Available online at www.sciencedirect.com
ScienceDirect
Diagnostic Microbiology and Infectious Disease 69 (2011) 98–104
DIAGNOSTIC MICROBIOLOGY AND INFECTIOUS DISEASE
www.elsevier.com/locate/diagmicrobio

Serotypes and extended-spectrum β-lactamase types of clinical isolates of *Shigella* spp. from the Zhejiang province of China

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Received 22 June 2010; accepted 25 August 2010

Abstract

To understand the distribution of the serotypes of *Shigella* spp. and to investigate the drug-resistant genes of extended-spectrum β-lactamases (ESBLs) in *Shigella* in Zhejiang province, we have collected clinically isolated *Shigella* isolates from 2 hospitals in Zhejiang province during August to December of 2006. There are 11 *Shigella flexneri* and 13 *Shigella sonnei* isolates, respectively. Among the 11 *S. flexneri*, 9 are serotype F4 and the remaining 2 are F1a and F2a. Antimicrobial susceptibility tests revealed that 20.8% of the isolates were resistant to cefotaxime and 20.8% of the isolates were intermediate to cefotaxime. Isoelectric focusing demonstrated that the ESBL-positive isolates and their transconjugants produce a single β-lactamase with a p*I* of 7.9 or 9.0. DNA sequence analysis demonstrated that they harbor either CTX-M-14 or CTX-M-15 gene. Pulsed-field gel electrophoresis analysis showed that 9 F4 *S. flexneri* isolates belong to the same clone and 13 *S. sonnei* isolates from different regions in Zhejiang province belong to different subclones of the same clone. In summary, resistance and reduced susceptibility to β-lactam antibiotics were mainly caused by the production of CTX-M-type ESBLs. This is the first report of CTX-M-15–type ESBLs in *S. sonnei* in China.

Keywords: *Shigella* spp.; Extended-spectrum β-lactamases; CTX-M type; Serotype

1. Introduction

Shigella species remain to be the major cause of gastrointestinal infectious illness throughout the world, especially in the developing countries. The annual number of *Shigella* episodes in developing country was estimated to be 163.2 million, with 1.1 million deaths (Kotloff et al., 1999). In China, incidence of shigellosis is ranked at the top among gastrointestinal infectious illness. The predominant pathogenic species responsible for shigellosis in China are *Shigella flexneri* and *Shigella sonnei*. One of the strategies to control shigellosis is to develop vaccines, especially against the *S. flexneri*, which accounts for >60% of shigellosis in the developing countries (Kotloff et al., 1999). However, because there are 13 serotypes in *S. flexneri* and the immune response is serotype specific, 2 immunization strategies have been developed to overcome this problem. One is to use the administration of multiple vaccine isolates and the other one is to construct multivalent *S. flexneri* vaccines to provide protection against multiple serotypes (Jennison et al., 2006). Nevertheless, neither one of the above strategies is able to provide a single vaccine that is effective against all the 13 serotypes. Therefore, it is necessary to understand the distribution of the specific serotypes of *Shigella* in a certain region to develop vaccines suitable for that region.

Equal important as vaccine development for shigellosis control is the daily clinical care of patients with shigellosis in hospital. Because of the occurrence of the multi-drug–resistant isolates of *Shigella* from different countries (Jin et al., 2010; Stelling et al., 2009; Wong et al., 2010; Xiong et al., 2010), shigellosis has become a serious threat to public

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doi:10.1016/j.diagmicrobio.2010.08.027

health. Third-generation cephalosporins so far have been commonly used to treat the infections caused by multi-drug–resistant isolates. However, since the initial report of SHV-11–type β-lactamases in *Shigella dysenteriae* from India in 1999 (Ahamed and Kundu, 1999), *Shigella* species producing CTX-M, SHV, and TEM-type ESBLs have been frequently identified from different regions of the globe (Lartigue et al., 2005; Nicolas et al., 2001; Sirot et al., 2001; Xiong et al., 2006a, 2006b), most of those genes being located on conjugable plasmids. Other β-lactamases including OXA type (Siu et al., 2000), AmpC (CMY-2) (Huang et al., 2005), and metallo-β-lactamase (MET-1/IMP-3) were also reported in *Shigella* isolates isolated from Hong Kong, Taiwan, and Japan, respectively (O'Hara et al., 1998). In contrast, only a few cases of ESBL-producing *Shigella* have been reported in China so far.

To understand the distribution of the serotypes of *Shigella* spp. to guide the potential vaccine development and to investigate the drug-resistant genes of *Shigella* in Zhejiang province, we have collected clinically isolated *Shigella* isolates, analyzed the serotype of those isolates by serum agglutination test and genetic relation of *Shigella* isolates from 2 hospitals located in 2 different geographical areas of the Zhejiang province by pulsed-field gel electrophoresis (PFGE), and investigated the genotypes of ESBLs and their potential pathway of ESBLs transfer in those clinical isolates. This is the first report documenting the production of CTX-M-15–type ESBLs by *S. sonnei* in China.

2. Materials and methods

2.1. Bacteria Isolates

Twenty-four *Shigella* strains were isolated from feces of patients with acute dysentery in 2 hospitals located in 2 different geographical areas of Zhejiang province. Among them, 11 *S. flexneri* isolates and 8 *S. sonnei* isolates were from the Health Care Hospital for Maternal and Children of XiaoShan from August to December of 2006; the other 5 *S. sonnei* isolates were from ShaoXing People's Hospital.

2.2. Isolate serotyping and antimicrobial susceptibility tests

Serotyping for *S. flexneri* isolates were determined by serum agglutination test by slide agglutination (Shigella Antisera, DenkaSeiken, Tokyo, Japan). Minimal inhibitory concentrations (MICs) of 12 antimicrobials agents (imipenem, meropenem, aztreonam, ampicillin, piperacillin, piperacillin/tazobactam, ceftazidime, cefotaxime, cefepime, cefoperazone/sulbactam, ciprofloxacin, gentamicin) were determined by agar dilution according to the criteria recommended by the Clinical Laboratory Standards Institute (CLSI, 2007). ESBL production was determined by CLSI for ESBL phenotypic confirmatory tests with cefotaxime and cefotaxime/clavulanic acid or ceftazidime and ceftazidime/clavulanic acid.

2.3. Conjugal transfer experiment

Conjugal transfer experiment was performed by broth culture conjugation method (Shannon et al., 1990). *Escherichia coli* EC600 was used as recipient (Lac−, Nal^R, Rif^R) in conjugal transfer experiment. Transconjugants were selected on Muller–Hinton agar containing rifampicin (512 μg/mL) and cefotaxime (1.0 μg/mL). The selected colonies were picked up and identified by Automatic Identification Systems (VITEK, Bio-Merieux, Marcy l'Etoile, France). Plasmids from *E. coli* transconjugants of *Shigella* isolates and clinical isolates were extracted using AxyPrep Plasmid Miniprep Kit (Axygen Scientific, Union City, CA) and electrophoresed.

2.4. Isoelectric focusing

Crude β-lactamase preparations were obtained by the sonication method. Isoelectric focusing (IEF) was performed on PhastGel polyacrylamide gel (pH, 3–9; Amersham Biosciences, Uppsala, Sweden) using the PhastSystem (Pharmacia Biotech, Uppsala, Sweden) by the method of Mathew et al. (1975). β-Lactamase activity was detected by staining the gel with Nitrocefin (Oxoid, Basingstoke, England). The isoelectric points were determined after comparison to known β-lactamases TEM-1 (p*I* 5.4), SHV-7 (p*I* 7.6), and ACT-1 (p*I* 9.0).

2.5. β-Lactamase type-specific polymerase chain reaction and DNA sequencing

The primers used to amplify β-lactamase genes are listed in Table 1. Plasmids from clinical isolates and their transconjugants were used as templates in polymerase chain reaction (PCR). PCR was performed using approximately 0.3 μg of the template DNA, 0.5 μmol/L each primer, 10 mmol/L Tris–HCl, 1.5 mmol/L $MgCl_2$, 50 mmol/L KCl, 0.2 mmol/L dNTP, and 2.5 U of Taq DNA polymerase (Promega, Madison, WI) in a total volume of 50 μL. The reaction was conducted in a GeneAmp PCR System 9600 thermal cycler (Applied Biosystems, Foster City, CA). The PCR products were directly sequenced using an ABI3730 Sequencer (Applied Biosystems), and the sequences were compared with the reported sequences from GenBank.

2.6. Pulse-field gel electrophoresis

The procedures were based on the protocols of CDC PulseNet (http://www.cdc.gov/pulsenet/protocols.htm) for *S. sonnei* with slight modification. Briefly, pure bacterial cultures on Muller–Hinton agar plate were embedded into plugs of 1% SeaKem Gold Agarose (Biowhittaker, Rockland, ME) and incubated in cell lysis buffer (50 mmol/L Tris: 50 mmol/L EDTA, pH 8.0 + 1% sarcosyl) with proteinase K in a 54°C shaker water bath for 2 h with constant agitation (170 rpm), then these plugs were washed 2 times with sterile reagent-grade water and 4 times with 1× TE buffer (10 mmol/L Tris: 1 mmol/L EDTA, pH 8.0), and incubated with 50 U restriction endonuclease *Xba*I (MBI Fermentas,