Differential expression of ompC and ompF in multidrug-resistant Shigella dysenteriae and Shigella flexneri by aqueous extract of Aegle marmelos, altering its susceptibility toward beta-lactam antibiotics

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Differential expression of *ompC* and *ompF* in multidrug-resistant *Shigella dysenteriae* and *Shigella flexneri* by aqueous extract of *Aegle marmelos*, altering its susceptibility toward β-lactam antibiotics

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Abstract

Steadily increasing resistance among *Shigella* to β-lactams, aminoglycosides, and tetracycline has compromised the utility of these commonly used antimicrobial agents. Also, undesirable side effects of certain antibiotics have triggered immense interest in search of alternative therapies using medicinal plants. One such medicinal plant used since ancient times to cure diarrhea is *Aegle marmelos*. The present study exemplifies the susceptibility of β-lactam–resistant *Shigella dysenteriae* and *Shigella flexneri* toward β-lactam antibiotics, when grown in the presence of aqueous extract of *A. marmelos* (AEAM), by altering porin channels. This was demonstrated by antibiotic sensitivity test using disc diffusion method and MIC test. Susceptibility toward β-lactam antibiotic is associated with changes in outer membrane porins OmpC (∼42 kDa) and OmpF (∼38 kDa) and cytosolic proteins of ∼26 kDa, OmpR, a transcriptional regulator. Expression of *ompF* is increased in *S. dysenteriae* and *S. flexneri* grown in the presence of AEAM due to down-regulation of *ompR*, which is conformed by reverse transcriptase polymerase chain reaction. In conclusion, AEAM influences susceptibility of β-lactam–resistant *Shigella* toward β-lactam antibiotics by altering porin channels. Hence, AEAM along with β-lactam can be used for treatment of multidrug-resistant *Shigella*. © 2008 Elsevier Inc. All rights reserved.

Keywords: *Shigella; Aegle marmelos; β-lactams; Multidrug resistant; OmpR; OmpC; OmpF; Porin channels*

1. Introduction

Bacillary dysentery is an acute inflammatory bowel disease caused by the enteroinvasive bacteria *Shigella*, leading to a condition known as shigellosis (Hale, 1998). Shigellae are Gram-negative bacteria that have the ability to invade the colonic and rectal epithelium in humans, causing the acute mucosal inflammation that characterizes the disease. Shigellosis is a major health problem worldwide, causing 1 million deaths and 163 million cases of dysentery annually, mainly in the developing countries, in areas where personal and general hygiene is inadequate. Almost 70% of the cases of shigellosis were found to occur in children below the age of 5 years (Kotlo et al., 1999). There are 4 species of *Shigella. Shigella dysenteriae* (*Shiga bacillus*) accounts for brisk and deadly epidemics in the poorest populations. *Shigella flexneri* accounts for the endemic form of the disease. *Shigella sonnei* is prevalent in the industrialized world, whereas *Shigella boydii* is only observed in the Indian subcontinent. *Shigella* is a highly contagious microorganism because as few as 10 to 100 bacteria can cause the disease in adult volunteers. After oral contamination, bacteria pass through the stomach and the small intestine before reaching the colon where they invade the mucosa, initiating the acute destructive rectocolitis that causes the dysenteric symptoms: fever, intestinal cramps, and emission of mucopurulent and bloody stools. Many factors such as the 220-kbp plasmid, invasive plasmid antigens, antioxidant enzymes, lipopolysaccharides, and outer membrane proteins (OMPs) contribute to its virulence. Frequency of strains resistant to ampicillin, trimethoprim–sulfamethoxazole, streptomycin, chloramphenicol, tetracycline, and cephalosporins (ceftriax-
one or ceftazidime) is a cause of growing concern (Bennish and Salam, 1992; Cohen, 1994; Sader et al., 2003). Bacterial resistance to these antibiotics is due to the following 4 factors: a) alteration of the drug targets (Spratt, 1994), b) degradation of the drug by enzymes such as β-lactamases (Livermore, 1995), c) decrease in permeability of the cell envelope (Lee et al., 1991, 1992; Masuda et al., 1995; Nikaido, 1994; Parr et al., 1987), and d) extrusion of the drugs by efflux pumps of broad specificity (Nikaido, 1996). The permeability factor controlling the access of the antibiotic to its target is often critical in determining the efficacy of β-lactam against a Gram-negative bacterium (Harder et al., 1981; Nikaido and Normark, 1987). Clinical isolates of Shigellae, which are lagging or having mutated porin OmpF (~39 kDa), were resistant to a number of β-lactam antibiotics (Kar et al., 1997; Poole, 2002). Expression of ompF and ompC play an important role in antibiotic resistance (Clarke et al., 2003). Hence, strategies aimed at outer membrane barriers can be effective at reversing antimicrobial resistance in these organisms.

The emergence of multiple drug resistance in Shigellae has necessitated a search for alternative therapeutic strategies. There is a growing interest in traditionally used medicinal plants that produce a variety of compounds having therapeutic properties (Chopra et al., 1992; Harborne and Baxter, 1995; Iyengar, 1985). Compounds that can either inhibit the growth of pathogens or kill them without harming host cells are considered candidates for developing new antimicrobial therapeutic strategies. In recent years, antimicrobial properties of medicinal plants are being increasingly reported from different parts of the world (David, 1997; Nimri et al., 1999; Saxena, 1997; Saxena and Sharma, 1999; Silva et al., 1996). It is expected that plant extracts showing positive virulence by modified Sereny test (Murayama et al., 1986) were chosen for the studies. A single colony from Luria–Bertani (LB) agar plate was inoculated in Luria–Bertani broth and incubated for 18 h at 37 °C, with constant shaking at 200 rev/min.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Clinical isolates of S. dysenteriae and S. flexneri were obtained from Dr. Mary V. Jesudason, Department of Microbiology, Christian Medical College, Vellore, India. Bacterial strains showing positive virulence by modified Sereny test (Murayama et al., 1986) were chosen for the studies. A single colony from Luria–Bertani (LB) agar plate was inoculated in Luria–Bertani broth and incubated for 18 h at 37 °C, with constant shaking at 200 rev/min.

2.2. Preparation of AEAM

Fruits of A. marmelos were collected from Vaniyambadi, Tamil Nadu, India. The fruits were shade dried, seeds were removed, and fruits were ground mechanically. Powdered fruit (500 g) were extracted with 1 L of water for 12 h. The resulting extract was filtered using Whatman No. 1 filter paper. The filtrate was evaporated in vacuum to give a residue. (yield, 2.98%).

2.3. Antibiotic sensitivity test by disc diffusion method

Antibiotic sensitivity of test strains was determined by the standard disc diffusion method (Baur et al., 1966) against ampicillin (A10), nalidixic acid (Na30), kanamycin (K30), gentamycin (G30), neomycin (N30), penicillin (P30), norfloxin (Nr30), carbenicillin (Cb100), vancomycin (Va30), erythromycin (E15), chloramphenicol (C30), novobiocin (Nv 30), tetracycline (T30), rifampicin (R5). All antibiotic discs were purchased from HiMedia Pvt., Mumbai, India.

2.4. Determination of MIC

The antimicrobial susceptibility test was done by the 2-fold broth macrodilution method according to the National Committee for Clinical Laboratory Standards (NCCLS, 1997). The wild bacteria and bacteria treated with AEAM at final concentrations of about 7 × 10^5 CFU/mL were separately inoculated into LB broth with various concentrations of ampicillin, penicillin, and vancomycin. The MIC of each antibiotic was determined by enumerating colonies on the agar plates after incubation for 18 h at 37 °C. The percentage of growth inhibition was calculated by comparing the growth of bacteria alone.

2.5. Plasmid isolation

Isolation of plasmid DNA was done using plasmid mini preparation kit obtained from GeNei™, Bangalore, according to the manufacturer’s instruction. Plasmids were detected
by electrophoresis in 0.7% agarose gel containing 0.5 μg of ethidium bromide per milliliter and photographed with ultraviolet light illumination (Sambrook et al., 1989).

2.6. Preparation of membrane and cytosol fractions

OMPs were isolated as described previously (Kumar et al., 2001). Overnight cultures were centrifuged for 5 min at 7000g. Pellets were washed once with 20 mmol/L Tris, 10 mmol/L EDTA, pH 8 (TE), then resuspended in the same buffer. Bacteria were disrupted by sonication for 1 min, followed by a 2-min rest, then an additional 1-min sonication. Samples were centrifuged for 5 min at 7000g to remove debris, and the resulting supernatant was centrifuged for 1 h at 60 000g at 4 °C. The clear supernatant was retained as cytosolic fraction. The pellet was resuspended in TE, and the protein concentration was estimated, and equal concentration of protein fraction were run on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The gels were stained with Coomassie brilliant blue R-250.

2.7. Isolation of RNA

Exponentially growing bacterial cell reaching the optical density (OD) of 1.0 at 540 nm was harvested by centrifugation (5 min, 3800g, 4 °C). For preparation of total RNA, the phenol–guanidinium thiocyanate-based Tri Reagent (GeNei™) was used. To 10⁸ bacterial cells, 1 mL of Tri reagent was added and lysed by repetitive pipetting and allowed to stand for 5 min followed by addition of 200 μL of chloroform for phase separation, and then vigorously vortexed for 15 s and allowed to stand for 15 min followed by centrifugation at 12 000g for 15 min at 4 °C. The upper aqueous layer containing RNA was transferred to a fresh sterile diethylpyrocarbonate (DEPC)-treated microfuge tube. To this, 250 μL of ice-cold isopropanol was added, gently mixed, and allowed to stand for 10 min and centrifuged at 12 000g for 15 min at 4 °C. The supernatant was discarded, and the RNA pellet was washed with 1 mL of 75% ethanol in DEPC-treated water and again centrifuged at 14 000g for 10 min at 4 °C to get the total RNA. This pellet was dissolved in 25 μL of sterile RNase-free water by heating at 55 °C for 20 min and stored at −20 °C until use.

Table 2
Antibiotic sensitivity test for S. dysenteriae and S. flexneri grown in the absence and presence of AEAM (inhibition zone in centimeter)

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>S. dysenteriae</th>
<th>S. dysenteriae grown in the presence of AEAM</th>
<th>S. flexneri</th>
<th>S. flexneri grown in the presence of AEAM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin (A10)</td>
<td>0.0</td>
<td>R</td>
<td>2.8</td>
<td>S</td>
</tr>
<tr>
<td>Nalidixic acid (Na30)</td>
<td>0.0</td>
<td>R</td>
<td>0.9</td>
<td>S</td>
</tr>
<tr>
<td>Kanamycin (K30)</td>
<td>1.6</td>
<td>I</td>
<td>2.4</td>
<td>S</td>
</tr>
<tr>
<td>Gentamicin (G30)</td>
<td>1.3</td>
<td>S</td>
<td>2.8</td>
<td>S</td>
</tr>
<tr>
<td>Neomycin (N30)</td>
<td>0.0</td>
<td>S</td>
<td>1.9</td>
<td>S</td>
</tr>
<tr>
<td>Penicillin (P 30)</td>
<td>0.0</td>
<td>R</td>
<td>2.8</td>
<td>S</td>
</tr>
<tr>
<td>Norfloxin (N30)</td>
<td>2.8</td>
<td>S</td>
<td>3.3</td>
<td>S</td>
</tr>
<tr>
<td>Carbenicillin (Cb100)</td>
<td>0.0</td>
<td>R</td>
<td>0.0</td>
<td>R</td>
</tr>
<tr>
<td>Vancomycin (Va30)</td>
<td>0.0</td>
<td>R</td>
<td>2.6</td>
<td>S</td>
</tr>
<tr>
<td>Erythromycin (E15)</td>
<td>2.1</td>
<td>I</td>
<td>3.4</td>
<td>S</td>
</tr>
<tr>
<td>Chloramphenicol (C30)</td>
<td>1.9</td>
<td>I</td>
<td>2.1</td>
<td>S</td>
</tr>
<tr>
<td>Novobiocin (Nv 30)</td>
<td>1.0</td>
<td>R</td>
<td>2.4</td>
<td>S</td>
</tr>
<tr>
<td>Tetracycline (T30)</td>
<td>2.1</td>
<td>S</td>
<td>3.0</td>
<td>S</td>
</tr>
<tr>
<td>Rifampicin (R5)</td>
<td>1.2</td>
<td>R</td>
<td>1.2</td>
<td>R</td>
</tr>
</tbody>
</table>

The above table represents inhibitory zones of S. dysenteriae and S. flexneri in the absence and presence of AEAM. R = resistance; S = sensitive. (Values in the above table represent the average of 3 experiments.)
2.8. Reverse transcriptase polymerase chain reaction

To synthesize cDNA, a reverse transcription reaction solution containing the following reagents, 1.0 μg total RNA in RNase/DNase-free water and 1.5 μL of random hexamer primer (GeNei™), were incubated for 10 min at 72 °C and chilled immediately. To this, 5.0 μL of premixed 10 mmol/L deoxy nucleotide triphosphate (dNTP) solution (GeNei™), 3.0 μL 10× Moloney Murine leukemia virus (M-MLV) reverse transcriptase (RT) buffer (GeNei™), and 1.0 μL (200 units/μL) M-MLV RT (GeNei™) were added and made up to 50 μL using sterile RNase/DNase-free water.

To amplify the cDNA, we used polymerase chain reaction (PCR) ready mix (GeNei™) according to the manufacturer’s instruction. All PCR samples were denatured at 94 °C for 5 min before cycling and were extended for 10 min at 72 °C after cycling. The PCR assay using primers was performed.

Table 3
Antibiotic sensitivity test for S. dysenteriae and S. flexneri grown in the absence and presence of AEAM (inhibition zone in centimeter)

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Penicillin</th>
<th>Ampicillin</th>
<th>Vancomycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria</td>
<td>S. dysenteriae</td>
<td>S. flexneri</td>
<td>S. dysenteriae</td>
</tr>
<tr>
<td>AEAM</td>
<td>Presence</td>
<td>Absence</td>
<td>Presence</td>
</tr>
<tr>
<td>Concentration (mg/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.025</td>
<td>0</td>
<td>19</td>
<td>0</td>
</tr>
<tr>
<td>0.05</td>
<td>0</td>
<td>45</td>
<td>0</td>
</tr>
<tr>
<td>0.1</td>
<td>0</td>
<td>90</td>
<td>0</td>
</tr>
<tr>
<td>0.2</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>0.4</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>0.8</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>1.6</td>
<td>6.6</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>3.2</td>
<td>17.8</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>6.4</td>
<td>33</td>
<td>100</td>
<td>6.6</td>
</tr>
<tr>
<td>12.5</td>
<td>36</td>
<td>100</td>
<td>17.8</td>
</tr>
<tr>
<td>50</td>
<td>49</td>
<td>100</td>
<td>33</td>
</tr>
<tr>
<td>100</td>
<td>56</td>
<td>100</td>
<td>36</td>
</tr>
<tr>
<td>200</td>
<td>69</td>
<td>100</td>
<td>49</td>
</tr>
<tr>
<td>400</td>
<td>79</td>
<td>100</td>
<td>56</td>
</tr>
<tr>
<td>800</td>
<td>91</td>
<td>100</td>
<td>69</td>
</tr>
<tr>
<td>1600</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>3200</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

The above table represents MIC of S. dysenteriae and S. flexneri in the absence and presence of AEAM. (Values in the above table represent the average of 3 experiments.)
for 39 cycles at 94 °C for 60 s, 60 °C for 60 s, and 72 °C for 60 s. Primers for ompF, ompC, ompA, ompR, envZ and 16s rRNA are shown in Table 1. Primers were designed using primer3 software available free on http://fokker.wi.mit.edu/primer3/input.htm, and nucleic acid sequence was accessed from http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?val=NC_007606. The primers were purchased from Integrated DNA Technologies (Coralville, IA).

3. Results

3.1. MIC of A. marmelos

MIC of A. marmelos was determined by the serial dilution experiment. The numbers of colonies (CFU/mL) were gradually decreased with increase in AEAM concentration, and the optimal inhibitory concentration of the extract was found to be 300 μg/mL. The concentration below this (i.e., 200 μg/mL) was used for further study.

3.2. Antibiotic sensitivity test by disc diffusion method

Antibiotic sensitivity of both wild- and AEAM-treated S. dysenteriae and S. flexneri was quantitatively and qualitatively assessed by the presence or absence of inhibition zone and zone diameter, respectively, as given in Table 2 and Fig. 1. Inhibition zones of S. dysenteriae and S. flexneri grown in the presence of AEAM are 2.8 and 2.6 cm, 2.8 and 2.4 cm, and 2.6 and 2.1 cm for penicillin, ampicillin, and vancomycin, respectively. The susceptibility tests were repeated for each antibiotic at least 3 times.

3.3. MIC of antibiotics

Table 3 shows MIC of penicillin, ampicillin, and vancomycin on wild and AEAM-treated S. dysenteriae and S. flexneri. MIC of penicillin for wild S. dysenteriae and S. flexneri was high, 0.4 and 1.6 mg/L, respectively. MIC of penicillin on AEAM-treated S. dysenteriae and S. flexneri was 0.012 and 0.05 mg/L, respectively.

For wild S. dysenteriae and S. flexneri, MIC for ampicillin was 0.1 and 0.4 mg/L, respectively. AEAM-treated S. dysenteriae and S. flexneri showed very low MIC for ampicillin, that is, 0.025 and 0.035 mg/L, respectively. MIC of vancomycin for wild S. dysenteriae and S. flexneri was high, 0.1 and 0.4 mg/L, respectively. MIC of vancomycin for AEAM-treated S. dysenteriae and S. flexneri were very low, 0.05 and 0.05 mg/L, respectively.

3.4. Plasmid profile

Plasmid profile did not show any significant difference in treated Shigella when compared with the wild type (data not shown).

3.5. Analysis of cytosolic, inner membrane, and OMPs

S. dysenteriae and S. flexneri were grown overnight at 37 °C in medium without and with AEAM (200 μg/mL). The proteins were fractionated and subjected to SDS-PAGE. SDS-PAGE of the cytosolic inner membrane and outer membrane were shown in Fig. 2A, B, and C, respectively. Cytosolic fraction of S. dysenteriae and S. flexneri grown in the presence of AEAM showed significant difference with decreased levels of ~26-kDa protein (Fig. 2A, lane 1 versus lane 2 for S. dysenteriae and lane 3 versus lane 4 for S. flexneri) (Laemmli, 1970).

The inner membrane fractions of S. dysenteriae and S. flexneri grown in the presence and in the absence of AEAM did not show any significant difference (Fig. 2B, lane 2 versus lane 1 for S. dysenteriae and lane 4 versus lane 3 for S. flexneri) (Laemmli, 1970). Fig. 2. (A) Cytosolic protein profile of S. dysenteriae and S. flexneri grown in the absence and presence of AEAM. SDS-PAGE, 12%. Lane 1 was loaded with molecular weight marker. Lanes 2 and 3 are S. dysenteriae grown in the absence and presence of AEAM, and lanes 4 and 5 are S. flexneri grown in the absence and presence of AEAM, respectively. (B) Inner membrane protein profile of S. dysenteriae and S. flexneri grown in the absence and presence of AEAM. SDS-PAGE, 12%. Lanes 1 and 2 are S. dysenteriae grown in the absence and presence of AEAM, and lanes 4 and 5 are S. flexneri grown in the absence and presence of AEAM, respectively. Lane 3 was loaded with molecular weight marker. (C) OMP profile of S. dysenteriae and S. flexneri grown in the absence and presence of AEAM. SDS-PAGE, 12%. Lanes 1 and 2 are S. dysenteriae grown in the absence and presence of AEAM, and lanes 3 and 4 are S. flexneri grown in the absence and presence of AEAM, respectively. Lane 5 was loaded with molecular weight marker.
lane 3 for *S. flexneri*). In the presence of AEAM, the outer membrane fraction of *S. dysenteriae* and *S. flexneri* showed significantly higher levels of ∼38-kDa protein and significantly lower levels of ∼42-kDa protein when compared with their levels in *Shigella* grown in the absence of AEAM (Fig. 2C, lane 2 versus lane 1 for *S. dysenteriae* and lane 4 versus lane 3 for *S. flexneri*). In addition, there were other minor differences between protein profiles of the bacteria grown in the presence and in the absence of the AEAM.

### 3.6. Expression analysis of ompF, ompC, ompA, ompR, envZ, and 16S rRNA

In our study, we used RT-PCR to determine the expression levels of the porin transcripts. This method potentially eliminates some of the difficulties associated with subjective interpretation of OMP profiles. *S. dysenteriae* and *S. flexneri* grown in presence of AEAM showed decreased amounts of RNA transcripts of the *ompC* and *ompR* genes compared with the *S. dysenteriae* and *S. flexneri* grown in the absence of AEAM (Fig. 3A), whereas levels of RNA transcript of *ompF* were increased in *S. dysenteriae* and *S. flexneri* grown in the presence of AEAM when compared with *S. dysenteriae* and *S. flexneri* grown in the absence of AEAM (Fig. 3B). *ompA* and *envZ* does not show any significant changes between *Shigella* grown in the presence and absence of AEAM. 16S rRNA was used as internal control.

### 4. Discussion

Emergence of multidrug resistance in pathogenic *Shigella* as well as undesirable side effects of certain antibiotics has triggered immense interest in the search for alternative antimicrobial strategies to combat these organisms. In the
present study, we show the synergistic effect of AEAM on β-lactam-resistant clinical isolate of *S. dysenteriae* and *S. flexneri*, which increases susceptibility toward β-lactam antibiotics. Initially, we compared the antibiotic susceptibility of *S. dysenteriae* and *S. flexneri* grown in the absence and in the presence of AEAM (200 μg/mL), which revealed marked difference in inhibition zones of ampicillin (A10), nalidixic acid (Na30), penicillin (P 30), carbencillin (Cb100), vancomycin (Va30), novobiocin (Nv 30), and tetracycline (T30).

Table 3 shows the MIC of antibiotics on *S. dysenteriae* and *S. flexneri* grown in the presence and absence of AEAM. The MIC of penicillin, ampicillin, and vancomycin for *S. dysenteriae* and *S. flexneri* grown in the presence of AEAM shifted to a lower concentration, which was higher in the absence of AEAM. This clearly shows that the presence of AEAM influences susceptibility toward β-lactam antibiotics.

The large 220-kb invasion plasmids of *Shigella* are necessary for their pathogenesis and antibiotic resistance (Honma et al., 2003; Watanabe and Nakamura, 1985). However, plasmid loss was not observed in both *S. dysenteriae* and *S. flexneri* grown in the presence of AEAM. Also, β-lactamase activity (O’Caflaghia et al., 1972) of the *S. dysenteriae* and *S. flexneri* grown in the presence and in the absence of AEAM was unchanged as determined by spectrophotometric assays when the chromogenic nitrocefin, β-lactam, was used as substrate (data not shown). This suggests that factors other than plasmid-encoded factors and β-lactamases were involved in observed β-lactam susceptibility.

Outer membrane profiles of *S. dysenteriae* and *S. flexneri* grown in the presence and absence of AEAM showed significant differences (Fig. 2C). In the presence of AEAM, the outer membrane fraction of *S. dysenteriae* and *S. flexneri* showed significantly higher levels of ~38-kDa protein and significantly lower levels of ~42-kDa protein when compared with *Shigella* grown in the absence of AEAM.

In *Shigella* and other bacteria, the absence or mutation of ~39-kDa porin affected largely the susceptibility to slowly penetrating β-lactams such as the dianionic moxalactam and aztreonam, as well as low permeability of hydrophilic antibiotics such as piperacillin and penicillin (Kar et al., 1997; Nakae, 1995; Pangon et al., 1989; Van de Klundert et al., 1988). This can be correlated to the observed resistance in *Shigella* grown in the absence of AEAM and the observed susceptibility due to expression of ~38-kDa protein in *Shigella* grown in the presence of AEAM. This ~38-kDa protein was confirmed as OmpF, a porin and ~42-kDa protein as OmpC, also a porin, by RT-PCR. Expression of these *ompF* and *ompC* at the transcriptional levels showed significant variations, that is, levels of RNA transcript of *ompF* were increased in *Shigella* grown in the presence of AEAM when compared with *Shigella* grown in absence of AEAM, whereas levels of RNA transcript of *ompC* was decreased as shown in Fig. 3A and B.

The reciprocal transcription of *ompF* and *ompC* is regulated by the active form of OmpR (Yoshida et al., 2006) and env Z, which phosphorylates OmpR by its histidine kinase and dephosphorylates by its phosphatase activity (Aiba et al., 1989). Differential expression of *ompF* and *ompC* is a direct consequence of the intracellular concentrations of the active form of OmpR (Russo and Silhavy, 1991). Overexpression of *ompF* is due to decreased expression of ~26-kDa protein by SDS-PAGE and RNA transcript of *ompF* by RT-PCR in *Shigella* grown in the presence of AEAM.

In conclusion, AEAM influences susceptibility of β-lactam-resistant *Shigella* toward β-lactam antibiotics by altering porin channels. Hence, combinational therapy for AEAM with β-lactam antibiotics can be used for treating multidrug-resistant *Shigella* without any risk of side effects.

**Acknowledgment**

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**References**


