



## The role of entero-aggregative *Escherichia coli* (EAEC) strains on diarrheic children in some southern States of Nigeria

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### ABSTRACT

The role of entero-aggregative *Escherichia coli* (EAEC) strains on diarrheic children in some southern States of Nigeria was carried out in this paired control study. *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 800 *E. coli* strains (2 strains for every test subject) from both 250 children with diarrhea and 150 apparently healthy controls were examined for aggregative adhesion using HEp-2 cells. Statistically significant ( $P < 0.05$ ) EAEC strains were obtained from the diarrheic children 40.6% as against 20% from the control. In this study, Bayelsa State recorded the highest isolate with 21.4 vs 12%, followed by Rivers State (10.8% vs 4.0%), then Imo State the least (8.4% vs 4.0%). Children within 4-8 years age range recorded the highest prevalence in the States studied. High prevalence of intestinal parasites were seen on both groups although the diarrheal group had a statistical significant (91% vs 36%) prevalence ( $P < 0.05$ ). The parasites were *Giardia lamblia*, *Ascaris lumbricoides*, hookworm and *Trichuris trichuria*. This showed that intestinal parasites are also important factor in the etiology of diarrhea in this area. There was mixed infection between parasites and EAEC in 5 test subjects in Bayelsa State, but none in the other States and also none from the control. The entero-aggregative *E. coli* showed marked resistance to conventional antimicrobial agents like cotrimoxazole, ampicillin and chloramphenicol with 80.3%, 81.2% and 61.6% respectively, while the cephalosporins: cefuroxime and ceftazidime showed low resistance with 3.3% and 4.3% respectively, indicating that the cephalosporins will be a good choice for the empirical treatment of bacterial infectious diarrhea in this region.

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**Keywords:** HEp-2 cells, Intestinal parasites, Mixed infection, Antimicrobial agents, Cephalosporins.

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### INTRODUCTION

*Escherichia coli* is the predominant facultative anaerobe of human colonic flora. Some clones of this species are pathogenic. One of the major clinical syndromes to result

from infection with pathogenic *E. coli* is enteric/gastrointestinal disease. Five major categories of diarrheagenic *E. coli* have been identified: enteropathogenic (EPEC), enterotoxigenic *E. coli* (ETEC),

enteroinvasive *E. coli* (EIEC), enterohemorrhagic *E. coli* (EHEC) and enteroaggregative *E. coli* (EAEC). These categories are differentiated on the basis of pathogenic mechanisms and clinical manifestations.

Amongst this diarrheagenic *E. coli* are the members of EAEC group which are defined as *E. coli* strains that adhere to HEp-2 cells in an aggregative pattern. Most *E. coli* strains that belong to EAEC category have been found to be serologically non typable using standard methods. EAEC strains belong to a large number of different serogroups such as O44, O55, O86, O111, O125, O126, and O128, heterogeneity both in serotypes and outer membrane protein (OMP) profiles suggested a great genetic diversity of EAEC (Yatsuyanagi et al., 2002).

Starting in early May, 2011, an outbreak of haemolytic uraemic syndrome and bloody diarrhea caused by *Escherichia coli* O104:H4 has spread throughout Germany (Frank et al., 2011). As of June 20, 2011, 810 cases of haemolytic ureamic syndrome, 2684 non-haemolytic ureamic syndrome cases, and 39 deaths have been reported by the Robert Koch Institute and European Centre for Disease Prevention and Control. The particular features of this outbreak are the predominant involvement of adult women and common severe neurological complications, such as encephalopathy and epileptic seizures (Frank et al., 2011).

The pathogenesis of EAEC infection is only partially understood. In several *in vivo* and *in vitro* models, EAEC strains adhere to the small and large bowel mucosal surface in a thick aggregating biofilm. In order to cause diarrheal disease, EAEC adheres to intestinal mucosa, forms a mucoid biofilm and induces toxic effects on the intestinal mucosa that result in diarrhea. The exact mechanism of pathogenesis is not fully understood; however,

adhesins, toxins and several other factors have been implicated. Certain strains carry a high molecular weight plasmid associated with the aggregative adherence, on which a number of virulence genes are located. The first studies with EAEC strains showed that this bacteria was associated with persistent ( $\geq 14$  days) diarrhea in children (Okeke and Nataro, 2001). However, Kahali et al. (2004) showed that EAEC may be associated with acute diarrhea. EAEC is also associated with a number of both nosocomial and community outbreaks worldwide. In addition, the participation of EAEC as the causative agent of diarrheal disease in human immunodeficiency virus (HIV)-infected adults in the developed world has also been described (Durrer et al., 2000).

The gold standard for identification of EAEC remains HEp-2, in which the bacteria adhere to the HEp-2 cells in an aggregative fashion, in a way that resembles stacked-brick configuration. This method was used in identification of EAEC in this work because classical EAEC strains are recognized by their characteristic aggregative adherence (AA) or "stacked-brick" adherence to HEp-2 culture cells monolayers (Vial et al., 1990).

The aim of this work however is to find the role EAEC plays amongst diarrheagenic children in some south- south States of Nigeria. Phylogenetic relations of the isolates and their antimicrobial susceptibilities were also determined.

## MATERIALS AND METHODS

### Subjects and strains

In this paired case-control study, a total of 400 children between a day old and 12 years of age were used. This included 250 children with diarrhea and 150 age-matched controls. The diarrheic children were outpatients attending medical and health centers, private and public hospitals in Rivers,

Bayelsa and Imo States of southern Nigeria. 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 December 2011. 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 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, ATCC 25922 for non-pathogenic *E. coli* served as controls. They were all from ATCC (Manassas, USA).

#### **Sensitivity test**

Antimicrobial Susceptibility testing was done on all the entero-aggregative 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).

#### **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.

## **RESULTS AND DISCUSSION**

Classical EAEC strains are recognized by their characteristic aggregative adherence (AA) or “stacked-brick” adherence to HEp-2 culture cells monolayers (Plate 1), while the non adherent strains showed no adhesion to the HEp-2 culture cells (Plate 2), In this study, there was a total of 40.6% vs 20% of EAEC for the test subjects and controls respectively, isolated from the various States. This was statistically significant (*P*<0.05). Bayelsa

State had the highest isolates of EAEC with 21.4% vs 12% for the test subjects and controls respectively. Bayelsa State was followed by Rivers and then Imo State (Table 1). All studies on EAEC in Africa have found it to be one of the most common diarrheagenic categories, and most have highlighted the EAEC strains' associations with diseases ranging from watery to invasive diarrhea, which may be acute or persistent (Okeke et al., 2000; Opintan et al., 2009). Also, enteroaggregative *Escherichia coli* (EAEC) was the most commonly isolated pathogenic *E. coli* group from patients with diarrhea in England (Wilson et al., 2002), and has emerged as an important pathogen in travellers' diarrhea (Adachi et al., 2002), diarrhea among children (Huang and Dupont, 2004) and in immune-compromised patients (Gassama-Sow et al., 2004). In this work, EAEC were also commonly recovered from asymptomatic individuals (controls) although statistically significant ( $P < 0.05$ ) to the test subjects (Table 1). 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.

Also from the data obtained from this work, Imo State had a low prevalence in both the adherent *E. coli* strains and intestinal parasites colonization (Tables 1, 2 and 3), compared to other works done on this in other States of Nigeria (Okeke et al., 2000; Okeke et al., 2006). This could be attributed to lots of 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 parents, informed awareness from the media like television, radio, paper, where there are

always information on the importance of basic things like hand washing, are also contributors to the low prevalence in Imo State.

The sensitivity pattern of the EAEC isolates were not favorable to the economy of the people in these States studied as the cheap and easily available drugs like ampicillin and cotrimoxazole showed marked resistance while chloramphenicol showed moderate resistance (Figure 1). In developing countries like Nigeria, ampicillin, chloramphenicol, and cotrimoxazole are widely used to treat diarrhea because of their low cost and ready availability.

Antibiotics have revolutionized the treatment of common bacterial infections and play a crucial role in reducing mortality. Antimicrobial therapy should be used in severe cases of diarrheal disease to reduce the duration of illness and may be used to prevent traveler's diarrhea (Nataro and Kaper, 1998). However, from this result, the progressive increase in antibiotic resistance among enteric pathogens in Nigeria and other developing countries is becoming a critical area of concern.

Although the cephalosporins; cefuroxime and ceftazidime, though not traditional diarrhea antimicrobial; they showed good sensitivity which makes them good candidates for empirical treatment of diarrhea 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., we have tested the susceptibilities EAEC strains to these antibiotics (Figure 1), since they could be empirically or incidentally used which is a common practice in developing nations like Nigeria and Vietnam, where according to the national household survey in Hanoi, Vietnam, FROM 1997 to 1998, on average, about two-

thirds of those who are ill treated themselves (Chuc, 2002).

The fluoroquinolone and quinolone antibiotics which are commonly used to treat infections, including diarrhea were not used in this work because they were contra-indicated in children, but they have been recommended for prophylaxis and treatment of traveler's diarrhea (Bhattacharya and Sur, 2003).

However, other studies have shown high prevalence of resistance to these antibiotics in enteric pathogens, especially diarrheagenic *E. coli* (Hoge et al., 1998; Prats et al., 2000). Also Vu Nguyen et al. (2005) in Vietnam, isolated more than 77% of diarrheagenic *E. coli* strains which were resistant to the commonly used antibiotics which were ampicillin and cotrimoxazole. Putnam et al., (2004) also show that most diarrheagenic *E. coli* strains that cause diarrhea in hospitalized children in Mexico are resistant to cotrimoxazole and ampicillin, which were the first line of drugs used to treat diarrhea there. Also, this year, Frank et al., (2011) observed that EAEC isolates which caused outbreaks in some parts of Europe with lots of fatalities have an extended-spectrum  $\beta$ -lactamase phenotype (i.e., resistance to all penicillins and cephalosporins; susceptibility to carbapenems). They are resistant to sulfamethoxazole-trimethoprim but remain susceptible to fluoro-quinolones and aminoglycosides. This resistance pattern is an emerging problem for Diarrheagenic *E. coli* (DEC) strains isolated from children in other developing countries and for other enterobacteria worldwide.

That means that the patients infected with these *E. coli* strains could risk a treatment failure. It is also indicated that the multi resistance of different categories of diarrheagenic *E. coli* strains is emerging in different developing countries where these antibiotics (both classical and new) have been

widely used (Gomi et al., 2011; Vila et al., 2009).

These ampicillin and cotrimoxazole are the first line choice for treating diarrhea as well as other non specific ailments in the States studied, 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 our 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 around here have not helped the scourge as every citizen here knows that an appreciable percentage of drugs used here are smuggled into the country, this smuggled drugs are outright fake or sub-standard. Although the appropriate agency; National Agency for Food and Drug Administration and Control (NAFDAC) is intensifying its effort to check this anomaly. High rate of intestinal parasites were also noted in this work, Bayelsa State also recorded the highest prevalence, with Imo State being the least, hookworm was the highest intestinal parasite encountered in the States studied for both the test subjects and controls. Others parasites were *Ascaris lumbricoides*, *Trichus trichuria* and *Giardia lamblia* (Table 2). Mixed infection between parasite and adherent EAEC were observed in seven test samples and none in the control group. Eighty six percent (86%) of this mixed infection were seen in Bayelsa State while the remaining 14% was in Rivers State, none was observed in Imo State. No mixed infection between two parasites was observed. These are all seen in Table 2.

There was no statistically significant difference ( $P>0.05$ ) between the males and the females in both the test subjects and the controls in the isolation of the EAEC strains

(Table 2). This proves the long known fact that there is no correlation between pathogenic enteric bacteria and sexes. The same cannot be said for the intestinal parasites which were statistically greater in the males (older) than the females ( $P < 0.05$ ) (Table 2). The fact that the highest prevalence of intestinal parasites were seen in older males in both the test subjects and controls can be attributed to the males natural recreational activities which include their mode of playing, fishing, farming and their general quest for adventure. These predispose them to the parasitic infestations especially hookworm infection which was the most prevalent parasite in the Bayelsa State, where as early as 6 years old, children are already fishing where they are possibly exposed to the hookworm larvae. Other intestinal parasites were also seen in the three States with *G. lamblia* being the most prevalent in Rivers and Imo State (Tables 2 and 3). Considering their modes of infection, sources of drinking water should be looked into in these States, What is also worthy of note is the high level of asymptomatic carriage of intestinal parasites in the States studied, especially Bayelsa State (Table 3), where some children were found to harbor multiple parasites. The presence of these parasites lead to malnutrition of the

affected individuals which in turn predisposes them to an unfavorable prognosis in diseases like malaria, pneumonia, to mention a few.

Also from the data obtained from this work, Imo State was the least prevalent in both the adherent EAEC strains and intestinal parasites colonization (Tables 1, 2 and 3). This could be attributed to lots of factors including the availability of pipe borne water in many of their local government areas. Other factors like hygiene level, literacy level of the children parents, informed awareness from the media like television, radio, paper, where there are always information on the importance of basic things like hand washing, are also contributors to the low prevalence in Imo State .

Mixed infection occurred in 7(2.8%) of patients and almost solely in Bayelsa State. The patients involved with these infections actually had severe diarrhea with marked level of dehydration and were all admitted in various hospitals. This is quite different from most of the patients that had single infection where the bouts of diarrhea and dehydration were not as severe as their mixed contemporary and most were not out-patients. This proves that intestinal parasites have a lot of contributory effects in the prognosis of diarrhea.

**Table 1:** Distribution (%) of EAEC isolated from the various age ranges.

	Rivers		Imo		Bayelsa	
	S	C	S	C	S	C
1-3 years	4.8	-	1.2	-	3.6	2.0
4-6 years	4.8	2.0	2.4	2.0	10.7	2.0
7-9 years	1.2	2.0	1.2	2.0	7.1	4.0
10-12 years	-	-	3.6	-	-	4.0
<b>Total</b>	<b>10.8</b>	<b>4.0</b>	<b>8.4</b>	<b>4.0</b>	<b>21.4</b>	<b>12</b>

S - Sample under study, C - Control

**Table 2:** Prevalence of sex on EAEC, parasites and mixed infection on the various States.

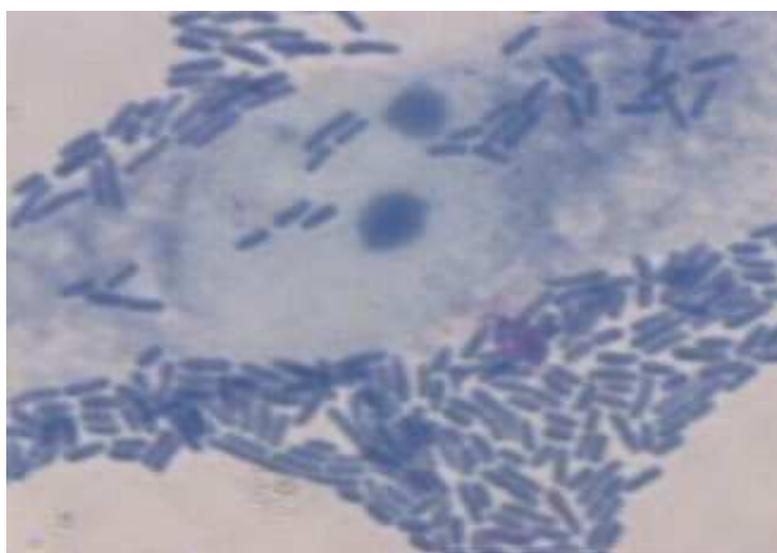
		<b>Rivers</b>	<b>Imo</b>	<b>Bayelsa</b>
EAEC	<b>M</b>	4	4	10
	<b>F</b>	5	3	8
Hookworm	<b>M</b>	7	2	12
	<b>F</b>	1	0	11
<i>G. lamblia</i>	<b>M</b>	4	4	4
	<b>F</b>	5	2	6
<i>Ascaris</i>	<b>M</b>	1	3	2
	<b>F</b>	2	1	2
<i>T. trichuria</i>	<b>M</b>	1	2	1
	<b>F</b>	1	2	1
Mixed Infection	<b>P/EAEC</b>	1	0	6
	<b>P/P</b>	0	0	0

M-Male, F - Female, G - *Giardia*, T- *Trichuris*, P-Parasite,

**Table 3:** Distribution (%) of intestinal parasites isolated from the States.

	<b>Rivers</b>		<b>Imo</b>		<b>Bayelsa</b>	
	<b>S</b>	<b>C</b>	<b>S</b>	<b>C</b>	<b>S</b>	<b>C</b>
<i>G. lamblia</i>	10.8	4.0	7.2	2.0	12.0	4.0
<i>A.lumbricoides</i>	3.6	2.0	4.8	-	3.6	2.0
hookworm	9.6	8.0	2.4	-	27.4	12.0
<i>T .trichuria</i>	2.4	2.0	4.8	-	2.4	-
<b>Total</b>	<b>26.4</b>	<b>16</b>	<b>19.2</b>	<b>2.0</b>	<b>45.4</b>	<b>18</b>

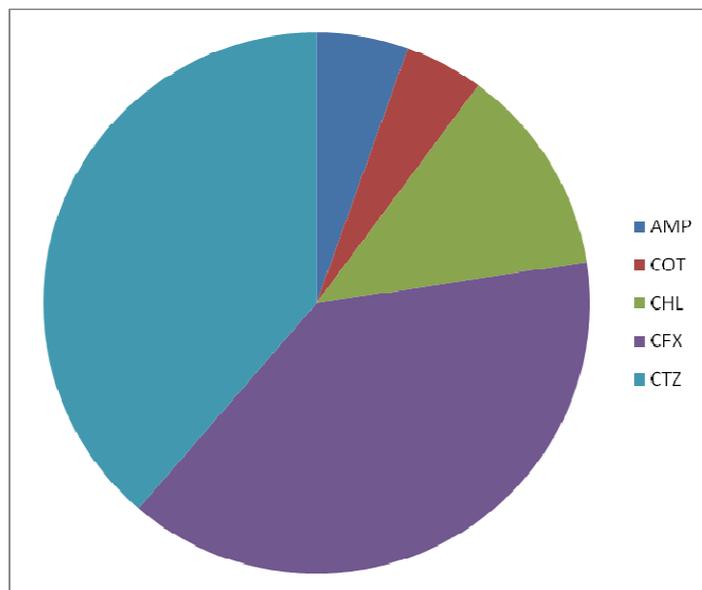
S - Sample under study, C - Control



**Plate 1:** Adherence patterns of EAEC to HEp-2 cells.



**Plate 2:** No Adherence of *E. coli* to HEp-2 cells.



**Figure 1:** Sensitivity pattern of the EAEC isolates.

## Conclusion

This work has revealed the worrisome emergence of antimicrobial resistance and high asymptomatic carriage rates for 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 diarrhea, so there is an urgent need for effective preventive measures based on detailed understanding of the epidemiology of these diarrhea, as the recent outbreak of EAEC in Europe and its corresponding fatalities have shown that these bacteria are extremely dangerous not only in children but in adults as well. This progressive increase in antibiotic resistance among enteric pathogens, particularly in developing countries, is becoming a special concern. Of greatest immediate concern is the need for an effective, inexpensive antimicrobial agent that can be used safely for treatment of children with diarrhea, especially in developing countries, such as Nigeria.

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