

IDENTIFICATION OF INTESTINAL MICROBES IN CHILDREN WITH DIARRHEA AND NON-DIARRHEA USING POLYMERASE CHAIN REACTION / ELECTROSPRAY IONIZATION-MASS SPECTROMETRY (PCR / ESI-MS)

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Abstract : Microbiota present in human intestinal are diverse, and imbalance in composition of intestinal flora may cause diarrhea. This study aimed to obtain a profile of intestinal bacteria in children with and without diarrhea and assess their presence with incidence of diarrhea. An analytical descriptive with cross sectional design study was carried out. A stool specimen was collected from each children of 2-12 years old with and without diarrhea who lived in North Jakarta. DNA extraction was performed prior to detection of microbes using Polymerase Chain Reaction/Electrospray Ionization-Mass Spectrometry.

Eighty stool specimens consisted of 33 and 47 specimens from children with and without diarrhea were included in the study. Thirty single and 6 multiple matches were detected in 30 specimens of the diarrhea group; 28 single and 8 multiple matches were found in 34 specimens of the non-diarrhea. *Escherichia coli* and *Klebsiella pneumoniae* were predominant in both groups. Firmicutes, Proteobacteria and Bacteroidetes were detected in the diarrhea group, while Actinobacteria, Proteobacteria and Verrucomicrobia were in the non-diarrhea. The relationship of incidence of diarrhea and the present of enteropathogens in the stool was not significant, however, there was a strong correlation of the risk of suffering diarrhea due to the presence of enteropathogens (OR = 0.724 with 95% CI: 0.237-2.215).

In conclusion, most bacteria detected in both groups were similar, nonetheless, Actinobacteria was present only in the non-diarrhea. The chance to have diarrhea was higher when enteropathogen was detected in the stool.

Keywords: Gut microbiota, diarrhea, PCR/ESI-MS

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INTRODUCTION

Gastrointestinal tract (GIT) is the most heavily colonized organ; the colon alone contains over 70% of all the microbes in the human body^{1,2}. The intestinal microbiota composed of more than 1000 species³. Almost all of these species (98%) belonged to only four bacterial phyla i.e. Firmicutes (64%), Bacteroidetes (23%), Proteobacteria (8%), and Actinobacteria (3%)⁴. Other species such as members of Verrucomicrobia, Fusobacteria and Cyanobacteria exist in small amount³. Microbes in small intestine were enriched with the Bacilli class of Firmicutes and Actinobacteria, while in colon, Bacteroidetes and the Lachnospiraceae, family of Firmicutes

were more prevalent⁴. Infection will affect on interaction among microbiota and trigger inflammation responses, and consequently influence the composition of intestinal microbiota⁵. Lupp C et al, 2007 reported that intestinal mucosal inflammatory process due to infection altered the resident obligate anaerobic bacteria and triggered excessive growth of commensal Enterobacteriaceae to become pathogenic⁵.

A variety of approaches which include full-length 16S rRNA sequencing⁶, denaturing gradient gel electrophoresis by Zoetendal et al⁷, and fluorescent in situ hybridization by Franks et al⁸ had been used in the study of intestinal microbial communities. Further, Polymerase Chain Reaction coupled with Electrospray Ionization/Mass Spectrometry (PCR/ESI-MS), a technology based on PCR technique, and dispersion technology of electro ionization mass spectrometry, has been utilized for a complete identification of a large number of microorganisms^{9,10}. It

has a capability to identify multiple organisms present in a sample up to species level simultaneously without prior cultivation^{9,11,12}.

In this study we employed the PCR/ESI-MS to identify intestinal microbes from stools of children aged 2-12 years with and without diarrhea in Jakarta, and analyse their relationship to incidence of diarrhea. Such information will be valuable for the development of management of diarrhea in the context of intestinal microbial balance.

MATERIALS AND METHODS

Clinical specimens

The research was an analytical descriptive study with cross sectional design. Stool samples were collected from September 2012 to December 2012. Diarrhea samples were collected from outpatients in Infectious Diseases Hospital (IDH) Prof Dr Sulianti Saroso and primary health centers highlighted the close relation area of study population in North Jakarta, Indonesia. Non-diarrhea samples were collected from healthy children in toddler classes and kindergartens in the same area as mentioned above. The samples were stored at 4°C for not more than 24 hours and immediately transported to laboratory using special coolbox. All samples were stored at -70°C until further process. Inclusion criteria for diarrhea sample were children aged 2-12 years which had an episode of acute diarrhea according to WHO criteria¹³ and has not received antibiotic. For non-diarrhea sample, were children aged 2-12 years, without episodes of diarrhea. This study was approved by Ethical Committee of Faculty of Medicine, University of Indonesia.

DNA extraction

Total DNA was extracted from 180-220 mg solid stool or 200 µl liquid stool. The extraction was performed by using QIAamp® DNA Stool Mini Kit according to the manufacturer's instructions with 200 µl of the elution. The elution was stored at -70°C until processed.

PCR-ESI/MS assay

Prior to PCR-ESI/MS, inhibitors in the samples were analyzed by conventional PCR. PCR-ESI/MS was performed by using PLEX-ID Broad Bacteria panel in accordance with manufacturer's instruction. The data threshold is 0.85 confidence, in that less than 0.85 were reported uninterpretable¹⁴.

Statistical analysis

Statistical analysis was performed by using SPSS ver 17 software. The relationship between enteropathogens and diarrhea cases was done using Chi-square test with a P-value of < 0.05 was considered significant. The risk relationship (Odds ratio) between those two was also measured.

RESULT

1. Subjects profiles

Overall 80 subjects were obtained in accordance with the inclusion criteria, consisted of 33 children who had diarrhea (diarrhea subjects) and 47 children who did not have diarrhea (non-diarrheal subjects). Diarrhea subjects consisted of 24 boys and 9 girls with an age range of 2 years to 9 years and the median age of 3 years. While the non-diarrheal subjects consisted of 27 boys and 20 girls with an age range of 2 years 3 months to 11 years with a median age of 4 years and 4 months.

2. PLEX-ID result

PLEX-ID positive detection means that the processor PLEX-ID reads one or more microorganisms with a Q score > 0.85 in accordance with the spectrometer database PLEX-ID. If more than one bacteria detected in one sample with a different Q score, means that there is more than one bacteria that has the same opportunities as the detected bacteria. PLEX-ID detection would give three kinds of result, as shown in Figure 1.

Of 33 samples in diarrhea group showed the presence of 30 single match bacteria and 6 multiple matches bacteria, while the 3 other samples showed no detectable bacteria. In the non-diarrhea group (47 samples) showed 28

single bacteria, 8 multiple matches and 13 samples showed no detectable bacteria.

3. Detection of bacteria in samples

Table 2 shows *Escherichia coli* dominated the diarrhea group, followed by *Klebsiella pneumoniae*, both from the phylum Proteobacteria. Another species were *Campylobacter jejuni*, *Acidovorax avenae* and *Aquaspirillum gracile*, all from Proteobacteria, while *Staphylococcus epidermidis*, *Clostridium perfringens*, *Streptococcus vestibularis* from Firmicutes and *Prevotella albensis* from Bacteroidetes.

The non-diarrhea group also dominated by *Escherichia coli* and *Klebsiella pneumoniae*. Another Proteobacteria detected was *Escherichia fergusonii*. Additionally it also detected the presence of Actinobacteria (*Bifidobacterium longum*) and Verrucomicrobia (*Akkermansia muciniphila*).

The diversity of bacteria detected in the diarrhea group (12 of 30 samples) was more than in non-diarrheal group (5 of 28). Figure 2 shows Firmicutes and Bacteroidetes were only detected in diarrhea group, while Actinobacteria and Verrucomicrobia were only detected in non-diarrhea group. Proteobacteria was detected in both groups by the number of samples in non diarrhea more than in diarrhea group (25 vs. 24).

The results of multiple matches were dominated by clusters of *Escherichia coli* / *Shigella flexneri* / *Shigella sonnei* in each sample group, 4 samples (D02, D13, D14 and D33) in the diarrhea group, and 4 samples (S10, S17, S23 and S38) in the non diarrhea group. Sample D11, D33 and S11 were also detected single match bacterial. *Streptococcus sp* on D11, *Aquaspirillum gracile* species on sample D33, and *Vibrio sp.* on sample S1 (Table 3).

In this study, enteropathogens were determined using the assumption that the organisms identified using PLEX-ID were organisms known as enteropathogens. In single match, they were *Campylobacter jejuni*, *Clostridium perfringens* and *E. coli*; in multiple matches, they were *Shigella spp* and *Vibrio spp*. Table 4 shows that of

all cases with enteropathogens, 48.2% (27/56) had diarrhea. While in the cases with non-enteropathogens, which had diarrhea was 56.5% (9/16). Using Chi-square test gave $P = 0.571$. It means there is no significant relationship between enteropathogens with the incidence of diarrhea. Odds ratio is used to see the strength of the relationship between the incident of diarrhea by enteropathogens (OR 0,724 [95% CI: 0,237-2,215]). This means that the chance of having diarrhea caused by enteropathogens was 0.724 fold than by non-enteropathogen.

DISCUSSION

This study was conducted in four health centers and one hospital in North Jakarta. The health centers were selected on the basis of the distance between the sites, which ranges from 4 km to IDH, to facilitate sample collection. In addition, it was assumed homogenization of social, economic, environmental and climate of the subjects. Specimens obtained were placed in a cooler box with temperatures around 8°C before transfer to a collection point in IDH on the day or the next day. From the published literature, stool specimens can be stored at 2-8°C prior to processing¹⁵.

Of the 33 diarrhea subjects, there were 24 boys and 9 girls with an age range of 2 years to 9 years. This is in accordance with the Basic Health Survey Indonesia in 2007 that claimed the prevalence of diarrhea in boys was higher than girls, as well as the prevalence by age group ie age of 24-48 monthshad a higher prevalence than the age of 48 months upwards¹⁶.

The use of PLEX - ID of the faecal samples directly, as far as we know just recently conducted in this study. In previous studies on blood culture, the suitability of the results obtained at the species level by standard methodswas 86.75 % (n = 234)⁹, while from clinical isolates was 74% (n = 156) identified appropriately, with only 9 % were incorrectly identified¹⁷. In this study, due to not using the comparison method, the results of which can be expressed,wasobtained 80 % (64 of 80)

detected the presence of bacteria, with 14 of 64 samples detection results were the result of multiple matches (Table 3). The detection results can not be distinguished further thus reported as a cluster (M. Rost, personal communication, April 12, 2013)¹⁸. Of the 14 multiple matches samples, dominated by a cluster of *Escherichia coli* and *Shigella sp.* This happens due to *E. coli* and *Shigella* is a bacterial species with close ties phylogenetic¹⁷.

Escherichia coli is the dominant bacteria in the gastrointestinal tract[19]. It is showed in both groups of the study that most of bacteria detected were *E. coli*. Detection of *Campylobacter jejuni* and *Shigella boydii* , which are enteropathogens, in the diarrhea group, distinguish diversity detection results with non-diarrhea group. Despite the similar study with different detection methods conducted by Bodhidatta (2010) in Thailand found that *Campylobacter* and *Shigella* were also found in non-diarrheal samples²⁰.

Moreover, it also detected the presence of the *Aquaspirillum gracile* which have a new name as *Hylemonella gracilis*. This bacterium is commonly found in aquatic ecosystems²¹. Other bacteria detected in the diarrhea group was *Acidovorax avenae*. This bacterium is a Gram-negative rod bacteria, not pigmented and do not ferment lactose . Usually found in soil and water, as well known as plant pathogens. There are case reports stating that the bacteria associated with the onset of catheter- related sepsis²². Although very rare, the role of environmental bacteria which are not usually found associated with diarrhea should be evaluated and studied in the future.

All bacteria that were detected in the group of non-diarrhea is normal intestinal flora. *Akkermansia muciniphila* colonizes in the intestinal mucosal lining and amounted to 3-5 % of the entire community of intestinal bacteria²³. *Bifidobacterium* species was intestinal commensal bacteria associated with the synthesis of compounds that affect a human. *B. longum* express serine protease inhibitor which plays a role in the function of immunomodulator²⁴.

The non diarrhea group, was not detected pathogen bacteria, such as *Campylobacter sp* and *Shigella sp.* While in the diarrhea group, no detection of probiotic bacteria that play a role in maintaining immunological conditions of children. There may be an imbalance in the microbiota of the gastrointestinal tract of children with diarrhea than non diarrhea, although it is more confirmed when the number of samples involved in this study is greater.

Detection of *E. coli* and *K. pneumonia* in both groups of samples as most bacteria detected, and the similarity in the results of multiple matches, implies similarities of the intestinal microbiota in the subjects studied. Likely, this is due to the similarity of subject demographics, which causes environmental, habits and eating patterns have similarities. In a study conducted by Yatsunenko et al (2012) stated that differences in cultural tradition also affect food, exposure to pets and livestock, and many other factors that could influence how and from where a gut microbiota/microbiome is acquired²⁵.

The dominant phyla in diarrhea and non-diarrhea group was Proteobacteria. Bacterial phyla were only detected in diarrhea group were Firmicutes and Bacteroidetes whereas that was only found in non-diarrheal group were Actinobacteria and Verrumicrobia. The composition of those bacterial phyla are intestinal microbial composition^{3,4}. In this study, analysis of the intestinal microbial composition were obtained from faecal samples only describe the intestinal microbial composition in general and do not describe the composition of intestinal microbes on the anatomical location. That is because most of the results obtained in each sample only one bacterium and to be able to know the different microbial composition at each anatomical location, samples should be obtained from the anatomical locations, such as the research conducted by Frank et al⁴, Monstein et al^[26] and Morteau et al²⁷.

Few studies noted that *E. coli*, *Campylobacter jejuni*, *Shigella spp* and *Vibrio spp* as a cause of diarrhea in children^{15,20,28}, while *Clostridium perfringens* is known to cause food

poisoning causes gastroenteritis^{29,30}. Investigation of pathogenic strains of *E. coli*, was not included in the study, thus the analysis it is to be assumed that all *E. coli* detected as a pathogen. In the study the relationship between enteropathogens and diarrhea incidence is not significant ($P = 0.571$). There are two possibilities that support this statistical analysis: (1) all *E. coli* detected (diarrhea = 18 and non-diarrhea = 21) were considered as enteropathogens without any investigation of pathogenic strains, (2) the calculation also includes multiple matches that likely contributed to the statistical analysis. The strength of the relationship between enteropathogens and diarrhea in a clinical need to be analyzed with the case-control design, using odds ratio. OR values obtained were 0.724 (95% CI: 0.237-2.215). It means there is a strong correlation between enteropathogens with the incident of diarrhea.

Limitation of study was the results of PCR-ESI/MS detection while not giving an overview of interaction between microbiota and enteropathogens populations in non-diarrhea and diarrhea children, but provide results that enteropathogens found only in cases of diarrhea. These limitations can still be overcome by further research, one of which is a comparison with other detection methods.

CONCLUSION

Bacteria detected in the diarrhea group were more diverse than in the non-diarrheagroup and similarity in the pattern of most detected bacteria in both sample groups. *Escherichia coli* and *Klebsiella pneumoniae* were two most detected bacteria in both groups, suggest the gut microbiota was influenced by environmental, habits and eating patterns. One of the well known probiotic bacteria i.e. *Bifidobacterium longum* were found only in non diarrhea group. Likely the chance of children with enteropathogen detected in the stool would have diarrhea 0.724 fold more than children with no enteropathogen detected.

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Figure

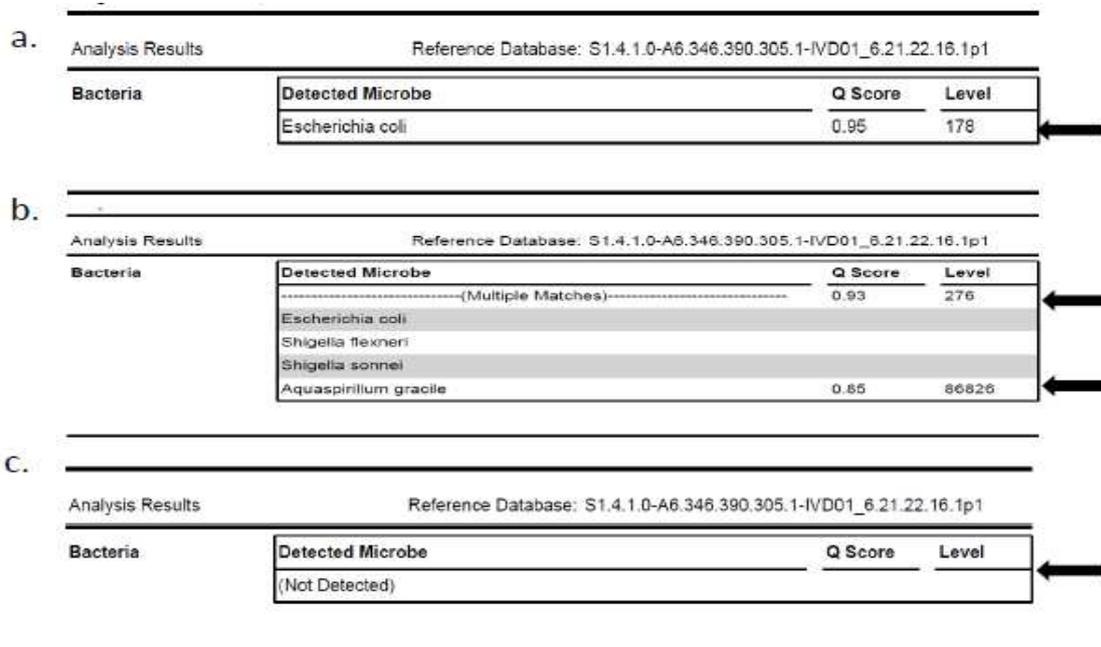


Figure 1. PLEX-ID result sheet which shows the detection of bacteria, with each having Q-score and the different levels (arrow). **a.** Shows the detection of a single bacterium, **b.** Shows the detection of the bacteria, and bacterial detection with the results of multiple matches and **c.** Shows no detectable.

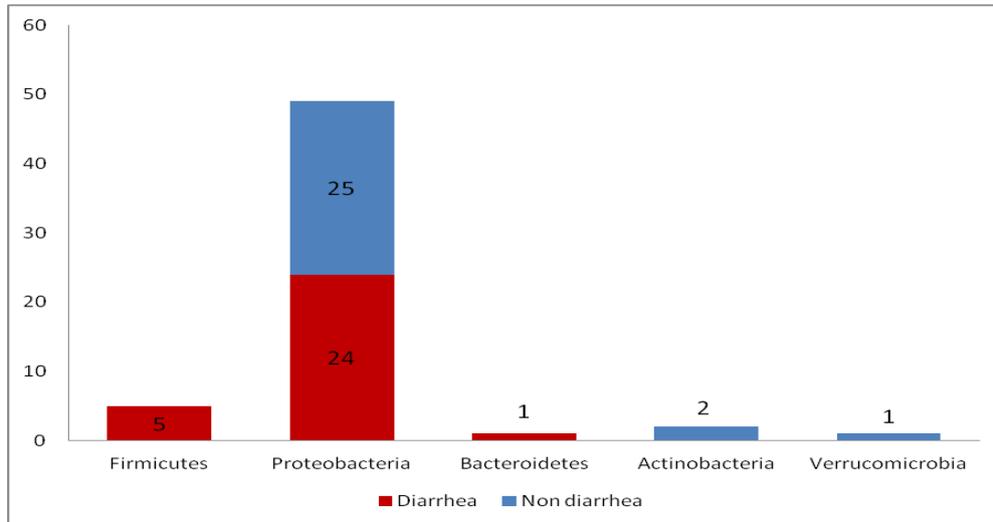


Figure 2. Phyla comparison chart based on the number of species bacteria detected in a sample group of diarrhea and non-diarrhea.

Tables

Table 1. Types of PLEX-ID detection results

	Detection of bacteria species		No detectable bacteria
	Single match*	Multiple matches detection	
Diarrhea (n =33)	30	6	3
Non diarrhea (n =47)	28	8	13
Total (n=80)	58	14	16

*Single match means detection of bacteria in sample with definitive species name, it can be a multiple (more than one bacteria detected) or together with multiple matches detection.

Table 2. Bacteria detected in samples group diarrhea and non-diarrhea

Bacteria in diarrhea samples	Number of samples	Family	Phylum	Bacteria in non diarrhea samples	Number of samples	Family	Phylum
Escherechia coli	18	Enterobacteriaceae	Proteobacteria	Escherechia coli	21	Enterobacteriaceae	Proteobacteria
Klabsiella pneumonia	2	Enterobacteriaceae	Proteobacteria	Klabsiella pneumonia	3	Enterobacteriaceae	Proteobacteria
Prevotella albensis	1	Bacteroidaceae	Bacteroidetes	Bifidobacterium longum	2	Bifidobacteriaceae	Actinobacteria
Staphylococcus epidermidis	1	Straphylococcaceae	Firmicutes	Eschenchia fergusonii	1	Enterobacteriaceae	Proteobacteria
Eubacterium rectale	1	Eubacteriaceae	Firmicutes	Akkermansia muciniphila	1	Verrucomicrobiaceae	Verrucomicrobia
Clostridium perfringens	1	Clostridiaceae	Firmicutes				
Streptococcus vestibularis	1	Streptococcaceae	Firmicutes				
Streptococcus sp	1	Streptococcaceae	Firmicutes				
Acidovorax avenae	1	Comamonadaceae	Proteobacteria				
Aquapinillum gracile	1	Comamonadaceae	Proteobacteria				
Campylobacter jejuni	1	Campylobacteraceae	Proteobacteria				
Shigella boydii	1	Enterobacteriaceae	Proteobacteria				

Table 3. The results of multiple matches

Diarrhea Sample code	Bacteria	Non diarrhea Sample code	Bacteria
D02	<i>E.coli/Shigella flexneri/S.sonnei</i>	S10	<i>E.coli/Shigella flexneri/S.sonnei</i>
D11	<i>E.coli/Shigella dysenteriae</i>	S11	<i>Vibrio proteolyticus/Vibrio sp.</i>
D13	<i>E.coli/Shigella flexneri/S.sonnei</i>	S14	<i>E.coli/Escherichia coli O157:not H7/Shigella sonnei</i>
D14	<i>E.coli/Shigella flexneri/S.sonnei</i>	S17	<i>E.coli/Shigella dysenteriae</i>
D15	<i>E.coli/Escherichia coli O157:not H7/Shigella boydii/Shigella sonnei</i>	S23	<i>E.coli/Shigella flexneri/S.sonnei</i>
D33	<i>E.coli/Shigella flexneri/S.sonnei</i>	S26	<i>E.coli/Shigella flexneri/S.sonnei</i>
		S38	<i>E.coli/Shigella flexneri/S.sonnei</i>
		S46	<i>E.coli/Escherichia coli O157:not H7/Shigella sonnei</i>

Table 4. Cross tabulation between enteropathogen and diarrhea incidence

		Enteropathogen		Total
		+	-	
diarrhea	+	27	9	36
	-	29	7	36
Total		56	16	72