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Detection of Virulence Factor Genes Associated with Diarrhoeagenic *Escherichia Coli* (DEC) from Aboriginal Children in the Northern Territory of Australia

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Abstract:
The aim of this study was to investigate the incidence of ten virulence genes associated with diarrhoeagenic Escherichia coli (DEC) among Aboriginal children in the Northern Territory (NT) of Australia. Stool samples from 64 Aboriginal children, from rural and remote areas, aged less than five years hospitalized at Royal Darwin Hospital (RDH) due to acute/persistent diarrhoea, were collected, cultured and analysed by PCR. Polymerase Chain Reaction (PCR) was used to detect ten different virulence genes and to identify specific DEC types namely enteroaggregative- (EAEC), enteropathogenic- (EPEC), enterotoxigenic- (ETEC), enteroinvasive- (EIEC), enterohemorghagic- (EHEC) and diffusely adherent Escherichia coli (DAEC). Potentially DAEC (53%), EAEC (75%) and STEC (12.5%) were the predominant DEC which may play a major role in the etiology of diarrhea in Aboriginal children while EIEC (1.6%) and EPEC (4.7%) played a minor role, leading to the question of whether or not they contribute for the diarrhoea of Aboriginal children. Aboriginal children with diarrhoea are suspected to suffer from DEC pathotypes infections which can be very dangerous in remote tropical areas, especially during wet season. Furthermore, these results might contribute to better understanding the predominance of DEC causing diarrhoea among Aboriginal children, particularly for those residing in remote areas of the NT of Australia.

1. Introduction

1.1. Aboriginal Health Overview

Currently, gastrointestinal infections are the significant cause of childhood morbidity and mortality worldwide, and diarrhoea is the most common symptom of gastrointestinal tract infections in children, especially, those living in the tropics (Rodríguez *et al*, 2011). In the Northern Territory (NT) of Australia, diarrhoea remains one of the most important clinical issue, and continue to contribute to the large admission rates to hospital per population and most of the patients are Aboriginal children (kukuruzovic *et al*, 2002).

According to an information report from the Australian Institute of Health and Welfare (1999), the second biggest killer of Aboriginal children under the age of 15 is infectious and parasitic disease with 12 times higher than those of non-Aboriginal children. Aboriginal children from rural and urban areas experience much higher rates of all GI infections due to poor environmental and domestic conditions that lead to symptoms such as diarrhoea and vomiting. Further complications result in dehydration and ineffective absorption of nutrients, which in turn cause inadequate weight gain or weight loss (Condon *et al*, 2001).

Previous studies on Aboriginal children hospitalized with diarrhoea reported that most of the children were admitted with high rates of malnutrition, electrolyte disturbances, and lactose intolerance and infected with more than one enteric pathogen (Kukuruzovic R., 2003). In addition, it has been observed that apparently healthy Aboriginal children suffer from intestinal mucosal damage due to heavy exposure and colonization by enteric organisms ingested with food and water. This condition is known variously as “tropical enteropathy syndrome”, and it is characterized by a range of severe manifestations of diarrhoeal

disease such as acidosis, hypokalemia, lactose malabsorption and severe dehydration which contribute to prolonged hospitalization (Currie and Brewster, 2001).
Diarrhoeal disease in northern Australian Aboriginal children is often complicated and in order to prevent this small bowel mucosal damage and severe diarrhoeal diseases, efforts need to focus on improving rapid diagnosis tests, personal hygiene, better dietary practices and decreasing household overcrowding in communities.

1.2. Diarrhoeagenic E. coli (DEC) in the NT
Previous studies in the NT Aboriginal children admitted to the hospital showed that the most frequently identified diarrhoeal pathogens were enteroaggregative *E. coli* (EAEC), rotavirus, enteropathogenic *E. coli* (EPEC), Salmonella species, Cryptosporidium *parvum* and Strongyloides *stercoralis*. (Kukuruzovic and Brewster, 2001). Detection and identification of DEC strains requires that these organisms have to be differentiated from pathogenic and nonpathogenic strains. The diagnosis is suggested by the clinical picture and confirmed by stool cultures. Since the categories of DEC are differentiated on the basis of pathogenic features, emphasis is given on the mechanisms of disease and the development of diagnostic techniques based on virulence factors (Nataro and Kaper, 1998).
In the present study, we investigated the incidence and prevalence of ten DEC virulence factor genes (*aaf1*, *aaf3*, EAEC, DAEC, EIEC, *hlyA*, *eaeA*, *bfpA*, *stx1* and *stx2*) among five of the six major groups of DEC strains found in Aboriginal children admitted to the Royal Darwin Hospital (RDH) in Northern Territory. The detection of pathogenic genes by PCR may be the best way to identify the category of DEC. For this reason, we performed PCR–based tests to detect common DEC virulence factors.

2. Methods

2.1. Specimens
Stool specimens were collected from 64 Aboriginal children (aged between 4 months to 5 years old) who were admitted with acute or severe diarrhoea were collected at RDH and examined for the presence of DEC. 2 mg of stool sample was inoculated into 5 ml of EC modified broth (Oxoid, Adelaide, Australia), stored at 4ºC and transported to our laboratory at Menzies School of Health Research (MSHR) once a week over the period of March 2003 to March 2004. Eight non-diarrheal controls were also included.

2.2. Bacterial sample and DNA preparation
In our laboratory, samples were cultured overnight shaking at 37C, 500 µl of the EC culture solution was aliquoted into a 1.5 ml microtube and pelleted in a bench top microfuge (Sigma, Sydney, Australia). The overlying media was removed and discarded. 200 µl of InstaGene matrix was added to the pellet, vortexed for 10 sec and then incubated at 56ºC for 30 min. The tube was vortexed again for 10 sec and then incubated at 95ºC for 8 min, followed by vortexing and storage at -20ºC prior PCR. For RFLP analysis, 30 ul of diluted *E. coli* culture was plated onto EC agar and grown aerobically overnight at 37ºC, single colonies were then "pick and patched" onto fresh EC agar and grown overnight at 37ºC. These struck out single colonies were then tested for virulence factors using colony taq buffer PCR (CTB-PCR) to ensure the isolated colony was the *E. coli* of interest.

2.3. PCR analysis of DEC Virulence Factor Genes
PCR was used to test for the presence of DEC virulence factor genes *aaf1*, *aaf3*, EAEC, DAEC, EIEC, *hlyA*, *eaeA*, *bfpA*, *stx1* and *stx2*. For all PCR analysis, we have selected specific primers (table 1) used to amplify conserved regions of the target DNA templates from a range of different bacterial species. The PCR tests directly on stool DNA were carried out in a 50 µl volume containing 3 mM MgCl2, 2 mM concentrations of each primer, 200 µM of each dNTP, and 1 U of Taq DNA polymerase and 2 µl InstaGene template. The PCR parameters used were 95°C for 3 min, 60°C for 30 sec, 72°C for 1 min, for 34 cycles. The amplified products were then resolved using agarose gel electrophoresis. The PCR was carried out by resuspending the bacteria in 200 µl CTB buffer in a 1.5 ml microtube and heated at 95ºC for 5 minutes. A 2 µl aliquot of this mix was subsequently used as PCR template. Colony Taq PCR was performed with the same conditions as previously described for normal PCR test but with an exception of the number of cycles, which were 36.

2.4. Restriction Fragment Length Polymorphism (RFLP)
Amplified PCR product was digested using 3 units of *hae*III (Roche, Mannhein, Germany) diluted in restriction buffer M (10x) to a final volume of 12.3 µl. The restriction enzyme was added into 30 µl of PCR product, and incubated at 37ºC for 3 hrs. Agarose gel (2%) electrophoresis was used to separate the restricted fragments and to identify their restriction pattern subtypes.

2.5. Randomly Amplified Polymorphic DNA (RAPD)
RAPD profiles were compared by using two primers (table 1). RAPD analysis were carried out in a 50 µl volume containing 1.5 µl InstaGene template prepared from identified PCR positive single *E. coli* colonies. The reaction included 3 mM MgCl2 (10x buffer), 2 mM primer, 200 µM of each dNTP, and 1 U of Taq DNA polymerase. The parameters were 95°C for 3 min and 40 sec then 39 cycles of 94°C for 30 sec, 38°C for 1min, and 72°C for 1 min. The amplified RAPD-PCR products were resolved by agarose gel electrophoresis. Estimated proportion of polymorphic DNA was carried out by applying the coefficient defined by pairwise DICE's similarities. Data recording and calculations were performed by using the 'BioNumerics' software.

3. Results

3.1. Detection of DEC by PCR

A total of 128 DEC samples were isolated from 64 Aboriginal children with diarrhoea and from 8 non-diarrhoeal children. The prevalence of DEC among Aboriginal children with diarrhoea was analysed by PCR assays and detected 34 (53%) DAEC isolates (*afaBC* PCR positive), 48 (75%) EAEC isolates (*aaf1*, *aaf3* and *pAgg* PCR positive), 16 (25%) atypical EPEC isolates (*eaeA* and/or *bfpA* PCR positive), 3 (4.7%) typical EPEC isolates (*bfpA-aeaA* PCR positive), 8 (12.5%) STEC isolates (*stx2* PCR positive), 3 (4.7%) atypical EHEC (*eaeA* PCR positive), and 1 (1.6%) EIEC isolates (*ipaC* PCR positive). The prevalence in non-diarrhoeal children were 2 (25%) DAEC and 2 (25%) EAEC isolates. Typical EHEC was not detected in this study.

3.2. Analysis of EIEC and EAEC PCR positive isolates by RFLP

The EIEC positive sample was isolated and plated for single colonies and analysed by CTB-PCR. None of the colonies were positive and we considered that the numbers of this pathogen was not significant for comparisons by RFLP and RAPD analysis. On the other hand, we compared EAEC bacteria using the RFLP to determine if the genes *aaf1*, *aaf3* and *pAgg* were homologous in children with the same infection. We examined all 48 EAEC isolates by restricting the amplified CTB-PCR products with *hae*III and demonstrated that *pAgg* and *aaf1* PCR positive colonies did not appear to have sequence variation that was detectable using the 4 base pair cutter (data not shown). In contrast, the *aaf3* PCR products exhibited clearly distinguishable RFLP patterns ranging from 1 to 3 band variations (fig. 1).

3.3. RAPD investigation of potential EAEC isolates

RAPD profiles of 63 single colonies isolated from 48 (75%) patients including controls were compared by using two primers (Table 1). To find out if EAEC isolates were clonally related, a result matrix of pairwise distances was used to generate a phenogram based on the unweighted pair-group method with arithmetic mean (UPGMA) method which resulted in various different clusters. Diversity and clonal relatedness variations observed by different EAEC clusters showed high prevalence of polymorphic DNA profiles.

Primer	Direction	Primer sequence (5' – 3')	Tm	Fragment size (bp)	Reference
Aaf1	Fwd	TTAGTCTTCTATCTAGGG	55	457	13
	Rev	AAATTAATTCCGGCATGG			
Aaf3	Fwd	GTTTGGAACCGGGAATTAACATTG	60	485	13
	Rev	ATACTTTAGATACCCCTCACGCAG			
EAEC	Fwd	CTGGCGAAAGACTGTATCAT	60	629	15
	Rev	CAATGTATAGAAATCCGCTGTT			
DAEC	Fwd	CATCAAGCTGTTTGTTCGTCCGCCG	60	793	13
	Rev	G CTGGGCAGCAAACTGATAACTCTC			
EIEC	Fwd	CTGGATGGTATGGTGAGG	60	320	7
	Rev	GGAGGCCAACAATTATTTCC			
Stx1	Fwd	ACACTGGATGATCTCAGTGG	55	602	19
	Rev	CTGAATCCCCCTCCATTATG			
Stx2	Fwd	CCATGACAACGGACAGCAGTT	60	842	19
	Rev	ACTGCACTTCAGCAAATCCG			
eaeA	Fwd	GCAAATTTAGGTGCGGGTCAGCGTT	60	494	13
	Rev	GGCTCAATTTGCTGAGACCACGGTT			
bfpA	Fwd	CAATGGTGCTTGCGCTTGCT	60	325	13
	Rev	GCCGCTTTATCCAACCTGGT			
hlyA	Fwd	AGCTGCAAGTGCGGGTCTG	55	569	16
	Rev	TACGGGTTATGCCTGCAAGTTCAC			
RAPD primer one		AAGAGCCCGT	38		17
RAPD primer two		TCACGATGCA	38		18

Table 1: Primer sequences used in this study. Tm is the melting temperature used for this primer set.

4. Discussion

Aboriginal children living in the NT of Australia suffer greater incidences of acute, chronic and severe diarrhoea than other children (kukuruzovic *et al*, 2002). The present study was performed to determine the predominant virulence factor genes belonging to pathotypes of DEC in hospitalized diarrheal Aboriginal children in Darwin, Australia. Identification of EIEC bacteria by PCR in only one stool sample demonstrated that this bacterium was not significantly associated with the aetiology of diarrhoea in Aboriginal children. Previous studies have reported that the incidence of EIEC worldwide is thought to be low and its identification depends on the population investigated and attributed to misidentification with non-pathogenic *E. coli* strains (Andrade *et al*, 2002 and Ponds J., 2001).

Typical EHEC bacteria which is identified by the presence of the EHEC-*hlyA*, were not found in this study. However, the presence of virulent genes (*aeaA* and *stx2*) may implicate atypical forms of EHEC. The presence of 12.5% of stool samples positive for stx2 is a much higher prevalence than in other studies (Paton and Paton, 1998). Overall, Aboriginal children

hospitalized with acute diarrhoea are apparently suspected to be susceptible in acquiring STEC infections than EHEC and further characterization of this bacteria is still needed.

Previous studies in the NT have shown cases of EPEC in diarrhoeal and non-diarrhoeal Aboriginal children (Ponds J., 2001). In our study, the incidence of typical EPEC was of 4.7% (3 of 64) for diarrheal patients and interestingly, EPEC bacteria were not found in controls as previously reported by Ponds et al (2001). Also, it was previously reported that children infected with EPEC are rarely infected with other enteric pathogens (Robins-Browne *et al*, 2004). However, our study revealed that 3 patients with potentially EPEC bacteria were infected with DAEC and/or EAEC, respectively. A possible explanation to this phenomenon is that enteroadherent pathogens could be highly spread in the Aboriginal children communities and could be associated with most of the cases of diarrhoea. In addition, malnourished Aboriginal children could be at high risk of suffering persistent diarrhea (Ponds J., 2001) which could be associated with traditional EPEC strains possessing an unknown virulence factor(s) (Robins-Browne *et al*, 2004). However, confirmation of malnutrition associated with persistent diarrhoea has not been reported in Aboriginal children yet.

Potentially DAEC was detected in 34 (53%) diarrheal patients and in 2 (25%) controls. The prevalence of DAEC does not indicate association of this pathogen with diarrhoea. However, it is suspected that DAEC may cause diarrhoea in children more than one years of age. It was previously reported that DAEC infections are in some way aged-dependent diarrhoea and the role of DAEC virulence factors causing diarrhoea is still under study (Scaletsky *et al*, (2002). Although we did not present any data related to the children's age in our results, these reports are highly supported by our findings. In this present study it was observed that most of the Aboriginal children infected with DAEC presented acute diarrhoea and were aged more than 1 year old which supports DAEC infections associated with age dependent diarrhoea. Although DAEC bacteria was very commonly detected our study (50% of 72 patients), the role of DAEC pathogenesis in diarrhoea among Aboriginal children communities is still unclear. However, this studies finding suggests further investigations should be carried out.

In the present study, the plasmid-borne genes *aaf1*, *aaf3* and *aggR* were used to detect EAEC. Several studies have implicated EAEC strains causing acute and persistent diarrhoea, indicating that this bacteria is a potential diarrheal pathogen in children and adults from developing countries (Bernier *et al*, 2002). The genes for these strains are located on an adherence plasmid (Moyo *et al*, 2007). In our study, positive EAEC bacteria was detected in 50 (69%) patients including two controls. EAEC infections in the NT seem to be very high affecting most of Aboriginal children with diarrhoea and a small proportion of children without diarrhoea. However, chances that EAEC may be associated with the aetiology of diarrhoea in indigenous children still remains unclear.

RFLP and RAPD analysis were performed on EAEC bacteria to determine the prevalence of different types of EAEC bacteria and its relation to virulence marker genes and plasmids found in the Aboriginal children. The variant products amplified by CTB-PCR and restricted by RFLP test, showed only 2 different restricted polymorphic fragments (fig. 1). In contrast, RAPD analysis of EAEC virulence factor genes (*aaf1, aaf3* and *aggA*) showed high prevalence of polymorphic DNA profiles. These different clusters indicate the presence of more than one EAEC bacteria type among NT Aboriginal children.

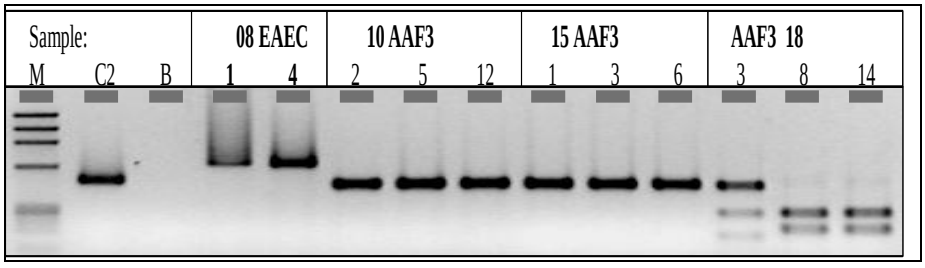

Figure 1

Figure 1: RFLP analysis of *aaf3* gene products resolved by electrophoresis. Lane M: molecular ladder. Lane C2: *aaf3* gene positive control. Lane B: blank. Lanes 08[¶] (1,4[¶¶]): *aggR* gene positive. Lanes 10[¶](2,5,12[¶¶]), 15[¶] (2,5,12[¶¶]), 18[¶] (3,8,14[¶¶]): *aaf3* gene positive.[¶] ID Number of the patient. [¶¶] ID Number of the isolates.

Overall, RFLP results shown in this study demonstrated variant *aaf* genes and also a potential variant of the adherence plasmid. RAPD results demonstrated that the adherence plasmids are very mobile in the *E. coli* population as seen in the large number of genetically different *E. coli* strains carrying the adherence plasmid (Moyo *et al*, 2007). Collectively, these results seem to suggest a greater association between DAEC, EAEC and diarrhoea.

To date, we are unable to state with certainty that EAEC play a role in the diarrhoeal infections among Aboriginal children. However, our finding suggests that different types of EAEC bacteria is being potentially spread among Aboriginal children and possibly causing diarrhoea. Finally, EIEC and EPEC strains were not predominant in this population indicating their limited role in childhood diarrhoea while a high proportion of DAEC, EAEC and EHEC predominated among Aboriginal children with diarrhoea.

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