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Emergence of ciprofloxacin-resistant extended-spectrum β-lactamase-producing enteric bacteria in hospital wastewater and clinical sources

Meenu Maheshwari, Nawar Hadi Yaser, Suraiya Naz, Mansha Fatima, Iqbal Ahmad *

Department of Agricultural Microbiology, Aligarh Muslim University, Aligarh 202002, India

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ABSTRACT

This study aimed to evaluate the incidence of ciprofloxacin-resistant extended-spectrum β-lactamase (ESBL)-producing enteric bacteria in hospital wastewater and clinical sources. Enteric bacteria, mainly *Escherichia coli*, were isolated from clinical sources (urinary tract and gastrointestinal tract infections; 80 isolates) and hospital wastewater (103 isolates). The antibiotic resistance profile and ESBL production of the isolates were investigated by disc diffusion assay and combined disc diffusion test, respectively. Plasmid profiling was performed by agarose gel electrophoresis, and elimination of resistance markers was performed by a plasmid curing experiment. Antibiotic susceptibility testing revealed a high incidence of β-lactam resistance, being highest to ampicillin (88.0%) followed by amoxicillin, ceftriaxone, cefpodoxime, cefotaxime, aztreonam, cefepime and ceftazidime. Among the non-β-lactam antibiotics, the highest resistance was recorded to nalidixic acid (85.7%). Moreover, 50.8% of enteric bacteria showed resistance to ciprofloxacin. Among 183 total enteric bacteria, 150 (82.0%) exhibited multidrug resistance. ESBL production was detected in 78 isolates (42.6%). A significantly higher incidence of ciprofloxacin resistance was observed among ESBL-producing enteric bacteria both in clinical ($P = 0.0015$) and environmental isolates ($P = 0.012$), clearly demonstrating a close association between ESBL production and ciprofloxacin resistance. Plasmid profiling of selected ESBL-positive strains indicated the presence of one or more plasmids of varying sizes. Plasmid curing resulted in loss of ciprofloxacin and cefotaxime resistance markers simultaneously from selected ESBL-positive isolates, indicating the close relationship of these markers. This study revealed a common occurrence of ciprofloxacin-resistant ESBL-producing enteric bacteria both in hospital wastewater and clinical sources, indicating a potential public health threat.

1. Introduction

Hospital wastewater is considered one of the major reservoirs of pathogenic bacteria. Wastewater or natural water supplies into which wastewater has been discharged are likely to contain pathogenic organisms mainly coming from human excreta [1]. Furthermore, antibiotics used in hospitals and their release into effluents and municipal sewage via patient excreta or direct deposition impose a selection pressure on bacteria leading to emergence of resistance to different classes of antibiotics in micro-organisms in the aquatic environment [2]. Besides this, wastewater from hospitals constitutes a route of dissemination of antibiotic-resistant bacteria to human and animal populations through various ecological modes of transmission including food, water and insects vectors. Moreover, in developing countries, the chance of transmission of pathogenic bacteria is more common owing to lack of adequate hygiene, poor water quality and inadequate management of human and hospital wastes [1]. Considering the impact of hospital-acquired infections, national evidence-based guidelines for preventing healthcare-associated infections in National Health Service (NHS) hospitals were commissioned by the Department of Health in England. These guidelines focused on hospital environmental hygiene, hand hygiene, the use of personal protective equipment, and the safe use and disposal of sharps; preventing infections associated with the use of short-term indwelling urethral catheters; and preventing infections associated with central venous catheters [3].

* Corresponding author. Tel.: +91 571 270 3516; fax: +91 571 270 3516.
E-mail address: ahmadiqbal8@yahoo.co.in (I. Ahmad).

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The occurrence of drug-resistant micro-organisms in hospital wastewater and clinical sources creates immense clinical and public health problems. Currently, extended-spectrum β-lactamase (ESBL)-producing enteric bacteria are globally recognised problematic multidrug-resistant (MDR) bacteria. ESBLs confer resistance to β-lactam antibiotics containing an oxyimino group (e.g. ceftazidime, cefotaxime and aztreonam). ESBL-producing organisms have been associated with various community- and hospital-acquired infections, including urinary tract infections, bacteraemia, peritonitis, nosocomial pneumonia, intra-abdominal infections and meningitis [4]. Ciprofloxacin has been regarded as a potent antimicrobial recommended for the treatment of various diseases caused by ESBL-producing enteric bacteria [4,5]. As the use of fluoroquinolones in treatment strategies is increasing, resistance to fluoroquinolones has emerged and has been documented globally. A recent surveillance study, the Study for Monitoring Antimicrobial Resistance Trends (SMART), included 38 hospitals from 10 countries in the Asia-Pacific region and found 48.6% resistance to ciprofloxacin with wide variation among different countries, being as high as 76.2% in Vietnam and 72.0% in China [6].

However, the incidence of fluoroquinolone resistance in ESBL-producing enteric bacteria is less explored in India [7]. Therefore, the aim of this study was to investigate the prevalence of ciprofloxacin resistance and ESBL production among enteric bacteria from hospital wastewater as well as clinical sources and to emphasise the impact of this potential problem on human health.

2. Materials and methods

2.1. Isolation and characterisation of enteric bacteria and growth conditions

Enteric bacteria from clinical sources were isolated from urine samples of patients with urinary tract infection and from stool samples of patients with gastrointestinal tract infection (mainly diarrhoea) at Jawaharlal Nehru Medical College, Aligarh Muslim University (Aligarh, India) and Ashok Pathology and Research Centre (Aligarh, India). Wastewater samples were collected from the Jawaharlal Nehru Medical College Hospital wastewater discharging point during the months December 2011 to February 2012. Fifty wastewater samples were taken using sterile bottles. All of the samples were transported to the laboratory within 1 h for bacterial isolation and were analysed as follows: 0.1 mL of appropriate dilutions were placed on MacConkey and eosin methylene blue agar plates (Hi-Media Laboratories Pvt. Ltd., Mumbai, India) in duplicate and the plates were incubated at 37 °C for 18 h. Following incubation, distinct colonies were screened and purified by repeated screening. The clinical isolates obtained were subcultured in the laboratory on MacConkey and eosin methylene blue agar plates. Pure cultures of all bacterial isolates were characterised biochemically and were identified according to standard protocols [8,9]. The isolated bacteria were stored in Luria–Bertani broth (Hi-Media Laboratories Pvt. Ltd.) with 40% glycerol at −70 °C until use.

2.2. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by the standard disc diffusion method on Mueller–Hinton agar medium (Hi-Media Laboratories Pvt. Ltd.). Minimum inhibitory concentrations (MICs) were determined by the broth macrodilution method according to the criteria established by the Clinical and Laboratory Standards Institute (CLSI) [10].

2.3. Detection of extended-spectrum β-lactamase production

All isolates were tested for ESBL production by the combined disc diffusion test according to the criteria established by the CLSI [10] using cefotaxime (30 μg) and ceftazidime (30 μg) alone and in combination with clavulanic acid (10 μg). A ≥5 mm increase in the diameter of the zone of inhibition of around the disc with antibiotic plus clavulanic acid compared with the disc with antibiotic without clavulanic acid was considered positive for ESBL production. *Escherichia coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC 700603 were used as control strains.

2.4. Isolation of plasmid DNA and agarose gel electrophoresis

Plasmids were isolated from drug-resistant enteric bacteria by the method of Sambrook and Russell [11] and were characterised by agarose gel electrophoresis in 0.7% agarose gel in a TAE (Tris–acetate–ethylene diamine tetra-acetic acid) buffer at room temperature at 7 V/cm for 4 h. Plasmid DNA bands were visualised and photographed using Gel Documentation with ultraviolet transilluminator (Gel Doc™; Bio-Rad, Hercules, CA).

2.5. Determination of plasmid-encoded antibiotic resistance by a plasmid curing experiment

Plasmid curing using ethidium bromide (EtBr) was carried out as described by Singh and Yadava [12]. The MIC of the curing agent was determined by the broth macrodilution method against the test isolates. The MIC was defined as the concentration of curing agent that inhibited visible growth of test organisms. A range of sub-MIC concentrations was selected to treat the culture. Briefly, 0.1 mL of overnight grown culture (diluted to 10^5 CFU/mL) was inoculated in each tube containing different concentrations of curing agent as well as a control broth tube without curing agent. The culture tubes were incubated at 37 °C for 18 h. Culture broth was diluted in sterile normal saline and was spread on nutrient agar plates and incubated at 37 °C overnight. Isolated colonies were replica plated onto nutrient agar plates containing cefotaxime (64 μg/mL) or ciprofloxacin (4 μg/mL). A plate without antibiotic was simultaneously inoculated as a control. The experiment was performed in duplicate. The curing frequency was calculated as the mean count of the colonies from antibiotic agar plates that did not grow/total mean colonies tested × 100.

2.6. Statistical analysis

The relationship between ESBL production and ciprofloxacin resistance was evaluated statistically using Fisher's exact χ^2 test.

3. Results

A total of 183 isolates comprising *E. coli* (n = 101), *K. pneumoniae* (n = 17), *Enterobacter* spp. (n = 21), *Salmonella* spp. (n = 18), *Shigella* spp. (n = 9) and *Proteus* spp. (n = 17) were isolated, of which 80 were from clinical sources and 103 were isolated from hospital wastewater (Supplementary Table S1).

Supplementary table related to this article can be found, in the online version, at doi:10.1016/j.jgar.2016.01.008.

The incidence of antibiotic resistance against individual antibiotics was determined for 16 antibacterial agents. Overall, the bacterial isolates showed the highest resistance to ampicillin (88.0%), followed by amoxicillin (83.1%), nalidixic acid (79.2%), ceftriaxone (78.7%), tetracycline (78.1%), cefpodoxime (76.5%), cefotaxime (72.7%), aztreonam (71.6%), cefepime (71.0%), doxycycline (70.5%), nitrofurantoin (57.9%), ceftazidime (55.7%), erythromycin (54.6%), ciprofloxacin (50.8%), gentamicin (40.4%) and