Iraqi Journal of Biotechnology, 2015, Vol. 14, No. 1 , 85-92

Detection of *E. coli* and Rotavirus in Diarrhea among Children Under Five Years Old

Arwa M. AL-Shuwaikh [1], Israa AJ. Ibrahim [2], Rana M. Al- Shwaikh [2]

[1] Department of Microbiology, College of Medicine, AL-Nahrain University

[2]Department of Biology, College of Education for Pure Science Ibn AL-Haitham, University of Baghdad

Received: February 16, 2015 / Accepted: March 29, 2015

Abstract: Ninety four fecal specimens were collected from children diarrhea less than five years old from Al-Emamain Al-Kadhemain Medical City Hospital in Baghdad province. Samples collection was carried out from 25 May to 16 July 2014. Our study showed 25.53 % *and* 17% of samples had positive tests for *Escherichia coli* and Rota virus respectively. Low number of *E. coli* isolates, 4/24 (16.6%) were produced β-lactamase and 10/24 (41.6%) produced biofilm. However, half *E. coli* isolates (50%) produced hemolysis. The *E. coli* isolates showed different degrees of sensitivity to different antibiotics. All *E. coli* isolates were 100% sensitive to Ciprofloxacin, Gentamycin, and Norfloxocin. However, 16.6% of *E. coli* isolates were sensitive for Carbenicillin and Amikacin. But 33.3% of *E. coli* isolates were sensitive to Amoxacillin- clavulanic and Aztreonam. Our study showed a number of plasmid range between 750 to 10,000 bp in size.

Key words: *E. coli*, Rota virus, antibiotic sensitivity, plasmid.

Corresponding author: should be addressed (Email: arwa_alshwaikh_2004@yahoo.com)

Introduction

Diarrheal disease is the second leading cause of death in children under five years old. Each year diarrhea kills around 760 000 children under five years (1). Ministry of health in Iraq record more than 212 diarrheal case under five years of age per 1000 of population in 2010 (2). Rotavirus and enterohaemorrhagic *E. coli* (EHEC) are considered to be the most common cause diarrhea in the world (3, 4). *Escherichia coli* is gram negative bacteria belong to enterobacteriaceae family, short bacilli, non-spore forming, facultative anaerobic and it is grow on simple media (5). Its significance as a public health problem was recognized in 1982, following an outbreak in the United States of America (2). There are six different intestinal *E. coli* pathotypes associated with diarrhea, including enteropathogenic *E. coli* (EPEC), enterohemorrhagic E. coli (EHEC), enterotoxigenic *E. coli* (ETEC), enteraggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), and diffusely adherent *E. coli* (DAEC) (6). "Plasmid profile analysis is useful in determining the epidemical strain in outbreaks caused by multiple species: *Escherichia*, *Klebsiella*, *Pseudomonas*, *Serratia*, S*treptococcus*, and so on" (7). "Plasmids size range from 1 kbp to 2,000 kbp resistance plasmids code for enzymes that can inactivate antibiotics,

prevent the uptake of an antibiotic, or pump out the particular antibiotic" (8).
Rotavirus, an icosahedral virus belonging to the family Reoviridae. It was first recognized as a diarrhea agent by Bishop *et al*. (9).The fully infectious Rotavirus particle consists of 3 protein layers and is also termed triple-layered particle (TLP). By electron-microscopy, TLPs resemble wheels (Latin Rota), and this appearance has led to the name of Rotavirus for the genus (10).Rotavirus remains the most common cause of severe childhood diarrhea worldwide and of diarrheal mortality in developing countries (11). The World Health Organization (WHO) estimates that 527,000 children under the age of five years die of rotavirus disease each year. Children in the poorest countries account for 82% of rotavirus deaths (12). Currently available rotavirus vaccines protected against severe Rotavirus gastroenteritis and were well tolerated; the implementation of immunization programs would be expected to reduce disease burden (13).
Most local previous studies detect the most common cause diarrhea in children as virus infection or bacterial infection in separate studies. This study design, to evaluate the characteristic of *E. coli*, and rotavirus in children diarrhea, and determine the most common cause of diarrhea in the test group.

Materials and Methods

Specimens

In this study, 94 stool specimens were collected from children diarrhea aged less than 5 years from Al-Emamain Al-Kadhemain Medical City Hospital in Baghdad province. Samples collection were carried out from 25 May to 16 July 2014. An informed consent was obtained from the parent (either mother or father) of the child before inclusion in the study. The parents were informed about the procedure, making certain that he/she was fully competent of understanding the procedure carried out to obtain the specimen from the child. From each case at least 5ml or 5g of stool was collected in a clean sterile container.

Isolation and Identification of *E. coli*

94 stool samples from children with diarrhea (aged under 5 years) were cultured on MacConkey agar and Eosin Methylene Blue (EMB). All enteric bacteria isolated were identified on the basis of colonial characteristics, Gram stain and biochemical tests, IMViC, Urea, Kligler Iron Agar (14).

Antibiotic Sensitivity Test

Antibiotic susceptibility test of *E. coli* isolates was determined by the standard Kirby-Bauer disk diffusion method (15). These antibiotics with their respective disk concentrations are Amoxicillin - clavulanic (30µg), Amikacin (10µg), Aztreonam (30µg), Carbenicillin (25µg), Cephalothin (30µg), Ciprofloxacin (10µg), Gentamycin (10µg), Norfloxacin (10µg) (Bioanalyse). Bacterial cultures suspension equivalent of 0.5 tube McFarland turbidity standards were spread on Muller-Hinton agar plates using sterile swabs and incubated aerobically at 37^0C for 24 hours, then inhibition zones diameter around antibiotic disks were measured. Results were expressed susceptible or resistant

according to the criteria recommended by the CLSI (16).

Detection of Hemolysin Production

The *E. coli* isolates tested for blood hemolysis were streaked on blood agar plates containing 5% (v/v) human blood and incubated aerobically at 37°C for 24 hours. The clear zones around the growth colonies indicate a positive reaction (17).

Detection of β-lactamase Production

All *E. coli* isolates were tested for production of β-lactamase enzyme using iodometeric test method (18).

Detection of Biofilm Formation

The ability of *E. coli* isolates to colonize a biotic surface was investigated by using Christensen *et al.,* (19) method. The *E. coli* isolates were cultivated in tubes with Tryptone soy broth and incubated aerobically at 37°C for 48 hours and thereafter the culture tubes were emptied carefully and stained with 1% crystal violet solution for 30 minutes, and then the tubes were rinsed with distilled water and left to dry at room temperature. Results were compared with negative control, and biofilm formations as a layer at the internal wall of the tubes noted by the naked eye indicate a positive result.

Plasmid DNA Isolated Procedure

All *E. coli* isolates were screened for plasmid content by the alkaline method of Brinboim and Doly (20) and separated on a 1% agarose, at 50 vol. for 1 hour and 1.30 hour. The DNA bands were visualized and photographed under UV light after the gel had been stained with ethidium bromide.

Detection of Rota Virus Rapid Chromatographic Immunoassay

The stool specimens were tested as soon as possible after collection, they were directly tested with One Step Rotavirus Test Device (DiaSpot, USA) for rotavirus antigen. This is a ready-to-use test based on the use of a homogenous membrane system which pre-coated with anti-rotavirus antibody on the test line region of the test. The tests were performed following manufacturer's instructions. Stool samples were prepared by adding 50mg or 50µl of the stool sample to the dilution buffer supplied with the test. After mixing by vortex, samples were centrifuged at 800 g for 10 minutes and supernatant was taken. 80µl of processed stool samples was dropped into immune-chromatographic well, positive and negative controls were performed with each batch of the tests. After 10 min. incubation at room temperature, the results of test were read by observation of colored indicator: Rotavirus negative: one red line appears only in the control line region marked with the letter C. Rotavirus positive: In addition to the red control line, a red line (Rotavirus test line) also appears in the test line region marked with the letter T (result line). Invalid: A total absence of the control red colored line regardless the appearance or not of the result lines.

Enzyme Linked Immunosorbent Assay (ELISA)

All positive samples and negative samples for rotavirus by Rapid

Chromatographic Immunoassay were also tested by ELISA (RIDASCREEN® Rotavirus, Germany). In this test, monoclonal antibodies are used in a sandwich-type method. Monoclonal antibodies against a capsid protein of gene 6 (VP6) of the rotaviruses are applied to the surface of the well of the microwell plate. The tests were performed following manufacturer's instructions. Briefly, fecal samples were diluted by adding 100mg or 100µl of the fecal sample to test tube containing 1 ml RIDASCREEN® sample dilution buffer supplied with the test. After mixing of stool suspension in a vortex mixer. Samples were leaving for a short time to settle down and centrifuged at 5000 rpm (approx. 2300 - 2500 g) for 5 minutes. Then the clarified supernatant of the stool suspension used directly in the test following manufacturer's instructions. The optical density (O D) obtained at wave length (450-630) nm. The cut-off was calculated by adding 0.15 extinction units to the measured extinction for the negative control. The net optical density of each sample was considered positive if their extinction is more than 10 % above the calculated cut-off. Samples were considered equivocal and must be repeated if their extinction is within ± 10 % of the cut-off. Samples with extinctions more than 10 % below the calculated cut-off were considered negative.

Statistical Analysis

The Data were presented as percentages using Statistical Package for the Social Sciences (SPSS), version 10.

Results and Discussion

"The incidence of infectious diarrhea and the prevalence of a given causal agent are strictly associated with socioeconomic factors such as nutrition, sanitation and habitat of the population" (21). Our study showed that 25.53% and 17% of samples were give positive results for *E. coli* and Rota virus respectively, 38(40.42%) of samples may be another cause of children diarrhea (Table 1). Local previous studies recorded in Basrah (2003) , Erbil (2006), Baghdad (2011), Ramadi (2012), and Babylon (2013) were 24%, 37%, 30%,39.26%, and 52.54% Rota virus infection in children (22,23,24,25,26).

Rotavirus vaccine was found to prevent in the first life of year, almost all (85% to 98%) rotavirus illness episodes that were severe and to prevent 74% to 87% of all rotavirus illness episodes (27). In 2009, the World Health Organization (WHO) recommended inclusion of rotavirus vaccination in all national immunization programs. According to the local information rotavirus vaccine used in Iraq later of 2013 that supposed decrease gastroenteritis caused by rotavirus.

Table (2) showed low number of *E. coli* isolates produced β-lactamase and biofilm, but half isolates produced β-hemolysis. Other recent local study also revealed the nearly result (28).

Table 1: The microbial spectrum in stool specimens from children diarrhea

Eschericha. coli isolates	*Klebsiella spp.*	Rotavirus ELISA	Rotavirus Rapid test	Negative culture	Total sample
24 (25,53%)	16 (17%)	16(17%)	14(14.89%)	38(40.42%)	94(100%)

Table 2: Number of *E. coli* isolates produce β-lactamase, β –hemolysis and Biofilm

No. of *E. coli* Isolates	β-lactamase	β -hemolysis	Biofilm
24	4 (16.66%)	12(50%)	10 (41.66%)

All *E. coli* isolates 100% sensitive to Ciprofloxacin, Gentamycin, and Norfloxocin. However, 16.6% of *E. coli* isolates were sensitive for Carbenicillin and Amikacin. But 33.3% of *E. coli* isolates were sensitive to Amoxacillin-clavulanic and Aztreonam. Figure (1). This is similar to the study of (29) in which gram negative bacteria were multi drug resistant to Ampicillin, Amoxicillin, Ceftizoxime, Cefepime, Tetracyclin. Many studies have shown that active efflux pump or produced beta lactamase enzyme, can be a mechanism of resistance for almost all antibiotics like penicillins, cephalosporine and carbapenems.

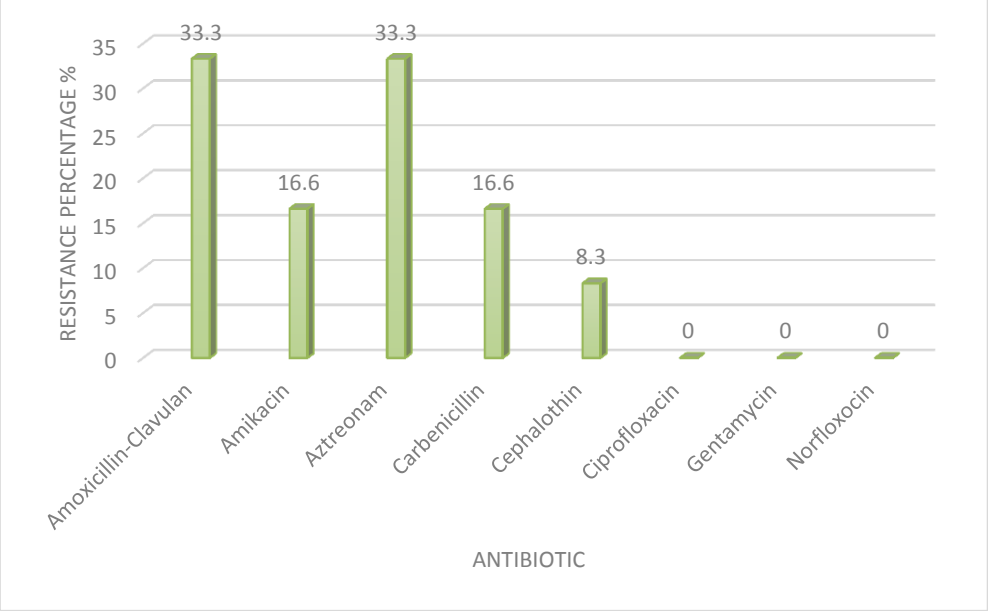

Figure 1: Percentage of *E. coli* isolates resistance for antimicrobial agents

Plasmid and chromosomal analysis is important to determine the site of virulence factor. The study showed a number of plasmid range between 750 to 10,000 bp. Plasmids size range from 1 kbp to 2,000 kbp resistance plasmids code for enzymes that can inactivate antibiotics, prevent the uptake of an antibiotic, or pump out the particular antibiotic (30). Also previous local study of children diarrhea showed that all *E. coli* isolates contained plasmids with molecular weight range between 4.507 kbp and 5.07 kbp(28). (Fig 2 ,3).

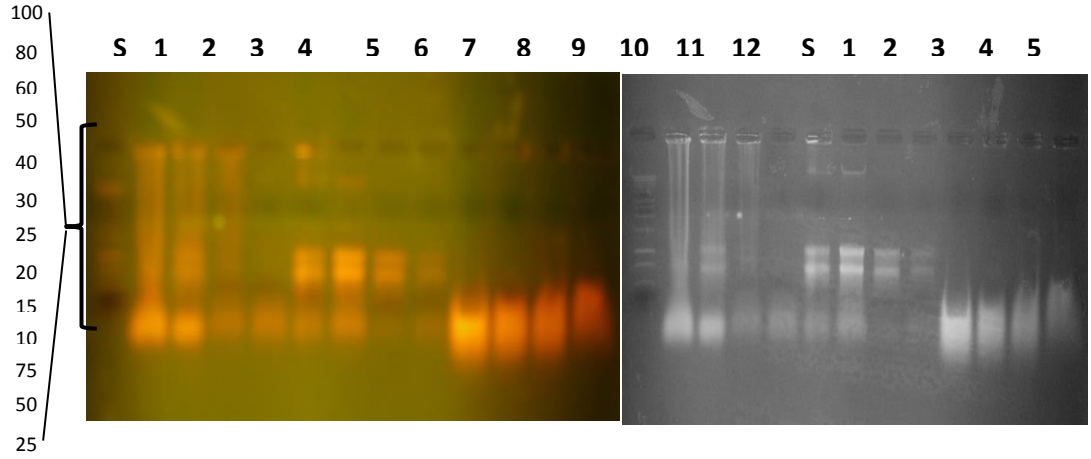

Figure 2: Agarose gel electrophoresis of plasmids extracted from clinical *E. coli* isolates (1-12), S. 1kbp (250-10,000 bp) DNA ladder (Promega); (1% agarose, 50 vol. 1 hour)

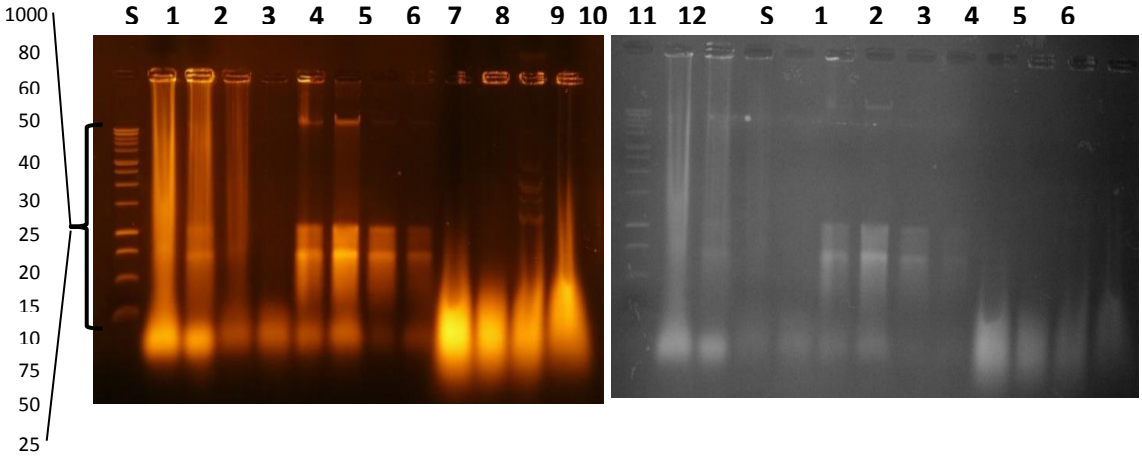

Figure 3: Agarose gel electrophoresis of plasmids extracted from clinical *E. coli* isolates (1-12), S. kbp (250-10,000 bp) DNA ladder (Promega); (1% agarose, 50 vol. 1.30 hours).

In conclusion, our study revealed that *E. coli* isolates are most prevalent than Rotavirus in children diarrhea. Thus need further study to determine the type of serotyping and genetic virulence marker to *E. coli* isolates.

References

1- WHO, (2013). Fact sheet N°330.
2- WHO, (2011). Fact sheet N°125.
3- Annual report of Ministry of Health (2010)/Republic of Iraq.
4- Parashar, U.D.; Bresee, J.S.; Gentsch, J. R. and Glass, R. I. (1998). *Rotavirus*. *Emerging Infectious Diseases*. 4:561-570.
5- Brooks, G.F.; Carroll, K.C.; Butel, J.S.; Morse, S. A. and Mietzner, T.A. (2010). Medical Microbiology. 25th edition, Jawetz, Melnick & Adelberg's, McGraw-Hill Companies, Inc. Pp.213-225.
6- Kaper, J.B.; Nataro, J. P. and Mobley, H.L.T. (2004). Pathology *Escherichia coli*. *Nat. Rev. Microbiol*. 2:123–140.
7- Wachsmuth, K. (1986). Molecular epidemiology of bacterial infections: examples of methodology and of investigations of outbreaks. *Rev. Infect. Dis*. 8:682–692.
8- Madigan, M.; Martinko, J. and Parker, J. (2003). Brock Biology of Microorganisms. 10th ed. Upper Saddle River, NJ., USA: Prentice Hall.
9- Bishop, R.F.; Davidson, G.P.; Holmes, I. H. and Ruck, B.J. (1973). Virus particles in epithelial cells of duodenal mucosa from children with acute non-bacterial gastroenteritis. *Lancet*. 2: 1281-1283.
10- Desselberger, U. (2014). Rotaviruses. *Virus Research*. 75–96.
11- Widdowson, M. A.; Steele, D.; Vojdani, J.; Wecker, J. and Parashar, U.D. (2009). Global rotavirus surveillance: preparing for the introduction of rotavirus vaccines. *J Infect Dis.* 200 (Suppl 1):S1-S8.
12- Khoury, H.; Ogilvie, I.; El Khoury, A. C.; Duan, Y. and Goetghebeur, M.M. (2011). Burden of rotavirus gastroenteritis in the Middle Eastern and North African pediatric population. *BMC. Infectious Diseases.* 11:9.
13- Grimwood, K. and Lambert, S.B. (2009). Rotavirus vaccines: opportunities and challenges. *Hum. Vaccin*.5(2):57-69
14- Forbes, B. A.; Sahm, D. F. and Weissfeld, A. S. (2002). Diagnostic Microbiology.11th edition, Bailey and Scotts. Mosby, Missouri.
15- Bauer, A..; Kirby, W.M.M. Sherris, J. C. and Truck, M. (1966). Antibiotic susceptibility testing by a standardized single disk method. *Am. J. Clin. Pathol*.43:493–496.
16- CLSI (2012). Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Second Informational Supplement. CLSI document M 100-S22. Wayne, PA: *Clinical and Laboratory Standards Institute.*
17- Senior, B.W. and Hughes, C. (1987). Production and properties of hemolysin from clinical isolates of *Proteus*. *J. Med. Microbiol*. 24:17–25.
18- Livermore, D.M. and Brown, D.F.J. (2001). Detection of β-lactamase-mediated resistance. *J. Antimicrob. Chemother*. 48:59–64.
19- Christense, G.D. Bisno, A.L. Parisi, J.T. McLaughlin, B. Hester, M. G. and Luther, R.W. (1982). Nosocomial septicemia due to multiply antibiotic resistant *Staphylococcus epidermidis*. *Ann. Intern. Med.* 96:1–10.
20- Brinboim, H. C. and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic. Acid Res*. 7(6):1513-1523.
21- Rodrigues, J.; Acosta, V.C.; Candeias, J.M.G.; Souza, L.O.; and Filho, F. J. C. (2002). Prevalence of diarrheogenic *Escherichia coli* and rotavirus among children from Botucatu, São Paulo State, Brazil. *Brazilian Journal of medical and biological research*. 35: 1311-1318.
22- Mahmood, D.A; and Feachem R.G. (1987). Clinical and epidemiological characteristics of rotavirus- and EPEC-associated hospitalized infantile diarrhea in Basrah, Iraq. *J. Trop. Pediatr*. 33:319–25.
23- Herish, M. A.; Coulter, J. B. S.; Nakagomi , O.; Hart, C. A.; Zaki , J. M.; Al-Rabaty, A. A.; Dove, W. and Cunliffe, N. A. (2006). Molecular Characterization of Rotavirus Gastroenteritis Strains, Iraqi Kurdistan *Emerging Infectious Diseases*. 12 (5): 824-826.
24- Abdulrazzaq, A.; Aljeboory, S.K.; Abdul Kareem, S. and Klena, J. (2011). Two different diagnostic methods for detection of rotavirus in Iraqi young Children. *Al-Anbar J. Vet. Sci.*, 4(1): 1999-6527.

25- Alani, Q.A. ; Al-Rawi , S. A. ; Salih, A. K. and Al-Mawla, S. O.G. (2012) Common Rota Virus Gastroenteritis in Children under 5 Years in Maternity and Children Teaching Hospital, western Iraq . *Anb. Med. J.* 10 (1): 1-7.

26- AL- Khafaji, Y. A-R. and AL-Jiboury, H. J. (2013). Detection of Rotavirus in diarrhea stool samples of children with acute gastroenteritis in Babylon governorate. *Iraq International Research Journal of Microbiology (IRJM)*. 4(3): 84-88.

27- Thomas R. F.; Harold W. J.; James W. S.; Stephen B. T. and Stephanie Z. (2014). CDC Central for Disease Control and prevalence. May 12.

28- Ibrahim I. A J., Al-Shwaikh R. M., and Ismael M.I. (2014). Virulence and antimicrobial resistance of *Escherichia coli* isolated from Tigris River and children diarrhea. *Infection and Drug Resistance.*7: 317–322.

29- Tyagi1, A. Singh, V. Bharadwaj, M. Kumar, A. and Thakur.K. (2011). Isolation and antibacterial susceptibility testing of multi drug resistant *Pseudomonas aeruginosa* causing urinary tract infections. *J.Chem.Pharm.Res*. 3 (4):342-347.

30- Madigan, M.; Martinko, J. and Parker, J. (2003). Brock Biology of Microorganisms. 10thed. Upper Saddle River, NJ, USA: Prentice Hall.