Molecular detection and antimicrobial resistance of diarrheagenic *Escherichia coli* strains isolated from diarrheal cases

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**ABSTRACT**

**Objectives:** To identify and classify Iranian isolates of diarrheagenic *Escherichia coli* (*E. coli*) on the basis of presence of virulence genes and to determine antibiotic susceptibility of isolated strains.

**Methods:** The current cross-sectional study was conducted in 2005 at the Pasteur Institute, Tehran, Iran. One hundred and ninety-three diarrheagenic *E. coli* isolated from diarrheal patients in different regions of Iran were included in current study. Virulence factor genes for diarrheagenic *E. coli* were detected by polymerase chain reaction.

**Results:** Of the 193 diarrheagenic *E. coli* detected by PCR, 86 (44.5%) were Shiga toxin-producing *E. coli* (STEC), 74 (38.4%) enteropathogenic *E. coli* (EPEC), 19 (9.8%) enteroaggregative *E. coli* isolates, and 14 (7.3%) enterotoxigenic *E. coli* isolates. Susceptibility to 12 clinically important antimicrobial agents was determined for 193 strains of diarrheagenic *E. coli*. A high incidence of resistance to tetracycline (63%), ampicillin (62%), streptomycin (56%), amoxicillin/clavulanic acid (44.5%), trimethoprim/sulfamethoxazole (39.5%), and cephalothin (37%) was observed.

**Conclusion:** The STEC and EPEC strains with high resistance to tetracycline and ampicillin, but highly susceptible to quinolones are among the most important causative agent of diarrhea in Iran. This study suggests that antimicrobial resistance is widespread among *E. coli* strains colonizing Iranian patients. Guidelines for appropriate use of antibiotics in developing countries require updating.

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Resistence to antibiotics is very common in bacterial isolates all around the world.\textsuperscript{1-4} Regular surveillance of antibiotic resistance provides data for antibiotic therapy and resistance control.\textsuperscript{5,6} Information on antimicrobial resistance patterns is important in choosing the appropriate antibiotic therapy, as even if microbiology laboratory services are accessible, antibiotic susceptibilities patterns will generally be known after 72 hours, and acute enteritis is an important cause of mortality and morbidity among children in developing countries. Diarrhea caused by multidrug-resistant bacteria is an important public health dilemma among children and is set as a research priority of the control of diarrheal disease program for developing countries by the World Health Organization. The strains of the different diarrheagenic \textit{Escherichia coli} (\textit{E. coli}), namely, Enterotoxigenic \textit{E. coli} (ETEC), Shiga toxin-producing \textit{E. coli} (STEC), Enteropathogenic \textit{E. coli} (EPEC), Enteroinvasive \textit{E. coli} (EIEC), and enterohaemorrhagic \textit{E. coli} (EAEC) are among the most important causes of acute enteritis.\textsuperscript{7} In Iran, based on WHO guidelines, children with diarrhea are treated by oral rehydration salt (ORS). However, in most cases, an antibiotic (currently ampicillin or co-trimoxazole) is also administered. Therefore, treatment of patients with antibiotics requires knowledge of the antimicrobial resistance patterns of the most prevalent diarrheagenic bacteria. Antimicrobial therapy should be restricted to severe diarrheal cases as well as traveler's diarrhea.\textsuperscript{8} However, because of overuse and misuse of antibiotics in the treatment of diarrhea, antibiotic resistance is progressively increasing among enteric pathogens in our country. The main purpose of this study was to identify and classify Iranian isolates of diarrheagenic \textit{E. coli} on the basis of presence of virulence genes and to determine antibiotic susceptibility of isolated strains.

\textbf{Methods.} \textit{Bacterial strains.} One hundred and ninety-three diarrheagenic \textit{E. coli} isolated from diarrheal patients in different regions of Iran were included in the current study. Ethical approval was obtained for this study. These strains included rural and urban isolates of Central, Western, and Northern parts of Iran including strains from Tehran, the capital city of Iran. The isolates were identified by a combination of conventional and molecular bacteriological methods at Pasteur Institute, Tehran, Iran in 2005.\textsuperscript{9}

\textit{PCR detection.} The organisms were classified as EAEC, EPEC, ETEC, and STEC based on the specific virulence genes detected by polymerase chain reaction (PCR). For DNA extraction, a loopful of bacterial growth was taken directly from the confluent area of the culture, suspended in 0.5 ml of sterile, distilled water and boiled for 20 minutes. These samples were subjected to 5 different PCR reactions targeting Shiga toxinogenic (\textit{stx}) gene,\textsuperscript{10} \textit{Escherichia coli} attaching and effacing (\textit{eae}) gene,\textsuperscript{11} heat-labile (\textit{LT}) enterotoxin (\textit{eltB}) encoding gene,\textsuperscript{12} heat-stable (\textit{ST}) enterotoxin (\textit{estA}) encoding gene,\textsuperscript{12} and pCVD 432 plasmid.\textsuperscript{13} The primers and amplification procedures are shown in Table 1. The \textit{E. coli} strains that carried \textit{eae} gene and were negative for \textit{stx} gene were considered as EPEC. The strains that were positive by PCR for pCVD432 were interpreted as EAEC and those positive for \textit{stx} gene and positive or negative for \textit{eae} gene as STEC. The \textit{E. coli} strains positive for \textit{LT}-producing gene, \textit{ST}-producing gene, or both, were considered to be ETEC. Positive controls were \textit{E. coli} ATCC 35401 (\textit{LT}+, \textit{ST}+), \textit{E. coli} ATCC 43894 (\textit{stx}+, \textit{eae}+), and \textit{E. coli} RH6420 (\textit{E. coli} 17-2, EAEC). The negative control was sterile distilled water.

\begin{table}[h]
\centering
\begin{tabular}{llll}
\hline
Pathogen & Target & Primer pair & Amplimer (No. of bp) & Reference \\
\hline
STEC & \textit{stx} & F: GAACGAAAATAATTATATGTR, TTTGTATTGTACAGTCAT & 900 & \textit{Lin} et al\textsuperscript{16} \\
EPEC & \textit{eae} & F: CATATTGGACGCCAGGTTT, R: ATTCTCTCGGACTGCTGTC & 790 & \textit{Beaudry} et al\textsuperscript{11} \\
ETEC-LT & \textit{eltB} & F: TCTCTATGGTAGCTACCGGAC, R: CCAATCTAATCGGCAAT & 322 & \textit{Blomen} et al\textsuperscript{12} \\
ETEC-ST & \textit{estA} & F: GCTAAACAGTAGAGTCACAAAAAT, R: CCCGCTACAGGAGCACTACAACA & 147 & \textit{Blomen} et al\textsuperscript{12} \\
EAEC & pCVD432 & F: CTTGCCAAAGAGCTTATCAT, R: CAATGTATGAAAATCCGCGT & 630 & \textit{Schmidt} et al\textsuperscript{13} \\
\hline
\end{tabular}
\caption{Amplimer and primers of PCR for diarrheagenic \textit{Escherichia coli}.}
\end{table}

Antimicrobial susceptibility testing. Antimicrobial susceptibility pattern was determined by Kirby-Bauer disk diffusion method according to recommendation proposed by the clinical and laboratory standards institute (CLSI). The antibiotics tested were ampicillin (AM-10), cefoxitin (FOX-30), chloramphenicol (C-30), ceftriaxone (CRO-30), cephalexin (CF-30), gentamicin (GM-10), nalidixic acid (NA-30), ciprofloxacin (CIP-5), tetracycline (Te-30), sulfamethoxazole/trimethoprim (SXT), amoxicillin/clavulanic acid (AmC-30) and streptomycin (S-10) (BD BBLTM Sensi-DiscTM). The E. coli ATCC 25922, ATCC 35218, and Pseudomonas aeruginosa ATCC 27853 were tested along with the isolates for quality control purposes. The result was documented according to performance range of inhibition zone of the control strains. It was considered that in vitro resistance to cephalothin, a commonly tested cephalosporin, may not predict resistance to other cephalosporins including cefazolin, cefuroxime, cefpodoxime, cefprozil, and loracarbef.

Data interpretation. Data were interpreted by susceptible, intermediate, or resistant percent of strains to an antimicrobial agent according to CLSI interpretative criteria. It was considered that in vitro resistance to cephalothin, a commonly tested cephalosporin, may not predict resistance to other cephalosporins including cefazolin, cefuroxime, cefpodoxime, cefprozil, and loracarbef.

Results. Distribution of strains. Of the 193 diarrheagenic E. coli detected by PCR, 86 (44.5%) were STEC, 74 (38.4%) EPEC, 19 (9.8%) EAEC, and 14 (7.3%) ETEC isolates. The 2 types of ETEC according to the type of enterotoxin synthesized was as follow: 7 strains (50%) of the ST toxin, 6 strains (42.9%) of the LT toxin, and one strain (7.1%) had both ST and LT genes.

Antimicrobial resistance. The results for the antibiotic susceptibility testing of the different group of diarrheagenic E. coli strains are shown in Tables 2 & 3. The results showed that the highest rates of resistance (resistance plus intermediate) were to chloramphenicol, tetracycline, amoxicillin/clavulanic acid, streptomycin, and ampicillin Table 2. The EPEC and STEC strains showed the highest rates of resistance to ampicillin and tetracycline. However, the rates of resistant strains among EAEC and ETEC strains were higher than that of EPEC and STEC strains. These antibiotics were tetracycline, amoxicillin/clavulanic acid and ampicillin Table 2. Of the 193 diarrheagenic E. coli isolates, 63% were resistant to Tetracycline, 62% were resistant to Ampicillin, 56% were resistant to Streptomycin, 44.5% were resistant to Amoxicillin/Clavulanic acid, 39.5% were resistant to SXT, 37% were resistant to Cephalothin, 31% were resistant to Chloramphenicol, 2.5% were resistant to Ciprofloxacin, and 2% were resistance to Nalidixic acid. Of the few E. coli strains resistant to Ciprofloxacin, one strain was EAEC and 5 strains were ETEC.

Table 2 - Antimicrobial susceptibility of diarrheagenic E. coli isolates from diarrheal cases.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Diarrheagenic E. coli</th>
<th>R</th>
<th>S</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>Ampicillin (Am-10)</td>
<td>120</td>
<td>62</td>
<td>73</td>
</tr>
<tr>
<td>Cefoxitin (Fox-30)</td>
<td>8</td>
<td>4</td>
<td>185</td>
</tr>
<tr>
<td>Chloramphenicol (C-30)</td>
<td>60</td>
<td>31</td>
<td>133</td>
</tr>
<tr>
<td>Ceftriaxone (CRO-30)</td>
<td>13</td>
<td>7</td>
<td>180</td>
</tr>
<tr>
<td>Cephalexin (CF-30)</td>
<td>72</td>
<td>37</td>
<td>121</td>
</tr>
<tr>
<td>Gentamicin (GM-10)</td>
<td>12</td>
<td>6</td>
<td>181</td>
</tr>
<tr>
<td>Nalidixic Acid (NA-30)</td>
<td>7</td>
<td>4</td>
<td>186</td>
</tr>
<tr>
<td>Ciprofloxacin (CIP-5)</td>
<td>6</td>
<td>3</td>
<td>187</td>
</tr>
<tr>
<td>Tetracycline (Te-30)</td>
<td>121</td>
<td>63</td>
<td>72</td>
</tr>
<tr>
<td>Sulfamethoxazole and Trimethoprim (SXT)</td>
<td>76</td>
<td>39</td>
<td>117</td>
</tr>
<tr>
<td>Amoxicillin/Clavulanic acid (AmC-30)</td>
<td>86</td>
<td>45</td>
<td>107</td>
</tr>
<tr>
<td>Streptomycin (S-10)</td>
<td>108</td>
<td>56</td>
<td>85</td>
</tr>
</tbody>
</table>

R - resistance, S - sensitive, E. coli - Escherichia coli

Discussion. Diarrheal diseases due to the different diarrheagenic E. coli are a health problem in many parts of the world. It has been estimated that infectious diseases cause 9.2 million deaths in the developing countries, and diarrheal diseases are the fourth most common cause of mortality. Most mild diarrhea cases are effectively treated by ORS therapy and only patients with severe or persistent diarrhea should receive antibiotics. Antimicrobial resistance of diarrheagenic E. coli is usually frequent towards beta-lactam antibiotics. Surveillance information about antimicrobial resistance should be up-to-date and used in clinical management and treatment guidelines. Of the 193 E. coli isolates studied, approximately 55% displayed resistance to one or more antibiotics, including ampicillin, tetracycline, streptomycin, sulfamethoxazole-trimethoprim, and cephalexin. These data are in accordance with previous studies indicating that use of these drugs played a key factor in the emergence of antimicrobial-resistant E. coli.

In this study, amplification of the eaeA gene was used to detect EPEC strains, and 74 (38.4%) strains were found to have this gene. Approximately 60% of EPEC strains were resistant to ampicillin and tetracycline.
Since these drugs are recommended for management of different infections in children, *E. coli* isolates should be monitored for further dissemination of ampicillin and tetracycline resistance. However, EPEC isolates from this study were susceptible to cephalosporins and ciprofloxacin that are important antimicrobials for treating infection caused by multi resistant EPEC strains.

The STEC strains are among the most important agents of diarrhea in Iran. Human infections with STEC strains are commonly transmitted from cattle or dairy products especially undercooked meat or unpasteurized milk and usually are connected with the rural environment. Resistance to 2 or more classes of antimicrobials was found in 45% of STEC strains. In the last decades since antimicrobial agents have been used for the aim of disease prevention or growth promotion in animals, livestock on farm is frequently exposed to antimicrobial substances, so the resistance phenotype can give a selective advantage to bacteria. As a result, humans are more likely to be exposed to these organisms via food and direct and indirect transmission from animals.

There is a controversy on the association of EAEC with acute diarrhea, however, many reports suggest that they are associated with persistent diarrhea. The highest frequencies of antimicrobial-resistant phenotype were observed for EAEC. Nearly more than 80% of strains were resistant to ampicillin, chloramphenicol, tetracycline, and amoxicillin/clavulanic acid. As long-term antimicrobial therapy is usually recommended for EAEC persistent diarrhea, the indirect selection for multiresistant strains will increase the antimicrobial-resistant pathogen, and also facilitate the spread of mobile resistance elements to these bacteria.

Antimicrobial resistance in ETEC as a cause of diarrhea in children was more frequent than that of other groups of diarrheagenic *E. coli*. The ETEC were significantly more resistant to ciprofloxacin and nalidixic–acid than EPEC and STEC. This group of *E. coli* also showed higher resistance to ampicillin, tetracycline, amoxicillin/clavulanic acid and sulfamethoxazole-trimethoprim.

Our results showed that application of antibiotics as chemoprophylactic agents or growth promotion in agriculture is important in the selection of antimicrobial-resistant phenotypes. In the current study, the presence of 75% resistant *E. coli* to ampicillin that showed a dual resistance to streptomycin and tetracycline could be indicative of spread of mobile genetic elements or plasmids. The results also suggest that antimicrobial resistance is widespread among diarrheagenic *E. coli* strains.

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References


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