphite agar, XLD and brilliant green MacConkey's agar and subjected to different biochemical reactions. MDR isolates were confirmed and identified to species level using API 20E. Antibiogram was done by Kirby Bauer disc diffusion method and the minimum inhibitory concentration (MIC) was determined using the microtiter broth dilution method. The MDR isolates were further investigated to determine the potential resistance mechanisms to β-lactam antibiotics, chloramphenicol and ciprofloxacin using EDTA disc synergy test, AmpC disc test and efflux pump test. This study revealed that imipenem 97.5% (39/40) was the most effective antibiotic, while cefepime 47.5% (19/40) was the least effective. Out of 23 isolates (MDR), one isolate 4.35% (1/23) was AmpC producer, three isolates 13% (3/23) were Metallo-β-lactamase (MBL) producers and nineteen isolates 82.61% (19/23) were efflux pump positive. This study suggests that efflux pump is an important mechanism for antimicrobial resistance among *Salmonella* spp.

Key words: *Salmonella* · Multidrug Resistance · Efflux Pump · EDTA Disc Synergy (EDS)

INTRODUCTION

Salmonella is a rod-shaped, mostly motile, non spore forming Gram-negative bacterium belonging to the family of *Enterobacteriaceae*. Poultry and poultry products have been implicated as a major source of *Salmonella* infections in human [1] and also environmental sources including water, soil, insects, factory surfaces, kitchen surfaces, animal feces, raw meats and raw seafood [2].

Salmonella infection of humans and animals continues to be a distressing health problem worldwide [3]. Salmonellosis is the major cause of food borne infections and the second most common food borne illness after *Campylobacter* infection [4].

Most *Salmonella* infections result in acute gastroenteritis and do not require antimicrobial therapy. However, antimicrobial agents are commonly prescribed for patients with salmonellosis, particularly very young, very old and immunosuppressed patients. Several lines of evidence demonstrate that the use of antimicrobial agents in animal's food contributes to the emergence and dissemination of antimicrobial resistance in food borne *Salmonella* [5].

The increase in antibiotic resistance among Gram-negative bacteria is a notable example of how bacteria can procure, maintain and express new genetic information that can confer resistance to one or several antibiotics [6]. *Salmonella enterica* serovar *arizona* (*S. enterica* subspecies IIIa) is naturally found in reptiles but also causes outbreaks of salmonellosis in turkeys and sheep and can produce both enteritis and serious disseminated disease in humans [7]. Multidrug resistant (MDR) *Salmonella* has been detected in many serotypes. The most common MDR pattern, first emerged in *S. typhimurium*, has been the ACSSuT pattern (resistance to ampicillin, chloramphenicol, streptomycin,

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sulphonamides and tetracycline). Some strains may also display resistance to gentamicin, kanamycin and trimethoprim/sulfamethoxazole. Resistance to third generation of cephalosporins in *Salmonella* is of interest because these are drugs of choice for treating salmonellosis in children, where fluoroquinolones are contraindicated [8]. One of the most important mechanisms of microbial resistance to β-lactam antibiotics in Gram-negative bacteria is their hydrolysis by β-lactamases. Among β-lactamases, carbapenemases especially transferrable metallo-β-lactamases (MBLs) are the most feared because of their ability to hydrolyze virtually all drugs in that class, including the carbapenems. Various methods are used for detection of MBLs, but ethylene diamine tetracetic acid (EDTA) disc synergy (EDS) test is a relatively simple and sensitive method for MBL detection. AmpC disk test is commonly used for detection of AmpC β-lactamases [9]. In many *Enterobacteriaceae*, AmpC expression is low but inducible in response to β-lactam exposure [10].

Another resistance mechanism in bacteria is the efflux pumps that contribute to intrinsic resistance to a wide range of antibiotics and often have a broad substrate range. The over expression of multidrug efflux pumps can lead to low-level multidrug resistance, which poses a clinical problem. To overcome this problem an efflux pump inhibitor such as reserpine had been used. It has been concluded that the reduction of MIC by reserpine indicates the involvement of an active efflux system and the multidrug resistance displayed was eliminated by reserpine [11]. Since efflux pump genes and proteins are present in both antibiotic-susceptible and antibiotic-resistant bacteria, some systems can be induced by their substrates so that an apparently susceptible strain can overproduce a pump and become resistant [12].

This study was done to study phenotypically the potential mechanisms of resistance which may be partially or completely responsible for the antimicrobial resistance pattern among *Salmonella* isolates included in this study and also to determine the most effective antimicrobial agent against our isolates based on results of antibiogram and MIC.

MATERIALS AND METHODS

Microorganisms: This study was performed on 40 bacterial isolates recovered from various food products such as raw meat, raw vegetables, mayonnaise and raw chickens which were obtained from hospitality industrially locations in Cairo, Alexandria and Sharm el sheikh in Egypt.

Materials: MacConkey's agar, Xylose Lysine Deoxycholate (XLD), brilliant green MacConkey's agar and bismuth sulphite agar were provided by HIMEDIA Laboratories, PVT Limited, India. Mueller-Hinton agar (MHA) and Mueller-Hinton Broth (MHB) were purchased from Difco laboratories (U.S.A.). API 20E was the product of Biomerieux (France). The following antibiotic discs: ceftazidime, cefepime; cefoperazone, ceftriaxone, chloramphenicol, doxycycline, cotrimoxazole, ticaracillin/clavulanic, imipenem, erythromycin, azithromycin, gentamicin, lomefloxacin, norfloxacin, ofloxacin and ciprofloxacin were obtained by HIMEDIA Laboratories, PVT Limited (India).

Methods

Identification of Bacterial Isolates: Food specimens were cultivated primarily on MacConkey's agar, then on XLD and brilliant green MacConkey's agar. The isolated colonies were subjected to microscopical examination using Gram stain technique. Bacterial isolates on MacConkey's agar were sub-cultured on bismuth sulphite agar and then subjected to various confirmatory biochemical tests for *Enterobacteriaceae* according to Mackie & McCartney [13]. All isolates were confirmed and identified to species level using API 20E.

Antimicrobial Susceptibility Test: Bacterial isolates were investigated for antimicrobial susceptibility by Kirby Bauer disc diffusion method according to Vandepitte *et al.* [14] using the following antibiotic discs: ceftazidime (CA 30 μg); cefepime (CPM 30 μg); cefoperazone (CS 75 μg); ceftriaxone (CL 30 μg); chloramphenicol (C 30 μg); doxycycline (DO 30 μg); ticaracillin/clavulanic (TC 75/10 μg); cotrimoxazole (CO 25 μg); imipenem (I 10 μg); erythromycin (E 15 μg); azithromycin (AT 15 μg); gentamicin (G 10 μg); lomefloxacin (LO 10 μg); norfloxacin (Nor 10 μg); ofloxacin (OF 5 μg) and ciprofloxacin (CIP 5 μg). *Escherichia coli* American Type Culture Collection (ATCC) 25922 was used as control during the performance of the antimicrobial susceptibility test. The test was performed by adjusting the inoculum to a standard turbidity (0.5 -1.0 McFarland), which was homogenously streaked by a swab all over the surface of MHA plate. The antimicrobial discs were placed on the inoculated plates gently with the flamed tip of sterile forceps to ensure good contact with the agar and the plates were incubated at 37°C for 18-24 hours. The results were interpreted according to CLSI [14].

Determination of Minimum Inhibitory Concentration (MIC): The MIC of the tested antibiotics, against isolates which exhibited high resistance pattern (23 out of 40), was determined by the microtiter plate broth dilution method using double strength Mueller-Hinton Broth (MHB) (Difco, U.S.A.) as a test medium. MIC was detected visually after incubation at 37°C for 18-24 hours. MIC was calculated based on the least antibiotic concentration inhibiting bacterial growth. The readings of the microtiter plates were detected using Microtiter Reader at wave length 600 nm., taking in consideration that the absorbance of the negative control must not exceed 0.01A° [15].

Amp C Disc Test: A suspension of *E. coli* ATCC 25922, from an overnight culture, adjusted to 0.5 McFarland standard was inoculated onto the surface of MHA plate. A 30 µg cefoxitin disc was kept on the surface of the agar. A blank disc (6 mm in diameter, Whatmann filter paper no. 1) was moistened with sterile saline and inoculated with a few colonies of the test strain. The inoculated disc was then placed beside the cefoxitin disc almost touching it. The plate was incubated overnight at 37°C. A flattening or indentation of the cefoxitin inhibition zone in the vicinity of the disc with test strain was interpreted as positive for the production of AmpC β-lactamase. An undistorted zone was considered as negative [9].

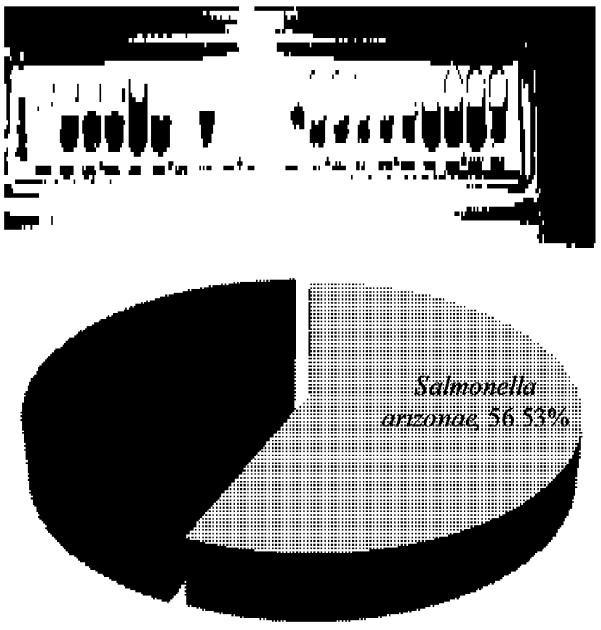
EDTA Disc Synergy Test: This test was performed using ceftazidime for detection of MBLs for the ceftazidime resistant isolates. A 0.5 M EDTA solution was prepared by dissolving 186.1 g of disodium EDTA.$2H_2O$ in 1 L of distilled water. The pH was adjusted to 8.0 by using NaOH and the solution was sterilized by autoclaving [16]. An overnight liquid culture of the test isolate was adjusted to a turbidity of 0.5 McFarland standard and spread on the surface of a MHA plate. A 10 µg ceftazidime disc was placed on the agar. A blank disc (6 mm in diameter, Whatmann filter paper no. 1) was kept on the inner surface of the lid of the MHA plate and 10 µl of 0.5 M EDTA solution was added. This EDTA disc was then transferred to the surface of the agar and was kept 10 mm edge to edge apart from the ceftazidime disc. After overnight incubation at 37°C, the presence of an expanded growth inhibition zone between the two discs was interpreted as positive for MBL production [9].

Phenotypic Detection of Efflux Pump: This test was accomplished by using reserpine as an efflux pump inhibitor (EPI) and ciprofloxacin [17, 18]. Minimum inhibitory concentration of ciprofloxacin was determined by the microtiter broth dilution method (with and without reserpine) according to Amsterdam [19] and Andrews [15]. This test was repeated by using antibiotics other than ciprofloxacin, which were cefepime, cefoperazone and chloramphenicol.

RESULTS

Identification of Bacterial Isolates: In this study, a total of 40 bacterial strains was isolated from food specimens. These isolates appeared to be non lactose fermenters upon culture on MacConkey's agar medium, xylose lysine deoxycholate agar (XLD) and brilliant green MacConkey's agar. These isolates produced black colonies on both xylose lysine deoxycholate agar and bismuth sulphite agar. Further investigations using different biochemical tests revealed that 100% (40/40) of isolates belonged to *Salmonella* spp. Regarding to species identification carried out on MDR isolates using API 20E, 56.53% (13/23) of isolates were identified as *Salmonella arizonae* and 43.47% (10/23) were identified as *Salmonella species*.

Antimicrobial Susceptibility Test: The antibiogram using Kirby-Bauer method revealed that 17.5% (7/40) of isolates were ceftazidime resistant, 47.5% (19/40) were cefepime resistant, 42.5% (17/40) were cefoperazone resistant, 15% (6/40) were ceftriaxone resistant, 22.5% (9/40) were chloramphenicol resistant, 20% (8/40) were tetracycline



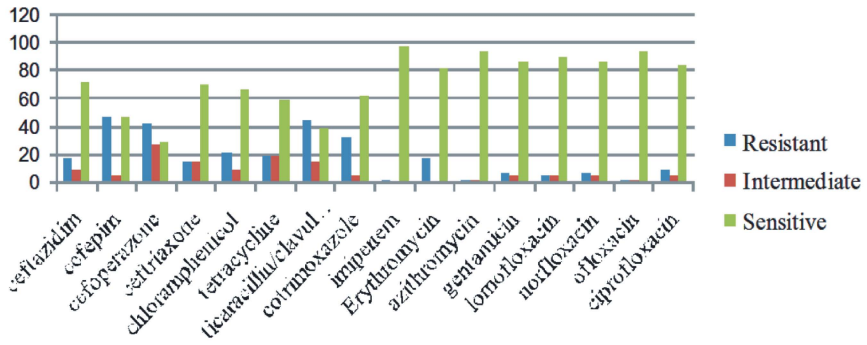


Fig. 1: Frequency of antimicrobial susceptibility among different *Salmonella* isolates.

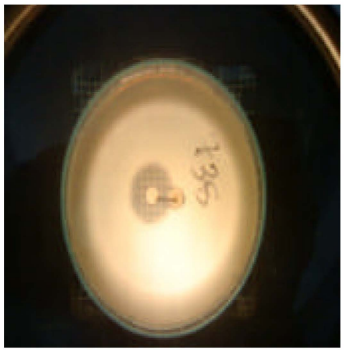


Fig. 2: Positive MBL

Fig. 3: Negative MBL

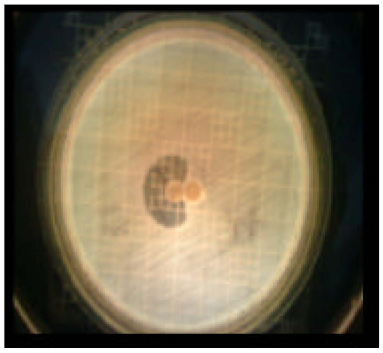


Fig. 4: Positive AmpC

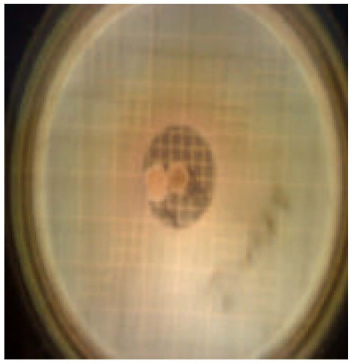


Fig. 5: Negative AmpC

resistant, 45% (18/40) were ticaracillin/clavulanic resistant, 32.5% (13/40) were cotrimoxazole resistant, 2.5% (1/40) were imipenem resistant, 17.5% (7/40) were erythromycin resistant, 2.5% (1/40) were azithromycin resistant, 7.5% (3/40) were gentamicin resistant, 5% (2/40) were lomofloxacin resistant, 7.5% (3/40) were norfloxacin resistant, 2.5% (1/40) were ofloxacin resistant and 10% (4/40) were ciprofloxacin resistant (Fig. 1). In this study, 57.5% of isolates (23/40) showing multi drug resistance pattern were subjected to MIC using microtiter broth dilution method which revealed that 4.35% (1/23) of MDR isolates was ceftazidime resistant, 47.82% (11/23) were cefepime resistant, 52.17% (12/23) were cefoperazone resistant, 26.1% (6/23) were ceftriaxone resistant, 39.13% (9/23) were chloramphenicol resistant, 8.69% (2/23) were tetracycline resistant, 34.78% (8/23) were cotrimoxazole resistant, 26.1% (6/23) were erythromycin resistant, 4.35% (1/23) was gentamicin resistant and 4.35% (1/23) was ciprofloxacin resistant. These results led us to the investigation for the potential resistant mechanisms of MDR isolates which revealed that 82.61% (19/23) of isolates express positive efflux pump (Fig. 6) while 13% (3/23) of isolates were MBL positive (Fig. 2, 3 & 6) and 4.35% (1/23) was AmpC positive (Fig. 4, 5 & 6).

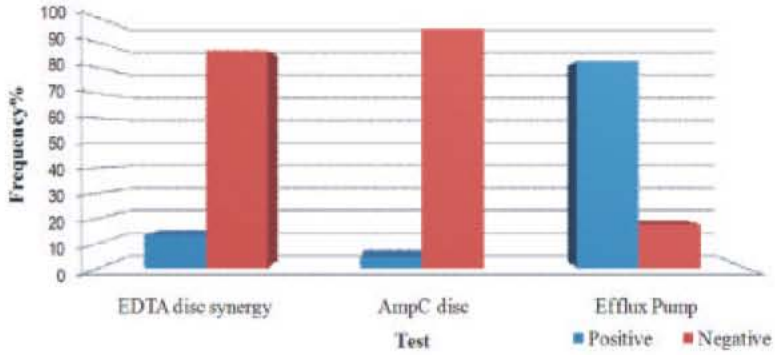


Fig. 6: Frequency of different antimicrobial resistance mechanisms among MDR *Salmonella* isolates

DISCUSSION

Gram-negative bacterial resistance possibly now equals or usurps that of Gram-positive bacterial resistance. Many isolates are resistant to one or more antibiotics and the choice of drugs should be based on susceptibility testing [6]. During this study antimicrobial resistance to more than three unrelated classes of antimicrobial agents was observed for many isolates so we aimed to investigate the susceptibility of these isolates against different classes of antimicrobial agents to detect the most effective agents against *Salmonella* isolates.

The present study revealed that the susceptibility pattern for ciprofloxacin, gentamicin and norfloxacin among *Salmonella* isolates was 85%, 87.5% and 87.5% respectively, these findings were relatively similar to the findings of studies carried out by Cardoso *et al*. [20], Oliveira *et al*. [21] and Molla *et al*. [22] who revealed that 100% of *Salmonella* isolates were susceptible to ciprofloxacin, gentamicin and norfloxacin. Also the study results are in consistent with Muthu *et al*. [23] who reported that 92.5% and 94% of *Salmonella* isolates were susceptible to norfloxacin and ciprofloxacin respectively. Angkititrakul *et al*. [24] also found that 82.8% of *Salmonella* strains were sensitive to norfloxacin.

In this study, 17.5% of *Salmonella* isolates proved to be erythromycin resistant. Higher results were obtained by Cardoso *et al*. [20] and Gunell *et al*. [25] who reported that 100% and 99.6% of the *Salmonella* isolates were resistant to erythromycin. In contrast, Chengappa *et al*. [26] reported that 100% of the *Salmonella* isolates were susceptible to erythromycin.

The levels of antibacterial resistance observed in this study emphasize that antibiotics must be used judiciously. Based on this fact we aimed to determine the appropriate effective concentration of these antimicrobial agents by determining the MIC using micro titter broth dilution method. Our study revealed that most *Salmonella isolates* were susceptible to ciprofloxacin with MICs ranging from 0.24-0.49 μg/ml. These results are relatively in agreement with Lunn *et al*. [27] who reported that all isolates were susceptible to ciprofloxacin with MICs ranging from 0.012–0.75 μg/ml, findings of Sekar *et al*. [28] who found that all isolates are susceptible to ciprofloxacin with MICs >0.5 μg/ml and Whichard *et al*. [29] who found that most isolates are susceptible to ciprofloxacin with MICs ranging from 0.12-0.5 μg/ml. On the other hand Yu *et al*. [30] found that 19.4% of *Salmonella* isolates were resistant to ciprofloxacin with MICs ≥ 4 μg/ml.

Regarding to susceptibility to gentamicin, it was found that 87.5% of *Salmonella* isolates were susceptible for gentamicin with MICs ranging from 0.98-1.95 μg/ml. These results are relatively in agreement with the findings of Mandal *et al*. [31, 32] who reported that all isolates were susceptible to gentamicin with MICs ranging from 0.75–2 μg/ml and from 0.01–4 μg/ml respectively and with those of Gautam *et al*. [33] who found that all isolates were susceptible to gentamicin with MICs ranging from 0.015–2 μg/ml.

The MICs of ceftriaxone in 70% of *Salmonella* isolates were 3.91-7.81 μg/ml, a result which is consistent with findings of Bhat *et al*. [34] who found that MICs of all isolates were ≤ 8μg/ml and relatively in agreement with Sekar *et al*. [28] who found that MICs of most isolates were >0.5 μg/ml.

Since efflux pump mechanism is an important mechanism of bacterial resistance to many antimicrobial agents, we aimed to investigate the role of this mechanism in resistance of MDR isolates using reserpine as an efflux pump inhibitor (EPI). This test showed that efflux pump mechanism was common mechanism of resistance among *Salmonella* isolates since 82.61% (19/23) were positive for efflux pump test. These results are in agreement with the observations

of Baucheron *et al*. [35] who reported that active efflux by the AcrAB-TolC efflux system is the main mechanism of resistance to quinolones in *Salmonella* isolates and with Ricci *et al*. [36] who found that efflux pump mechanism is responsible for ciprofloxacin resistance in *Salmonella* isolates suggesting a role for *ramA* in multiple antibiotic resistance (MAR).

Other antibiotic resistance mechanisms, to β-lactam antibiotics, were detected in this study such as MBL and AmpC β-lactamase production. Only one out of 23 *Salmonella* isolates (4.35%) proved to be AmpC positive. These results are relatively consistent with those of some investigators who found that, in many *Enterobacteriaceae* (*Salmonella*) AmpC expression is low but inducible in response to β-lactam exposure and that *Salmonella* spp. lack a chromosomal *bla*AmpC gene [10, 37]. Black *et al*. [38] also reported that 69% of isolates were negative AmpC disk test and Batchelor *et al*. [39] found that only 8.5% of *Salmonella* isolates were AmpC disk test positive. AmpC-mediated resistance has been reported for non typhoidal *Salmonella* isolates [30]. These findings are in disagreement with Carattoli *et al*. [40] who revealed that in species that do not produce AmpC β-lactamases, such as *Salmonella* spp. the resistance to β-lactams is mediated predominantly by plasmid-encoded enzymes of the ambler class A. Finally, our study revealed that 13% of *Salmonella* isolates were metallo-β-lactamases (MBL) producers. Simm *et al*. [41] observed that all *Salmonella* isolates possessed an L1-like metallo-beta-lactamase. MBL have been identified in *Salmonella enterica* serovar *typhimurium* where MBL gene (*bla*SPM-1) has been detected [6, 42].

It can be concluded that imipenem remains the most effective β-lactam antibiotic against *Salmonella* infection and efflux pump is the most important mechanism of resistance while Metallo-β-lactamase plays a role in cephalosporin resistance and AmpC β-lactamase is a contributory factor for cephalosporin resistance among *Salmonella* isolates in our study.

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