INNSPUB

Journal of Biodiversity and Environmental Sciences (JBES)
ISSN: 2220-6663 (Print) 2222-3045 (Online)
Vol. 14, No. 5, p. 37-44, 2019
http://www.innspub.net

RESEARCH PAPER OPEN ACCESS

PCR base identification of *Shigella* in children with diarrhea at Noshki, Balochistan

Aziz Ullah[1], Muhammad Kamran Taj[*1], Zafar Ahmed[1], Zahoor Ahmed[2], Imran Taj[1], Zohra Samreen[4], Nargis Taj[4], Mahrukh Naseem[3], Azmat Ullah[3], Abdul Rehman Sherani[4], Ghulam Muhammad[2], Qazi Dad Muhammad Anwar[2], Saima Azam[1]

[1]*Center for Advanced Studies in Vaccinology and Biotechnology (CASVAB), University of Balochistan, Pakistan*

[2]*Department of Livestock, University of Balochistan, Balochistan, Pakistan*

[3]*Department of Zoology, University of Balochistan, Pakistan*

[4]*Bolan University of Medical and Health Sciences, Balochistan, Pakistan*

Article published on May 30, 2019

Key words: *Shigellosis,* Age, Sex, Noshki, Balochistan.

Abstract

A total of 200 samples were collected out of which 76% were found *Shigella* negative and 24% were found positive. The district head quarter hospital Noshki, different basic health unit rural and basic health unit urban results showed that, district head quarter hospital Noshki have *Shigella* 11%, basic health unit urban has *Shigella* 07% and basic health unit rural have *Shigella* 06%. However, in our study sex wise ratio showed that males were (13%) more affected as compared to female (11%). Microbial infection leading to *Shigella* diarrhoea is a major contributor to neonatal deaths in developing world. Whereas age wise distribution showed 9% in one year, 7% in two years, 6% in three years, 1% in four years and 1% in five years of age. The culture media composition is very important for bacterial growth. Most of the previous studies on *Shigella* development and growth have been performed under optimal conditions. *Shigella* best growth was recorded on *Salmonella Shigella* Agar and Xylose Lysine Deoxycholase Agar. In this study bacteria grow on pH ranges from 6-8 and temperature of 04-47^{o}C. *Shigella* showed sensitivity to most of the antibiotics classes, whereas multi drug resistance against Ampicillin, Penicillin, Chloramphenicol, Tetracycline classes. Apolymerase chain reaction indicates that the rapidity and high specificity of the *Shigella rfp B gen* -specific assays provide optimal efficiency without the need for additional hybridization. While conformation through PCR sample shows clear of 211bp of *Shigella rfp B* gene.

***Corresponding Author:** Muhammad Kamran Taj ✉ kamrancasvab@yahoo.com

Introduction

Shigella causes *bacillary dysentery*, diarrhea, vomiting, headache, fever, stiff neck, convulsions and joint pain which remains a significant threat to public health (Niyogi *et al.,* 2005). It is a rod-shaped, non-motile, nonspore-forming, facultative anaerobic, gram-negative and lactose fermenting bacteria. The genus *Shigella* was first described by Kiyoshi Shiga in 1897 (Hiranrattana *et al.,* 2005). The natural host of *Shigella* is only humans and mode of transmission is fecal oral or by contact with contaminated food and water (Elliott *et al.,* 2007). *Shigella* causes *Shigellosis* in human. *Shigellosis* also known as acute *bacillary dysentery*, is characterized by the passage of loose stools mixed with blood and mucus and accompanied by fever, abdominal cramps and tenesmus. *Shigella* causes deadly epidemics in many of the poor countries. The pathogenic species of *Shigella* to humans includes *S. dysenteriae, S. boydii, S. flexneri and S. sonnei.* The site of infection is colonel mucosa, but sometimes it causes extra-intestinal complications including bacteremia, urogenital and neurologic manifestations (Singh *et al.,* 2011). The pathogenic organisms attach to epithelial cells of the intestinal mucosa, multiply in the cells and spread to neighboring epithelial cells, causing destruction, the characteristic pathology of *Shigella* (Timaeus *et al.,* 1995).

Sometimes the patient may develop severe hemolytic hematuria when infected by Shiga toxin strain, which may cause renal failure and ultimately death (Oyofo *et al.,* 2002). The *Shigella* become highly specific children pathogen with variable epidemiological and pathological features. The most severe forms encountered with *Shigella*. In children under 5 years of age, lead to a mortality rate of 10 to 30% during outbreaks (Sur *et al.,* 2004). *Shigellosis* kill about 11 million children each year while acute diarrheal diseases account for 3.1 million deaths in children, of which 6,00,000 deaths annually are contributed by *shigellosis* alone (Steins *et al.,* 2003). It may be associated with a number of complications of which haemolytic uraemic syndrome is the most serious. *Shigella* has been documented to survive in salt water for 12-30 hours, in kitchen wastes for 1-4 days, in fresh water for 5-11 days, in sour milk for four weeks, in dust at room temperature for six weeks and in soiled linen for up to seven weeks (Levine *et al.,* 1977). *Shigella* species have been found in most of sewage, water surface, crops and food contaminated by human feces (Levine *et al.,* 1989).

Diarrheal disease is recognized as a major cause of morbidity and mortality in the community. Since an etiological diagnosis of diarrheal disease with susceptibility results is almost near available to assist in the selection of prompt therapy, necessary for diarrheal infection, the initial approach to the treatment is largely empirical and in ordinary guided by the risk category of the patient. *Shigella* infection to be associated with mortality in children presenting with diarrhoea. Treatment strategies targeting *shigella* infections might reduce diarrhoea associated mortality. Because a range of antibiotics have shown efficacy in treating children with s*higella* infections, antibiotic treatment of high risk groups of children might be an effective addition to the current guidance.

People of Pakistan face health hazards because of poor sanitation practices. The present research study is aimed at evaluating and analyzing infestation of *Shigella* (Ahmed *et al*., 2003). The purpose of this study is to evaluate the most prevalent pathogen responsible for diarrhea in children and to study the descriptive epidemiology of *shigellosis* in children suffering from diarrhea in Noshki Balochistan. The majority of *Shigella* mortality studies reported a significant association with death when compared with other causes of diarrhoea. The different populations, clinical management strategies, study designs, and enrolment periods resulted in marked heterogeneity in the magnitude of association across the studies. Most studies used standard culture to detect *Shigella* infection. Our study focus on molecular methods to detect *Shigella* infection which can triple the detection rate by detecting burden infections.

The outbreaks of these diseases are disasters for the children of area and may cause death imposing excessive human losses.

So it is quite necessary to prevent these infectious diseases to ensure wellbeing. For this isolation of organism and examination the fecal samples of patients with suspect gastroenteritis was taken. The sample were brought to the CASVAB from District Headquarter Hospital Noshki and other basic health units, in Noshki, was investigated for common enter pathogenic bacteria.

Material and methods

Collection of Sample and Area of Study

Present study was conducted in the Center for Advanced Studies in Vaccinology and Biotechnology (CASVAB) University of Balochistan, Quetta. To isolate and identify the *Shigella* bacteria from stool samples of the children of different age, sex groups from District Head Quarter Hospital Noshki, Basic Health Unit Urban Noshki and Basic Health Unit Rural Noshki District of Balochistan.

Size of Sampling and Procedure

Two hundred suspected samples were collected from Children of different age, sex during year 2015 to 2016 from Balochistan, Pakistan. Sample were collected from clinically confirm infected one from Government DHQ Hospital Noshki, BHU, Urban Noshki and BHU, Rural Noshki District of Balochistan. All the stool samples were collected in sterile disposable plastic containers and transported to the laboratory for further processing (Getnet *et al.*, 2014).

Identification and Isolation of Shigella

The isolation of organism was performed on Xylose Lysine Deoxycholase agar, Salmonella Shigella Agar, MacConkey's agar, Eosin Methylene Blue Agar. The isolates were identified on the basis of cultural, morphological, biochemical characteristics and PCR (Cowan *et al.*, 1993).

Antibiogram Test

Standardized antibiotic sensitivity experiment was done on Mueller Hinton agar by means of disc diffusion Bauer technique and McFarland Turbidity Standard technique (0.5) following CLSI procedures. Isolates were measured as sensitive and resistant to a specific antibiotic on the basis of inhibitory zone (Ghosh *et al.*, 2007).

Results

Total 200 suspected stool samples were collected in which 24% were *Shigella* positive and 76% were *Shigella* negative as shown in Fig.-1. Result revealed that 11% samples were positive from District Head Quarter Hospital, 7% samples were from Basic Health Unit Urban Noshki and 6% samples were from Basic Health Unit Rural Noshki as shown in Fig.-2. A predominance of male infant is apparent in almost all studies of *Shigella* diarrhoea in newborns. While in our study sex wise ratio showed that males were (13%) more affected as compared to female (11%) as shown in Fig. 3. Microbial infection leading to *Shigella* diarrhoea is a major contributor to neonatal deaths in developing world. Whereas age wise distribution showed 9% in one year, 7% in two years, 6% in three years, 1% in four years and 1% in five years of age as shown in Fig. 4.

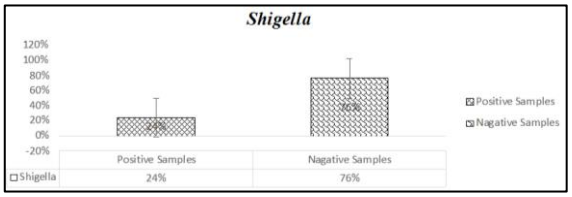

Fig. 1. Over all percentage of *Shigella* isolated from Diarrheal patients of Noshki District.

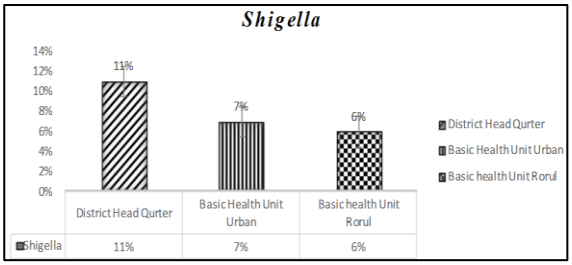

Fig. 2. The percentage of *Shigella* isolated from patients in different units.

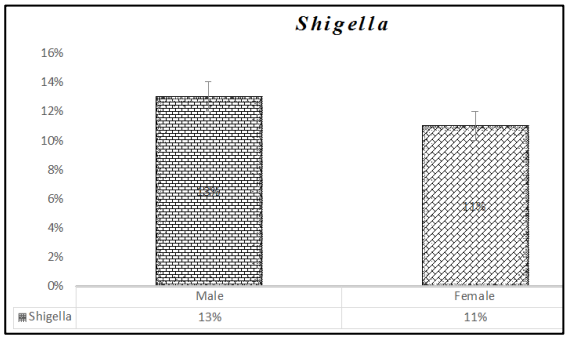

Fig. 3. Sex wise distribution of *Shigella* isolated from patients of Noshki district.

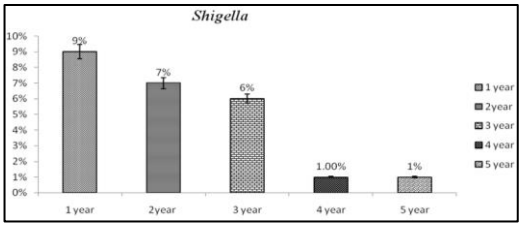

Fig. 4. Age wise distribution of *Shigella* in different age groups.

Temperature and pH effect

The temperature is one of the most important environmental factors that affect many aspects of the biological system of *Shigella*. *Shigella* grow between from 04 to 47**°**C as shown in Table 1. The pH is another standard growth factor that affect many aspects of the biological system of *Shigella*. The *Shigella* show growth from 6-8 pH as shown in Table 2.

Table 1. Affect of Temperature on *Shigella* growth.

Temperature °C	
0	-
4	+
10	+
15	+
20	++
25	++
30	+++
37	++++
40	++
45	+
47	+

Table 2. Affect of pH *Shigella* growth.

pH	
3	-
4	-
5	-
6	+
7	+
8	+

Affect of Differentiate Media

The isolate organism was grown on Salmonella Shigella Agar media, Xylose Lysine Deoxycholase (XLD) Agar media, Eosin Methylene Blue (EMB) Agar media and Mac Conkey's media.

The strong growth was recorded on Salmonella Shigella Agar media and XLD Agar media while Moderate growth was observed on MacConkey's media. Whereas poor growth was observed on EMB Agar media as shown in Table -3.

Biochemical Tests

Biochemical tests were fulfilled for the further confirmation of bacteria. *Shigella* was confirm by gram staining and concluded different biochemical tests (IMIVIC, sugar fermentation tests, catalase test and oxidase test) as shown Table 4.

Table 4. Different Biochemical tests for identification of *Shigella*.

Biochemical Tests For Identification Of *Shigella*	
Biochemical Tests	*Shigella*
Catalase Test	+
Oxidase Test	-
Motality Test	-
Methyl Red Test	+
Voges Proskauer Test	-
Indole Production Test	+
Simmon Citrate Test	-
Urea Hydrolysis Test	-
Sugar Fermentation Test	
Dextrose Fermentation Test	+
Dulcitol Fermentation Test	-
Maltose Fermentation Test	+
Mannitol Fermentation Test	+
Sorbitol Fermentation Test	+
Xylose Fermentation Test	-

Table-5. Antibiotic trail against *Shigella* isolated from patients.

S#	Classes	Antibiotics		Zone for Resistance (mm)		Zone for Suspect (mm)
				R	IM	S
1	β-Lactams	(a)Cephalosporins	Cefixime (CFM)	≤15	16-18	≥19
			Ceftriaxone (CRO)	≤10	11-13	≥14
			Cephradine (CE)	≤07	14-12	≥14
		(b) Penicillin	Ampicillin (AM)	≤17	12-14	≥15
		(c) Penicillin-β-Lactamase Inhibitor Combinations	Ampicillin (SAM)	≤13	14-16	≥17
			Amoxicillin (AMC)	≤13	14-17	≥18
2	Sulfonamide	Sulphamethoxazole	Sulphamethoxazole (TMP)	≤10	11-15	≥16
3	Quinolones	Nalidixic acid	Nalidixic acid (NA)	≤13	14-18	≥19
		Ciprofloxacin	Ciprofloxacin (CIP)	≤20	21-30	≥31
4	Fluoroquinolone	Enrofloxacin	Enrofloxacin (ENR)	≤16	17-19	≥20
		Norfloxacin	Norfloxacin (NOR)	≤12	13-16	≥17
5	Phenicols	Chloramphenicol	Chloramphenicol (C)	≤12	13-17	≥18
6	Tetracycline	Doxycycline	Doxycycline (DO)	≤11	12-14	≥15
		Tetracycline	Tetracycline (TE)	≤12	11-13	≥16
7	Aminoglycosides	Vancomycin	Vancomycin (VA)	≤14	15-16	≥17

Antibiogram Test

Antimicrobial resistance and sensitivity test for the isolates of Shigella. Shigella was sensitive to Azithromycin, Ciprofloxacin, Gentamicin, Cefotaxime and Chloramphenicol. Resistant to Amoxicillin, Co-trimoxazole, Norfloxacin, Cephalexin, Perfloxacin, Tetracycline, Nalidixic acid, Sulphamethaxole and Metronidazole classes as shown in the Table -5 and Fig.-5.

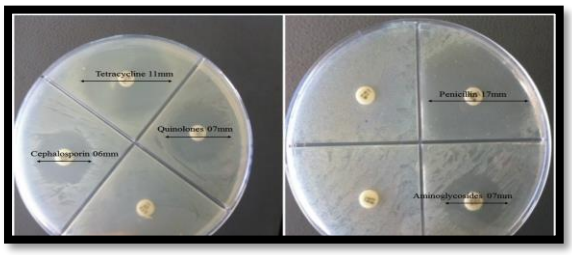

Fig.-5. Antibiogram against *Shigella* isolated from patients of Noshki District.

Genomic Dna Extraction

Excellent result by using genomic DNA isolation kit was obtained as shown in Fig.-6.

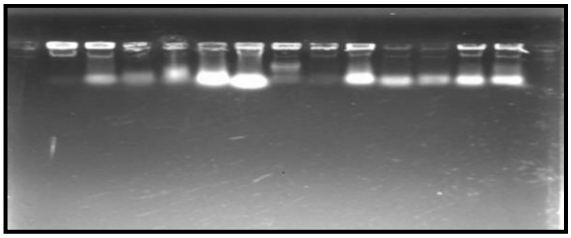

Fig. 6. Band of DNA *Shigella* through genomic DNA isolation kit method.

Polymerase Chain Reaction

The DNA of all bacterial isolate was amplified using primers, SDYSDF1- TCTCAATAATAGGGAACACAGC*, and SDYSDR1,* CATAAATCACCAGCAAGGTT. *The isolates showed amplicons of 211bp as shown in Fig.-7.*

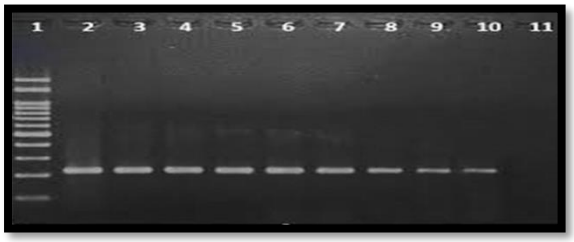

Fig. 7. *Gel showing PCR* product for detection of *Shigella* (rfp B).

Discussion

The aim of this study was to isolate and identify *Shigella* from children who were admitted in hospitals of District Hospital Noshki, Basic Health Unit Rural and Basic Health Unit Urban at District Noshki Balochistan, has clinical as well as social significance with shigellosis. Any disease can be treated if we come to be sure about real cause of that disease. Most objective of this research was to confirm the causal agent of *Shigella* is hospitalized children. Different antibiotics were also checked for microbial sensitivity and resistance. Checking susceptibility and resistance of *Shigella* several antibiotics were lead us towards control of this detrimental causal agent and consequently reduction of infection and contamination in masses. Being socially significant this investigation will help us to reduce infant mortality rate thus having a healthy society. In addition, identification of *Shigella* was done by performing different biochemical tests and Polymerase Chain Reaction. 200 stool samples were collected from Diarrhea suffered children from three main Unit District Head Quarter Hospital Noshki, Basic Health Unit Rural Noshki and Basic Health Unit Urban Noshki at District Noshki Balochistan. From test results 24% cases were confirmed for having *Shigella* as a causal agent for Diarrhea. Within these 24% *Shigella* positive patients 11% samples were found to be from District Head Quarter Hospital Noshki. Percentage of samples taken from Basic Health Unit Urban was 7% and percentage of positive cases from Basic Health Unit Rural was 6%. Microbial infection leading to *Shigella* diarrhoea is a major contributor to neonatal deaths in developing world. As an examination of age wise distribution, it was found that age wise distribution showed 9% in one year, 7% in two years, 6% children were belonging to age group from three years, 1% in four years and 1% in five years of age. In age wise distribution we see a constant increase in percentage with the decrease in age. Statistics show that the most *Shigella* affected children were from lower age group i.e. from 1 day to 1 year. Negative correlation was exhibited between age and percentage of infected children diarrhoea is a leading killer of children, accounting for approximately 8 per

cent of all deaths among children under age 5 worldwide in 2016. This translates to over 1,300 young children dying each day, or about 480,000 children a year. Most deaths from diarrhoea occur among children less than 2 years of age living in Africa (Vogt *et al.,* 2005). From 2000 to 2016, the total annual number of deaths from diarrhoea among children under 5 decreased by 60 per cent. Many more children could be saved through basic interventions. Diarrhoea is the third commonest cause of neonatal death disease of children under five years in pakistan. According to the study of Unicef diarrhoeal disease date from the year 2000 to 2016 the overall neonatal death to post neonatal death of children in pakistan is (4366,218) and responsible for neonatal death and post neonatal death of children among children under five years of age Available statistics from the Ministry of Health indicate 284000 annual deaths in pakistan attributed to diarrhoeal disease with about 25 per cent being children under five years of age *(WHO and Maternal Child Epidemiology Estimation of Child Cause Death Diarrhoea MCEE Group Unicef February 2018)*. This incidence of 25% is comparable to inpatient data from other provinces of the country but much high as compared to similar studies from other provinces of the country. Diarrhoeal disease is continuously to be an important public health concern in Balochistan among the other provinces of the country greater understanding of the bacteriology, epidemiology and public health aspects associated with this disease. Mortality aside, the long term effects of diarrhoeal disease on child health are particularly serious with far reaching negative consequences on cognitive and growth development which impacts on school performance (Saima *et al.,* 2018). This deemed to be sufficiently determine that youngest children have weakest immunity and when are mistreated with contaminated equipment like ventilators, catheters and contaminated hands in hospitals and other health care setting, they tend to be more vulnerable to micro-organisms and become infected very soon. In Pakistan, infant mortality rate is already reflecting alarming situation which should be controlled to secure prosperous future of the nation. Pakistan is the seventh country in the world where every year 480,000 children die at the age of from one month to five years due to Diarrheal disease. Pakistan Pediatric Association (PPA) while observing the Bloody and watery Diarrheal declared that the health situation has improved in Punjab and Khyber-Pakhtunkhwa but not in Sindh and Balochistan. Noshki is being the part of district of Balochistan shares the same living conditions and health care settings. Affirmative actions should be taken to bring out Pakistan form this alarming situation. This investigation will help us to reduce infant mortality rate thus having a healthy environment to neonatal to post neonatal birth bovine. Identification of Shigella and related bacteria remains a challenge to many laboratories. PCR is the best way to identified the Shigella from area field samples.

This study is highlighting the burden of diarrheal disease in children under the five years of age, especially those from infant neonatal to post neonatal. Foodborne diseases are being under documented in developing countries like Pakistan, India, Sirlanka, Bangladesh, although treatment have been given to the affected individual the sources are not been keen to the public. In general, most of the food related disease outbreaks are mainly depending upon the route and load of contamination of microbes. *Shigella* are being continuously reported from the many foodborne outbreaks throughout the world. Even though outbreaks are documented sources are not being highlighted. Many methods had been adapted for the isolation of these pathogens. In this study the tremendous growth of *Shigella* was recorded at 37°C to 47°C where as poor growth was observed at lower temperature 0°C to 25°C. The pH of media was also found affecting Shigella survival. Our result showed that the best pH for the growth of *Shigella* from 6 to 8 pH. Polymerase chain reaction indicated a clear band of 211bp of *rfp BShigella* gene and from Bacterial isolates DNA was amplified using primers SDYSDF1- TCTCAATAATAGGGAACACAGC, SDYSDR1- CATAAATCACCAGCAAGGTT. Sensitivity test was performed to check the resistance and sensitivity of *Shigella* species against a range of antibiotic drugs.

After performing confirmation and identification tests, several antibiotic range was also checked against the sensitivity and resistance of *Shigella* species.

Conclusion

Diarrheal disease is an important disease of infant neonatal to post neonatal children from the year of under five years to ten years in the remote areas as well urban areas of Balochistan pakistan. This disease is mostly devastating to population of remote areas where is health facility availability is equal to zero. Where the facility of health clinically and preventive practices are very poor and self-medication management is common. The high level of Shigella species prevalence was observed during the month of April-June. This study revealed that the use of raw animal manure as fertilizer, irrigation of vegetables with fecal contaminated water, a poor sanitary system and improper treatment of water supplies can increase potential risks to the consumer. *so, proper management will provide protection to the infant neonatal health to post neonatal health from under five years old ten years old of the region.* Adaptation and application of Hazard Analysis and Critical Control Point (HACCP) can decrease the possibility of contamination and eliminate pathogenic microorganisms. Awareness regarding communicable diseases also helps control shigellosis and other diarrheal disease. Shigella species are frequently associated with food and water borne infections leading to acute invasive enteric infections.

Acknowledgements

The author is thanking full to District Health Officer Dr. Abdul Rehman Sherani to highly facilitate visit and sample collection at District Head Quarter Hospital and BHU's of District Noshki of Balochistan Province. Researcher also wishes to thank the staff of anaerobic laboratory CASVAB Mr. Abdullah khan& Mr. Aurangzeb khanwho helped to do this research.

Reference

Ahmed K, Abdul RS, Farah RS. 2003. A etiology of *shigellosis* in northern Pakistan. Journal Health Popul Nutr **21(1),** 32-9.

Cowan, Steel. 1993. Manual for the identification of medical bacteria, 3rd edition, Cambridge University press.

Elliott EJ. 2007. Acute gastroenteritis. British Medical Journal **33(4),** 35-40.

Getnet AG. 2014. Prevalence and Antimicrobial Susceptibility Patterns of *Shigella* and *Salmonella* Species among Patients with Diarrhea Attending Gondar Town Health Institutions, Northwest Ethiopia. Science Journal Public Health **5(2),** 469-475.

Ghosh M, Wahi S, Kumar M, Ganguli A. 2007. Prevalence of *Enterotoxigenic Staphylococcus aureus* and *Shigella spp*. in some raw street vended Indian foods. International Journal of Environmental Health Research **17(2),** 151-156.

Hiranrattana A, Mekmullica J, Chatsuwan T, Pancharoen C, Thisyakorn U. 2005. "Childhood *shigellosis* at King Chulalongkorn Memorial Hospital, Bangkok, Thailand: a 5-year review (1996-2000)," South east Asian Journal of Tropical medicine and Public Health, **36(3),** 683-685.

Levine MM, Caplan ES, Waterman D, Hornick R, Snyder MJ. 1977. Diarrhea caused by *Escherichia coli* that produce only heat-stable enterotoxin. Journal of Infection and Immunity **1(7),** 78-82.

Levine MM, Du PHL, Hornick RB. 1989. Inoculum size in shigellosis and implication for expected mode of transmission. Journal of Infectious Disease **15(9),** 1126-1128.

Niyogi SK. 2005. Increasing antimicrobial resistance-an emerging problem in the treatment of shigellosis. Journal of microbiology **43(2),** 133-143.

Oyofo BA, Lesmana M, & Subekti D. 2002. "Surveillance of bacterial pathogens of diarrhea disease in Indonesia, "Diagnostic Microbiology and Infectious Disease **44(3),** 227-234.

Singh KKB, Ojha SC, Deris ZZ, Rahman RA. 2011. "A 9-year study of *shigellosis* in North east Malaysia antimicrobial susceptibility and shifting species dominance, "Journal of Public Health **19(3),** 231-236.

Steins LH, Valentiner BP, Grewal HM, Gaastra W. 2003. Development and evaluation of genotypic assays for the deletion and characterization of Enterotoxigenic E. coli. Journal of Diagnosis of Microbial Infections **4(5),** 97-105.

Sur DT, Ramamurthy JD, Bhattacharya SK. 2004. "*Shigellosis* challenges & management issues," The Indian Journal of Medical Research **120(5),** 454-457.

Timaeus IM, Lush L. 1995. Intra-urban differentials in child health. Health Transition Reviews **5(1),** 163-190.

Vogt RL, Dippold L. 2005. *Escherichia coli* O157:H7 outbreak associated with consumed ground June-July 2002. Public Health Reports **120(2),** 174-8.