

Phenotypic assay of adherent *E. coli* strains using hep-2 cells on diarrheic children in Rivers State of Nigeria

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ABSTRACT

In this paired case-control study of children with diarrhea in Rivers state, the association between HEp-2-adherent *Escherichia coli* strains and diarrhea was examined. *Escherichia coli* isolates from stool specimens of children with diarrhea 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 diarrhea 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 diarrheic children 18 (21.7%) as against 5 (10%) from the control. While EPEC (3.6% vs 0%) and EAEC (10.8% vs 4%) strains were significantly associated with diarrhea ($P < 0.05$), EPEC was isolated only in children <3 years old while EAEC was more distributed on the age ranges studied. There was no significant association of DAEC (7.2% vs 2%) strains isolated from the test subjects and the control group ($P > 0.05$). High prevalence of parasites were seen on both groups although the diarrheal group had a statistical significant (26.5% vs 16%) prevalence ($P < 0.05$), this showed that intestinal parasites are also important factor in the etiology of diarrhea 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 isolates showed poor sensitivity to traditional drugs like Ampicillin, Cotrimoxazole and Chloramphenicol, while Cefuroxime and Ceftazidime showed good sensitivity. These data suggest that EAEC may be a pathotype that is increasing in incidence as a cause of diarrhea in children in these areas.

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Keywords: Adherent *E. coli* (DEC); Enteropathogenic *E. coli* (EPEC); Enteroaggregative *E. coli* (EAEC); Diffusely adherent *E. coli* (DAEC); Mixed Infections; Intestinal Parasites.

INTRODUCTION

Among the bacterial causes of diarrhea is *Escherichia coli*. The diarrheagenic *E. coli*

(DEC) is the most important bacterial etiologic agent of childhood diarrhea and constitutes a major public health problem in

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developing countries (Okeke, 2009). 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).

HEp-2-adherent *Escherichia coli* strains that show localized adherence (LA), aggregative adherence (AA), diffuse adherence (DA), and localized adherence-like (LAL) patterns have been implicated as diarrheal pathogen (Scaletsky et al., 2002).

The Localized Adherence (LA) shown by typical EPEC is mediated by an inducible bundle-forming pilus, which correlates with the presence of a plasmid, designated the EPEC adherence factor (*eaf*) plasmid (Giron et al. 1993). EPEC strains also cause attaching and effacing lesions on eukaryotic cells that involve a 94 kDa protein encoded by the chromosomal *E. coli* attaching and effacing (*eae*) gene (Okeke, 2009).

Two factors, F1845 and AIDA-I, were found to encode Diffuse Adherence (DA) in DAEC (Bilge et al., 1993). Several studies have implicated DAEC strains as agents of diarrhea (Germani et al., 1996), while other studies have not recovered DAEC strains more frequently from diarrheal patients than from asymptomatic controls (Rappelli et al., 2005).

EAEC strains are an emergent pathogen causing diarrhoea. Classical EAEC strains are recognized by their characteristic aggregative adherence (AA) or “stacked-brick” adherence to HEp-2 culture cells monolayers (Frank et al., 2011). The adherence of many EAEC strains requires the presence of a plasmid with localized genes coding for Aggregative Adherence (AA) (Nataro and Kaper, 1998).

The epidemiological significance of each childhood diarrhea varies with geographical area. Very few studies, if any, have investigated the microbiology of

adherent *E. coli* especially in south-south Nigeria, by using HEp-2 cells adherent assay the aims of this work however are to find the association between adherent DEC, intestinal parasites and diarrhea in this region.

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 diarrhea and 50 age-matched controls. The diarrheic children were outpatients attending medical and health centers, private and public hospitals in Rivers States. Patients were enrolled in the study if they had diarrhea 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 diarrheic 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 diarrhea. This study was carried out from February 2009 through June 2010. Informed consents were obtained from the children parents or guardians.

Specimen collection and processing

Samples were inoculated onto MacConkey agar (Oxoid, Cambridge, U.K.) for colonies isolation. Fresh stools 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 13 mm 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 AND DISCUSSION

Statistically significant adherent strains were isolated from the test subjects 18

(21.7%) compared to the control 5 (10%) ($P<0.05$) (Table 1). Figure 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.

Table 1 also showed that Localized Adherence (LA) which is typified by EPEC only occurs in children less than 3 years old, no LA was seen in the control group (3.6% vs 0%) demonstrating that EPEC continues to be an important cause of diarrheal disease in Nigeria. In this study, those strains were significantly isolated from diarrheal stools ($P<0.05$).

EAEC which was represented by *E. coli* strains exhibiting AA was the highest in the adherent test with 9 (10.8%) vs 2 (4%) for the control, they showed statistical significant correlation to diarrhea 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 which exhibited DA showed no statistically significant correlation with diarrhea with 7.2% vs 6% ($P>0.05$).

High prevalence of parasites were seen on both groups (Table 2) although the diarrheal group had a statistical significant prevalence (26.5% vs 16%) ($P<0.05$), this showed that intestinal parasites are also important factor in the etiology of diarrhea in this area. The parasites were mostly seen in older children from both groups and this can 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 resistance pattern of the AEC isolates were not favorable to the economy of the people in this area as the cheap and easily available drugs like ampicillin and cotrimoxazole showed marked resistance while chloramphenicol showed moderate resistance (Table 3). These drugs are the first line choice for treating diarrhea as well as non specific ailments in these areas and this

Table 1: Prevalence (%) of Adherent *E. coli* in Rivers State.

Age	1-3		4-6		7-9		10-12		TOTAL	
	S	C	S	C	S	C	S	C	S	C
LA	3.6	-	-	-	-	-	-	-	3.6	-
AA	4.8	-	4.8	2.0	1.2	2.0	-	-	10.8	4.0
DA	-	2.0	1.2	2.0	4.8	-	1.2	2.0	7.2	6.0
Total	8.4	2.0	6.0	4.0	6.0	2.0	1.2	2.0	21.6	10.0

Key: s-sample under study, c- control

Table 2: Prevalence (%) of Parasites in Rivers State.

Age	1-3		4-6		7-9		10-12		TOTAL	
	S	C	S	C	S	C	S	C	S	C
<i>G</i>	-	-	1.2	-	4.8	-	4.8	4.0	10.8	4.0
<i>A</i>	-	-	-	-	1.2	2.0	2.4	-	3.6	2.0
<i>H</i>	-	-	-	-	1.2	-	8.4	8.0	9.6	8.0
<i>T</i>	-	-	1.2	-	-	-	1.2	2.0	2.4	2.0
Total	-	-	2.4	-	7.2	2.0	16.9	14.0	26.4	16.0

KEY: S-Sample Under Study, C- Control, G- *Giardia lamblia*, T- *Trichuris trichuria*,

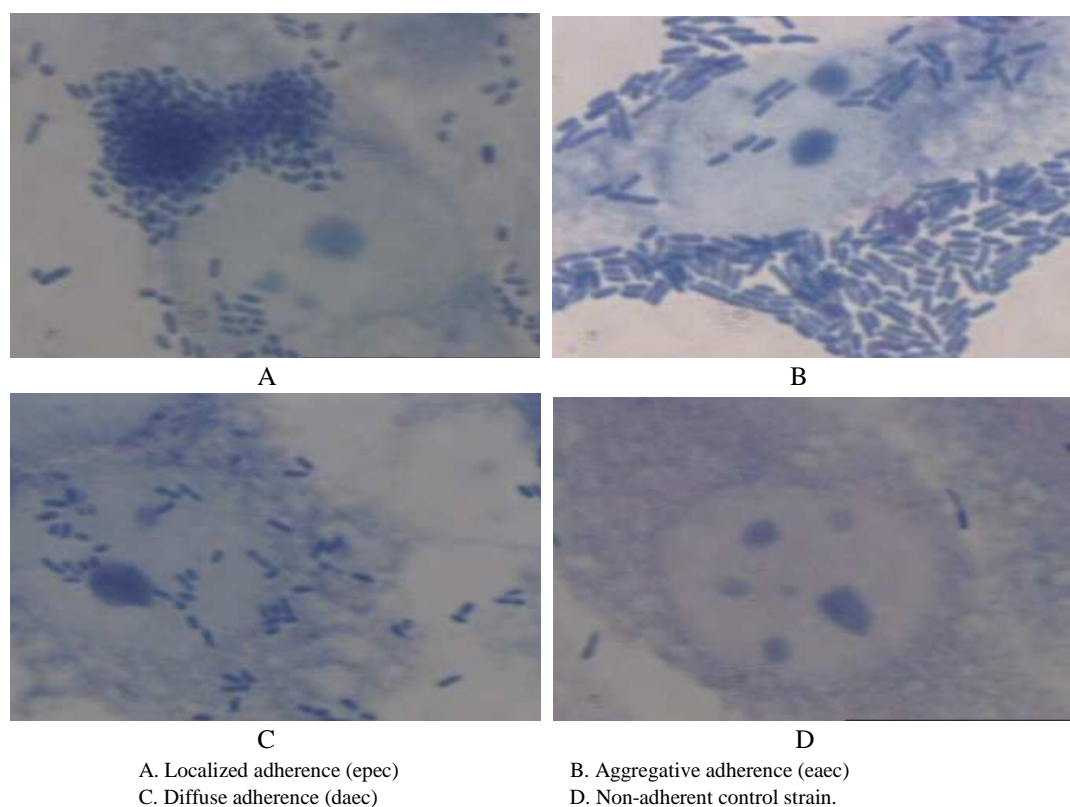


Figure 1: Adherence patterns of diarrheagenic *E. coli* to hep-2 cells.

Table 3: Sensitivity pattern of the adherent isolates.

	No. sensitive	Percentage sensitive (%)	Percentage resistant (%)
Ampicillin	3	16.6	83.4
Cotrimoxazole	1	5.5	94.5
Chloramphenicol	6	33.3	66.7
Cefuroxime	14	77.7	22.3
Ceftazidime	14	77.7	22.3

probably led to the marked resistance as they were prone to abuse by both medical practitioners and general public. Also the menaces of fake drugs around here have not helped the scourge. The cephalosporins; cefuroxime and ceftazidime, though not traditional diarrhea drugs showed good sensitivity which makes them good candidates for empirical treatment of diarrhea in this region.

This work has revealed the worrisome emergence of antimicrobial resistance and high asymptomatic carriage rates for adherent diarrheagenic *E. coli* but bacterial and host factors that predispose to disease, as well as non-human reservoirs, are largely unknown. Future diarrheal disease research needs to focus on broadening the repertoire of pathogens sought in epidemiological surveys to include multiple categories of diarrheagenic *E. coli* while building capacity to detect these pathogens in local reference laboratories.

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