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DETECTION OF SHIGELLA SP ON STOOL CHILDREN WITH DIARRHEA USING CULTURE METHODE AND PCR

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ABSTRACT

Introduction: *Diarrhea is one health problem in the world with mortality and morbidity rates are high, both in developed and developing countries. This research aims to detect Shigella sp in the stool of children with diarrhea using culture* method *and PCR.* ***Methods:*** *This research is a descriptive with cross-sectional design. A total of 50 samples were obtained from four health centers in Makassar city, i.e Barabaraya, Pampang, Antang Perumnas and Tamangapa from May - July 2016 with rectal swab* method*. For culture test, the samples were inoculated on medium SSA. While with PCR test, primer icsA (526 bp) was used.* ***Results:*** *The results indicate that, from 50 samples tested by using culture method, there is no sample (0%) positive detected Shigella sp. While the PCR* method *there 6 samples (24%) positive detected Shigella sp.* ***Conclusion:*** The sensitivity of PCR in the detection of *Shigella sp* from the level of dilution 10^0 - 10^{-3}*. Al together, it can be inferred that PCR* method *is more accurate* method *detect Shigellasp than other culture* methode*.*

Keywords: Diarrhea, Shigellasp, Culture, PCR

INTRODUCTION

Diarrheal disease still remains a health problem of the world till today, both in developed and developing countries. Diarrhea is a disease with morbidity and mortality occur in children under 5 years old and about 18% of cases of infant deaths in the world each year are caused by diarrhea.[1]

Ongoing study in eight hospitals in Indonesia put *Shigella sp.* (27.3%) as a pathogen with of the 2nd most high-frequency in causing of diarrhea after *Vibrio cholerae* (37.1%), and by *Salmonella sp.* (17.7%).[2]

The cause of diarrhea is usually caused by infection from viruses, bacteria and other parasites. *Shigella sp* is one of the bacteria that can causes diarrhea. Based on the biochemical properties and its antigen, distinguished on S. dysenteriae, S. flexneri, S. boydii and S. sonnei. All kinds of Shigella are able to infect humans and cause diarrhea, from mild diarrhea to severe diarrhea.[3]

A common method used to detect *Shigella sp* is culture method. But the culture method is relatively inefficient, it takes about 48-72 hours, and sometimes generated erroneous diagnosis.[4] To overcome the shortage of culture method other diagnostic method are needed with better sensitivity and specificity. The use of biomolecular in the medical field is constantly increasing. Various types of new diagnostic method have been used to diagnose infectious diseases such as PCR method. PCR is a method that is based on the reproduce copies of DNA template (target).

Several studies have shown that detection of Shigella using DNA amplification technique is better than using conventional method (culture). Research conducted by Thiem et.al, showed as much as 46% of diarrheal stool

samples were cultured showed negative results, but with PCR method especially quantitative PCR (qPCR) showed positive results for the Shigella.[5] Under these conditions, this study will look at the effectiveness of culture and PCR method in diagnosing diarrhea caused by Shigella sp. This research aims to detect *Shigella sp* in the stool of children with diarrhea with culture and PCR method.

METHODS

Research Sites

Research location was distinguished based on the location of sampling and sampling inspection. Sampling was conducted in four health centers in Makassar that is Barabaraya, Antang Perumnas, Tamangapa and Pampang. Test of the samples carried out in the Laboratory of Microbiology, Unhas Hospital Makassar.

Population and Sample

The population in this research is all diarrhea patients who came to the sampling site. Total sample as many as 50 children with diarrhea fulfilled the inclusion criteria, such as diarrhea patients, aged 0-59 months, do has recently taken antibiotics and are willing to sign an informed consent form from the Research Ethics Committee of Faculty of Medicine Unhas.

Sample Collection

Samples were obtained by taking rectal swabs from 50 children with diarrhea. Subject was laid sideways position to the left, the position of the right leg above and the left foot below. The anal canal is opened using the left hand, then put a cotton swab into the anus previously soaked in a solution of NaCl and swab rod is rotated 360º. Tip swab removed by twisting and immediately put in a transport medium is Cary-Blair.

Culture method

Samples of Cary-Blair medium were inoculated on the BHIB fertilizer medium and incubated at 37ºC for 24 hours. 1 loop ose taken and inoculated on media saucer Salmonella-Shigella agar. Samples were incubated for 24 hours at a temperature of 37ºC. Colonies that gives the appearance of dull or colorless was taken and followed by biochemical tests. Biochemical tests that was done using Triple Sugar Iron Agar (TSIA) test, Sulfate Indole Motility (SIM), Methyl Red, Vogas Proskaurer (VP), Simmon citrate, urease test, fermentation of carbohydrates test. After incubation for 24 hours at a temperature of 37°C, seeing the reaction that occurs in every *test*.

PCR method

DNA extraction

DNA extraction process is done by using Genead Presto™ DNA / RNA Extraction Kit Protocol.

DNA Amplification

DNA amplification is done using a primer ICSA (forward: 5'-ATG TTC TAA GCA CAG AAA TGG - 3', reverse: 5'-GTG ACA CCC TTC TGT AGG CG -3). Making PCR regen mix was made by mixing 12.5 mL green master mix (dNTP, DNA polymerase tag, MgCl2), 0.5 mL of forward primer, reverse primer 0.5 mL, Nuclease free water 6.5 mL, 5.0 mL of product DNA, and inserted into the eppendorf tube. Amplification program was carried out in several cycles. The first cycle is done, by denaturation at a temperature of 94°C for 3 minutes. Furthermore, a total of 35 cycles of denaturation next performed at a temperature of 94°C for 1 minute, annealing at a temperat temperature of 56°C for 45 seconds and extension at 72°C for 1 minute andthe third cycle do the final extension at 72°C for 5 minutes.

Agarose Gel Electrophoresis

Analysis of the results of PCR amplification products is done by using a 2% agarose gel electrophoresis, and then visualized with the Gel Doc receipts. A positive result is indicated by the presence of DNA fragment bands were formed in accordance with the target band (526 bp).

RESULTS

Characteristics of children with diarrhea in the research samples was differentiated by age range and gender. A total of 50 child patients in this study were consisted of 28 males (56%) and 22 females (44%) with an age range of children with diarrhea highest row is aged 12-35 months were 29 cases (58%), then followed by age 36 - 59 months with 12 cases (24%) and aged 0-11 months were 9 cases (18%) (Table 1).

Results of detection by using cultures of 50 samples were inoculated at SSA medium

Table 1. Diarrhea patient distribution by age and sex

Characteristic	N	Percentage (%)
Age		
0 – 11 month	9	18
12 – 35 month	29	58
36 – 59 month	12	24
Sex		
Male	28	56
Female	22	44

and has conducted biochemical test sample obtained from 0 (0%) positive Shigella sp. Characteristic of *Shigella sp*-suspected colonies that grow on the SSA medium is uncoloured colony (transparent). While by using the PCR method, 6 samples (24%) were positive *Shigella sp* (Table 2).

PCR test results after visualized by 2% agarose gel electrophoresis showed formed fragment of DNA band with a size of 526 bp.

Table 2. The result of *Shigella sp* detection by culture and PCR Method

Method	Result (%)	
	Positive	Negative
Culture	0 (0%)	50 (100%)
PCR	6 (24%)	44 (76%)

DNA fragments that are formed in accordance with the size of the target DNA fragment. It indicates that the sample was contained with Shigella sp. While the sample which is not form fragment of DNA indicates that there were no bacteria *Shigella sp* (Figure 1-3).

The sensitivity of PCR in the detection of *Shigella sp* was measured by dilution method storied. Dilution is done in the positive control from the level of dilution of 10^0 - 10^{-6} (Figure 4). DNA electrophoresis results of dilution of positive control gave positive results from the level of dilution of 10^0 - 10^{-3}which marked with the formation of the band corresponding to the target band (526 bp). While the dilution rate of 10^{-4} - 10^{-6} is not formed DNA fragments that showed *Shigella sp* targets can not be detected at the level of the dilution.

DISCUSSION

Diarrhea is a disease characterized by increased frequency of defecation more than normal (> 3 times / 24 hours) and a complete change in stool consistency to a liquid with or without blood and mucus.[6] Shigella sp. is the pathogen most frequently isolated from feces

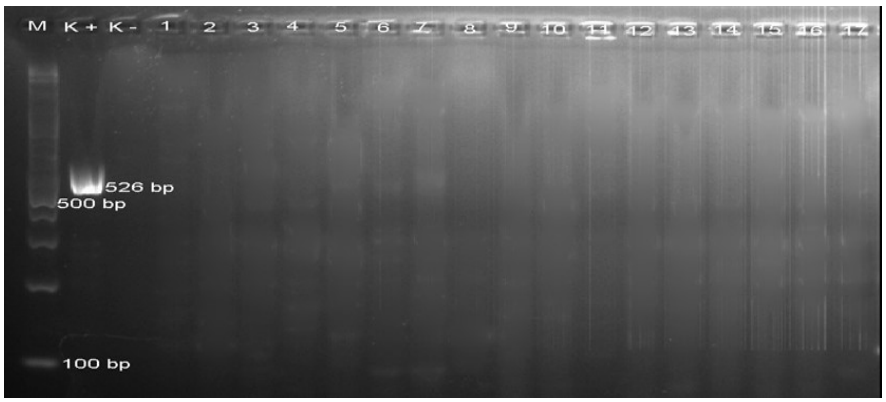

Figure 1.
Agarose gel electrophoresis of PCR products sample code of 1-17 amplified with *Shigella* spp. icsA gene primers, M: Marker, K+ : Positive control; K-: negative control

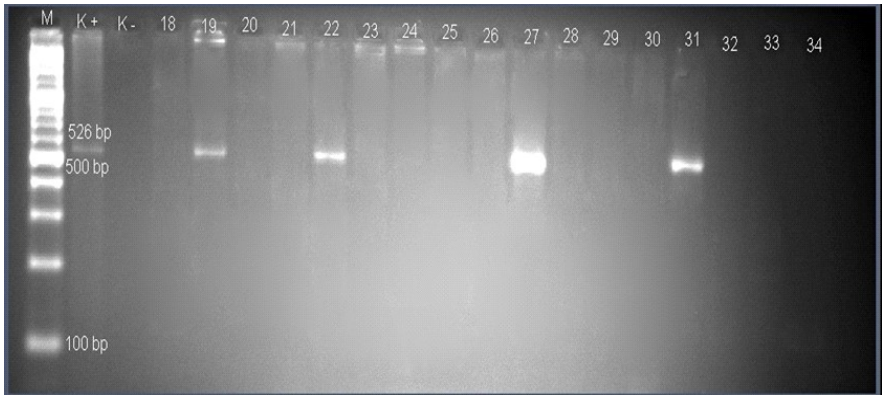

Figure 2.
Agarose gel electrophoresis of PCR products sample code of 18-34 amplified with *Shigella* spp. icsA gene primers, M: Marker, K+: Positive control; K-: negative control

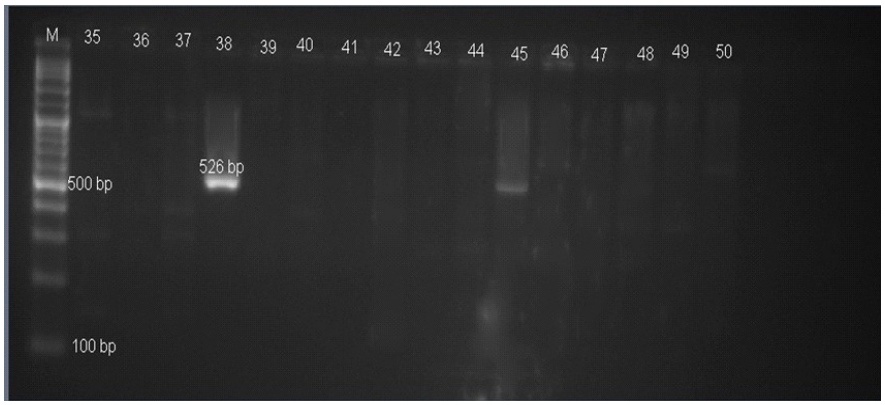

Figure 3.
Agarose gel electrophoresis of PCR products 35-50 amplified with *Shigella* spp. icsA gene primers. M: Marker

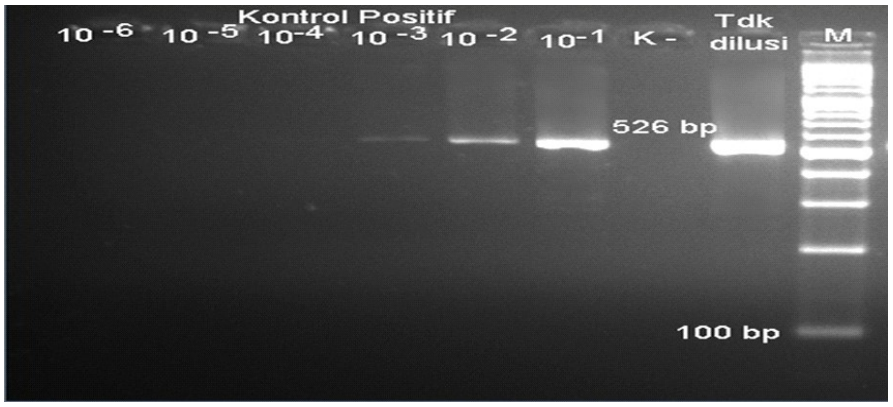

Figure 4.
Agarose gel electrophoresis of *Shigella* PCR products obtained by the analysis of dilution of different concentrations of *S. flexeneri* strain (ATCC 12022).

in children with bloody diarrhea in developing countries. Only with 10 -100 these bacterial in the body, it can make infections in humans.[7] The disease usually begins with fever and malaise, followed by watery diarrhea and painful abdominal cramps. On the second day of the disease, blood and mucus usually appears in the stool, and tenesmus became a prominent symptom.[8]

The high incidence of diarrhea in children aged 12 to 35 months (toddler), could be covered by children at this age have began actively playing.[9] In addition, children under the age of two years are in the oral phase where at this phase children have a habit of entering items around them into the mouth so that it can increase the risk of diarrhea.[10] Meanwhile, the high proportion of cases of diarrhea in boys than girls because of the tendency activities more active by playing wider area coverage so it's easier to be infected by the causative agent of diarrhea.[10]

The standard procedures to identify *Shigella sp,* are using the culture on selective media and agglutination tests with anti-sera.[11] In this study the detection by using culture method, which samples were inoculated on medium SSA.

In this study, there are no positive results *Shigella sp* with culture method. Characteristic of Shigella colonies that grow on the medium SSA is shaped convex (convex) with a diameter of 2-3 mm and transparent colony color (Colourless).[12] In this study, 50 stool samples containing no blood. This can happen because of number of bacteria *Shigella sp* contained in stool bloody diarrhea patients more in comparison with the stool in patients with diarrhea without blood.[5]

The presence of blood in the stool is not always an infection by Shigella sp. The bacteria that can cause bloody diarrhea is Shigella and invasive E. coli, Salmonella, Campylobacter jejuni and coli Balantidium.[13]

In addition to using culture method, this study also used the PCR method to detect Shigella sp. PCR is an enzymatic method to double strand DNA without using a living organism. Results of *Shigella sp* detection by PCR after electrophoresis obtained as much as 6 samples (24%) were detected as positive bacterium Shigella sp, namely the sample code F 19, F 22, F 27, F 31, F 28, and F. 45 is marked with a band formed in accordance with the size of the target band that is 526 bp.

There being a different diagnosis between culture and PCR method wherein the culture method detected Shigella negative to positive samples detected by PCR. This is because the number of *Shigella sp* in the sample is low and the competition from other commensal organisms.[4]

This is according to research conducted by Lindsay et al, that of 887 stool samples of children suffering from diarrhea were tested by culture method and the PCR was obtained 90 positive samples of Shigella with the culture method and 155 samples were positive by PCR with primers ipaA.[14] Another study in India showed similar results where the method of PCR (primer Ipah) more positive samples detected Shigella compared with culture method.[15]

CONCLUSIONS

From the results of this study concluded that more effective PCR method to detect shigela sp in stool of children with diarrhea compared with culture method. Based on this study, it can be suggested that more research is needed with a number of more samples and on different populations, besides the city of Makassar.

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REFERENCES

1. Bryce J, Boschi-Pinto C, Shibuya K, Black RE, Group WCHER. WHO estimates of the causes of death in children. The Lancet. 2005;365(9465):1147-1152.
2. Tjaniadi P, Lesmana M, Subekti D, Machpud N, Komalarini S, Santoso W, et al. Antimicrobial resistance of bacterial pathogens associated with diarrheal patients in Indonesia. The American journal of tropical medicine and hygiene. 2003;68(6):666-670.
3. Levine MM, Kotloff KL, Barry EM, Pasetti MF, Sztein MB. Clinical trials of Shigella vaccines: two steps forward and one step back on a long, hard road. Nature Reviews Microbiology. 2007;5(7):540-553.
4. Ojha SC, Yean Yean C, Ismail A, Banga Singh K-K. A pentaplex PCR assay for the detection and differentiation of Shigella species. BioMed research international. 2013; 2013.
5. Thiem VD, Sethabutr O, von Seidlein L, Van Tung T, Chien BT, Lee H, et al. Detection of Shigella by a PCR assay targeting the ipaH gene suggests increased prevalence of shigellosis in Nha Trang, Vietnam. Journal of clinical microbiology. 2004;42(5): 2031-2035
6. Sudaryat S. Gastroenterologi Anak. Lab/ SMF Ilmu Kesehatan Anak Fakultas Kedokteran Universitas Udayana. 2010.
7. Nascimento AM, Van Der Sand ST. Detection of the virulence plasmid pINV, using inv E gene in shigella sp. in sewage samples by PCR assay. Biociências (Online). 2008;16(1).
8. Wyllie R, Hyams JS, Kay M. Pediatric gastrointestinal and liver disease: Elsevier Health Sciences; 2015.
9. Kemenkes R. Buletin jendeta data dan informasi kesehatan: situasi diare di Indonesia. Jakarta: Pusat Data dan Informasi Kementrian Kesehatan RI Hlm. 2011:1-6.
10. Palupi A, Hadi H, Soenarto SS. Status gizi dan hubungannya dengan kejadian diare pada anak diare akut di ruang rawat inap RSUP Dr. Sardjito Yogyakarta. Jurnal Gizi Klinik Indonesia. 2009;6(1):1-7.
11. Alipour M, Talebjannat M, Nabiuni M. Polymerase chain reaction method for the rapid detection of virulent Shigella spp. 2012.
12. Gaurav A, Singh S, Gill J, Kumar R, Kumar D. Isolation and identification of Shigella spp. from human fecal samples collected from Pantnagar, India. Vet World. 2013; 6:376-379.
13. Bhutta ZA. Acute gastroenteritis in children. Nelson textbook of pediatrics. 2007; 19:1323-1339.
14. Lindsay B, Ochieng JB, Ikumapayi UN, Toure A, Ahmed D, Li S, et al. Quantitative PCR for detection of Shigella improves as-

certainment of Shigella burden in children with moderate-to-severe diarrhea in low-income countries. Journal of clinical microbiology. 2013;51(6):1740-1746.

15. Dutta S, Chatterjee A, Dutta P, Rajendran K, Roy S, Pramanik K, et al. Sensitivity and performance characteristics of a direct PCR with stool samples in comparison to conventional techniques for diagnosis of Shigella and enteroinvasive Escherichia coli infection in children with acute diarrhoea in Calcutta, India. Journal of medical microbiology. 2001;50(8):667-674.