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Effect of Fetal Bovine Serum Concentration on Detection and Morphological Identification of *Blastocystis hominis* in vitro

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Abstract

Diarrhea significantly contributes to the high rates of illness and death among young children. Diarrhea can be caused by bacterial infections, viruses, or even parasites. *Blastocystis hominis* causes parasitic diarrhea, which can be identified by microscopy, culture, and molecular methods. Previous reports have modified the Jones' culture medium using three different serums, such as human plasma, donkey serum, and horse serum (in Jones' medium). This research replaces horse serum with fetal bovine serum for detection tests, morphological observation, and diagnosis of *B. hominis*. The research encompasses five experimental groups, each subjected to varying concentrations of fetal bovine serum: 2%, 10%, 20%, 30%, and 40%. Detection analysis is conducted using the Mc-Nemar test, while the Wilcoxon test is applied to evaluate ordinal data from morphological assessments. Diagnostic tests and metrics such as accuracy, sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) are performed using MedCalc® software. The findings demonstrate that serum concentrations of 2%, 10%, 20%, and 30% produced effective results in detection tests, morphological identification, and diagnostic evaluations of B. hominis, exhibiting high sensitivity, specificity, PPV, NPV, and accuracy. Fetal bovine serum can be used at a concentration of 2% in a Jones' medium that has been modified. This depends on the results of detection tests, morphology, and diagnosis.

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INTRODUCTION

Diarrhea is a medical condition characterized by increased intestinal activity and the frequent production of feces with a loose consistency, occurring at least three times a day.[1] Diarrhea represents a major public health challenge, especially among young children, for whom it remains one of the primary contributors to both morbidity and mortality. It is recognized as the second most common cause of death in this age group, surpassed only by pneumonia [2]. It is responsible for significant child mortality globally, with 444,000 deaths annually, equating to 1,200 deaths each day [3]. Based on the Indonesian Health Profile 2022, diarrhea caused a mortality rate of 6.6% in children aged 29 days to 11 months and a mortality rate of 5.8% in youths aged 12-59 years [4]. The incidence of diarrhea among adults aged 70 years and above was approximately half that of infants aged below 5 years [5]. Diarrhea during early infancy can significantly impair the absorption of essential nutrients, such as fats, vitamins, proteins, carbohydrates, electrolytes, minerals, and water, potentially leading to malnutrition [6 7]. This disruption in nutrient absorption can have severe consequences for the growth and development of infants, highlighting the importance of addressing the underlying causes of diarrhea to prevent malnutrition and ensure the well-being of young children. In the past, severe dehydration and significant fluid loss were the primary causes of mortality connected to diarrhea. Presently, there is a potential that infection will play a more substantial role in the overall number of deaths associated with diarrhea [8].

Diarrhea, an infectious condition caused by various agents such as bacteria, viruses, and parasites, is often associated with the intestinal protozoan Blastocystis hominis, which commonly infects humans and animals [910]. It is known that *B. hominis* comprises up to 22 subtypes, which can overlap between humans and animals [11]. According to the CDC (2019), the life cycle of *B. hominis* remains a subject of debate [12]. Infections caused by *B. hominis* have shown increased transmission rates due to poor sanitation, close contact with pets, reliance on water supplies directly sourced from wells and rivers [13]. Recent studies indicate that in Europe, the majority of human *Blastocystis* infections are attributable to subtypes ST1, ST2, ST3, and ST4, which collectively represent approximately 90% of detected cases. On a global level, ST3 emerges as the most commonly identified subtype, particularly among individuals presenting with symptoms, while ST1 and ST2 also occur frequently but at lower rates [14].

Blastocystis hominis is classified as a protist due to its cellular structure, which includes one or more nuclei, rough and smooth endoplasmic reticulum, Golgi apparatus, and organelles such as mitochondria; it also exhibits sensitivity to antiprotozoal medications [15]. There are 6 forms of *B. hominis*, namely vacuolar, avacuolar, multivacuolar, ameboid, granular, and cyst. The pathological progression of the disease, which transforms the condition of patients from asymptomatic to symptomatic, occurs due to the morphological shift of *B. hominis* from the vacuolar form to the amoeboid form [16]. Appropriate management can be provided if the etiology and clinical manifestations in patients with diarrhea are clearly identified [17]. Inadequate management may allow *B. hominis* to persist, leading to chronic diarrhea and further complications such as malnutrition.

Confirmatory tests that can be conducted to validate the diagnosis of *B. hominis* using microscopy, culture, immunoserological, and molecular [18].

Previous reports have demonstrated that the combination of culture methods and immunoserological assays is the most sensitive approach for detecting *B. hominis* [19]. Sari et al., (2018) found that the level of culture sensitivity in Jones' medium is greater when compared to polymerase chain reaction (PCR) [20]. Modification culture in Jones' medium was done by Hassan et al. (2016) by culturing the samples in different culture media supplemented with human plasma, donkey serum, and horse serum, with horse serum as the primary serum. This research modified the use of horse serum using fetal bovine serum [21].

Fetal bovine serum is the most prevalent serum used for cell culture in laboratories worldwide [22]. This serum is commonly employed in cell culture because of its high concentration of growth factors, making fetal bovine serum more prevalent in cancer cell culture, such as of colorectal cancer and breast cancer [23]. Fetal bovine serum has high levels of growth hormones and low levels of γ-globulins, which restrict cell proliferation [24]. Currently, there is a lack of studies regarding the optimal dosage that should be used for the Jones' culture medium. Improper levels of fetal bovine serum can complicate research by causing wrinkles, which hinder the identification, detection, and analysis of *B. hominis* morphology. This investigation aimed to determine the most effective concentration of fetal bovine serum for the in vitro cultivation of *B. hominis*. The study concentrated on several critical components, such as parasite detection, comprehensive morphological analysis, and the assessment of the culture method's sensitivity and specificity. By systematically varying the concentrations of fetal bovine serum, the study aimed to establish the most effective conditions for maintaining the viability and integrity of *B. hominis* in a controlled laboratory environment.

MATERIALS AND METHODS

The specimens were collected from the regional public hospital and the community health clinic in Buleleng, between May and November 2023. Research was conducted on 35 samples of diarrhea patients. Participants in this study gave informed consent, consenting to the collection of their fecal samples immediately following defecation, which were then deposited into sterile containers. These specimens were then transported in an ice box directly to the Parasitology Laboratory at the Faculty of Medicine, Universitas Pendidikan Ganesha, for further processing, including direct smear and in vitro culture.

The research begins with preparing the stock solution based on Hassan et al. (2016) by mixing 1.244 grams of disodium phosphate (Na_2HPO_4) into 131.25 mL of distilled water, and 0.397 grams of monopotassium phosphate (KH_2PO_4) into 43.75 mL of distilled water. Fetal bovine serum (HiMedia Laboratories Pvt. Ltd, Brazil) at various concentrations was added to the prepared stock solution. Table 1 shows the different treatment variations of bovine fetal serum concentrations based on adjustments made to the Jones' medium. The cultures were observed for 24, 48, and 72 hours. The samples were examined under a microscope using high-power duplicates (400x).

Preparations of *B. hominis* culture will be observed under a microscope and verified immediately by the parasitological analysts. The morphological identification was evaluated in 100 fields of view by morphological observations that were categorized as: (1) absence of parasite; (2)

presence of parasites with morphology characterized by wrinkled walls; and (3) presence of parasites with perfectly rounded wall morphologies. Diagnostic testing is conducted on culture findings that display the ideal morphology of *B. hominis*. The culture findings will be assessed by five observers utilizing five duplicate samples, resulting in a total of 125 test samples for the optimal culture test group. A direct microscopic examination with identical replication would be compared with the diagnostic test.

Table 1. Modification of the concentration of fetal bovine serum in *B. hominis* in vitro culture

Test Groups	Concentration of Fetal bovine serum
P1	2% (0.1 mL)
P2	10% (0.5 mL)
P3	20% (1.0 mL)
P4	30% (1.5 mL)
P5	40% (2.0 mL)
K (-)	Aquades
K (+)	Fecal + examined microscopically

Data Analysis

The Mc-Nemar test method is used to analyze the detection test in this study. The Wilcoxon test will be used to examine the ordinal data obtained from the morphological test. MedCalc® software is utilized to conduct diagnostic tests and assess accuracy, sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV). A variable is deemed statistically significant when the $P < 0.05$.

RESULTS AND DISCUSSION

Of the 35 samples that were confirmed positive for diarrhea, five of them specifically gave positive results on microscopic examination for *B. hominis*. These samples were sent for testing by five competent laboratories. The testing process extended over a period of 24, 48, and 72 hours. The samples underwent a series of tests, including a detection test, a morphological test, and a diagnostic test.

Table 2. Detection of *B. hominis* in vitro using different concentrations of fetal bovine serum

Test Groups	Negative N (%)	Positive N (%)	P Value*
Positive control**	0 (0)	25 (100)	-
Negative control	25 (100)	0 (0)	<0.01
P1 2%	1 (4)	24 (96)	1.00
P2 10%	2 (8)	23 (92)	0.50
P3 20%	1 (4)	24 (96)	1.00
P4 30%	2 (8)	23 (92)	0.50
P5 40%	15 (60)	10 (40)	<0.01

*Difference in proportion of detection test results using Mc-Nemar test

According to the data presented in Table 2, the morphological outcomes for the P1, P2, P3, and P4 test groups with the golden standard did not differ ($P > 0.05$). Statistically significant differences were seen in the P5 test groups ($P < 0.01$). The P1, P2, P3, and P4 test groups exhibited no visible differences in morphology compared to the positive control group. The only group that exhibited significant differences.

Table 3 indicates that the morphological characteristics of B. hominis cell walls in the P1, P2, P3, and P4 test groups did not significantly differ from those observed in the gold standard group ($P > 0.05$). These test groups showed no discernible morphological differences when compared to the positive control group. However, statistically significant differences were noted in the P5 test group ($P < 0.01$).

Table 3. Morphological observations of *B. hominis* in in vitro cultures were conducted using varying concentrations of fetal bovine serum.

Test groups	Negative N (%)	Wrin-kled N (%)	Ideal N (%)	P Value*
Positive control**	0 (0)	0 (0)	25 (100)	-
Negative control	25 (100)	0 (0)	0 (0)	<0.01
P1 2%	1 (4)	0 (0)	24 (96)	0.317
P2 10%	2 (8)	0 (0)	23 (92)	0.157
P3 20%	1 (4)	0 (0)	24 (96)	0.317
P4 30%	2 (8)	0 (0)	23 (92)	0.157
P5 40%	15 (60)	10 (40)	0 (0)	<0.01

*Difference in proportion of detection test results using the Wilcoxon test
**This positive control is used as the gold standard

Table 4 demonstrates that the P1, P2, P3, and P4 test groups achieved the highest values in sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and overall accuracy in the in vitro diagnostic test of *B. hominis*. In contrast, the P5 group showed the lowest sensitivity and accuracy compared to the other test groups.

Table 4. Sensitivity and specificity of the *B. hominis* in vitro diagnostic test using different concentrations

Test Groups	Sn (%)[a]	Sp (%)[b]	PPV[c]	NPV[d]	Acc[e] (%)
Positive control	**	**	**	**	**
Negative control	-	-	-	-	-
P1 2%	100	100	100	100	100
P2 10%	100	100	100	100	100
P3 20%	100	100	100	100	100
P4 30%	100	100	100	100	100
P5 40%	40	~	100	0	40

~ = untestable value, [a]Sn=Sensitivity, [b]Sp=Specificity, [c]PPV=positive predictive value, [d]NPV=negative predictive value, [e]Acc=Accuracy. **Used as a gold standard

In this study, differences in pH levels were observed among the test groups. The P5 group exhibited a pH of 6.15, which was more acidic compared to the P1 and P2 groups, with pH values of 7.23, and the P3 and P4 groups, with pH values of 7.15. A prior research conducted by Farah Haziqah et al [25] highlighted the critical role of acidity or pH levels in influencing the viability and morphology of *B. hominis* cell walls. Physiologically, *B. hominis* requires a neutral pH environment for optimal growth, typically ranging from 7.0 to 7.5 [26] Deviations from this optimal pH range, whether too acidic or too alkaline, can adversely affect the growth and morphology of this microorganism. Extreme pH levels can disrupt cell membrane integrity, leading to leakage of intracellular components and organelle dysfunction [27].

At extreme pH levels, essential metabolic enzymes critical for the survival of *Blastocystis* may undergo denaturation or reduced activity, leading to impaired growth and replication [28]. In acidic environments, the activity of digestive enzymes tends to increase, creating stress conditions for organisms such as *B. hominis* [29]. In this study, low pH levels resulted in significant changes in cell wall morphology, which appeared wrinkled and irregular. These wrinkled cell walls indicate structural damage caused by prolonged exposure to unstable environmental conditions. Such changes reflect the parasite's inability to maintain cell membrane integrity under suboptimal conditions. These findings are particularly important as they suggest that increased acidity significantly impacts the viability and morphological structure of *Blastocystis hominis*. The significant variations in pH levels observed could account for the discrepancies in detection outcomes within the P5 group compared to other groups. This underscores the pivotal

role of environmental factors, particularly pH, in preserving the viability and morphological features of *B. hominis*.

STRENGTH AND LIMITATION

The study presents significant strengths, serving as an innovative and valuable contribution to parasitology by offering a practical, efficient, and cost-effective diagnostic alternative. Its streamlined methodology facilitates implementation, particularly in resource-constrained regions. However, the research has limitations as it concentrates just on general morphological characteristics such as complete grown structures and wrinkled cell walls, excluding more intricate morphological aspects.

CONCLUSIONS

Fetal bovine serum exhibits good detection and identification capabilities for *B. hominis* morphology at concentrations of 2%, 10%, 20%, and 30%. Fetal bovine serum can be used as a modality to diagnose *B. hominis* . Based on the clinical findings from detection, morphological, and diagnostic tests, it is recommended to utilize fetal bovine serum in modified medium at a concentration of 2% (0.1 mL). 2% concentration offers significant advantages in terms of material use efficiency and practicality in applications. Despite that, for an extensive review of morphology, a serum concentration of 20% (1.0 mL) is the ideal medium to stimulate growth. This research aims to serve as a reference for future studies and as a clinical guide for diagnosing *B. hominis* in patients with diarrhea.

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ETHICAL CLEARANCE

The Ethics Committee of Universitas Pendidikan Ganesha approved the research protocol, as indicated by reference number 099/UN48.24.11/LT/2024.

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CONFLICT OF INTEREST

No conflicts of interest are declared by the authors. Furthermore, the funding agencies had no involvement in any stage of the research process. This includes the planning and design of the study, the implementation and execution of the research methods, and the preparation of the manuscript.

AUTHOR CONTRIBUTION

The research design was conceptualised by PSAJ and MBP. PSAJ, KES, KIAS, and KIM conducted the clinical study and data collection. PSAJ, KIAS, and KIM wrote the article. The article was reviewed and revised by MBP and MKWG.

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