

**Original Article**

**The Role of Hep-2 Cell Adherent *Escherichia coli* Strains on Diarrheic Children in Imo State of Nigeria**

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**ABSTRACT**

The role of adherent *Escherichia coli* strains on diarrhoeic children in Imo state of Nigeria was carried out in this paired Control study. *Escherichia coli* isolates from stool specimens of children with diarrhoea were matched with Controls and tested in HEp-2 cell adherence assay. A total of 266 *E. coli* strains (2 strains for every test subject) from both 83 children with diarrhoea and 50 apparently healthy Controls were examined for virulent traits using HEp-2 cells. Statistically, significant ( $P < 0.05$ ) adherent strains were obtained from the diarrhoeic children, 14.4%, as against 6% from the Control. In this study, Enteropathogenic *E. coli* (EPEC) (1.2% vs 0%), Enteroaggregative *E. coli* (EAEC) (8.4% vs 4.0%) and Diffusely adherent *E. coli* (DAEC) (4.8% vs 2.0%) strains were significantly associated with diarrhoea ( $P < 0.05$ ), EPEC was isolated only in children  $< 3$  years old while EAEC and DAEC were more distributed on the age ranges studied. High prevalence of parasites were seen on both groups although the diarrhoeal group had a statistically significant (19.2% vs 2.0%) prevalence ( $P < 0.05$ ), The parasites were *Giardia lamblia* (7.2% vs 2.0%), *Ascaris lumbricoides* (4.8% vs 0%), hookworm (2.4% vs 0%) and *Trichuris trichuria* (4.8% vs 0). This showed that intestinal parasites constitute an important factor in the etiology of diarrhoea in this area. The parasites were mostly seen in older children from both groups and this might be attributed to their wandering, playing and eating habits. No mixed infection between parasites, parasite and adherent *E. coli* or between two adherents *E. coli* was observed. The adherent *E. coli* showed marked resistance to conventional antimicrobial agents like Cotrimoxazole, Ampicillin and Chloramphenicol with 83.3%, 83.3% and 66.6%, respectively, while the Cephalosporins: Cefuroxime and Ceftazidime showed low resistance with 8.3% and 0%, respectively, indicating that the Cephalosporins will be a good choice for the empirical treatment of bacterial infectious diarrhoea in this region.

**Key words:** Intestinal parasites; Diarrhoeaic children; Antimicrobial agents; Cephalosporins;

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**INTRODUCTION**

Diarrhoea is one of the leading causes of morbidity and mortality among children in the developing world (Bern *et al.*, 2005). Besides the classic bacterial pathogens like *Shigella*, *Salmonella*, *Yersinia*, *Vibrio* and *Campylobacter* spp., at least six different categories of *Escherichia coli* may cause diarrhoea worldwide. These Diarrhoeagenic *E. coli* (DEC) are: Enteroaggregative *E. coli* (EAEC), Enterohemorrhagic *E. coli* (EHEC), Enteroinvasive *E. coli* (EIEC), Enteropathogenic *E. coli* (EPEC), Enterotoxigenic *E. coli* (ETEC) and Diffusely adherent *E. coli* (DAEC) (Nataro and Kaper, 1998). Classification is based on the presence of different chromosomal or plasmid-encoded virulence genes in *E. coli* enteropathogens that are absent in most commensal strains, as well as their pattern of interaction with epithelial cells and tissue culture monolayers (Nataro and Kaper, 1998). On the basis of patterns of adherence to tissue culture cells (HEp-2 or HeLa), *E. coli* strains can be classified into four groups: localized, localized-like, diffuse, and aggregative (Scaletsky *et al.*, 2002).

One of the most useful phenotypic assays for the diagnosis of diarrhoeagenic *E. coli* is the HEp-2 adherence assay. This assay was first described by Cravioto *et al.* (1979) and remains the "gold standard" for the diagnosis of EAEC, EPEC and DAEC (Nataro and Kaper, 1998).

Infectious diarrhoea can be caused by viruses and bacteria as well as parasites, and patients may have mixed infections. Few studies look for major pathogens in all classes and even fewer studies seek supposedly minor pathogens so that the true contribution of many agents to disease is uncertain. Biomedical and clinical researches have focused on those agents that are mostly likely to cause life-threatening illness, to spread rapidly, or to be vaccine controllable. Epidemiological research is also biased towards those agents that are most easily detected. Rotavirus, *Salmonella* spp. and *Shigella* are among the most investigated

etiologic agents, particularly in Africa, for many of the aforementioned reasons, and diarrhoeagenic *E. coli*, which are difficult to differentiate from commensals, are less frequently sought. By using sensitive and specific methods, hopefully, this work will help to determine the epidemiological significance of adherent *E. coli* in childhood diarrhoea in Imo state, because current researches have shown that morbidity and mortality caused by adherent *E. coli* are becoming worrisome, with the recent epidemic in Europe last year with its corresponding fatalities, as a case study. Also the sensitivity patterns of the offending *E. coli* will be determined so as to have a prior knowledge of the anti-microbial pattern in case of an epidemic in this area. The prevalence of intestinal parasites in the areas studied was also determined.

## MATERIALS AND METHODS

### Subjects and Strains

In this paired case-control study, a total of 133 children between a day old and 12 years of age were used. This included 83 children with diarrhoea and 50 age-matched Controls. The diarrhoeic children were outpatients attending medical and health centers, private and public hospitals in Imo State. Patients were enrolled in the study if they had diarrhoea characterized by frequent watery stools (>3 times/day) with or without blood or mucus and they had not taken any antimicrobial agent in the week preceding sampling. The child also must not harbor traditional diarrhoeic agents like *Shigella* spp, *Salmonellae* spp and *Campylobacter* spp. The Control subjects were children with similar age distribution drawn from the same hospitals for other health reasons other than diarrhoea. This study was carried out from February 2009 through June 2011. Informed consents were obtained from the children's parents or guardians. This work was approved by the Ethical Committee of Post Graduate School of Rivers State University of Science and Technology, Nkpolu, Rivers State.

### Specimen Collection and Processing

Samples were inoculated onto MacConkey agar (Oxoid, Cambridge, U.K.) for colonies isolation. Fresh stool specimens obtained were examined microscopically for blood, leukocytes, erythrocytes, cysts and ova. Specimens collected at centers distant from the laboratory were inoculated into Cary-Blair transport media (Oxoid, Cambridge, U.K.) and plated out as soon as possible. After identification of *E. coli* using standard microbiological methods, two colonies from the same subject were preserved in nutrient agar stabs covered in Mineral oil until required for the adherent test.

### HEp-2 Adherence Assay

All *E. coli* isolates were subjected to HEp-2 adherence tests in the presence of D-mannose by the method modified by Vial *et al.* (1990). Two ml HEp-2 cells were grown overnight to 50% confluence in Dulbecco's modified Eagle medium (DMEM) (Gibco BRL, Gaithersburg, Md.) containing 0.1ml 10mg/ml streptomycin and 10% fetal bovine serum in 24-well plates (Becton, Dickinson and Company, Franklin Lakes, NJ) with one sterile round 13mm glass coverslip deposited in each well. Bacterial strains were grown in Nutrient broth (Difco Laboratories, Detroit, MI) for 16 h -18 h at 37°C. Cell monolayers were infected with 50 µL of appropriate bacterial cultures added to 1 mL of DMEM and incubated at 37°C for 3 h. The infected monolayers were washed with sterile phosphate-buffered saline, fixed with methanol, stained with Giemsa stain, and examined under a microscope.

### Control Strains

The reference strains, RKI 17-2 for EAEC, DSM 8698 for EPEC, and ATCC 25922 for non-pathogenic *E. coli* served as Controls. They were all from ATCC (Manassas, USA)

### Data Analysis

A two-tailed chi-square test was used to determine the statistical significance of the data, a *P* value of <0.05 was considered significant.

### Sensitivity Test

Antimicrobial Susceptibility testing was done on all the adherent strains isolated from the test group using Kirby-Bauer disc diffusion method according to the protocols of the National Committee for Clinical Laboratory Standards (NCCLS) (Wayne, 1998).

## RESULTS

In this paired Control study, a total of 14.4% of Adherent *E. coli* (AEC) were isolated from the diarrhoeic children, while 6% AEC were isolated from their Control counterparts. This was statistically significant ( $P < 0.05$ ) (Table 1). Plate 1 showed the morphological appearance of the adherent strains with A, B, C, D representing Localized Adherence (LA), Aggregative Adherence (AA), Diffuse Adherence (DA) and non adherent (NA) strains, respectively. EAEC (AA) had the highest prevalence in both the Control and the test subjects with 8.4% and 4.0%, respectively. They showed statistical significant correlation to diarrhoea compared to the control ( $P < 0.05$ ). There was no predilection of this strain to any of the age range as almost all the age ranges had AA (Table 1). DAEC (DA) was the second highest AEC isolated from this study with 4.8% vs 2.0% for the sample and Control, respectively (Table 1), this also showed statistical significant correlation to diarrhoea ( $P < 0.05$ ). EPEC (LA) had the least prevalence with 1.2% isolated only from the diarrhoea group (Table 1). This occurred solely from 1-3 years age range.

Intestinal parasites were also observed from both the diarrhoeic subjects and the control

Table 1: Prevalence (%) of Adherent *E. coli* in some Hospitals in Imo State

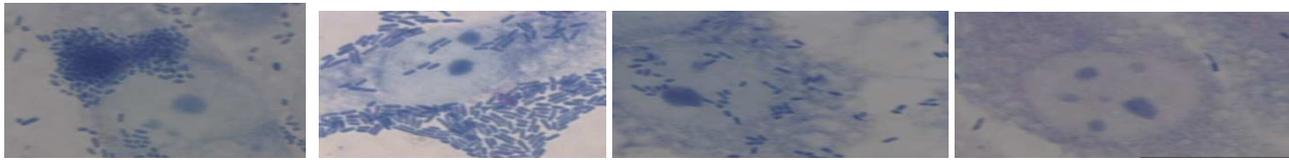
Age	1-3		4-6		7-9		10-12		TOTAL	
	S	C	S	C	S	C	S	C	S	C
<i>Localized Adherence</i>	1.2	-	-	-	-	-	-	-	1.2	-
<i>Aggregative Adherence</i>	1.2	-	2.4	2.0	1.2	2.0	3.6	-	8.4	4.0
<i>Diffuse Adherence</i>	-	-	1.2	-	2.4	2.0	1.2	-	4.8	2.0
Total	2.4	-	3.6	2.0	3.6	4.0	4.8	-	14.4	6.0

KEY: S=Sample under study, C= Control

Table 2: Prevalence (%) of Parasites in some Hospitals in Imo State

Age	1-3		4-6		7-9		10-12		TOTAL	
	S	C	S	C	S	C	S	C	S	C
<i>Giardia lamblia</i>	-	-	1.2	-	2.4	-	3.6	2.0	7.2	2.0
<i>Ascaris lumbricoides</i>	-	-	1.2	-	1.2	-	2.4	-	4.8	-
Hookworm	-	-	-	-	1.2	-	1.2	-	2.4	-
<i>Trichuris trichuria</i>	-	-	2.4	-	1.2	-	1.2	-	4.8	-
Total	-	-	4.8	-	6.0	-	8.4	2.0	19.2	2.0

KEY: S=Sample under study, C= Control



A

B

C

D

A. Localized Adherence (EPEC)

B. Aggregative Adherence (EAEC)

C. Diffuse Adherence (DAEC)

D. Non-Adherent Control Strain.

Plate i: Adherence patterns of diarrhoeagenic *E. coli* to Hep-2 cells

(19.2% vs 2.0%), respectively (Table 2). This was statistically significant ( $P < 0.05$ ). The parasites in their decreasing order of prevalence were *Giardia lamblia* 7.2% vs 2%, *Ascaris lumbricoides* 4.8% vs 0%, hookworm 4.8% vs 0% and *Trichuris trichuria* 2.4% vs 0%. No mixed infection amongst parasites, between parasites and adherent *E. coli* or between two adherents *E. coli* was observed. The adherent *E. coli* showed marked resistance to conventional drugs, with Cotrimoxazole and Ampicillin showing the highest resistance with 83.3% each, followed by Chloramphenicol, Cefuroxime and Ceftazidime with 66.6%, 8.3%, and 0%, respectively.

## DISCUSSION

EAEC strains are emerging pathogens in the sense that they have only recently become recognized. All studies on EAEC in Africa have found it to be one of the most common diarrhoeagenic categories, and most have highlighted the EAEC strains' associations with diseases ranging from watery to

invasive diarrhoea, which may be acute or persistent (Okeke *et al.*, 2000; Opintan *et al.*, 2009).

In this work, EAEC were also commonly recovered from asymptomatic individuals (Controls) although statistically significant to the test subjects, this according to Flores and Okhuysen (2009) partly reflects strain heterogeneity which may be related to infection load and also arises from inter-individual variation in susceptibility. Thus, individuals carrying EAEC strains may be reservoirs for bacteria that could cause disease in more susceptible individuals.

DAEC were statistically significant in this work ( $P < 0.05$ ). The roles of DAEC in diarrhoea has been controversial, some studies like those of Giron *et al.* (1991), and Germani *et al.* (1996) found correlation of DAEC to diarrhoea while other researchers like Gomes *et al.* (1989) and Gunzburg *et al.* (1993) were of contrary opinion.

EPEC has long been considered the putative dominant *E. coli* pathogen and has been shown to be associated with infantile diarrhoea in several paired case-control studies of children < 1 year of age

in developing countries (Okeke *et al.*, 2000; Scaletsky *et al.*, 2002). The infectious dose in naturally transmitted infection among infants is not known but is presumed to be low. The reason(s) for the relative resistance of adults and older children is not known, but loss of specific receptors with age is one possibility.

Also from the data obtained in this work, Imo State had a low prevalence in both the adherent *E. coli* strains and intestinal parasites colonization compared to similar works done in other states of Nigeria (Okeke *et al.*, 2000). This could be attributed several factors, including the availability of pipe-borne water in many of their Local Government Areas and its corresponding increase in hygiene. Literacy level of the children's parents, informed awareness from the media like television, radio, paper, where there are always information on the importance of basic good hygiene practices like hand washing, may be contributed to the low prevalence in Imo State.

The sensitivity pattern of the AEC isolates may not be favorable to the economy of the people in the study area as the cheap and easily available drugs like Ampicillin and Cotrimoxazole showed marked resistance while Chloramphenicol showed moderate resistance. In developing countries like Nigeria, Ampicillin, Chloramphenicol, and Cotrimoxazole are widely used to treat diarrhoea because of their low cost, and ready availability.

Ampicillin and Cotrimoxazole are the first line choice for treating diarrhoea as well as other non specific ailments in Imo state, as well as the whole country and this probably led to the marked resistance as they were prone to abuse by both medical practitioners and general populace. Secondly, the attitudes of the people in patronizing quacks (Chemists and sundry) who usually give these drugs in inappropriate doses and

duration have contributed immensely in propagating this menace of resistance. Also, the menace of fake drugs have not helped the scourge. Although the appropriate agency (NAFDAC) is intensifying its effort to check this anomaly. Susceptibilities of Adherent *E. coli* strains were also tested on the Cephalosporins; Cefuroxime and Ceftazidime, though not traditional diarrhoea antimicrobial; they showed good sensitivity which makes them good candidates for empirical treatment of diarrhoea in children in these regions. But these drugs are not commonly available, they are expensive and there could be problem of anaphylaxis reactions though they are mostly given in hospital settings.

This work has revealed the worrisome emergence of antimicrobial resistance and high asymptomatic carriage rates for adherent diarrhoeagenic *E. coli* (EPEC, DAEC and EAEC) but bacterial and host factors that predispose to disease, as well as non-human reservoirs, are largely unknown. There was also strong association between use of conventional antimicrobial and colonization with these antibiotic resistant *E. coli*, suggesting a trend which could be dangerous in the management of other enteric infections since there is evidence of horizontal transfer of genes between these organisms. Although, parasitic infections can be effectively treated; there is no specific treatment for diarrhoea, so there is an urgent need for effective preventive measures based on detailed understanding of the epidemiology of these diarrhoea, as the recent outbreak of EAEC in Europe (Frank *et al.*, 2011) and its corresponding fatalities have shown that these bacteria are extremely dangerous not only in children but in adults as well.

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