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MODIFIED CULTURE METHODOLOGY FOR SPECIFIC DETECTION OF *BLASTOCYSTIS HOMINIS* IN STOOL SAMPLES

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Abstract

Blastocystis hominis provides major challenges for laboratory diagnosis due to its polymorphic nature in wet mounts which can result in confusion with other protozoa, yeast or even fat globules. Studies revealed that simple smears were less sensitive than in vitro cultivation using different media for the detection of *B. hominis* in stool specimens. Cultures of *B. hominis* are usually enriched by different types of sera to enhance growth and multiplication of the parasite. The aim of this study is to assess the use of two sera types other than horse serum that is commonly used in culture media for the growth, multiplication and detection of *B. hominis* in examined stool samples and comparing the results with those obtained using horse serum.

Fifty stool samples were collected from patients suffering from different colonic manifestations attending Cairo University Hospitals. The samples were freshly cultured in three different culture media using horse serum (in Jones' medium), donkey serum (as a modification in Jones' medium) and human plasma (in modified Pavlova's medium) in adequate preparations. Cultures were then left for incubation and examined by direct microscopy to detect *Blastocystis hominis*.

The results showed of 50 stool samples studied, 18 samples (36%) were positive results for *B. hominis.* The number of positive results obtained by horse serum, donkey serum and human plasma were 13,18and 11 respectively. Paired comparisons were made between each 2 cultures with each culture set as a reference once to detect the most appropriate one for diagnosis. When horse was set as the reference method, donkey serum showed a sensitivity of 100% and specificity of 86.5% with a 90% agreement between the 2 methods. While human plasma showed a sensitivity of 46.2% and specificity of 86.5% with an agreement of 76%. In addition, the vacuolar form was the commonest pattern observed in this study throughout all the three cultures.

Key Words: *Blastocystis hominis,* culture, horse serum, donkey serum, human plasma.

Introduction

Blastocystis hominis is a rising enteric protozoan with zoonotic potential. It is one of the commonest parasites affecting human gut, with an incidence rate varying between 10% of the population in the developed countries and 50% in developing countries (Wong *et al*, 2008). It is considered as the most common eukaryotic organism reported in human fecal samples (Tan *et al,* 1997). The capability of *B. hominis* to cause infection is currently a subject of extreme argue. Most of the confusion arises from several clinical and epidemiological studies that either link or free the parasite as a cause for intestinal disease (Tan *et al*, 2002). However, latest researches proposed that *B. hominis* is pathogenic or related to a variety of intestinal disorders (Barahona *et al,* 2003; Hailemariam *et al*, 2004; Cassano *et al*, 2005; Andiran *et al*, 2006; Puthia *et al*, 2008; Duda *et al,* 2014).

Direct microscopic examination of faecal samples, with or without addition of Lugol's iodine solution, as well as permanent smears stained with trichrome had been recommended for diagnosis of *Blastocystis spp*. infection (Amato Neto *et al.,*2003). *Blastocystis* is a polymorphic protozoan with four main forms being described in literature. The several forms observed within in-vitro culture comprise vacuolar, granular, amoeboid, cyst, avacuolar, multi-vacuolar forms. However, vacuolar, granular, amoeboid and

cyst are the most commonly observed forms (Barua *et al,* 2015). Different epidemiological studies for diagnosis of *Blastocystis* spp. suggested that the *Blastocystis* spp. multiply rapidly in culture medium enhanced with serum after 24-48h of cultivation and considered it the most sensitive method for diagnosis of *Blastocystis* infection (Nascimento and Moitinho, 2005).

This study was conducted to assess the use of donkey serum and human plasma in addition to horse serum that is commonly used in culture medium, to detect growth, multiplication and diagnosis of *B. hominis* in stool samples, and compare results with horse serum.

Material and Methods

Study design: This is a cross sectional study aiming to compare between three culture media to detect *B. hominis* in stool samples.

Sample collection and processing: Fifty stool samples were collected from the patients suffering from different colonic manifestations, attending outpatient clinics of Internal Medicine and Tropical Medicine Departments at Kasr-Alainy Teaching Hospital, Cairo University.

All fifty samples were collected in clean, dry, labeled containers and were freshly cultured on three different media, left for incubation for up to 72 hours. Cultures were examined by direct microscopic examination in the Diagnostic and Research Unit of the Medical Parasitology Department, Faculty of Medicine, Cairo University. Stool Samples were examined under both a high power and oil immersion lens (40X & 100X) of microscope to detect the possible *B. hominis* infection.

Culture preparation: Three different liquid media were used as follows: 1- The first medium (referred to as Horse) is the well-known Jones' medium with horse serum (Jones, 1946). For preparation; 1.244g of disodium phosphate (Na_2HPO_4) was dissolved in 131.25ml distilled water. In another container 0.397g of monopotassium phosphate (KH_2PO_4) was dissolved in 43.75ml distilled water, and in a third one 7.07g of NaCl was dissolved in 787.50ml distilled water. The previously mentioned preparations were mixed together giving a total volume of 962.5ml. 12.5ml from the solution was discarded, so 950ml was left. In another container, 1g of yeast extract was dissolved in100ml of the left solution. This 1% yeast preparation was added back to the remaining of the solution (850ml). The final volume reaches 950ml with approximately 0.01% yeast. The solution was then aliquoted into 100ml bottles and autoclaved at 121ºC overnight. Ten ml of inactivated horse serum was added to 90ml of the above sterile medium under the UVR-Laminar flow sterilized hood.5ml of the medium was aliquoted into sterile 7ml culture tube using sterile pipette and was kept at -20ºC until used. 2- The second medium (referred to as donkey) is a new modification to Jone's medium; in which donkey serum was used instead of horse serum to evaluate the ability of *B. hominis* to utilize the components in the donkey serum and grow in this modified medium. The donkey serum was immersed in water bath at 56ºC to inactivate unwanted enzymes (HyClone, 1996). Culture media was prepared using the same steps used in Jones' medium but using donkey serum instead of horse serum. 3- Third medium (referred to as human) was prepared using human plasma in a slightly modified Pavlova's medium (Zerpa *et al,* 2000; [sodium acid phosphate 12 H_2O, 8.95g; potassium phosphate, 1.15g; chloride sodium, 20g; yeast extract, 4g; and distilled water, 2,750mL. Besides, 5% human plasma was added. Then 2.75g of sterile rice starch was added. The medium was distributed in sterile glass tubes, 7mL each. Stool was added to all three culture media and was incubated at 37ºC. Samples were examined 24, 48 & 72h post culture.

Statistical analysis: Data were analyzed using Statistical Package for Social Sciences (SPSS) version 17 (Chicago, IL, USA). Pos-

itive rates were expressed as percentages. Paired observations with sensitivities and specificities for each method were calculated.

Ethical considerations: All procedures performed throughout the study involving human participants were in accordance with the ethical standards of ethical committee of Faculty of Medicine, Cairo University and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Participation in the present study was optional and participants or their guardians signed an informed consent for sample collection and subsequent analysis according to national guidelines.

Conflict of Interest: The present authors declare that they have neither competing interests nor reserved financial support

Results

In the present study *B. hominis* was detected in 18 out of the 50 stool samples (36%). Donkey serum could detect 18 positive samples while horse serum and human plasma detected 13 & 11 samples respectively. The sensitivity and specificity of each culture medium were different. However, all three assays were in agreement for 70 % of samples (35/50: 6 positive & 29 negative). Number of samples were either positive (0 by horse, 3 by donkey, and 3 by human) or negative (2 by horse, 0 by donkey, and 7 by human) by only one of the three assays, distribution of positive and negative results by the three methods for detection of *B. hominis* in Venn diagram (Fig. 1). Among the positive samples, 13 samples were positive by horse serum; which were all also positive by donkey serum and only 6 were positive by human serum. Within the negative samples, 37 samples were negative by the horse serum of which 32 were also negative by donkey serum and 32 by human serum.

The comparative sensitivity and specificity of each of the three methods for detection of *B. hominis* are presented (Tab. 1). Within each comparison, each method was considered as the reference method (e.g., Horse is compared to donkey once with horse as reference method and once with donkey as reference method). In each of the three comparisons (A: horse vs. donkey; B: horse vs. human and C: donkey vs. human) both methods served as reference standard.

Table 1: Comparison of sensitivity and specificity of *B. hominis* detection by three different methods

A1		Horse			A2	Reference	
		Negative	Positive	Total		Donkey	Horse
Donkey	Negative	32	0	32	Sensitivity	72.2	100
	Positive	5	13	18	Specificity	100	86.5
	Total	37	13	50			
B1		Horse			B2	Reference	
		Negative	Positive	Total		Human	Horse
Human	Negative	32	7	39	sensitivity	54.5	46.2
	Positive	5	6	11	specificity	82.1	86.5
	Total	37	13	50			
C1		Donkey			C2	Reference	
		Negative	Positive	Total		Human	Donkey
Human	Negative	29	10	39	sensitivity	72.7	44.4
	Positive	3	8	11	specificity	74.4	90.6
	Total	32	18	50			

Paired observations (horse vs. donkey, horse vs. human and donkey vs. human) were available for all 50 stool samples. When Horse is compared to Donkey with Horse as the reference method, sensitivity is 100 %. Specificity also is high (86.5%); although they agree with each other well (90%). When Donkey is used as the reference method in this comparison Horse has a specificity of 100% & sensitivity of 72.2%.
When horse was compared to human; again, with horse as the reference method, human

sensitivity was low (46.2%) and specificity was high (86.5%), while, when human was considered the reference standard, horse sensitivity was (54.5%) whereas specificity was high (82.1%). The overall agreement between the two methods was 76%.

When donkey was compared to human; donkey was set as the reference method, human sensitivity was low (44.4%) and specificity was high (90.6%). However, when human was considered as the reference standard, the donkey's sensitivity was (72.7%) whereas the specificity was high (74.4%). The overall agreement between the two methods was 74%. Microscopic examination revealed that the vacuolar form of *B. hominis* was the predominant form detected in the positive samples in the three types of cultures (Fig. 2 A & B).

Discussion

Blastocystis spp. have a widespread geographic distribution all over the world (Tan, 2008). Due to insufficient evidence to designate new species of *Blastocystis* from humans without further biochemical and epidemiological data, *B. hominis* is the only species of *Blastocystis* infecting human hosts (Barua *et al,* 2015). *B. hominis* is a cosmopolitan protozoon which parasitizes the human large intestine. A positive diagnosis of *B. hominis* infection depends on the presence of vacuolar, granular, or amoebic forms in diarrheic stool, or the detection of the cyst form predominantly in formed feces under light microscopy using wet mount smears, iodine staining, or permanent dye as trichrome staining (Stensvold *et al,* 2007) or iron hematoxylin staining (Noel *et al,* 2003). However, the misidentification of *Blastocystis* with other organisms or even yeasts is a familiar problem and may bewilder studies limited to the use of microscopy alone (Stark *et al,* 2006). In addition, the organism might also be present in low numbers in the tested stool samples (Suresh and Smith, 2004).

In order to gain more sensitive diagnostic tool, *in vitro* cultivation of fresh specimens for this protozoan prior to light microscopy has been described and widely used (Zierdt, 1991, Clark and Diamond, 2002; Suresh and Smith, 2004; Tan, 2004; 2008). Most of these culture media require addition of serum for animals or humans and contain different types of salts and other materials. Moreover, culture techniques are more cost-effective and need less expertise to perform than those of molecular biological and immunological techniques for the laboratory diagnosis (Termmathurapoj *et al,* 2004; Su *et al,* 2007).

There are many types of cultures used for this parasite detection such as Jones' solution medium (Jones, 1946), Loffler medium, ringer solution medium (Stenzel and Boreham, 1996), modified whole-egg Slant medium, Locke solution (Zierdt, 1991), Xenic culture (Dogruman-Al *et al,* 2010) and methods made by Zerpa *et al.* (2000) who made a new culture modification in Pavlova's medium using human plasma. Saksiris ampant *et al.* (2010) deduced that Locke egg medium (LE medium) has the advantage of lower cost than that of the Jones' medium. Since the hen egg consisting in LE medium is cheaper than yeast extract, which must be imported. Moreover, they stated that hen egg could eliminate concerns about expiration dates of yeast extracts. Barua *et al.* (2015) reported that Xenic culture could be good medium but it contained many substances. Many studies used Jones medium successfully as the choice medium to culture *Blastocystis*. (Leelayoova *et al,* 2002; Suresh and Smith, 2004; Parker *et al,* 2007).

In the present work, in vitro culture using three different culture media was employed to detect which medium could be more successfully used to diagnose *B hominis in* stool samples according to the obtained results. The media used included Jones' medium using Horse serum, Jones' medium using Donkey serum as a modification of the procedure and finally human plasma in a slightly modified Pavlova's medium (Zerba *et al,*

2000). All three culture media were incubated and examined by direct microscopy.

Fifty stool samples were collected from patients suffering from GIT manifestations attending to Cairo University Hospitals and were freshly cultured on the three media types. The results revealed that 36% of the samples were positive for *B. hominis.* A similar study was done to investigate the presence of *B hominis* in persons with GIT symptoms in Sydney and showed a percentage of 19% (Roberts *et al,* 2011). Also, studies from Germany, Thailand, and China reported incidences of 17.9%, 13.3%, and 32.6%, respectively (Yoshikawa *et al,* 2004; Li *et al,* 2007). These lower values than the present data might be attributed to the fact that all these countries are considered as developing countries with adequate personal hygiene, proper environmental sanitation and satisfactory health awareness. However, the attendees of Cairo University Hospitals usually come from peri-urban or rural areas of high population densities and low socio-economic class populations aiding in protozoa transmission. On the other hand other studies have reported *Blastocystis* in much higher frequencies in which results were obtained on the basis of microscopy only 53.8% in Zambia (Graczyk *et al,* 2005), 52.3% in Malaysia (Noor Azian *et al,* 2007) and 30% in travelers in Nepal (Sohail and Fischer, 2005). These high frequencies may be accredited to the misidentification of *Blastocystis* with other organisms such as cysts or trophozoites of other protozoa, yeast or even fat cells, or may be due to low number of organisms in the sample (Stark *et al,* 2006; Suresh and Smith, 2004).

The current study revealed that the vacuolar form was the most common form found throughout all three types of culture media used. This agreed with Abdel Hafez *et al.* (2015) and Zhang *et al.* (2007) who stated that the vacuolar form was the typical *Blastocystis* cell form, and it was the most regularly used microbiological evidence for diagnosis of *Blastocystis* spp. infection.

Abu El Naga and Negm (2001) reported that the cyst forms of *Blastocystis* spp. were found chiefly in fecal maters stored for several days before being fixed. Granular forms mostly appear in older cultures of isolates or might be due to increased serum in culture media (Soriano *et al,* 2001; Katsarou-Katsari *et al,* 2008).

In this study, although each medium showed different sensitivity and specificity yet, they were all in agreement in 70% of the samples. Paired observations were offered for all samples so that within each comparison each method was considered as the reference method once. This was actually necessary since Horse serum in Jones' medium is still considered the 'gold standard', however its sensitivity and specificity for *B. hominis* detection is still imperfect i.e. not 100% each (Poirier *et al,* 2011; Elghareeb *et al,* 2015)

When horse serum was compared to donkey serum with horse serum as the reference method, sensitivity was perfect (100%), indicating that donkey serum detects all positive cases detected by horse serum. Specificity was also high (86.5%). Both sera types showed agreement in 90% of the samples. When the donkey serum was the one used as the reference horse serum gave a specificity (100%) and sensitivity (72.2%).

On comparing horse serum with human serum with each one as the reference once, the results indicated that interchanging the reference methods here had little effect on the sensitivities (46.2% & 54.5% for human and horse serum respectively) and specificities (86.5% & 82.1% for human and horse serum respectively) obtained thus deducing that both methods were similarly sensitive and specific for *B. hominis* detection with an overall agreement of 76%. On comparing donkey serum with human serum, there was a low sensitivity(44.4%) but a high specificity (90.6%) of human serum indicating that its use couldn't detect all positive samples that were detected by donkey serum.

To the best of the present knowledge this is the first study in literature to use donkey serum in a culture medium for the growth and multiplication of *B homins.* This proximity in sensitivity, specificity in addition to overall agreement obtained by both horse and donkey sera could attract our attention to the use of donkey serum as an alternative to horse serum in the culture media used for diagnosis of this protozoan. Also, the obvious morphological pattern was obtained by the donkey serum which was not at all less clear than those obtained by horse serum medium. This was not much astonishing as both animals belong to the equines which make them liable to share similar biological and biochemical features. Actually speaking, the use of donkey serum in research laboratories could be of great value especially in developing countries and in donkey rearing countries where this method could be cost effective and easy to use. Unlike horses, donkeys can thrive on very marginal forage, which makes them very economical to keep. Human plasma in culture medium by Zerba *et al.* (2000) showed *B. hominis* in 70% of stool samples vs. 21% with wet mount examination. This method was cost-effective as neither required horse serum nor an aerobiotic system and recommended an alternative to known standard methods. The present study showed lower sensitivities and specificities when compared to other 2 procedures when set as reference methods.

Conclusion

The results propose the use of donkey serum as good alternative to horse serum in culture media for *Blastocystis hominis*. Donkey serum could be preferably used in developing countries where donkeys are commonly bred, of less economic burden and much more available than horses. Modified Pavlova's medium using human plasma showed lower sensitivities and specificities when compared to the other 2 media.

Recommendations

Other types of animal sera could also be used in other studies for detection of this protozoan. Additional studies may be needed to detect which type of culture becomes positive more quickly for feasible identification after 24 hours. Also, since protozoan infection can be an indicator of poor personal and community hygiene, improvement of sanitation is crucial to prevent, not only *B. hominis* but also for other intestinal protozoa.

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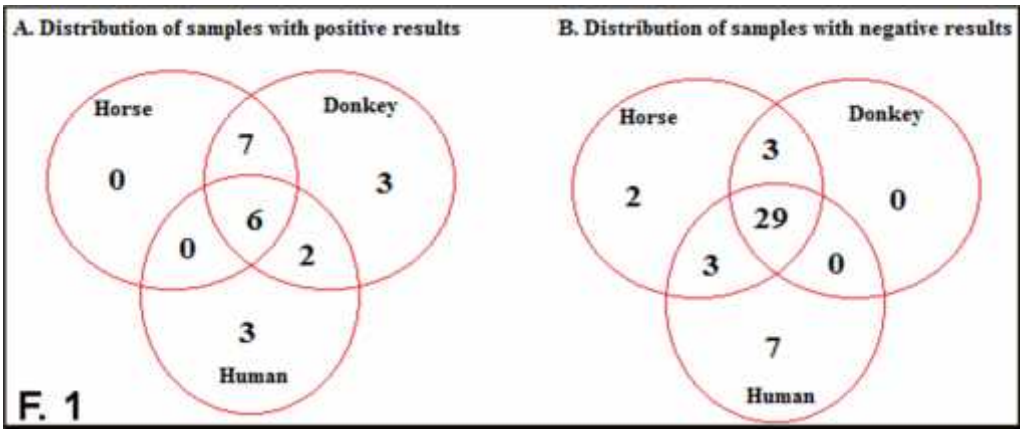

Fig 1: Venn diagram comparing results from horse, donkey, and human media for detection of *B. hominis*.

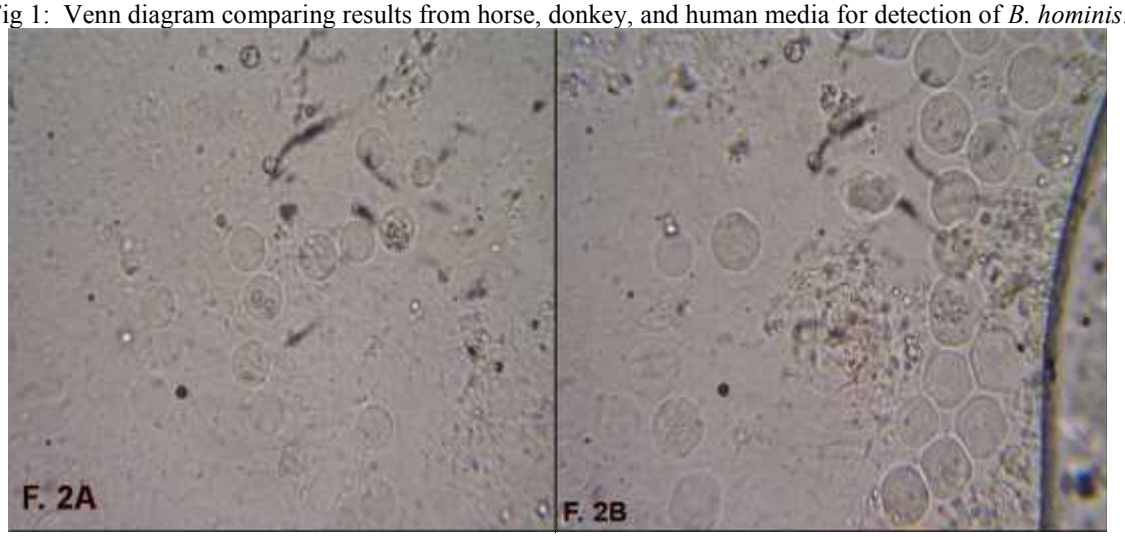

Fig 2: Light microscopy of *B. hominis* showing predominance of vacuolar forms in (A) horse serum enriched culture and (B) donkey serum enriched culture (40X).