Detection of colistin resistant *Escherichia coli* in children at Pediatric Hospital of Assiut University, using phenotypic and genotypic methods

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**ABSTRACT**

**Background:** The emergence of colistin-resistant strains is considered a great threat for the children suffering from diarrhea. This study aimed to screen for the presence of *mcr-1* in *Escherichia coli* (*E. coli*) isolates collected from children with diarrhea and to compare between genotypic and phenotypic methods for detection of colistin resistant *E. coli* carrying *mcr-1* gene. **Methods:** Isolation of *E. coli* was done followed by antimicrobial susceptibility test. Kirby-Baur disc diffusion was used to determine antimicrobial susceptibility, whereas broth microdilution (BMD) and the double disc synergy test (DDST) were used to determine colistin resistance. The screening for *mcr-1* was used to investigate one probable mechanism of colistin resistance by PCR. **Results:** All *mcr-1* *E. coli* isolates were resistant to ampicillin, while resistance to ampicillin/sulbactam, cefazolin, cefoxitin, ceftriaxone and trimethoprim-sulphamethoxazol was 94.1% (32/34), 94.1% (32/34), 94.1% (32/34), 85.3% (29/34) and 70.6% (24/34) respectively. All *mcr-1* carrying *E. coli* strains were sensitive to tobramycin, amikacin and imipemem. Moderate resistance was noticed to piperacillin/tazobactam (23/34) 67.6%, gentamycin (47.1% (16/34), and ciprofloxacin (44.1% (15/34). Thirty-one (91.2 %) *mcr-1* positive *E. coli* strains were multidrug resistant (MDR). Forty five out of 95 (47.4%) of *E. coli* isolates were positive for *mcr-1* by DDST and 34/95 (35.78%) of *E. coli* isolates were positive for *mcr-1* by PCR. **Conclusions:** This study reported a high prevalence of colistin resistant *E. coli* harboring *mcr-1* gene in young children in Pediatric Hospital of Assiut University. Broth microdilution is more accurate than DDST in detection of colistin resistance.

**Introduction**

Antimicrobial resistance is a multi-sectoral problem which is now recognized as one of the most serious threats to human health globally. Because of their ubiquity in the environment and animal systems, and their increased potential to acquire antibiotic resistance determinants through mobile genetic elements, *Enterobacteriaceae* resistance trends are particularly concerning [1].

Antibiotic resistance among *Enterobacteriaceae* is steadily increasing, posing a serious threat, particularly in developing countries. The most pressing need, especially in developing nations, is for an effective, low-cost antibacterial agent that may be administered safely to treat children with diarrhea [2].

Carbapenemase-producing *Enterobacteriaceae* are among clinically important multidrug-resistant (MDR) pathogens. These bacteria usually remain susceptible to polymyxins, an old family of antimicrobial agents which were of little use in the 1970s due to their potential toxicity, attention for polymyxins (polymyxin B and colistin) has been...
Repeated around the world [3]. However, the uncontrolled use of colistin nowadays explains why colistin resistance acquisition is added to the problem of carbapenem resistant Enterobacteriaceae [4].

However, as the number of carbapenem-resistant Enterobacteriaceae (CRE) strains has grown, polymyxin (colistin) antibiotics with relatively high nephrotoxicity (particularly in children) have grown in popularity as a last choice for treating clinical CRE infections [5].

Resistance to colistin and other polymyxins has many molecular mechanisms that have been characterized in different species of bacteria, given the fact that the mechanism underlying resistance is unknown. It’s been claimed that resistance to this antibiotic is linked to several forms of LPS modification. These include (a) specific modifications to outer membrane porins as well as generalized decrease in the amount of porins in the membrane that is may be caused by plasmid mediated mcr-1 gene (b) overexpression of efflux pump systems, (c) overproduction of capsule polysaccharide, and (d) enzymatic pathways of resistance (colistinase) [6].

The mechanism of resistance of the “mobilized colistin resistance” “mcr” gene is a phosphatidylethanolamine residue transferase. The enzyme transfers a phosphoethanolamine residue to the lipid A present in the cell membrane of gram-negative bacteria. This type of resistance is known as target modification of lipopolysaccharide [7]. Addition of phospho-ethanolamine, 4-amino-l-arabinose cationic groups, or both to lipopolysaccharide decreases polymyxin binding to the bacterial outer membrane. This addition caused by chromosomal-encoded mutations in the PmrAB or PhoPQ two-component systems, or changes in the mgrB gene [7]. The altered lipid A has much lower affinity for colistin and related polymyxins resulting in reduced activity of the antimicrobial [8].

The addition of phosphoethanolamine may be due to plasmid encoded mcr-1 gene, which provides the first identified plasmid-mediated colistin resistance in animal and human isolates, according to a recent study [9].

The first polymyxin resistance gene found to be horizontally spread between bacterial strains is mcr-1. Escherichia coli, Klebsiella pneumoniae, Salmonella, Shigella sonnet, Enterobacter cloacae, Enterobacter aerogenes, Klyuyera species, Citrobacter species, Raoultella ornithinolytica and Cronobacter sakazakii are among the Enterobacteriaceae species that contain this gene [9]. Escherichia coli is the most common bacteria found in clinical samples from patients suffering from diarrhea, and it has a high rate of drug resistance. Furthermore, there are just a few recent reports on antibiotic resistance among E. coli and Shigella strains isolated from children with diarrhea [2].

More recently, the mcr-1 gene was identified in several plasmid backbones, mostly in E. coli [10-12]. Escherichia coli with mcr gene variants has been found in animals, humans, food and the environment all around the world. Only the mcr-1 gene has been found in animals, people, and saltwater samples in Algeria, according to a few researches. [11-14]. Also, it was discovered in E.coli and Klebsiella pneumoniae in previous studies in China [15,16] and was then discovered in a variety of bacteria from many different nations [17,18]. Clinicians may face very limited treatment options as a result of selection and spread of MDR and extensively drug resistant (XDR) strains [18].

Colistin-resistant E. coli has emerged as a result of widespread usage of the antibiotic in veterinary medicine, disease prevention, and growth promotion as reported by Drali et al. [14]. The mcr-1 gene has also been detected in bacteria isolated from animals, animal products as chicken and pork meat [9]. It was acquired in the community [16] by humans, i.e., sick and asymptomatic persons [13]. Therefore, we intended to make screening about these E. coli strains present in children which have not been exposed previously to this type of antibacterial agents (colistin). This study aimed to screen for the presence of mcr-1 in E. coli isolates collected from children with diarrhea at Pediatric Hospital of Assiut University and also to compare between genotypic and phenotypic methods for detection of colistin resistant E.coli.

Materials and Methods

Study subjects
This cross-sectional descriptive study was conducted in Assiut University, Pediatric Hospital in Assiut city starting from August 2020 till March 2021. This study was carried out in the Department of Medical Microbiology & Immunology, Faculty of Medicine. It included 210 non repetitive stool samples collected from children suffering from diarrhea admitted at Pediatric Hospital. Bacterial identification and antimicrobial susceptibility testing were routinely carried out.
Written informed consent was obtained from the parents or guardian of the child before recruitment. The research was carried out in accordance with the (Declaration of Helsinki). The Ethical Committee of Faculty of Medicine, Assiut University approved the research proposal with IRB number 17300441 dated 26/7/2020. Full history was taken including demographic and clinical data as name, age, sex, type of feeding (breast feeding or artificial feeding) and history of vomiting, diarrhea, abdominal pain and fever

Identification of E. coli

Identification of E. coli was based on standard microbiology laboratory techniques. All samples were cultured on MacConkey’s agar and Eosin Methylene Blue (EMB) agar and were incubated for 24 hrs at 37°C. Isolated colonies were further identified according to morphology of colonies, fermentation of lactose, standard biochemical tests as oxidase, catalase, urease, indole, triple sugar iron tests and Gram-staining. Pure colonies of E. coli were stored on glycerol at 4°C [19].

Antimicrobial susceptibility testing by Kirby-Bauer disc diffusion method [20]

The following antibiotic discs were used: ampicillin sulbactam (SAM) (30 µg), ampicillin (AMP) (20 µg), piperacillin/ tazobactam (PRL/TAZ) (40µg), cefepime (FEB) (30 µg), gentamicin (CN) (10µg) cefazolin (CZ) (30 µg), cefoxitin (FOX) (30 µg),ceftriaxone (CRO) (30 µg), cefazidime (CAZ) (30 µg), meropenem (MEM) (10 µg), ciprofloxacin (CIP) (5 µg), levofloxacin (LEV) (5 µg), amikacin (AK) (30 µg), tobramycin (TOB) (10 µg), nitrofurantoin (FM)(50µg) and sulfamethoxazole/trimethoprim (SXT) (5 µg), (Oxoid; Basingstoke, UK).

The MDR strain is defined as non-susceptibility to at least one antimicrobial agent in three or more antimicrobial classes, whereas XDR strain is defined as non-susceptibility to at least one antimicrobial agent in all antimicrobial classes. The non-susceptibility of bacteria to all antimicrobial drugs in all antimicrobial categories is known as pandrug-resistance (PDR) [18].

In accordance to the Clinical Laboratory Standards Institute (CLSI) 2018 guidelines, isolates were classed as sensitive, intermediate, or resistant after interpretation of inhibition zones’ diameters [21]. Isolates were then classified as MDR, XDR and PDR [22] according to the principles described by Machado et al. [23] previously.

Phenotypic methods for colistin resistance detection

Determination of minimal inhibitory concentration (MIC) of colistin

Colistin MIC was determined by the broth microdilution method (BMD) [24]. The BMD was carried out with colistin sulphate (Arab co.med., Egypt) in untreated polystyrene plates, with no surfactant added (polysorbate 80). The dilutions ranged from 0.125 to 256 µg/ml according to Turlej-Rogacka et al. [25]. The isolates confirmed to be colistin resistant according to CLSI (2018) if the MIC is ≥ 4µg/ml [22]. Bacterial inoculum of 5 × 10^5 CFU/ml in Mueller-Hinton broth (Oxoid; Basingstoke, UK) was used for broth MIC testing. Two-fold serial dilutions were prepared in Mueller-Hinton broth ranging from 0.125 to 256 µg/ml colistin concentrations, 0.05 ml of each dilution was distributed over a 96-well polystyrene microwell-plate [26].

Screening tests based on the inhibition of mcr-1 activity

1- Double disk synergy test (DDST) for mcr-1 detection

This test is based on inhibition of mcr-1 phosphoethanolamine transferase by EDTA (ethylenediaminetetraacetic acid). By Comparing the inhibition zones of colistin (10µg) and colistin (10µg) plus EDTA (100 mM), an increase of ≥ 3mm in the size of inhibition zones around of colistin-plus EDTA in comparison to the inhibition zones of colistin without EDTA indicated a mcr-1 positive E. coli [27].

Genotypic detection methods (polymerase chain reaction)

DNA extraction

The DNA was extracted from an overnight culture of E. coli [28]. A bacterial pellet suspension was boiled for 10 minutes. The supernatant from the centrifuge was then used in the polymerase chain reaction (PCR) after checking for purity and concentration.

Detection of (mcr-1) gene by conventional PCR: Isolates that exhibited MIC values for colistin >2µg/mL were further investigated by PCR. Detection of mcr-1 gene was performed by PCR using the primers:

mcr-1-F (5’AGTCCGTTTTGTCTTTTGTTGCG-3’)

mcr-1-R (5’CCATGCTCTTCCATTGACCATTTG-3’).
**mcr-1-R** (5'-AGATCCTTGGTCTCGGCTTG-3') of amplicon size, 320bp [29].

Polymere chain reaction consisted of 12.5 μL DreamTaq Green PCR Master Mix (Thermo Fisher Scientifc, United States), 5.5 μL of nuclease-free water, 0.5 μL of each primer solution (10 μM), and 2 μL DNA lysate. The condition was: 1 cycle of denaturation at 94°C for 15 min, then 25 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 90 s and elongation at 72°C for 60 s, and a final cycle of elongation at 72°C for 10 min. Electrophoresis on a 1% agarose gel at 120 Volt for 1 hr was used to visualize the amplification, which was followed by ethidium-bromide staining [29].

**Statistical analysis**

The Statistical Package for Social Sciences, version 16 (SPSS Inc., Chicago, USA) was used to conduct the statistical analysis. The Chi-square test was employed to compare categorical and continuous variables. A *p*-value of less than 0.05 was considered statistically significant.

**Results**

**Demographic data and colistin resistant *E. coli***

Ninety-five *E. coli* strains were isolated by conventional culture method from 210 stool samples. Table 1 summarizes the demographic data of these 210 patients. The ages of these 210 patients ranged from 3 months up to 7 years. All patients did not use colistin. There was no significant correlation between the *mcr-1* fecal carrying rate and the patient’s age. There was no significant difference in sex between colistin resistant and colistin sensitive groups. Although there was significant difference with *p* value (0.005**) in the group of breast feeding between colistin resistant and colistin sensitive groups, there was higher number of artificial feeders among colistin resistant group with no significant difference. There were significant differences in presence of abdominal pain with *p* value (0.001**), vomiting with *p* value (0.021*), and low and high grade fever with *p* value (0.016*) between colistin resistant and colistin sensitive groups with higher incidence among colistin resistant group. There were significant differences in number of diarrhea with *p* value (0.024*) between colistin resistant and colistin sensitive groups with higher number among colistin resistant group.

**Escherichia coli** isolation and antibiotic susceptibility by Kirby-Bauer method

Antimicrobial susceptibility of *E. coli* isolates was shown in figure (1). *Escherichia coli* were highly resistant to ampicillin (96.8 %), followed by ampicillin/ sulbactam (77.9%), cefazolin (72.6%) and cefoxitin (68.4%). Moderate resistance was observed against ceftazidime (52.6%) trimethoprim-sulphamethoxazol (44.2 %) and piperacillin/tazobactam (41.1%). Furthermore, low resistance was shown against gentamycin (23.2%), ciprofloxacine (16.8%), ceftriaxone (14.7%), nitrofurantoin (10.5 %), cefepime (9.5%) and levofloxacin (8.4%). They were completely sensitive to imipenem, tobramycin and amikacin (Figure 1). Out of 95, 54 isolates (56.8 %) were classified as MDR.

**Results of MIC of colistin**

It was found that 17/95 (17.9%) and 12/95 (12.6%) isolates showed resistance to colistin antibiotic with high MIC= 256 and 128 μg/mL respectively. Moreover, 23/95(24.2%) isolates were resistant to colistin with MIC≥4μg/mL (ranged from 4 to 64µg/mL) and 43/95 (45.3%) isolates were sensitive to colistin at MIC 0.125 to less than 4 μg/ml as shown in table (2). Fifty-two (54.7%) out of 95 isolates, were confirmed phenotypically as Colistin Resistant (CR) (MIC range from 4-256 μg/mL). Figure 2 shows antibiotic resistance pattern of all *mcr-1* positive *E. Coli* isolates (34) using disc diffusion method.

**Determination of mcr-1 gene by DDST**

The *mcr-1* gene was detected phenotypically in colistin resistant isolates by DDST, with diameter variations of 3mm or more between the inhibition zones of colistin/EDTA and colistin discs. The results showed that 45/95 (47.4%) isolates exhibited an increase in the diameter of the inhibition zones around the colistin/EDTA disc by 3 mm up to 10 mm more than that around colistin disc alone (Figure 3).

**Detection of mcr-1 gene by PCR**

*mcr-1* gene detection by PCR revealed that 34(35.8%) out of 95 *E. coli* isolates were positive for *mcr-1* as shown in figure (4). All (100%) of *mcr-1* positive isolates were positive for DDST. *mcr-1* positive *E. coli* strains were found to be resistant to colistin and have MIC range from 32 to 256 μg/µL, as shown in table (2). *mcr-1* negative isolates 61 (64.2%) had MIC values for colistin ranging from 0.125 to 32 µg/mL, as shown in table (2). There were 18 isolates, were *mcr-1* negative (9 isolates at MIC (4 µg/mL), 4 isolates at MIC (16 µg/mL), 4 isolates at MIC (8 µg/mL), 1 isolate at MIC (32 µg/mL) as shown in table (2).
Antibiotic susceptibility of mcr-1 positive E. coli isolates

This study reported that 100% of mcr-1 E.coli isolates were resistant to ampicillin, while resistance to ampicillin/sulbactam, cefazolin, cefotixin, ceftazidime and trimethoprim-sulphamethoxazol was 94.1% (32/34), 94.1% (32/34), 94.1% (32/34), 85.3% (29/34) and 70.6% (24/34) respectively. All the mcr-1carrying E. coli strains were sensitive to tobramycin, amikacin and imipenem. Moderate resistance was noticed to piperacillin/ tazobactam (23/34) 67.6%, gentamycin 47.1% (16/34), and ciprofloxacin 44.1% (15/34). Low resistance was noticed to levofloxacin 23.5% (8/34), nitrofurantoin 23.5% (8/34) and cefepime 17.6% (6/34). It was noted that these 31/34 (91.2 %) mcr-1 postive E. coli strains were MDR with resistance varying from 3 to 7 different antibiotic categories as shown in figure (2).

Comparison between genotypic and phenotypic methods

The prevalence of colistin resistance among patient stool samples was 24.8% (52 /210). Only 45 (47.4%) out of these 95 isolates showed positive DDST phenotypically, Therefore, Minimal inhibitory concentration by broth microdilution is more accurate than DDST, (Table 3), as there were 9 isolates gave negative results for colistin resistance by DDST but, they were resistant for colistin by MIC. Also, there are 2 isolates gave false positive results by DDST, while by MIC gave negative results interpreted by low MIC at 0.5 and 2 µg/mL respectively as shown in table (2).

Table 1. Demographic characteristics of children with colistin resistant and colistin sensitive E coli by MIC using BMD.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Colistin resistant E coli</th>
<th>Colistin sensitive E coli</th>
<th>p value (Chi-squared test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (Months) &lt;6m</td>
<td>23 (57.50%)</td>
<td>17 (42.50%)</td>
<td>0.367</td>
</tr>
<tr>
<td>7-12m</td>
<td>24 (53.30%)</td>
<td>21 (46.70%)</td>
<td></td>
</tr>
<tr>
<td>&gt;12m</td>
<td>5 (50.00%)</td>
<td>5 (50.00%)</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>20 (50.00%)</td>
<td>20 (50.00%)</td>
<td>0.109</td>
</tr>
<tr>
<td>Female</td>
<td>32 (58.20%)</td>
<td>23 (41.80%)</td>
<td></td>
</tr>
<tr>
<td>Vomiting</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>34 (52.30%)</td>
<td>31 (47.70%)</td>
<td>0.021*</td>
</tr>
<tr>
<td>No</td>
<td>18 (60.00%)</td>
<td>12 (40.00%)</td>
<td></td>
</tr>
<tr>
<td>Breast feeding</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>35 (50.00%)</td>
<td>35 (50.00%)</td>
<td>0.005**</td>
</tr>
<tr>
<td>No</td>
<td>17 (68.00%)</td>
<td>8 (32.00%)</td>
<td></td>
</tr>
<tr>
<td>Artificial feeding</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>24 (60.00%)</td>
<td>16 (40.00%)</td>
<td>0.102</td>
</tr>
<tr>
<td>No</td>
<td>28 (50.90%)</td>
<td>27 (49.10%)</td>
<td></td>
</tr>
<tr>
<td>Diarrhea</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>2 (40.00%)</td>
<td>3 (60.00%)</td>
<td>0.024*</td>
</tr>
<tr>
<td>2</td>
<td>2 (40.00%)</td>
<td>3 (60.00%)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>18 (51.40%)</td>
<td>17 (48.60%)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>10 (66.70%)</td>
<td>5 (33.30%)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>10 (66.70%)</td>
<td>5 (33.30%)</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>1 (20.00%)</td>
<td>4 (80.00%)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>9 (60.00%)</td>
<td>6 (40.00%)</td>
<td></td>
</tr>
<tr>
<td>Abdominal pain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>44 (55.00%)</td>
<td>36 (45.00%)</td>
<td>0.001***</td>
</tr>
<tr>
<td>No</td>
<td>8 (53.30%)</td>
<td>7 (46.70%)</td>
<td></td>
</tr>
<tr>
<td>Low grade fever</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>14 (46.70%)</td>
<td>16 (53.30%)</td>
<td>0.016*</td>
</tr>
<tr>
<td>No</td>
<td>38 (58.50%)</td>
<td>27 (41.50%)</td>
<td></td>
</tr>
<tr>
<td>High grade fever</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>38 (58.50%)</td>
<td>27 (41.50%)</td>
<td>0.016*</td>
</tr>
<tr>
<td>No</td>
<td>14 (46.70%)</td>
<td>16 (53.30%)</td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Comparison between BMD, PCR and DDST methods.

<table>
<thead>
<tr>
<th>MIC range/μg/mL</th>
<th>0.125</th>
<th>0.25</th>
<th>0.5</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>8</th>
<th>16</th>
<th>32</th>
<th>64</th>
<th>128</th>
<th>256</th>
<th>Total</th>
<th>p value (Chi-squared test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of samples positive at this conc/</td>
<td>23 (4.2%)</td>
<td>5 (5.3)</td>
<td>5 (5.3)</td>
<td>6 (6.3)</td>
<td>9 (9.5)</td>
<td>4 (4.2)</td>
<td>4 (4.2)</td>
<td>2 (2.1)</td>
<td>4 (4.2)</td>
<td>12 (12.6)</td>
<td>17 (17.9)</td>
<td>52 (from 4-256)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mcr-1 Positive</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>1 (100%)</td>
<td>4 (100%)</td>
<td>12 (100%)</td>
<td>17 (100%)</td>
<td>34 (35.8%)</td>
<td>&lt;0.0001***</td>
<td></td>
</tr>
<tr>
<td>Mcr-1 Negative</td>
<td>23 (100%)</td>
<td>5 (100%)</td>
<td>5 (100%)</td>
<td>4 (100%)</td>
<td>6 (100%)</td>
<td>4 (100%)</td>
<td>4 (100%)</td>
<td>1 (50%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>61 (64.2%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DDST positive</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>1 (20%)</td>
<td>0 (0%)</td>
<td>1 (16.7%)</td>
<td>5 (55.6%)</td>
<td>1 (25%)</td>
<td>2 (50%)</td>
<td>2 (100%)</td>
<td>12 (100%)</td>
<td>17 (100%)</td>
<td>45 (47.4%)</td>
<td>&lt;0.0001***</td>
<td></td>
</tr>
<tr>
<td>DDST negative</td>
<td>23 (100%)</td>
<td>5 (100%)</td>
<td>4 (80%)</td>
<td>4 (100%)</td>
<td>5 (83.3%)</td>
<td>4 (44.4%)</td>
<td>3 (75%)</td>
<td>2 (50%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>50 (52.6%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Percentages of colistin resistant and sensitive E. coli by PCR, DDST and BMD

<table>
<thead>
<tr>
<th></th>
<th>PCR (mcr -1)</th>
<th>DDST</th>
<th>p value (Chi-squared test)</th>
<th>BMD</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of positive samples</td>
<td>34/95 (35.8%)</td>
<td>45/95 (47.4%)</td>
<td>0.08</td>
<td>52 (54.7%) at MIC 4 to 256 μg/ml</td>
</tr>
<tr>
<td>No. of negative samples</td>
<td>61/95 (64.2%)</td>
<td>50/95 (52.6%)</td>
<td>0.09</td>
<td>43 (45.3%) at MIC 0.125 to 4 μg/ml</td>
</tr>
</tbody>
</table>

Figure 1. Antibiotic resistance pattern of all isolated E. coli (95) isolates.
Figure 2. Antibiotic resistance pattern of all mcr-1 positive E. coli (34) isolates.

Figure 3. Phenotypic detection form CR positive isolates by (DDST).

(A): mcr-1 positive strain showed an increase in the zone diameter of discs with colistin and EDTA≥3mm in comparison to colistin alone. (B): mcr-1 negative isolate showed slight change in the inhibition zone diameter of colistin and EDTA disc in comparison to colistin alone.

Figure 4. Gel electrophoresis of PCR product of mcr-1 positive E. coli isolates.

Lanes 1, 3 & 4: colistin-resistant mcr-1 positive strains. Lane 2: colistin sensitive mcr-1 negative isolate.
Discussion

Bacterial antimicrobial resistance is a global emerging problem of public health concern. In the current study, a high percentage of E. coli strains were resistant to ampicillin, ampicillin/sulbactam, cefazolin and cefoxitin. Most of E. coli isolates were sensitive to levofloxacin, ciprofloxacin, and ceftriaxone, this was in agreement with previous reports conducted in Egypt by Moawad et al. [30].

In this study, (54/95) 56.8% of E. coli isolates were multidrug resistant. This is in agreement with prior study reported that the frequency of MDR E. coli in diarrheal patients was 61.0% [29]. The resistance to other antimicrobial agents was variable while all isolates were sensitive to imipenem, tobramycin, and amikacin in our study.

This study detected high prevalence of mcr-1 gene in stool samples isolated from children (34/210) 16.2% of samples and mcr-1 gene was detected in (35.8%) of E. coli isolates. Elnahriry et al. [31] found only one mcr-1 positive E. coli isolate from a hospitalized patient’s sputum admitted to the intensive care unit of a Cairo City hospital due to bacteremia with no history of traveling abroad. This strain was one of 241 Gram-negative bacterial isolates collected from many hospitals in different cities in Egypt during 2015. The MIC of this isolate was 16 mg/L for colistin.

Considering the frequent use of colistin in animal production and the importance of this antimicrobial agent for the control of multidrug resistant Gram-negative nosocomial infections in humans, monitoring the dissemination of resistance to colistin is mandatory. Animals and their products may be possible sources of mcr-1 acquisition in humans, given the high prevalence of mcr-1 in animal isolates compared to human clinical isolates around the world. The increased prevalence of mcr-1 in bacteria isolated from animals and animal products may be due to the abuse of colistin in the poultry industry and agriculture. All authorised authorities should address this problem by prohibiting the uncontrolled use of colistin in agriculture [9].

In agreement with our study, Gianini et al. [2] showed no variations in age and sex between children harbouring mcr-1-positive Enterobacterales and children who did not have mcr-1.

Out of 34 mcr-1 positive E. coli by PCR, 31(91.2 %) were MDR. These isolates were resistant to 3-7 different antibiotic categories. However, IMP was effective against all 34 mcr-1 positive strains, suggesting that IMP could be utilized to treat infections caused by colistin-resistant E. coli. This appears to be an alarming trend for a populous country like Egypt where infection control is becoming increasingly challenging due to the rise of multidrug resistance among members of the family Enterobacteriaceae, including E. coli [32,33]. The minimal identification of IMP resistance in MDR E. coli isolates in our study is similar with recent reports from Guangzhou, China [34], Germany [35], and Vietnam [36] respectively.

The difference in colistin susceptibility depending on the presence or absence of zinc chelators was used to screen for mcr-1. In our study, colistin resistance was detected in accordance to the results of MICs followed by screening for the presence of mcr-1 using phenotypic and genotypic methods. The fact that mcr-1 phosphoethanolamine is a zinc metalloprotein is required for phenotypic methods (DDST). As a result, any reduction in zinc in the media caused by metal chelators (EDTA) will decrease colistin MICs in isolates positive for mcr-1. Recently it was reported that the metal chelators (EDTA) potentiate the activity of colistin against mcr-1-producing strains [8]. The results of MICs revealed that DDST detected only 45 (47.4%) isolates as phenotypically CR strains, while genetic detection of mcr-1, revealed 34 (35.8%) as mcr-1 positive isolates.

Diffusion methods based on antibiotic diffusion in agar, whether with the Kirby–Bauer disc diffusion or with gradient strips, are unreliable for polymyxin testing and should be avoided, according to a vast number of research which reported high errors rates [37]. In this study the DDST gave some false results as it depends on antibiotic diffusion in agar, the only appropriate method for colistin MIC determination is BMD as reported by (CLSI) and (EUCAST) [22,24,37]. This is in agreement with Ibrahim et al. [38] which reported that MIC determinations by BMD and conventional PCR are now used to identify colistin resistance.

There were 18 mcr-1 negative isolates, had MIC values for colistin ranging from 0.125 to 32 μg /ml. They were resistant phenotypically, but did not have mcr-1 gene, their colistin resistance may be contributed to other resistance mechanisms. The presence of polymyxin resistance is indicated by
phenotypic methods, but they do not specify the mechanism or the risk of transmission [39]. As a result, phenotypic and molecular approaches are complimentary in detecting colistin resistance and analyzing the behavior of the clinical isolate, and they should be used in parallel [39].

The transmission of colistin resistance gene mcr-1 has been reported to be associated with the food chain Zurfluh et al. [40]. Thus, the high prevalence of mcr-1 in these diarrheal patients might be associated with food producing animals.

The use of colistin in children should be carefully considered due to its nephrotoxicity [5]. Our detection of a high prevalence of mcr-1 in isolated E coli strains among children is striking considering, because this antibiotic is not approved for clinical use, and this in agreement with previous study in China for colistin resistance in children [5].

Attempting to eradicate mcr-1 positive microorganisms by restricting the utility of colistin to animals appears to be too late. If therapeutic use of colistin is permitted in the future, plasmids expressing colistin resistance determinants could quickly extend across the hospital environment. Moreover, using colistin to treat colistin-resistant E. coli infections could lead to a rapid choice for organisms to be resistant to carbapenems and colistin. The only successful approach for extending the utility of colistin for the treatment of life-threatening bacterial infections may be the development of efficient inhibitors for mcr-1 or intervention measures to disrupt the transmission of these plasmids [5].

In conclusion, this study reported a high prevalence of colistin resistant E. coli harboring mcr-1 gene in young children in Pediatric Hospital of Assiut University. This finding supports prior reports of the plasmid-mediated gene mcr-1 spreading at an alarming rate. Broth microdilution is more accurate than DDST in detection of colistin resistance.

Recommendation

It is recommended that all authorised authorities should address this problem by prohibiting the uncontrolled use of colistin in agriculture. Significant resistance was detected to penicillin, cephalosporins, sulphonamides, quinolones and monobactams. So, be alert because these resistant germs may be resistant to all available antimicrobials or solely to hazardous ones like colistin, the health-care team may be left with few alternatives in the treatment of serious infections caused by MDR E.coli.

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