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Short Communication

Detection of extended spectrum beta lactamases in typhoidal salmonellae by phenotypic methods

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Extended spectrum beta lactamase (ESBL) producing members of the family Enterobacteriaceae are a major health problem in the hospitals and community. These enzymes confer bacterial resistance to penicillins, first, second and third generation cephalosporins and aztreonam. The prevalence of ESBLs varies from organism to organism and is increasing day by day. The study was designed to test typhoidal salmonellae for production of ESBL. One hundred and fifty eight (158) isolates of typhoidal salmonellae; *Salmonella* Typhi (n=126), *Salmonella* Paratyphi A (n=26) and *Salmonella* Paratyphi B (n=6) were collected from different hospitals of Lahore and Gujranwala. The isolates were identified morphologically, biochemically (API-20E) and serologically (BD Difco, USA). ESBL production has been tested by three methods; CLSI screening method, double disk diffusion synergistic method and CLSI phenotypic confirmatory method. CLSI screening method detected twenty two (22) strains as ESBL producers. However, when tested by the CLSI confirmatory method and by disk diffusion synergistic method, none proved to be ESBL producer. Based on our study, we concluded that the extended spectrum beta lactamase enzyme does not exist in tested clinical isolates of typhoidal salmonellae, however the isolates suspected to be ESBL by phenotypic methods must be subjected further for molecular analysis.

Key words: ESBL, typhoidal salmonellae, isolate.

INTRODUCTION

Salmonella enterica subspecies *enterica* is an important genus of the family Enterobacteriaceae. The genus Salmonella was named after an American microbiologist Daniel Elmer Salmon. *Salmonella* Typhi was isolated by Gaffky in Germany in 1884 and in 1886 by Salmon and Smith in United States (Winn et al., 2006).

Extended spectrum beta lactamases (ESBL) are enzymes that mediate resistance to the extended-spectrum cephalosporins; ceftazidime, cefotaxime, and ceftriaxone and monobactams like aztreonam but do not affect cephamycins. These ESBL enzymes also affect carbapenems; imipenem and meropenem (Coque et al., 2008) and are commonly inhibited by beta lactamase-inhibitors such as clavulinic acid (Coque et al., 2008), sulbactam and tazobactam. Plasmids responsible for ESBL production tend to be large (80 Kb or more in size) and carry resistance against several antimicrobials that are aminoglycosides, fluoroquinolones, tetracyclines, chloramphenicol and trimethoprim-sulfamethoxazole (Coque et al., 2008; Nathisuwan et al., 2001). This fact imparts an important limitation in the design of treatment alternatives (Jacoby and Medeiros, 1991).

ESBL enzyme production by *Salmonella* spp. was first detected in 1988 (Hammami et al., 1991). The prevalence

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Abbreviations: API 20E, Analytical profile index 20 Enterobactericeae; **ATCC,** American Type Culture Collection; **CLSI,** Clinical Laboratory Standards Institute; **ESBL,** extended spectrum beta lastamase; **MDR,** multi drug resistant.

of ESBL producing salmonellae in Kuwait is 17.6% out of which *S.* Typhi strains are 4%, while this prevalence is 15.5% in UAE and 5.3% of them are *S.* Typhi (Rotimi et al., 2008). A strain of *S.* Typhi producing CTX-M type of ESBL enzyme was reported from Germany in August 2009 (Pfeifer et al., 2009). Same year, there was another report of ESBL producing *Salmonella enterica* from Iran (Hamidian et al., 2009).

The objective of the present study was to screen the isolates of typhoidal salmonellae for ESBL production. The present study is focused on isolates from our local population to see the frequency of ESBLs in these isolates using phenotypic methods, that is, CLSI screening method, double disk diffusion synergistic method, and CLSI phenotypic confirmatory method.

MATERIALS AND METHODS

Bacterial strains

One hundred and fifty eight clinical isolates of typhoidal salmonellae were obtained from different hospitals of Lahore and Gujranwala; Sheikh Zayed Medical Complex Lahore (n=50), Fatima Memorial Hospital Lahore (n=25), Combined Military Hospital Lahore (n=22), Services Hospital Lahore (n=15), Ittefaq Hospital Lahore (n=14), Shaukat Khanum Memorial Cancer Hospital and Research Centre Lahore (n=12), and Fazil Memorial Hospital Gujranwala (n=20) from 2007 to 2011. The study was conducted in the Department of Microbiology, University of Health Sciences, Lahore.

BioMerieux, France, API-20E and BD Difco, USA antisera were used. Oxoid disks; Ceftazidime (30µg), Ceftriaxone (30µg), Aztreonam (30µg), Cefotaxime (30µg), Co-amoxiclav (clavulanate 10 µg + amoxicillin 20 µg), and Ceftazidime + Clavulanic acid (30 + 10 µg) were used.

The isolates were received on Nutrient agar slopes and subcultured on Nutrient agar. Isolates were identified by standard morphological and biochemical characteristics. Gram staining, Catalase and Oxidase tests were performed. Identification of isolates was done by API 20E (BioMerieux, France) and confirmed by antisera (BD Difco, USA). Antimicrobial susceptibility pattern was performed by Kirby Bauer disc diffusion method using Oxoid disks; Ceftazidime (30µg), Ceftriaxone (30µg), Aztreonam (30µg), Cefotaxime (30µg), Co-amoxiclav (clavulanate 10 µg + amoxicillin 20 µg), and Ceftazidime + Clavulanic acid (30 + 10 µg).

Method

For the detection of ESBL, three methods: CLSI screening method, CLSI phenotypic confirmatory method and double disk diffusion synergistic method were used.

CLSI screening method

Ceftazidime, ceftriaxone and aztreonam disks were placed on a MHA plate at appropriate distance. The plates were incubated aerobically overnight (18-24 hours/35°C). The strains showing ≤ 22 mm zone of inhibition around ceftazidime, ≤ 25 mm around ceftriaxone and ≤ 27 mm around aztreonam disks were suspected to be ESBL producers.

CLSI phenotypic confirmatory method

Strains that were suspected to be ESBL producer by screening method were confirmed for enzyme production by phenotypic confirmatory disk diffusion method.

Ceftazidime disk without clavulanic acid and ceftazidime with clavulanic acid combination disk were placed on the same plate. The plates with disks were incubated aerobically overnight (18-24 hours/35°C). The isolates showing an increase in zone size of 5 mm or more around ceftazidime with clavulanic acid as compared to ceftazidime alone were confirmed to be ESBL producer. No enhancement of zone indicates ESBL non-producer isolates (Cockerill et al., 2013).

Double disk diffusion synergistic method

A co-amoxiclav disk was placed in the center of MHA plate and ceftazidime, cefotaxime, ceftriaxone, and aztreonam, were placed at 20 mm distance, center to center, from co-amoxiclav disk. Plates were incubated aerobically overnight (18-24 hours/35°C). The isolates which showed an increase in zone of inhibition greater than 5 mm on co-amoxiclav side of the disk compared to the results seen on the side without co-amoxiclav were confirmed as ESBL producer. Whereas, no enhancement of zone on co-amoxiclav disk side indicates ESBL non-producer isolates (Jarlier et al., 1988).

RESULTS

Out of 158 isolates of typhoidal salmonellae i.e., *S.* Typhi (n=126), *S.* Paratyphi A (n=26) and *S.* Paratyphi B (n=6). No ESBL was detected. Initially 14% isolates were suspected to be ESBL producers when screened by initial screening disk diffusion method. These isolates were finally confirmed to be non-ESBL producers when tested with the disk diffusion phenotypic confirmatory method and by double disk diffusion synergistic method.

DISCUSSION

Among our 158 isolates of typhoidal salmonellae, no ESBL was detected. In another comparative study of screening methods for ESBL detection was done on 38 isolates comprising of 30 reference strains, that is, *E. coli* and *K. pneumoniae* already defined ESBL producers by Isoelectric focusing and DNA sequencing, and eight laboratory isolates not confirmed to be ESBL producer, no reduced susceptibility pattern to extended spectrum cephalosporins and aztreonam was seen, according to this study, only 52% ESBL producing isolates were detected by disk diffusion screening method (Vercauteren et al., 1997). In another reported study, 55% of ESBL producing isolates were detected by disk diffusion screening method (Goyal et al., 2008). Ceftazidime proved to be the best single indicator antibiotic for ESBL detection, however none of the isolates when subjected to molecular analysis produced any type of ESBL, that is, TEM-4, TEM 12, SHV-2, SHV-3 and SHV-5 (Vercauteren et al., 1997). Standard disk diffusion susceptibility test detected only 48% of ESBL producing reference strains (Vercauteren et al., 1997). Thus in agreement with other studies, it is evident that an additional testing method is

required for the confirmation of ESBL (Jacoby and Han, 1996; Katsanis et al., 1994; Thomson and Sanders, 1992). Several possibilities for these false negative results are known; among them one possibility is that TEM-type ESBLs with single amino acid substitutions have only low level oxyimino beta lactam activity (Jacoby, 1994). Other ESBL enzymes have relatively greater oxyimino beta lactam activity but lack intrinsic extended spectrum enzyme efficiency (Bush and Singer, 1989). In our study, 22 isolates were suspected to be ESBL producer by initial screening method, but were confirmed by phenotypic confirmatory disk diffusion method as recommended by CLSI (2013), to be non-ESBL producer. In another study described by Goyal et al. (2008) frequency of ESBL, detected by disk potentiation method, was 64.5%. Phenotypic confirmatory method cannot detect the ESBL in those bacterial isolates which also produce other classes of beta lactamases, AmpCs and TEMs. The probability for this false negative result could be masking effect of enzymes on inhibitory effect of clavulanic acid (Srisangkaew and Vorachit, 2004). According to Datta et al. (2004), 90% ESBL producers were detected correctly by disk potentiation method.

These 22 isolates were also confirmed by another phenotypic method described by Jalier et al. (1988). According to another study, this double disk synergy method, described by Jalier et al. (1988) detected 95% of isolates to be confirmed ESBL by CLSI phenotypic method (Goyal et al., 2008).

In our study and current settings, we only had the facility of phenotypic methods however these 22 isolates if subjected to molecular analysis could be confirmed to be ESBL producer or non producer.

ESBL producers also have the capability to take up quinolone resistance genes along with them, therefore ESBL producer become resistant to fluoroquinolones as well, which restricts the choice of treatment for typhoid patients (Cattoir et al., 2007).

The frequency of ESBL enzyme in clinical isolates of typhoidal salmonellae is reported as zero percent in our study. The possibility that ESBL producing typhoidal salmonellae have not been detected in study is because there are no recommendations by CLSI for ESBL detection methods in typhoidal salmonellae.

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