Analysis Gut Microbiota in Pregnant Women Trimester Three and Their Babies as Diagnostic Test of Degenerative Diseases

Nurisyah[1], Ratnasari Dewi[2], Mudyawati Kamaruddin[3], Miftahul Khairiyah Zamzam[4], Afra Anisah Purwadi[5], Abdur Rahman[6], Ana Hidayati Mukaromah[7], Andi Fatmawati[8]

[1,2,8]Politeknik Muhammadiyah Makassar, South Sulawesi 90132, Indonesia
[3,4,5,6,7]Universitas Muhammadiyah Semarang, Semarang, Central Java 50273, Indonesia
mudyawati@unimus.ac.id

Abstract. This study aims to evaluate gut microbiota colonization in the first two years of a child's life and explore the potential of the gut microbiota for the development of in vivo diagnostics (IVD) of degenerative diseases by reconstructing the dynamics of early colonization of gut microbiota at a genomic scale for the development of degenerative disease diagnostic kits. Data is being collected from pregnant ladies in their third trimester who will be used as research samples. This information comprises a health history, food (nutrition), and the way of delivering breast milk or formula milk for moms who will give birth to their babies, as well as the manner of giving birth, which is either normal or caesarean section. Interviews and questionnaires were used to acquire the sample data. Following the third stage of data processing from respondents, samples (whole blood, urine, and feces) were collected from healthy mothers and newborn babies. Whole blood, urine, and feces samples were obtained from pregnant women in their third trimester and newborns born in various locations, and each was labeled with the sample ID number. In the laboratory, samples were screened for Toxoplasma gondii microorganisms using the Rapid Diagnostic Test (RDT) to determine the presence of antibodies against T. gondii in serum and plasma samples (DALF Toxo-CA), and DNA was extracted and isolated from feces. Thus, amplifying the sample DNA with Polymerase Chain Reactions (PCR) and proceeding with sequencing.

Keywords: Gut Microbiota, Metagenomics, Pregnant Women in Their Third Trimester.

1. Introduction

The rise in degenerative diseases in Indonesia has resulted in an increase in the number of fatalities. Indeed, alterations in the epidemiological transition of disease patterns have been documented, such that degenerative diseases have become comorbid or concomitant

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diseases, causing severity and death in patients [1]. According to data from the Ministry of Health, four large groups of degenerative diseases, namely stroke, heart disease, cancer, and diabetes mellitus [2], are responsible for 60% of morbidity and death, and this is expected to rise significantly by 2030 due to population growth and lifestyle changes [3]. Degenerative disease is a medical disorder in which the function of cells, organs, or tissues deteriorates with time. As a result, aged people (seniors) are more likely than younger people to suffer from numerous sorts of degenerative diseases. However, degenerative diseases are now being discovered and experienced by people who are younger and more productive (under 45 years of age). Given these circumstances, it is obvious that strategic efforts must be made to avoid the formation of degenerative diseases from an early age by researching the various causes of disease transitions. Dybiosis, defined as an imbalance in the makeup of the microbiota in the intestine, is thought to be one of the causes of noncommunicable diseases and conditions (NCDs) or degenerative diseases. This is related to the important role of microbiota in regulating the body's biological and physiological processes, both of which play a role in immunity, nutrition, infection risk regulation, inflammation regulation, and human development, so dysbiosis conditions of the intestinal microbiota will have an impact on the pathogenesis of intestinal and extra-intestinal disorders. The purpose of this study is to assess the dynamics of intestinal microbiota colonization in a child's first two years of life and to investigate the potential of this microbiota for the development of in vivo diagnostics (IVD) of degenerative disorders [4].

So yet, only case control studies have been conducted on gut microbiota and degenerative illnesses. Of course, this is a crucial first step in determining whether there is a link between degenerative illness and microbiota, but it has yet to produce proof of a causal association. As a result, this study focuses on the early dynamics of gut microbiota colonization as a prospective cohort study in which samples of the relationship between the baby's immune response and the type of gut microbiota composition are analyzed for genes and metabolites produced, correlated with case-control study data for metagenomic data analysis using a gut microbiota gene catalogue [5].

The human gut microbiota colonizes during pregnancy [6], and the composition of the gut microbiota during this period is influenced by interactions between a number of internal and external factors that regulate the composition and function of the microbiota [7], ranging from mode of birth to diet. Thus, exposure to bacteria during the birth process is critical for the establishment and maturation of the gut microbiota in the early years of a child's life [8].

The mode of newborn delivery has important implications for the gut microbiota, as Dominguez-Belo's research [9] found that vaginal newborns had microbiota similar to their mothers' vaginal microbiota, whereas cesarean newborns had similar microbial communities. Staphylococcus, which is comparable to human skin bacteria, predominates.

The composition of the baby's food, whether breast milk (ASI) or formula milk, has a significant impact on differences in the dominance of the intestinal microbiota. Combining breast milk and formula milk to babies resulted in differences in the intestinal microbiota compared to the intestinal microbiota in the group of kids who were only given breast milk [1]. Furthermore, breast milk oligosaccharide compounds that cannot be digested by the human intestine but are hydrolyzed by the intestinal microbiota can enhance the population of good bacteria like Bifidobacterium and Lactobacillus [10]. Formula-fed babies, on the other hand, have a higher frequency of Clostridium, Enterobacteriaceae, and Enterococcus than breastfeeding babies [1][11]. Similarly, during the weaning period, the introduction of breast milk substitutes (MP-ASI) can influence the microbial community. The gut microbiota of European and African children with different diets, which can influence the composition of the gut microbiota of European and African children. microbiota that are substantially distinct [12]. Based on the findings of the preceding investigations, data on the dynamics of initial colonization of the gut microbiota is critical for understanding the causal relationship between the gut microbiota and the host.

Metabolic syndrome is a risk factor for degenerative illnesses because it is characterized by complex metabolic abnormalities such as increased insulin resistance, blood pressure, obesity, atherogenic, protothrombic, and proinflammatory states [13][14]. Many investigations on gut microbiota and metabolic syndrome illnesses have been undertaken. Obesity research founded on changes between intestinal microbiota components and obesity, specifically between the two main phyla in the human intestine, Bacteroidetes and Firmicutes, and it was discovered that there was an increase in Firmicutes in the intestinal microbiota of obese sufferers [15].

The same investigation could not substantiate the changing ratio between the two major phyla [16]. It is important to remember that the Phylum Bacteroidetes/Firmicutes ratio is a rough estimate of the microbiota composition in the human intestine. The phylum Firmicutes contains numerous plainly harmful species, such as Clostridium botulinum and Listeria monocytogenes, as well as those that are helpful to the host. Meanwhile, intestinal microbiota products in the form of Short Chain Fatty Acid (SCFA), which had a function in obese patients [17].

2. Research Methods

The first stage of this research is obtaining research permits for two locations, namely rural areas (Bulukumba Regency) and urban areas (Makassar City), and this permit is one of the facilities for making an ethical clearance letter from the Politeknik Kesehatan Makassar of the Ministry of Public Health with permit number 0363/O/KEPK-PTKMS/III/2023. The second stage involves gathering information from respondents who will be used as research samples, specifically pregnant women in their third trimester. This information comprises

a health history, food (nutrition), and the way of delivering breast milk or formula milk to moms who will give birth to their babies, as well as the manner of giving birth, which can be either naturally or via caesarean section.

Interviews and questionnaires were used to acquire the sample data. Following the third stage of data processing from respondents, samples (blood plasma, urine, and feces) were collected from healthy mothers and newborn newborns. The fourth step involved collecting blood plasma, urine, and feces samples from third trimester pregnant women and newborns born in various locations, each of which was labeled with the sample ID number. In the fifth stage, samples are treated in the laboratory by screening for Toxoplasma gondii microbes using the Rapid Diagnostic Test (RDT) to detect the presence of antibodies against T. gondii in serum and plasma samples (DALF Toxo-CA), as well as extracting and isolating DNA from fecal samples. The sixth stage involves amplifying the sample DNA with Polymerase Chain Reactions (PCR) and proceeding with sequencing.

3. Results and Discussion

3.1. Responden Analyze

Analysis of respondent data in the form of age group, education, occupation, sanitation, pets, baby diet methods, and pregnant women's delivery methods.

According to the data collected, respondents were 20 pregnant women divided into 10 samples from rural areas (Bulukumba Regency) and 10 samples from urban areas, in this case represented by the city of Makassar, as indicated in Table 1.

Table 1. Distribution of research respondents

No.	Characteristics	Frequency (n)	Percentage (%)
1	Aged		
	15-20 years	3	15
	21-25 years	7	35
	26-30 years	5	25
	31-35 years	5	25
2	Education		
	Primary school	1	5
	High school	13	65
	University	6	30

3	Profession		
	Unemployed	15	75
	Employee	5	25
4	Pet		
	No	6	30
	Yes	14	70
5	Baby diet method		
	Breast milk	18	90
	Formula milk	2	10
6	Birth method		
	Cesar section	0	0
	Normal	20	100
7	Sanitation		
	No toilet available	1	5
	Toilet available	19	95

According to the data distribution, the 21 - 25 year age group has 35% of the respondents, followed by the 31 - 35 (25%), 26 - 30 (25%), and 15 - 20 year (15%) groups. Secondary school graduates had the most educational data, with 13 responses (65%), followed by 8 university graduates (30%) and 1 elementary school (5%) respondents. The jobs of respondents were dominated by 15 respondents (75%) who did not work compared to 5 respondents (25%). A survey about each respondent's pets and sanitation was also undertaken as part of this study. It was discovered that respondents kept more than one maintenance animal, with 14 respondents (70%) keeping animals and 6 respondents (30%) not having one. Almost all respondents' sanitary conditions have toilets 19 (95%), indicating that all respondents follow the guidelines for keeping cleanliness. Breast milk (ASI) 18 (90%) and formula milk 2 (10%) are used to provide the first diet to newborns. Each respondent's delivery method was 100% usual delivery.

3.2. Serology Test

Whole blood, urine, and feces were obtained from each respondent. Whole blood is centrifuged to obtain serum, which is then used for microbiological analysis, in this example of the parasite Toxoplasma gondii. 70% of those polled have pets in their households. The T. gondii parasite examination seeks to detect parasite antigens in the serum of each respondent who owns a pet. T. gondii screening is done using an IgG/IgM rapid diagnostic test (RDT) (DALF Toxo-CA).

Based on the RDT screening results, two blood samples were confirmed to be positive for IgG antibodies (Figure 1). This indicates that the respondent was infected with Toxoplasma gondii for an extended period of time. After synchronizing the parasite-positive samples, they discovered that they all had pets in the form of cows and cats.

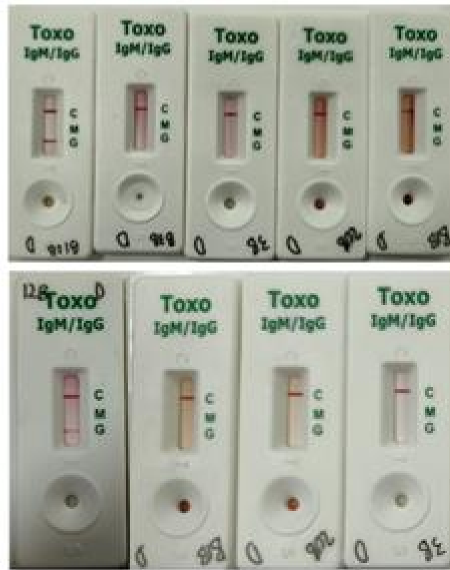

Fig. 1. Representative serological test performed on blood samples using RDT

Meanwhile, as demonstrated in Figure 2, there is no IgG or IgM antibodies were detected from urine samples. Although urine samples can be used to identify infection, their use is quite rare.

Fig. 2. Representative serological test performed on urine samples using RDT

3.3. Molecular Analysis

Extraction and isolation of fecal DNA. The main premise of DNA extraction is to break the cell walls and membranes before removing the DNA from the nucleus without damaging the DNA. A Nanodrop spectrophotometer was then used to determine the purity of the isolated genomic DNA. Fragmented DNA cannot actively travel through the gel, resulting in poor DNA band formation. Table 2 shows the purity and concentration of the results of isolating genomic DNA from isolates using nanodrop. According to Table 2, the highest concentration level was in the B12B DNA sample with a concentration of 320ng/l and the lowest concentration level was in the B3B DNA sample with a concentration of 57.5ng/l at a wavelength of 260nm.

Table 2. The purity and concentration of fecal DNA obtained using nanodrop.

Sample	A260/280	A260/230	Concentration (ng/μL)
1	2.289	1.852	46.97
2	2.081	1.961	50.79
3	2.123	1.974	88.32
4	2.174	1.822	50.85
5	2.096	1 974	41.02
6	2.015	1.07	40.68
7	2.092	1.83	98.33
8	2.015	1.854	44.22
9	2.089	1.853	45.99
10	2.189	1858	15.52
11	2.11	1.845	24.65
12	2.144	1.89	25,43
13	2.111	1.845	93.98
14	2.287	1930	32.03
15	2.173	1980	38.24
16	2.032	1.871	50.44
17	2.047	1.834	31.38
18	2.087	1828	52.91
19	2.172	1 024	92,37

20	2.099	1,037	86,92

A nanodrop spectrophotometer is used to assess the quality and yield of DNA. The aromatic rings in nitrogen bases (purines and pyrimidines) found in DNA are absorbed by ultraviolet (UV) light at λ = 260 nm, while the aromatic rings in the amino acid tryptophan, which is an amino acid in proteins, are absorbed by UV light at λ=280 nm. DNA is deemed to be pure if its λ 260/ ratio is between 1.8 and 2.0 ng/l. If the absorbance ratio is less than 1.8 ng/l, it indicates that protein has contaminated the DNA. It is contaminated with RNA if it is larger than 1.8 ng/l [18][19]. The purity of this study sample demonstrates that the results are pure and that the process can proceed to the amplification step.

Amplification of the 16S rRNA Gene by PCR. The primers used are universal primers for the bacterial domain in the form of forward primer 63F (5'-CAG GCC TAA CAC ATG CAA GTC-3') and reverse primer 1387R (5'-GGG CGG WGT GTA CAA GGC-3') [20]. All reaction components are mixed into a microtube and inserted into the PCR machine. The PCR stages consist of pre-denaturation 94°C, 2 minutes, denaturation stage 92°C, 30 seconds annealing stage 55°C 30 seconds, elongation stage 72°C for 1 minute. The PCR process consists of 30 cycles. Next, post PCR at a temperature of 75°C for 20 minutes and the stop PCR stage at a temperature of 4°C. PCR results are stored at -20°C or directly electrophoresed.

DNA analysis of feces samples is carried out using the PCR method. In general, PCR consists of three steps carried out simultaneously, namely: (1) initial denaturation, namely initial heating at a temperature of 95°C to completely denature the DNA complex. (2) Attachment of primers. (primary annealing) at a temperature of 55-72°C, (3) Polymerization of the new DNA strand by DNA polymerase, which is normally carried out at a temperature of 72°C (is the optimal temperature for Taq DNA polymerase). The number of cycles required by most PCRs is 25-40 cycles [21].

Electrophoresis. Gel electrophoresis was prepared with 0.8% agarose in 1x TAE buffer (0.24 gr agarose in 30 ml 1xTAE) (50x tris acetate EDTA (TAE): 1 l dH20, 242 gr Tris base, 37.2 gr Na2EDTA, and 57 1 ml glacial acetic acid), heated and after dissolving cooled to 50oC then poured into a gel mold. The container containing the gel is given sufficient 1x TAE buffer, then insert the digestion sample into the gel wells and a marker or DNA molecule marker in the first well. Electrophoresis was carried out at conditions of 30-40 Volts and 28 – 29 mA and ended after the bromophenol reached the bottom edge of the gel [1][2].

4. Conclusions

Based on the Rapid Diagnostic Test screening results, two blood samples were confirmed to be positive for IgG antibodies. This indicates that the respondent was infected with Toxoplasma gondii for an extended period of time. After synchronizing the parasite-positive samples, they discovered that they all had pets in the form of cows and cats.

Meanwhile, there is no IgG or IgM antibodies were detected from urine samples. Although urine samples can be used to identify infection, their use is quite rare

Authors' Contributions. All authors contributed equally to this work. MKZ, AAP, and AR did sample collection from respondents. MDK, RD, NH, and AHM designed and writing manuscript. MDK, and MKZ analysed data.

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