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ORIGINAL PAPER

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The usefulness of short-term in vitro cultivation for the detection and molecular study of *Blastocystis hominis* in stool specimens

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Abstract When in vitro cultivation was used as the 'gold standard' for the detection of *Blastocystis hominis* in stool specimens, simple smear and trichrome staining showed sensitivities of 16.7% and 40.2% and specificities of 94% and 80.4%, respectively. In vitro cultivation also enhanced PCR amplification for the detection of *B. hominis* in stool specimens. Our data show the usefulness of in vitro cultivation for the detection and molecular study of *B. hominis* in stool specimens.

Introduction

Blastocystis hominis is an intestinal protozoan commonly found in humans with a prevalence between 30% and 50% in the developing countries (Hussain Qadri et al. 1989; Nimri 1993; Ashford and Atkinson 1994; Leelayoova et al. 2002; Taamasri et al. 2002). Routine diagnosis is usually performed by a simple smear in normal saline or iodine solution. Several forms of *B. hominis* can be found in stool specimens, i.e. vacuolar, multivacuolar, avacuolar, granular, ameboid and cyst forms (Stenzel and Boreham 1996). However, most laboratories recognize only the vacuolar form as the diagnostic stage since it can be easily distinguished from other protozoa. As the result, the prevalence determined by wet mount preparation may be underreported. In vitro cultivation methods have been used to enhance detection; however, the usefulness of these methods is still controversial. Our recent report showed that simple smears were less sensitive than short-term in vitro cultivation in Jones medium for the detection of *B. hominis* in stool specimens (Leelayoova et al. 2002). Permanent staining by trichrome is a standard method for the diagnosis of *B. hominis* infection in most laboratories. However, the procedure used for trichrome staining might not be suitable for making field assessments. The aim of this study was to compare trichrome staining and in vitro cultivation for the detection of *B. hominis* in stool specimens. Our knowledge of the epidemiology and pathogenicity of *B. hominis* infection is still unclear. Genotypic characterization using PCR has been a useful tool for the study of such infection; however, PCR amplification using stool specimens is rather insensitive. One of the methods prior to PCR detection was cultivation. Thus, we also evaluated the usefulness of in vitro cultivation for enhancing DNA detection of *B. hominis* in stool specimens.

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Materials and methods

We evaluated the effectiveness of the three diagnostic methods including simple smear, permanent trichrome staining and short-term in vitro cultivation in terms of their sensitivities and specificities. The research protocol was approved by the Ethics Committee of the Medical Department, the Royal Thai Army. A total of 337 stool specimens from conscripts who lived on an army base in Prachinburi province, Thailand, during February 2003, were used for a comparative study of the three detection

methods. Simple smears were made using normal saline and iodine stain, then examined under the 40× objective of a light microscope. Permanently stained smears were prepared using the trichrome modification procedure (Garcia and Bruckner1997) and examined under a 100× objective for 20 oil immersion fields per slide. Short-term in vitro cultivation was performed using Jones medium supplemented with 10% horse serum and incubated at 37°C for 48 h (Jones 1946; Leelayoova et al. 2002). Examination was done by light microscopy under a 40× objective. Using in vitro cultivation as 'the gold standard', the sensitivities and specificities of simple smear and trichrome staining were determined using version 6.01 of the Epi Info software package (Centers for Disease Control and Prevention, Atlanta, Ga.). Pairwise comparisons of the sensitivities and specificities of simple smear and trichrome staining were calculated by χ^2-tests, and receiver operating characteristic (ROC) curves were generated using MedCalc (MedCalc software, Mariakerke, Belgium).

A total of 23 *B. hominis*-positive stool specimens were processed for the DNA extraction by QIAamp DNA Stool Mini Kit (QIAGEN, Germany). These 23 specimens were also cultured in Jones medium for 48 h before DNA was extracted. Successful DNA extraction was demonstrated by nested-PCR amplification for ssu rDNA. Genomic DNA and a primary primer pair (RD5, 5′-GGAAGCTTATC TGGTTGATCCTGCCAGTA-3′; RD3, 5′-GGGATCCTGATCCTTCCGCAGGTT CACCTAC-3′) were used in PCR amplification under the conditions described by Clark (1997). PCR product (1 μl) from the primary amplification and a secondary primer pair (forward, 5′-GGAGGTAGTGACAATAA ATC-3′; reverse, 5′-CGTTCATGATGAACAATT-3′) were used as described by Böhm-Gloning et al. (1997). PCR amplification was performed using a Perkin Elmer 480 Thermal Cycler. A 10-μl PCR product from each reaction mixture was run on a 2% agarose gel (FMC Bioproducts, USA) with 1.5% Tris/borate/EDTA buffer. Gels were stained with ethidium bromide, visualized under UV light and documented on high density printing paper using a UV-save gel documentation system I (Uvitech, UK).

Results and discussion

As shown in Table 1, the prevalence of *B. hominis* using in vitro cultivation was 30.3% (95%CI, 23.4–35.2), which was six times higher and twice as high as those detected by simple smears and trichrome staining, respectively. When cultivation was used as the gold standard, simple smears and trichrome staining had sensitivities of 16.7% and 40.2%, respectively. The specificities of simple smears and trichrome staining were 94% and 80.4%, respectively ($P = 0.001$). The positive predictive values of simple smears (54.8%) and of trichrome staining (47.1%) were lower than the corresponding negative predictive values (69.7% and 80.4%, respectively). Compared to the cultivation method, the sensitivity of trichrome staining showed no significant difference ($P = 0.176$), while simple smears were significantly different ($P < 0.0001$). In addition, the area under the ROC curve of simple smears and trichrome staining was 0.55 (95%CI, 0.48–0.62) and 0.60 (95%CI, 0.53–0.67), respectively (Fig. 1). The 95% confidence interval of the area under the ROC curve for trichrome staining was significantly higher than 0.5. Thus this method can possibly predict the positive outcome even though the difference of the area under the curves between trichrome staining and simple smears was not significant ($P = 0.274$). Although there was no significant difference between in vitro cultivation and trichrome staining, we recommend in vitro cultivation using Jones medium to

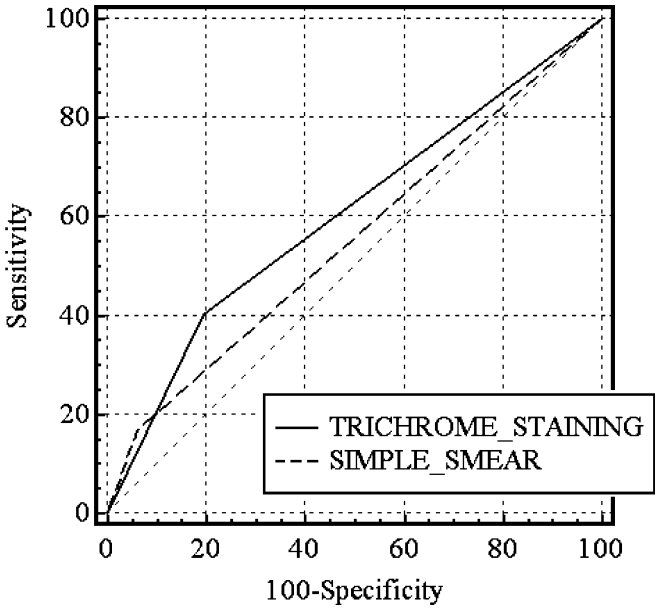

Fig. 1 ROC curve for simple smears and trichrome staining compared to in vitro cultivation

Table 1 The detection of *Blastocystis hominis* in stool specimens by simple smears and trichrome staining compared to in vitro cultivation

Detection methods		Culture		
		Positive (%)	Negative (%)	Total (%)
Simple smears	Positive	17 (16.7)	14 (6)	31 (9.2)
	Negative	85 (83.3)	221 (94)	306 (90.8)
Total		102 (30.3)	235 (69.7)	337 (100)
Trichrome staining	Positive	41 (40.2)	46 (19.6)	87 (25.8)
	Negative	61 (59.8)	189 (80.4)	250 (74.2)
Total		102 (30.3)	235 (69.7)	337 (100)

study the prevalence of *B. hominis* infection because of its high sensitivity, convenience and simplicity for a large number of samples, especially for field studies. In addition, for trichrome staining to be performed and examined correctly, it needs experienced technicians. However, if the actual number and forms of *B. hominis* in fresh specimens need to be determined, trichrome staining should be done before cultivation.

B. hominis is genetically highly variable, as shown by different molecular techniques, mostly PCR (Böhm-Gloning et al. 1997; Clark 1997; Noël et al. 2003; Thathaisong et al. 2003). The molecular study of *B. hominis* in stool specimens using these techniques will provide better epidemiological data and might explain its pathogenicity. However, PCR detection of *B. hominis* in stool specimens is rather insensitive. Thus the development of an efficient method for the isolation and extraction of DNA is required prior to PCR. Here we show the usefulness of in vitro cultivation for the PCR study of *B. hominis*. Of 23 specimens, 22 (98%) cultured and 12 (52%) direct stool specimens gave the positive band at 1,100 bp for ssu rDNA. These data show the advantages of short-term in vitro cultivation, which eases use and facilitates PCR detection of *B. hominis* without any partial purification steps. DNA extracted directly from the cultivated specimens can provide a specific and reproducible PCR method for further molecular characterization.

In summary, in vitro cultivation is useful for the detection of *B. hominis* by light microscopy and also for molecular studies using PCR because of increased numbers of the organism available.

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