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Improved Detection of *Shigella* Species in Diarrheic Children in Ghana Using Invasion Plasmid Antigen H-based Polymerase Chain Reaction Technique

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Abstract: *Shigella* species play an important role in the morbidity and mortality of children <5 years of age in low and middle-income countries. Previous surveillance studies to evaluate the burden of *Shigella* disease in Ghana involved conventional culture method which most probably resulted in underestimated prevalence. As efforts are being made globally to introduce vaccines against *Shigella* and *Enterotoxigenic Escherichia coli* (ETEC), this study sought to establish *Shigella* burden of disease in children <5 years of age for the implementation of appropriate public health measures to control diarrheal disease in Ghana. The study reports data of a collaborative research between Noguchi Memorial Institute for Medical Research (NMIMR), University of Ghana and Tel Aviv University (TAU), Israel, under the STOPENTERICS FP7 programme. STOPENTERICS FP7 programme aims to provide novel prophylactic solutions by imposing a two-fold paradigm switch in the development of vaccine candidates against *Shigella* and ETEC. Bloody diarrheal stool samples were collected from children (cases) (n=269) and from healthy children (controls) (n=38) aged <5 years and tested by traditional culture method in the department of Bacteriology, NMIMR. Samples were shipped and tested using invasion plasmid antigen H-based (*ipaH*-based) molecular method in TAU. All cases and controls tended *Shigella* culture-negative at NMIMR. Retesting by *ipaH* PCR assay in TAU identified *Shigella* in 31.2% (n=84) of 269 cases and 2.6% (n=1) of 38 controls. The males represented 63.1% (n=53) whilst females represented 36.9% (n=32) of cases (p=0.009). The single asymptomatic carrier (n=1) of the 38 controls, was a 3-month old male child. The asymptomatic carrier in the control group may be regarded as a potential transmitter of disease to vulnerable children of the household. Sanger sequencing confirmed *ipaH* in 10% of the positive samples. The prevalence of >30% of shigellosis indicates a substantial contribution of *Shigella* to diarrheal burden in children <5 years in Ghana. The most appropriate diagnosis of shigellosis should be PCR which is capable of detecting small amounts of nucleic acid. Furthermore, molecular screening for the detection of *Shigella* must be carried out in conjunction with the traditional culture method since isolation alone, may underestimate the prevalence of *Shigella*. Continuous surveillance will be useful in making evidence-based decisions on the introduction of vaccines against *Shigella* and *ETEC* in Ghana.

Keywords: *Shigella*, Children, Diarrhea, Ghana, Invasive Plasmid Antigen H (*IpaH)*

1. Introduction

Diarrhea is one of the leading causes of childhood mortality in Ghana causing approximately 3,660 deaths in children <5 years of age in 2015 [1]. One of the most effective ways of preventing diarrheal disease is vaccination. Among the top diarrheal pathogens, only rotavirus has licensed vaccines [2]. Vaccination against rotavirus diarrhea has led to a significant reduction in pediatric diarrhea-related hospitalization and mortality observed in Ghana [3]. Despite the significant reduction in the burden of severe childhood gastroenteritis, bacillary dysentery also known as shigellosis and *Enterotoxigenic Escherichia coli* (ETEC) have been identified among the top five major causes of moderate to severe diarrhea in children <5 years of age in Africa and Asia [4]. Shigellosis caused by *Shigella* spp. is responsible for 75,000 deaths among children <5 years and 270,000 deaths among all ages [5].

2. Theoretical Background

Shigella is a genus of Gram-negative, facultative anaerobic, non-spore forming, nonmotile, non-lactose fermenting, rod-shaped bacteria. *Shigella* species (spp.) are conventionally identified by isolation in culture. They are considered as fastidious pathogens for bacteriological isolation because they escape detection by traditional culture method [6]. Even though isolation in culture is regarded as the gold standard for detection of *Shigella* spp., naturally occurring microflora and competition from other commensal organisms make detection less plausible resulting in underestimated prevalence [6]. The advent of PCR-based molecular methods for the detection of microorganisms have made bacteria detection possible without the need for traditional culture [7]. PCR is considered a rapid, highly sensitive, specific and reliable method for the detection of bacterial pathogens such as *Shigella* spp. [8].

3. Research Objectives

This study was under the STOPENTERICS FP7 programme which aimed to provide novel prophylactic solutions by imposing a two-fold paradigm switch in the development of vaccine candidates against *Shigella* and ETEC. Most of the previous research on *Shigella* in Ghana involved conventional culture method of isolating the bacteria [9-10] resulting probably in underestimated prevalence. As global efforts are geared towards the introduction of vaccines against *Shigella* and *Enterotoxigenic Escherichia coli* (ETEC) [11], this study sought to establish *Shigella* burden of disease in children <5 years of age for the implementation of appropriate interventions to control diarrheal disease in Ghana. Between 2012 and 2013, children <5 years of age hospitalised for dysentery at the Asutsuare and Akuse Health facilities (in the Greater Accra and Eastern Regions respectively) of Ghana were enrolled in a collaborative research study between Noguchi Memorial Institute for Medical Research (NMIMR), University of Ghana and Tel Aviv University (TAU), Israel. Healthy children within the same age group living in the same compound with the hospitalized children were recruited as controls for the study.

4. Materials and Methods

4.1. Ethical Statement and Isolation of Shigella Species

The study was approved by the Institutional Review Board, NMIMR, University of Ghana, and informed consent was obtained from parents/guardians of children enrolled in the study. Bloody diarrheal stool samples collected from children (n=269) and non-diarrhoeic healthy children (n=38) <5 years were sent to the department of Bacteriology, NMIMR for culture. Samples were inoculated onto MacConkey agar, Deoxycholate citrate agar and *Salmonella-Shigella* agar for the isolation of *Shigella* spp. After overnight incubation at 37ºC the plates were checked for non-lactose fermenting colonies for the identification of suspected *Shigella* colonies.

4.2. DNA Extraction and Molecular Characterization of Shigella Species

Stool samples were shipped to TAU for testing by invasion plasmid antigen H-based (*ipaH*-based) PCR assay, based on the amplification of the *ipaH* gene sequence used for the diagnosis of dysentery [12]. DNA was extracted from the bloody diarrheal stool samples and the controls. The DNA was amplified using published primer pair specific for genes targeting *ipaH* for *Shigella* spp [12]. Ten percent of the *Shigella* positive samples were randomly selected and characterised by Sanger sequencing to confirm the accuracy of the results. Data were entered and analyzed using Stata software version 13.0 (StataCorp, College Station, Texas). The prevalence of *Shigell*a was determined using the Chi-square test. $P < 0.05$ at 95 % confidence interval was considered as statistically significant.

5. Results

5.1. Isolation of Shigella Species

Of the 269 cases recruited for the study, 52.8% (142/269) were males and 127 (47.2%) were females. The controls comprised 16 males and 22 females. After overnight incubation of plates at 37ºC, no non-lactose-fermenting colonies were seen. All the bloody diarrheal samples as well as the controls tended *Shigella* culture-negative (unpublished data).

5.2. Molecular Characterization of Shigella Species

Of the 269 cases subjected to PCR, ipaH was detected in 31.2% (n=84) consisting of 53 males (63.1%) and 31 females

(36.9%) (p-value=0.023) (Table 1). The study participants were further divided into six age groups (Table 1). Of the 84 *Shigella* positives in the bloody diarrheal group, 83.3% (n=70) were children within the age group of 1-18 months. *Shigella* infection was less common in children older than 37 months (Table 1). Of the 38 controls, 42.1% (n=16) were males and 57.9% (n=22) were females (Table 1). The controls were further divided into two age groups (0-6 months and 7-12 months) (Table 1). *IpaH* was detected in 2.6% (n=1) of the 0-6 months old children in the control group (Table 1). The randomly selected PCR positive samples were confirmed as positives by Sanger sequencing (unpublished data).

Table 1. *Age distribution of hospitalized children with bloody diarrhea and healthy children without diarrhea.*

Age distribution	Number (%)		
	Positive (*ipaH*)	Negative	Total
Age for bloody diarrhea cases (months)			
0-6	21 (28.8)	52	73
7-12	30 (29.7)	71	101
13-18	19 (33.3)	38	57
19-24	6 (46.1)	7	13
25-36	6 (35.3)	11	17
37-60	2 (25.0)	6	8
Total	84 (31.2)	185	269
Gender for Bloody diarrhea cases Positive (*ipaH*) Negative Total			
Male	53 (37.3)	89	142
Female	31 (24.4)	96	127
Total	84 (31.2)	185	269
Age for controls (months) Positive (*ipaH*) Negative Total			
0-6	1 (2.8)	35	36
7-12	0 (0.0)	2	2
Total	1 (2.7)	37	38
Gender for control group Positive (*ipaH*) Negative Total			
Male	1 (6.2)	15	16
Female	0 (0.0)	22	22
Total	1(2.6)	37	38

6. Discussion

Previous burden of disease studies in Ghana using traditional culture method documented low prevalence of *Shigella* in stool samples [9-10]. Diagnosis of bacterial diseases by conventional culture method usually takes days or weeks after sampling and is not always successful. The overall detection of *Shigella* in 31.2% of diarrheal cases by *ipaH*-based PCR in this study is higher than what was reported in Ghana previously [9-10]. PCR has the ability to rapidly identify specific pathogens that are difficult to culture *in vitro* or require a long cultivation period. PCR is highly sensitive than culture and requires very small amounts of genetic material in order to detect organisms in a sample. The failure to identify *Shigella* by conventional culture-based testing in this study could be due to over the counter use of antibiotics [13] before sampling. Improved detection of *Shigella* pathogens in this study by *ipaH*-based PCR conforms with reports from studies conducted in other countries such as India [14]. The diarrheal group recorded a higher rate of *Shigella* infection in males than in females which is similar to studies in India [15]. Fish (2008) asserted that females reportedly mount stronger humoral and cellular immune responses to infection or antigenic stimulation than do males [16]. *Shigella* infection was higher among children < 18 months in the diarrheal group. This could be attributed to the immature immune system, incapable of building an effective immunological response in this age group [17]. Other contributory factors to the observed high prevalence of *Shigella* in this age group include; licking contaminated fingers and fomites during teething [15]. This study sought to establish the burden of *Shigella* disease in children <5 years of age for the execution of timely interventions to control diarrheal disease in Ghana. Asymptomatic carriage for a particular disease can impact on the efficiency of interventions targeting susceptible individuals, as these carriers can be difficult to distinguish from vulnerable ones [18]. The 3-month old asymptomatic carrier in this study may be regarded as potential transmitter of disease to vulnerable children of his household.

7. Conclusions

The study has shown that *Shigella* remains a common pathogen associated with bloody diarrhea in children <5 years and it is an important public health problem in Ghana. This study evaluated and updated *Shigella* disease burden in children <5 years of age in Ghana using molecular method. The results of the study suggested that molecular screening for the detection of *Shigella* should be done alongside the traditional stool culture method since the traditional identification of *Shigella* using only culture method may underestimate the disease prevalence. The results generated from this study will form a baseline data for further studies. The study highlights the importance of continuous surveillance to establish *Shigella* disease burden which will be useful in making evidence-based decisions on the introduction of vaccines against *Shigella* and ETEC into the Expanded Programme on Immunization (EPI) in Ghana.

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Conflicts of Interest

All the authors do not have any possible conflicts of interest.

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