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RESEARCH NOTE:

A NEW MEDIUM FOR ISOLATION, CULTURE AND METRONIDAZOLE-SENSITIVITY TESTING OF *Helicobacter pylori:* DIAGNOSTIC VALUE FOR EARLY ERADICATION OF *H.pylori* INFECTION

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ABSTRACT

In the Philippines resistance to metronidazole has become a problem in the eradication of *Helicobacter pylori*, a bacterium now known to be the cause for chronic gastritis and peptic ulcer disease. In this study, a new medium was designed to ensure optimum growth of the bacteria for antimicrobial susceptibility testing. This complex basal medium used Columbia Blood agar base, urea agar base plus supplements such as horse serum, polyvitex, and peptone. For culture, antibiotics such as vancomycin, polymixin B and trimethoprim lactate were added. Since the medium does not contain whole blood but has urea agar base in its composition it is suitable for isolating *H. pylori* as well as direct testing of urease activity. This medium was tested using the six local isolates of *H. pylori* (as previously reported by the RBD Microbiology Laboratory) plus *H. pylori* ATCC 49503 strain as reference and other gram negative bacteria such as *Proteus vulgaris* ATCC 13315 and *Klebsiella pneumoniae*. Agar plates which contained this medium was inoculated with the different isolates and bacterial strains then incubated at 37°C in a microaerophilic environment (5% O_2, 10 CO_2 and 85% N_2) for 7 days. Confirmation of *H. pylori* was done by Gram staining and biochemical tests for catalase, oxidase and urease.

Keywords: *Helicobacter pylori*, metronidazole-resistant, urease, catalase, oxidase, microaerophilic, urea agar base, vancomycin, polymixin B, trimethoprim lactate.

INTRODUCTION

Helicobacter pylori is now accepted as one of the most frequent bacterial infections in the world. Ninety-five percent of chronic gastritis is attributed to this bacterium. It is also strongly associated with peptic ulcers and gastric carcinoma.

Patients infected with this bacterium are treated with antibiotics such as amoxycillin, tetracycline and metronidazole. Metronidazole together with omeprazol is the most common antibiotic used in the treatment of *H. pylori* (Beex et al., 1990). However with the emergence of *H. pylori* strains resistant to metronidazole and other 5-nitroimidazoles (Borody et al., 1995), this has become a problem.

The Philippine College of Physicians in their annual convention discussed "the challenges posed by *H. pylori* infection eradication." They attributed the high recurrence rate of infection in the Philippines to metronidazole resistance (Cellini et al., 1992). Thus, it is important that antimicrobial susceptibilty testing be done before treatment to ensure that antibiotics would be effective.

Since culture of *H. pylori* is very difficult even with known selective and non-selective media, the RBD Microbiology Laboratory developed a medium that is useful for culture and antimicrobial susceptibility testing. This new medium is a modified version of the one developed by Cellini in 1992.

MATERIALS AND METHODS

Bacterial Isolates

Five isolates of *H. pylori* isolated by the RBD Microbiology Laboratory from 1995-1999 were used in developing the modified medium in this study. The five isolates namely Hpg-03, Hpg-05, Hp-40, Hp-42 and Hp-43 were stored at -80°C in BHI with 10% glycerol before use. The isolates came from gastric biopsies of patients diagnosed with duodenal ulcers or gastritis.

Reference and Control Strains

H. pylori ATCC 49503, *Proteus vulgaris* ATCC 13315 and *Klebsiella pneumoniae* acquired from the culture collection of the Institute of Tropical Medicine, Nagasaki University were used as reference and control organisms, respectively. These strains were also stored at -80°C in cryobanks.

Media

Four media were developed and compared (Table 1) :

(1) HpNM1 consists of 4.4% Columbia Blood Agar base, 2.9 % Urea Agar base, 2.3% peptone, 0.1% Dextrose, 1% (vol/vol) Polyvitex and 7%(vol/vol) Horse serum.

(2) HpNM2, 1.5% of Bacto agar replaced CBA. Polyvitex and dextrose were eliminated.

(3) HpNM3, 0.3% yeast extract, 0.5% Beef extract and 0.1% glucose were added.

(4) HpNM4 is the same as HpNM3 except that horse serum was not added to the medium.

Table 1. Composition of the four media developed for *H. pylori*

Ingredients	MEDIA			
	HpNM1	HpNM2	HpNM3	HpNM4
Urea agar base	2.9%	2.9%	2.9%	2.9%
Proteose peptone	2.3%	2.3%	2.3%	2.3%
Polyvitex	1.0%	-	-	-
Dextrose	0.1%	-	-	-
Horse Serum	7%	7.0%	7.0%	-
Columbia Blood Agar	4.4%	-	-	-
Yeast Extract	-		0.3%	0.3%
Beef Extract	-		0.5%	0.5%
Glucose	-		0.1%	0.1%
Bacto-agar	-	1.5%	1.5%	1.5%

- Antibiotics were added to the media for culture:
  Vancomycin – 2.5 mg/250 ml
  Polymixin B - 1.25 mg/250 ml
  Trimethoprim- 625 units/250 ml

For culture, the following antibiotics were added: vancomycin (2.5 mg/ 250ml), polymixin B (1.25 mg/250ml) and trimethoprim (2.5mg/250ml).

Antimicrobial Susceptibility Testing

Local isolate Hp-40, which was found to be metronidazole-resistant, was inoculated into the HpNM1 medium and CBA with 7% laked horse blood. A 5 μg metronidazole disc was placed on top of the agar.

RESULTS AND DISCUSSIONS

The frozen local *H. pylori* isolates were thawed and rapidly seeded onto HpNM1, HpNM2, HpNM3 and HpNM4 and incubated at 37°C in a microaerobic atmosphere for 3-4 days. At the same time, the reference and control strains were cultured under the same conditions as the local *H. pylori* isolates for comparative studies of urease activity.

Upon inoculation of the isolates and control strains into the different media, color change in the media was observed every 15 min, 1 h, 6 h and 24 h and on the 3rd and 4th day. Observation was made before and after the plates were placed in a microaerophilic atmosphere.

Rapid color change was observed after 15 min at room temperature in Hp-40, Hp-42 and Hp ATCC 49503 (Table 2). The remaining three isolates, Hpg-03,

Table 2. Qualitative growth of H. pylori isolates on the different media and color change after different hours and days.

	HPNM1			HPNM2			HPNM3			HPNM4		
	15 mins. / 1 hr	6 hrs. / 1 day	3 days / 4 days	15 mins. / 1 hr.	6 hrs. / 1 day	3 days / 4 days	15 mins. / 1 hr.	6 hrs. / 1 day	3 days / 4 days	15 mins. / 1 hr.	6 hrs. / 1 day	3 days / 4 days
HPG-03	−* / −*	+* / −*	− / +*	−* / −*	−* / −*	− / −*	−* / −*	−* / −*	− / −*	−* / −*	−* / −*	−* / −*
HPG-05	− / −*	−* / −*	+* / +*	− / −*	−* / −*	−* / −*	− / −*	−* / −*	−* / −*	− / −*	−* / −*	−* / −*
HP-40	−* / −*	−* / −*	+* / +*	−* / −*	−* / −*	−* / −*	−* / −*	−* / −*	−* / −*	−* / −*	−* / −*	−* / −*
HP-42	−* / −*	−* / −*	+* / +*	−* / −*	−* / −*	−* / −*	−* / −*	−* / −*	−* / −*	−* / −*	−* / −*	−* / −*
HP-43	− / −*	−* / −*	+* / +*	−* / −*	−* / −*	−* / −*	− / −*	−* / −*	−* / −*	− / −*	−* / −*	−* / −*
ATCC 49503	−* / −*	−* / −*	+* / +	−* / −*	−* / −*	−* / −*	−* / −*	−* / −*	−* / −*	−* / −*	−* / −*	−* / −*
Klebsiella pneumonide	− / −	− / +*	+* / +*	− / −	− / −*	+* / −*	− / −	− / −*	+* / −*	− / −	− / −*	+* / −*
Proteus vulgaris	− / −	− / +*	+* / −*	− / −	− / −	+* / −*	− / −	− / +*	+* / +*	− / −	− / −*	+* / −*

LEGEND:

(+) Presence of growth

(--) Negative for growth

* color change from yellow to red

Hpg-05 and Hp-43 displayed a red color reaction after 1 h in the microaerophilc environment at 37°C. A wider area of color change was observed in all the plates with *H. pylori* samples after 6 h of incubation. The medium became completely red after 24 h and remained stable after the 3rd and 4th days. *Klebsiella pneumoniae* and *Proteus vulgaris* ATCC 13315 showed a color change of the media at 24 h. The slow color change eliminates possibilities of false positive results. Since urease production is one of the major characteristics essential in identifying the bacterium, immediate reaction of the inoculum to urea would shorten identification time. This is beneficial as it leads to rapid diagnosis and at the same time reduces the possibility of false positives.

Growth of *H. pylori* in the different media was observed qualitatively. Good growth of *H. pylori* was observed in the HpNM1 medium. Visible colonies were seen after 3 days of incubation. HpNM2, HpNM3 and HpNM4 medium showed no growth at all. Growth of *H. pylori* in HpNM1 medium is the same as compared to its growth in CBA containing 7% laked horse blood.

Antimicrobial susceptibility testing using HpNM1 and CBA showed the same results in both media. Local isolate, Hp-40, which is metronidazole- resistant gave the same growth rate and reaction in these two media. HpNM1 had urease activity of the bacteria immediately, identification time was shortened. Bacterial growth in this study was confirmed by modified Gram staining, catalase and oxidase test.

In conclusion, the HpNM1 medium is a good substitute for known selective media for growth of *H. pylori*. It was able to sustain good growth as compared to CBA with blood and its immediate reaction to urease facilitated identification of the bacteria. Since other urease producers like *Proteus vulgaris* and *Klebsiella pnuemoniae* did not have an immediate reaction, then false- positive results were eliminated.

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