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An evaluation of the usefulness of a culture system for the detection of *Ureaplasma urealyticum* in the endotracheal aspirates of neonates

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Abstract:

In this study, we evaluated the usefulness of a culture system used in our laboratory for the detection of ***Ureaplasma urealyticum*** in the endotracheal aspirates of neonates. Ureaplasmal broth was used to enhance the growth of ***U. urealyticum*** followed by observation of colonies on A7 agar. Of the 68 samples of endotracheal aspirates tested, 60 gave positive indication of urease activity by the broth. However, only 14 yielded ***U. urealyticum*** colonies on A7 agar medium. Polymerase chain reaction detected U. ***urealyticum*** in *21* samples. The use of Ureaplasmal broth was therefore not specific for the diagnosis of U. ***urealyticum.*** We suggest that subculture onto agar medium or PCR is essential for definite identification of ***U. urealyticum.***

Key words: evaluation, detection, ***Ureaplasma urealyticum,*** PCR

INTRODUCTION

Ureaplasma urealyticum is an inhabitant of the human lower genital tract and has been associated in a number of human diseases, which include nongonococcal urethritis and chorioamnionitis.[1] It has also been implicated in meningitis, respiratory disease, and death in neonates.[2,3,4] It was the most common organism isolated from the endotracheal aspirates of neonates with respiratory diseases in several studies.[5,6,7]

Ureaplasmas are distinct from other Mycoplasmas in their ability to hydrolyze urea to carbon dioxide and ammonia by the action of enzyme urease. This ability is a key function of their metabolism, as inhibition of the urease or a lack of urea in the environment arrests growth.[8] This feature has been used for the presumptive identification of the organism from clinical specimens. Ureaplasmas are very fragile and fastidious in growth requirement such as horse serum, yeast extract and other supplements. Many ureaplasma strains do not grow ***in vitro*** and an estimated 70% of all mycoplasmas cannot be subcultured.[9,10]

The advent of polymerase chain reaction (PCR) technologies has allowed the detection of many fastidious organisms. PCR primers, intended for diagnostic use, that amplify a sequence from all Ureaplasmal strains but not from any other mycoplasmas or urease-positive bacteria have been developed in recent years.[11]

In this study, we compared the detection rate of ***U. urealyticum*** from the endotracheal aspirates of Malaysian neonates using an ***in-house*** Ureaplasmal broth. Confirmation of ***U. urealyticum*** was done with 2 methods: (i) conventional subculture on A7 agar medium, and (ii) a PCR system.

MATERIAL AND METHODS

Culture technique

Samples of endotracheal aspirates of 0.1-0.5 ml volume were collected from neonates and inoculated into a tube containing 2.5 ml of Ureaplasmal broth and transported to our laboratory immediately for incubation at 37°C. The Ureaplasmal broth was composed of PPLO broth (Difco Laboratories, Detroit, USA), a final concentration of 10% horse serum, 2.5% yeast extract, 2.5% urea, 0.1% phenol red, 1 mg/ml ampicillin, 0.01% L-cysteine hydrochloride and 1% Isovitalex enrichment (BBL, USA). The pH changes of the medium was observed daily. Broth was kept for 14 days before discard. Any broth with pH changes to alkali (from yellow to pink) were inoculated without delay onto A7 agar (Sanofi, France). The plates were incubated anaerobically for a minimum of 48 hours and examined microscopically under low power (10X) for characteristic colonial morphology. A laboratory strain of ***U. urealyticum*** was used to test the quality of A7

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agar medium. A7 agar plates that were negative were reincubated for another 48 hours before discard. Parts of the culture broth were kept in liquid nitrogen immediately when pH changes was observed or after 14 days, for subsequent PCR testing.

Preparation of samples for PCR

A volume of 500 µl broth cultures were spun at 15,000 rpm for 10 min. The supernatant was removed as much as possible and the pellet was washed once in phosphate buffered saline, pH 7.2 before the addition of 100 µl lysis buffer (50mM KCl, 10mM Tris-Cl, pH 9.0, 0.1% Triton-X 100, 200 µg/ml proteinase K) into each preparation. The mixture was incubated at 56°C for 2 hours. This was followed by heating the mixture at 95°C for 10 minutes. This mixture was then used as DNA template. A laboratory strain of U. *urealyticum* was grown in Ureaplasmal broth and subjected to the same treatment and this was used as the positive control.

PCR technique

Primers were synthesized based on the published sequences of the urease structural genes:'' primers 14b 5'-CCAGGACCCCTAGTACCAGGAGC-3' and primers c72b, 5'-CTCCTAATCTAACGCTATCACC-3' were used. A 50 p1 PCR solution containing 200 µM each of the dNTPs, 10 pmol primers, and 1 U *Taq* polymerase (Boehringer Mannheim) was prepared and subjected to amplification in a DNA thermal cycler (ThermojeT, Belgium). A 10 µl lysed sample was used as DNA template. PCR was performed for 30 cycles with denaturation at 94°C for 30s, annealing at 55°C for 30s and elongation at 68°C for 90s. The amplification products were electrophoresed on 1.5% agarose gel which was then stained with ethidium bromide and observed under ultraviolet transillumination. When the 458 bp-specific band was detectable, the sample was designated positive.

RESULTS

Of the 68 specimens inoculated into Ureaplasmal broth, 60 were observed with pH changes to alkali (an indication for urease activity), 14 grew U. *urealyticum* upon subculturing on A7 agar and 21 were positive by PCR (Table 1). Fig. 1 shows the specific PCR amplification product of 458 bp. No amplification was obtained from Ureaplasmal broth without pH changes.

Using the results of the PCR test as the gold standard, the calculated sensitivity and specificity of the culture system using Ureaplasmal broth for the diagnosis of U. *urealyticum* was 100% and 17.0% respectively. Similarly, the calculated sensitivity and specificity of A7 agar was 66.7 and 100% respectively.

DISCUSSION

There are several problems encountered in the isolation of ureaplasmas from clinical specimens. These include types of specimens, method of collection, transportation and storage of specimens, media used, conditions of incubation, and experience in identifying isolates from clinical materials. To enhance isolation, fresh specimens should be inoculated into suitable media and subjected to optimal incubation conditions.

The use of Ureaplasmal broth is necessary to enhance the growth of U. *urealyticum* especially when the number of this bacteria present in the clinical specimens is low. In our study, an in-house prepared Ureaplasmal broth which supported the growth of U. *urealyticum* was used to transport the specimen to our laboratory.

TABLE 1: The use of Ureaplasmal broth, A7 agar and polymerase chain reaction for the detection of *Ureaplasma urealyticum* in the endotracheal aspirates of neonates

Ureaplasmal broth	No. tested	Growth on A7 agar	No. PCR positive
Negative	8	0	0
Positive	60	14	21
Total	68	14	21

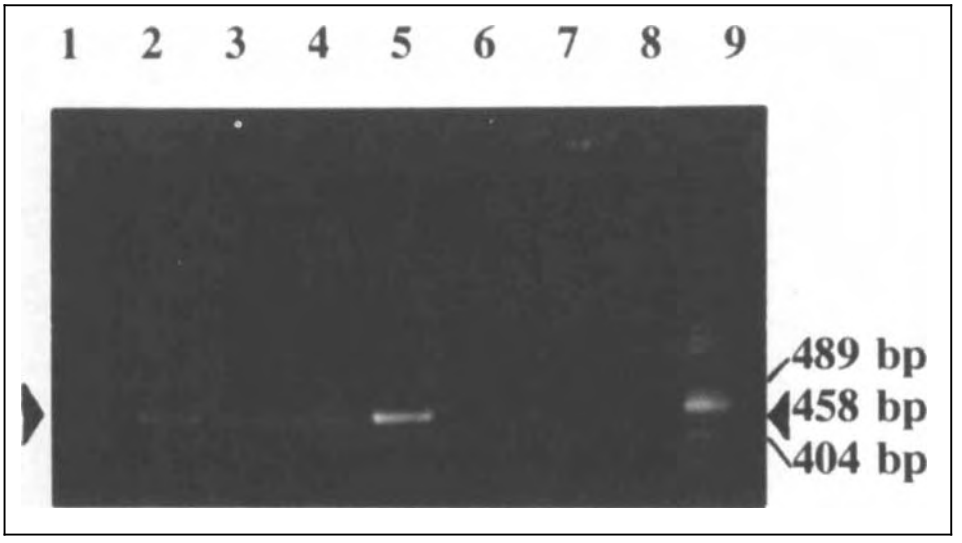

FIG. 1: Detection of *Ureaplasma urealyticum* in Ureaplasmal broth inoculated with endotracheal aspirates of neonates by polymerase chain reaction. Positive specimens (lane 1, 2, 3, 4); negative specimens (lane 6,7, 8); positive control (lane 5); DNA molecular weight marker VIII (Boehringer Mannheim) (lane 9).

The specimens were incubated at 37°C until pH changes were obtained before subculturing onto A7 agar. The urea in the broth was hydrolyzed by the bacterial ureases with resultant release of ammonia which raised the pH of the broth. In the presence of this alkaline environment, positive cultures were indicated by the phenol red in the broth which changed the colour of the broth from yellow to pink.

In some studies, the identification of U. ***urealyticum*** was based on isolation of the organisms by subculturing the Ureaplasmal broth onto blood agar and incubating aerobically to exclude bacterial contamination. If growth occurred on the subculture, the organism was inoculated into fresh Ureaplasmal broth and again incubated aerobically to check for urease activity. If no growth occurred on the subculture or if the isolate did not demonstrate urease activity, the colour change of the Ureaplasmal broth was ascribed to ***U. urealyticum.***[12]

However, there are several limitations with this system.[10,13] Despite the growth of ampicillin-resistant and urease-positive strains of bacteria, the presence of epithelia1 cells producing urease may result in alkali reaction of the broth.[14] In addition, the lack of an airtight seal to the glass vial and prolonged incubation may change the pH to alkali. The broth inoculated onto blood agar might result with no growth and this could be misinterpreted as culture positive for ***U. urealyticum.*** Subculture of the broth with positive indication onto a specific agar medium such as A7 agar was therefore important for the identification of U. ***urealyticum.***

In this study, 14 isolates were grown on A7 agar whereas an additional seven strains were detected by PCR method. Our study showed that the rate of detection of ***U. urealyticum*** by PCR technique was much higher. The procedure to prepare DNA templates used in our study was fast, simple and convenient to use. The use of phosphate buffer saline was important to remove the excess of remnant culture fluid which could inhibit PCR. The oligonucleotide primers used, based on the urease structural genes of ***U. urealyticum,*** had been shown previously to be highly specific for ***U. urealyticum*** and under optimal conditions, to allow detection of <10 cfu of each serotype of the organism."

PCR can detect the ***U. urealyticum*** organism even when present in lower number as compared with the culture technique which could detect U. ***urealyticum*** only when the organisms were present in larger number. Ampicillin-resistant bacteria may overgrow ***U. urealyticum*** and prevent the growth of ***U. urealyticum*** on agar medium. In this study, bacterial and fungal contamination on A7 agar media was also observed, especially after prolonged incubation. PCR also allows the detection of Ureaplasmas which failed to grow due to lack of certain requirements in the agar medium. The PCR

method was fast with results being available within 1-2 days.

The isolation rates of U. ***urealyticum*** vary with the media used. There have been much controversy on the suitability of media for isolation of U. ***urealyticwn.*** Some investigations found broth media superior to agar media for the detection of genital mycoplasmas, whereas other studies reported similar recovery rates from both types of media.[15] Although the combined use of liquid and solid media is considered to be the most sensitive culture method available, it detected only 80% of samples infected with U. ***urealyticum.***[16] Isolates which have lost their viability during incubation in the alkali condition of the broth could not be detected. Since relying on subculture for identification of U. ***urealyticum*** may result in a large number of these fastidious organisms being underdetected, the use of PCR may therefore improve laboratory diagnosis of U. ***urealyticum.*** Our study showed that using the Ureaplasmal broth alone was not specific for the detection of U. ***urealyticwn.*** We suggest that subculture onto agar medium or PCR is essential for definite identification of U. ***urealyticum.***

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