IDENTIFICATION OF NON-TYPHOIDAL *SALMONELLA* FROM DIARRHEAL PEDIATRIC PATIENTS IN SURABAYA, INDONESIA

Juniastuti[1,2,3*], Alpha Fardah Athiyyah[4*], Andy Darma[4*], Laura Navika Yamani[2,3,5], Virginia Ayu Ferandra[2,3], Nur Syamsiyatul Fajar[2,3], Sugeng Hariyono[1], Mochamad Amin[2,3], Takako Utsumi[2,6], Reza Ranuh[4], Subijanto M Sudarmo[4] and Maria Inge Lusida[1,2,3]

[1]Department of Microbiology, School of Medicine, [2]Institute of Tropical Disease, [3]Airlangga Health Science Institute, [4]Division of Gastroenterology, Department of Child Health, School of Medicine, Universitas Airlangga/Dr Soetomo General Hospital, [5]Department of Epidemiology, Faculty of Public Health, Universitas Airlangga, Surabaya, Indonesia; [6]Center for Infectious Diseases, Kobe University Graduate School of Medicine, Kobe, Japan

Abstract. *Salmonella* Typhi and *Salmonella* Paratyphi are the predominant agents of diarrheal disease. However, non-typhoidal *Salmonella* (NTS) serovars are becoming increasingly global. Indonesia is endemic for typhoid fever, but data on NTS is limited. The study employed multiplex PCR (mPCR) to identify NTS in diarrheal children. Bacterial cultures grown on Salmonella Shigella agar from 80 fecal samples of diarrheal pediatric patients in Dr Soetomo General Hospital, Surabaya, Indonesia were characterized by mPCR as NTS Enteritidis, Infantis, Thompson, and Typhimurium. Confirmation by direct amplicon sequencing and by conventional biochemical and serological tests revealed only one NTS as Infantis and one isolate each of *S*. Paratyphi A, *S*. Paratyphi C and *S*. Typhi. The clinical manifestation of *S*. Infantis infection was milder than that of *S*. Paratyphi or *S*. Typhi infection. Thus confirmation tests should be conducted to confirm NTS identification by mPCR.

Keywords: non-typhoidal *Salmonella*, diarrhea, pediatric patient, Indonesia

INTRODUCTION

Diarrheal disease is an important public health problem in developing countries and a prominent cause of morbidity and mortality worldwide (WHO, 2016). Genus *Salmonella*, a member of family Enterobacteriaceae, remains one of the major contributors to acute enteric infections in Asia, especially *Salmonella* Typhi *and S*. Paratyphi (Ochiai *et al*, 2008). However, non-typhoidal *Salmonella* (NTS) serovars are becoming increasingly a threat to human health globally, responsible for an estimated 94 million cases of gastroenteritis each year and upwards of 150,000 deaths (Majowicz *et al*, 2010). Among NTS, *S*. Enteritidis and *S*. Typhimurium are epidemiologically the most predominant

Correspondence: Juniastuti, Department of Microbiology, School of Medicine, Universitas Airlangga, Jl Mayjen. Prof Dr Moestopo 47, Surabaya 60131, East Java, Indonesia.
Tel: +62 31 5030252: Fax: +62 31 5022472
E-mail: koeraisindewi@yahoo.co.id
*Contributed equally to the work.

serovars, with poultry as a major source of infection (ECDC, 2005; Saravanan *et al*, 2015).

In most cases, NTS infection is a self-limiting disease, causing mild gastroenteritis; however it can lead to a wide spectrum of complications including bacteremia and severe infections such as meningitis (Fleisher and Sieff, 2010; Bhutta, 2011). In developing countries, especially in Africa, NTS is a major source of severe clinical illness in children (Graham *et al*, 2000). In Bangladesh, cases of NTS bacteremia present a more acute diarrhea in children with malnutrition compared to *Salmonella* Typhi bacteremia, and are more likely to have co-morbidities (Shahunja *et al*, 2015). In Africa and Thailand, the prevalence of NTS is higher in children with a median age of 13 months (Thamlikitkul *et al*, 1996; MacLennan *et al*, 2008).

Conventional methods recommended for detection of *Salmonella*, such as serotyping, are generally cumbersome, inefficient, labor intensive and time consuming (D'aoust, 1989; Wattiau *et al*, 2011). More recently, PCR-based methods have been employed as the techniques are cost-effective, less laborious, highly sensitive, and specific (Jamshidi *et al*, 2009; Lee *et al*, 2009; Tennant *et al*, 2010). A multiplex PCR (mPCR) approach allows simultaneous detection of several pathogens and has been developed to identify *Salmonella* serovars (Jamshidi *et al*, 2009; Lee *et al*, 2009).

Although typhoid fever is endemic in many developing areas in Asia, including Indonesia (Ochiai *et al*, 2008; Pickering *et al*, 2012), but data on NTS are limited. Hence, NTS in diarrheal children admitted to a hospital in Surabaya, Indonesia were initially identified using mPCR, together with their clinical features, followed by confirmation by conventional and sequencing methods as mPCR has not been widely used to identify NTS from human samples.

MATERIALS AND METHODS

Patients

Diarrheal pediatric patients were enrolled between November 2016 and March 2017 at Dr Soetomo General Hospital, Surabaya, Indonesia. Data of patient characteristics (age and nutrition status) and clinical picture (acute or chronic diarrhea, level of dehydration and any complication) were retrieved from medical records. Acute diarrhea, is defined as passage of ≥ 3 of abnormally loose or watery stools in the preceding 24 hours or as a new onset of diarrhea in a patient without a history of diarrhea in the previous 14 days. Chronic diarrhea is defined as an episode of diarrhea lasting at least 14 days. Level of dehydration and nutrition status followed WHO guidelines (WHO, 2005; WHO, 2010).

Ethical clearance for the study was obtained from the Ethics Committee of Dr Soetomo General Hospital, Surabaya (ref. no. 07/Panke.KKE/I/2017, extension of ethical clearance ref. no. 188/Panke.KKE/VIII/2012). Prior informed consent was obtained from parents or legal guardian of each participating child.

Bacteria isolation

Fecal sample was directly added to Selenite broth (OXOID, Basingstoke, UK), an enrichment medium, and incubated at 35°C for 24 hours before streaking onto Salmonella Shigella agar (SS) (OXOID) and incubating at 35°C for a further 24 hours. Black colonies of suspected *Salmonella* were maintained as stocks.

mPCR

DNA was extracted from a single colony using QIAamp DNA Mini Kit

(QIAGEN, Tokyo, Japan). Amplification by mPCR employed a set of five primer pairs (Table 1). Reaction mixture (20 μl) consisted of 2 μl of DNA template, 0.5 μl of 0.2 μM each primer (except 0.8 μM for H: set), 12.5 μl of 10X *Taq* PCR master mix kit (QIAGEN, Hilden, Germany) and appropriate volume of double-distilled water. Thermocycling was carried out in a Veriti thermal cycler (Applied Biosystems, Singapore) as follows: 95ºC for 6 minutes; followed by 30 cycles of 95ºC for 60 seconds, 63ºC for 12 seconds and 72ºC for 60 seconds; and a final step of 72ºC for 4 minutes (Shimizu *et al*, 2014). Amplicons were separated by 2% agarose gel-electrophoresis containing ethidium bromide and visualized under UV illumination. A strain generating amplicons from primer pairs P1 and P2, P3, P4, or P5 was identified as NTS.

Confirmation tests

Confirmation of presence P3 target gene was undertaken using uniplex PCR under an optimal annealing temperature of 56°C (Kardos *et al*, 2007). The obtained amplicons were subjected to direct nucleotide sequencing using Big Dye Terminator Cycle Sequencing v.1.1 kit and an ABI Prism 310 Sequencer (Applied Biosystems, Foster City, CA). Nucleotide sequence was aligned using Genetyx-Win version 9.0 (Genetyx, Tokyo, Japan) and, if necessary, by the program Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov) to find highest similarity to all reported genes deposited at NCBI database. UVA Fasta Server (fasta.bioch.virginia.edu) was also used to determine regions of similarities of sequences determined using forward and reverse primers and the corresponding reported sequences. All sequences obtained from the study were deposited at DDBJ database, accession nos. LC474099-LC474100.

Biochemical and serologic tests were also conducted to confirm the results of the mPCR. Colonies were placed onto triple-sugar-iron (TSI) agar (OXOID) slants, incubated at 35°C for 24 hours and

Table 1
Primers used in multiplex PCR for detection of non-typhoidal *Salmonella*.

Primer name	Sequence (5′→3′)	Amplicon size (bp)	Target gene	Serovar
invA-F[a]	GCCATGGTATGGATTTGTCC	118	*invA*	*Salmonella* specific
invA-R[a]	GTCACGATAAAACCG			
sdfF[b]	TGTGTTTTATCTGATGCAAGAGG	333	*sdf*	*S.* Enteritidis
sdfR[b]	CGTTCTTCTGGTACTTACGATGAC			
878f[c]	TTGCTTCAGCAGATGCTAAG	413	*fliC-r*	*S.* Infantis
1275r[c]	CCACCTGCGCCAACGCT			
H:for[d]	ACTCAGGCTTCCCGTAACGC	551	*fliC-i*	*S.* Typhimurium
H:I[d]	ATAGCCATTTACCAGTTCC			
H:k-F[e]	AACGACGGTATCTCCATTGC	658	*fliC-k*	*S.* Thompson
H:k-R[e]	CAGCCGAACTCGGTGTATTT			

[a]P1 (Shimizu *et al*, 2014). [b]P2 (Agron *et al*, 2001). [c]P3 (Kardos *et al*, 2007). [d]P4 (Tennant *et al*, 2010). [e]P5 (Shimizu *et al*, 2014).

observed for acid, gas and H_2S production, then tested for other biochemical properties, such as indole production, motility and citrate production (Walker *et al*, 2015). In addition, ELISA using *Salmonella* antiserum (Biofarma, Bandung, Indonesia) was performed to complete the identification of *Salmonella* serovars.

RESULTS

Patients' characteristics

Fecal samples were collected from 80 children with diarrhea, 1.5 months - 15 years of age (median 1.4 years) (Table 2). The majority of the patients were classified as in good nutrition status, followed by mild to moderate and lastly severe malnutrition status. Most had acute diarrhea and mild to moderate dehydration. Two patients died from complications of cardiomyopathy, acute liver failure or sepsis (data not shown).

mPCR assay and and confirmation tests

Among the 80 fecal samples, 38 produced on SS agar black putative *Salmonella* colonies, 11 of which were positive by mPCR only for *Salmonella* (Fig 1A). Subsequent uniplex PCR identified one isolate (RI 41) as *S.* Infantis (Fig 1B). Analysis of the sequence of sample RI 41 amplified by *Salmonella*-specific P1 primer pair demonstrated 100% identity to a *S.* Typhimurium M90846 *invA* fragment (Fig 2A) (Shimizu *et al*, 2014), but, surprisingly, that of P3 amplicon was only 26% similar to *S.* Infantis AY353282 *fliC-r* fragment (expected target gene) and 100% similar to *S.* Infantis CFSAN003307 sodium-potassium/proton antiporter ChaA gene fragment, nt 2,147,247-2,147,621 (Fig 2B). Fasta sequence analysis showed the sequence of *S.* Infantis CFSAN003307 ChaA gene had a 77% similarity to the sequence from P3 reverse primer and 50% similarity to the sequence from P3 forward primer (Fig 2B).

Biochemical and serological tests identified, among the ten remaining mPCR-positive isolates, *S.* Paratyphi A

Table 2
Characteristics of diarrheal patients with *Salmonella*-positive fecal samples.

Patient ID	Age	Sex	Presenting sign/ symptom	Nutritional status	*Salmonella* serovar
RI 1	9.5 y	F	Chronic watery diarrhea with prolonged fever, undehydration	Mild to moderate malnutrition	*S.* Paratyphi A
RI 14	4 m	M	Chronic watery diarrhea, severely wasted	Severe malnutrition	*S.* Paratyphi C
RI 24	7 m	F	Acute watery diarrhea, undehydration, complicated with pericardium effusion	Good nutritional status	*S.* Typhi
RI 41	9 m	F	Acute watery diarrhea, mild to moderate dehydration	Good nutritional status	*S.* Infantis

F, female; M, male; m, months; y, years.

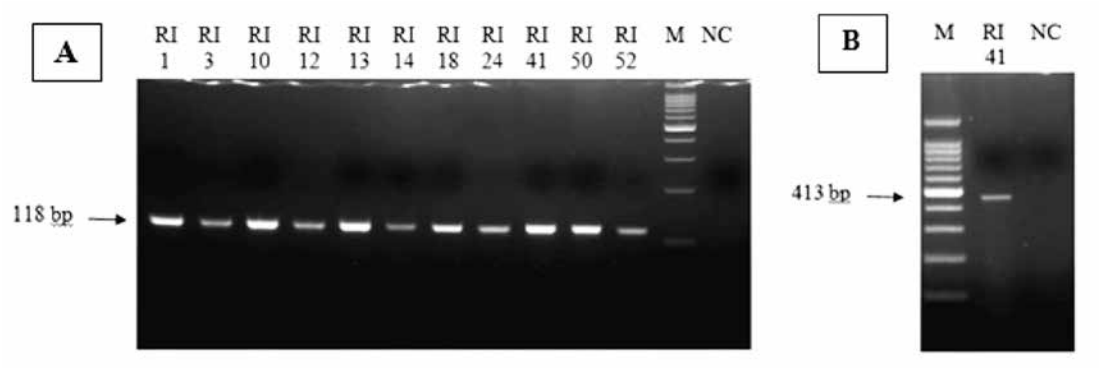

Fig 1-Electrophoresis profile of (A) multiplex and (B) uniplex PCR amplicons of *Salmonella* from fecal samples of diarrheal children admitted to Surabaya, Indonesia. Multiplex PCR was carried out using primer pairs P1-P5 and uniplex PCR with P3 primer pair (Table 1). RI, patient ID; M, 100 bp size markers; NC, negative control.

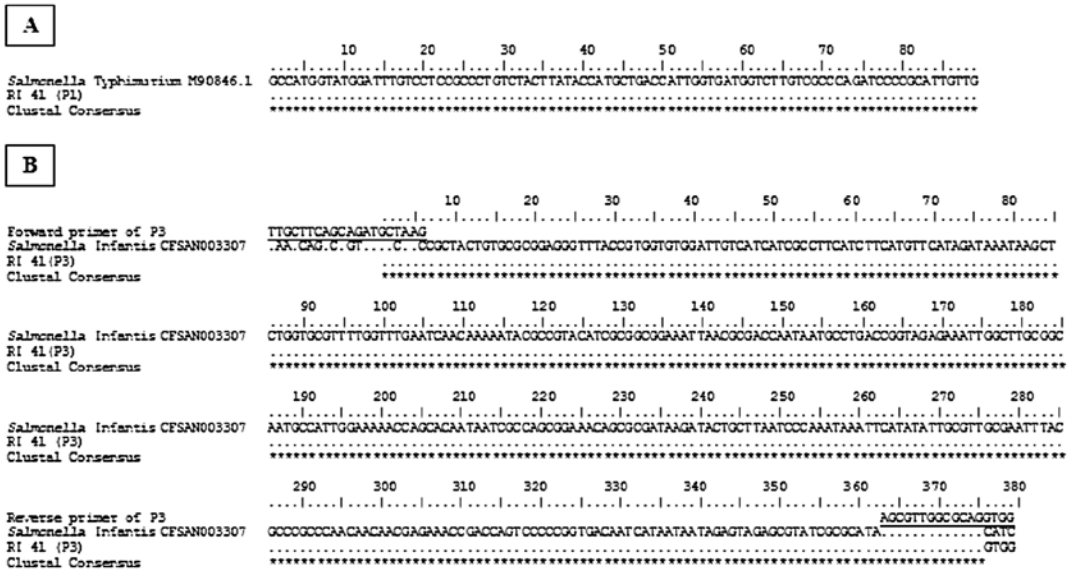

Fig 2-Multiple alignments between (A) sequence of sample RI 41 amplicon using P1 primer pair and that of *Salmonella* Typhimurium M90846 *invA*, and between (B) sequence of sample RI 41 amplicon using P3 primer pair and that of *S*. Infantis CFSAN003307. PCR was carried out using primer pairs listed in Table 1. P3 forward and P3 reverse primer sequences are underlined. *, identical nucleotide from Clustal analysis.

(one isolate), *S*. Paratyphi C (one isolate), *S*. Typhi (one isolate), and seven non-*Salmonella* isolates, namely, *Citrobacter* spp (four isolates), *Klebsiella pneumoniae* (two isolates) and *Proteus* sp (one isolate).

Clinical picture of *Salmonella*-infected diarrheal patients

Among the four diarrheal patients with *Salmonella*-positive fecal samples, two had chronic watery diarrhea together

with malnutrition and the other two, with good nutrition status, had acute watery diarrhea (Table 2). The patient with NTS Infantis presented a milder clinical picture compared to the three patients.

DISCUSSION

Shimizu *et al* (2014) developed a fast and technically simple mPCR method to detect NTS serovars. In our hands, using DNA from putative *Salmonella* colonies grown on SS agar, mPCR (employing five sets of primer pairs) only generated amplicons arising from *Salmonella*-specific primer pair. As only a single annealing temperature (63°C) was used in mPCR thermocycling, this indicated that this protocol favored amplification of the *Salmonella*-specific target *InvA*, which was confirmed by direct sequencing. However, sequencing of an amplicon generated by a primer pair targeting NTS Infantis *fliC-r* (Kardos *et al*, 2007) revealed the amplicon to be that of *S*. Infantis ChaA gene (analyzed by BLAST). Thus, it is important to confirm the identity of an amplicon by sequencing to obtain an unequivocal result.

Conventional biochemical and serological tests proved to be more useful in identifying the putative *Salmonella* colonies grown on SS agar. Vantarakis *et al* (2000) and Shimizu e*t al* (2014) noted the primer pair used for "specifically" amplifying *Salmonella invA* (Chiu and Ou, 1996) can cross hybridize with other Enterobacteriaceae members.

Among the four diarrheal patients with *Salmonella* detected in their feces, the patient with NTS Infantis manifested acute but mild acute and not the chronic symptoms typical of typhoid infection. The patient had a good nutritional status, and condition improved with antibiotic and supportive therapy (data not shown). *S*. Paratyphi A also produces a milder infection but is frequently associated with a lengthened fever clearance time (Thompson, 2015). In infants ≤1 year of age, breastfeeding is a significant protective factor against *Salmonella* infection (Bassal *et al*, 2014).

In summary, among 80 fecal samples from diarrheal children, 1 isolate of non-typhoidal *Salmonella* Infantis was identified by PCR and sequencing of amplicons. Typhoidal *S*. Paratyphi A, *S*. Paratyphi C and *S*. Typhi (as well as other Enterobacteriaceae members) were also identified by conventional biochemical and serological tests. Although multiplex PCR is suggested to aid in simultaneous identification of several *Salmonella* serovars, without judicious selection of the annealing temperature, the technique might not be as discriminatory as anticipated. Confirmation by conventional biochemical and serological tests should be undertaken. Mild clinical presentation was indicative of non-typhoidal *Salmonella* infection.

ACKNOWLEDGEMENTS

The authors thank all participants and GC Pandango, AFW Putri, LP Dewi, VV Pasaribu, and RA Satjadibrata, Dr Soetomo General Hospital, Surabaya for their kindness in collecting specimens. The study was supported by a grant from Airlangga Health Science Institute, Universitas Airlangga.

REFERENCES

Agron PG, Walker RL, Kinde H, *et al*. Identification by subtractive hybridization of sequences specific for *Salmonella enterica* serovar Enteritidis. *Appl Environ Microbiol* 2001; 67: 4984-91.

Bassal R, Reisfeld A, Nissan I, Agmon V. Risk

factors for sporadic infection with *Salmonella* Infantis: a matched case-control study. *Epidemiol Infect* 2014; 142: 820-5.

Bhutta SA. Salmonella. In: Kliegman R, Stanton B, St Geme J, Schor N, Behrman R, eds. Nelson textbook of pediatrics. 19th ed. Philadelphia: Elsevier Saunders, 2011: 948-59.

Chiu CH, Ou JT. Rapid identification of *Salmonella* serovars in feces by specific detection of virulence genes, invA and spvC, by an enrichment broth culture-multiplex PCR combination assay. *J Clin Microbiol* 1996; 34: 2619-22.

D' Aoust JY. *Salmonella*. In: Doyle MP, ed. Food borne bacterial pathogens. New York: Marcel Dekker, 1989: 327-445.

European Centre for Disease Prevention and Control (ECDC). Surveillance of enteric pathogens in Europe and beyond-Enternet annual report 2005. Solna: ECDC, 2005.

Fleisher GR, Sieff CA. Infectious disease emergencies. In: Fleisher GR, Ludwig SL, eds. Textbook of pediatric emergency medicine. 6th ed. Ch 92. Philadelphia: Lippincott, Williams and Wilkins, 2010.

Graham SM, Walsh AL, Molyneux EM, Phiri AJ, Molyneux ME. Clinical presentation of non-typhoidal *Salmonella* bacteraemia in Malawian children. *Trans R Soc Trop Med Hyg* 2000; 94: 310-4.

Jamshidi A, Bassami MR, Afshari-Nic S. Identification of *Salmonella* spp. and *Salmonella* Typhimurium by a multiplex PCR-based assay from poultry carcasses in Mashhad-Iran. *Iran J Vet Med* 2009; 3: 43-8.

Kardos G, Farkas T, Antal M, Nógrády N, Kiss I. Novel PCR assay for identification of *Salmonella enterica* serovar Infantis. *Lett Appl Microbiol* 2007; 45: 421-5.

Lee SH, Jung BY, Rayamahji N, *et al*. A multiplex real-time PCR for differential detection and quantification of *Salmonella* spp., *Salmonella enterica* serovar Typhimurium and Enteritidis in meats. *J Vet Sci* 2009; 10: 43-51.

MacLennan CA, Gondwe EN, Msefula CL, *et al*. The neglected role of antibody in protection against bacteremia caused by nontyphoidal strains of *Salmonella* in African children. *J Clin Invest* 2008; 118: 1553-62.

Majowicz SE, Musto J, Scallan E, *et al*; for the International Collaboration on Enteric Disease 'Burden of Illness' Studies. The global burden of nontyphoidal *Salmonella* gastroenteritis. *Clin Infect Dis* 2010; 50: 882-9.

Ochiai RL, Acosta CJ, Danovaro-Holliday MC, *et al*; the Domi Tyhphoid Study Group. A study of typhoid fever in five Asian countries: disease burden and implications for controls. *Bull World Health Organ* 2008; 86: 260-8.

Pickering LK, Baker CJ, Kimberlin DW, Long SS, eds. *Salmonella* infections. In: Red Book: Report of the Committee on Infectious Diseases. 29th ed. Grove Village: American Academy of Pediatrics, 2012: 635-41.

Saravanan S, Purushothaman V, Murthy TRGK, *et al*. Molecular epidemiology of nontyphoidal *Salmonella* in poultry and poultry products in India: implications for human health. *Indian J Microbiol* 2015; 55: 319-26.

Shahunja KM, Leung DT, Ahmed T, *et al*. Factors associated with non-typhoidal *Salmonella* bacteremia versus typhoidal *Salmonella* bacteremia in patients presenting for care in an urban diarrheal disease hospital in Bangladesh. *PLOS Negl Trop Dis* 2015; 9: e0004066.

Shimizu R, Osawa K, Shigemura K, *et al*. Development of multiplex PCR for rapid identification of four *Salmonella* serovars most commonly isolated in Japan. *Southeast Asian J Trop Med Public Health* 2014; 45: 654-61.

Tennant SM, Diallo S, Levy H, *et al*. Identification by PCR of non-typhoidal *Salmonella enterica* serovars associated with invasive infections among febrile patients in Mali. *PLOS Negl Trop Dis* 2010; 4: e621.

Thamlikitkul V, Dhiraputra C, Paisarnsinsup T, Chareandee C. Non-typhoidal *Salmonella*

bacteraemia: clinical features and risk factors. *Trop Med Int Health* 1996; 1: 443-8.

Thompson C. The burden of *Salmonella* Paratyphi A in Kathmandu: epidemiological observations from a decade of clinical trials. Bali: 9th International Conference on Typhoid and Invasive NTS Disease, April 30-May 3, 2015. [Cited 2017 Jun 2]. Available from: http://www.coalitionagainsttyphoid.org/wp-content/ uploads/2015/05/15.50-16.15-PM-CorinneThompson_Saturday2May_350pm. pdf

Vantarakis A, Komninou G, Venieri D, Papapetropoulou M. Development of a multiplex PCR for detection of *Salmonella* spp. and *Shigella* spp. in mussels. *Lett Appl Microbiol* 2000; 31: 105-9.

Walker KE, Mahon CR, Lehman D, Manuselis G. Enterobacteriaceae. In: Mahon CR, Lehman DC, Manuselis G, eds. Textbook of diagnostic microbiology. 5th ed. Ch. 19. Maryland Heights: Saunders, 2015: 420-52.

Wattiau P, Boland C, Bertrand S. Methodologies for *Salmonella enterica* subsp. *enterica* subtyping: gold standards and alternatives. *Appl Environ Microbiol* 2011; 77: 7877-85.

World Health Organization (WHO). The treatment of diarrhoea: a manual for physicians and other senior health workers. 4th ed. Geneva : WHO, 2005.

World Health Organization (WHO). Nutrition Landscape Information System (NLIS) country profile indicators: interpretation guide. Geneva: WHO, 2010.

World Health Organization (WHO). Diarrhoeal disease. Geneva: WHO, 2016. [Cited 2017 Aug 10]. Available from: https://www.who.int/news-room/fact-sheets/detail/diarrhoeal-disease