BioChemMed

Technium BioChemMed Vol. 8, pp.83-90 (2024)
ISSN: 2734-7990
www.techniumscience.com

Advancements in *Helicobacter pylori*: A Novel Culture AA-Medium for *Helicobacter pylori* Detection

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Abstract

This research provides crucial insights into the detection and culturing of *Helicobacter pylori*, a significant pathogen associated with gastric diseases. New culture media were prepared as X1, X2 and X3 in comparison with Columbia blood agar. Culturing success varied between Columbia blood agar and the novel X1-media which named as AA-media, while it failed on X2-media and X3-media. 48.8% of the positive samples were successfully cultured on Columbia blood agar and AA-media. Remarkably, colonies on AA-media displayed larger and distinct features within the first 24 hours, a stage achieved on Columbia blood agar after 48 hours. This research underscores the importance of considering alternative culture media to improve culture processing and obtaining rapid and distinctive results for the accurate diagnosis of *Helicobacter pylori*-associated diseases.

Introduction

Helicobacter pylori (H. pylori) stands as a primary contributor for chronic gastritis, peptic ulcers and is classified by the World Health Organization (WHO) as a class 1 carcinogen, significantly elevating the risk of gastric cancer. *H. pylori* infection remains highly widespread with its prevalence surpassing 50% globally (Bashir & Khan, 2023; Farthing et al., 2021), and in Iraq has a prevalence of 180/210 (85.7%) as reported by Salman *et al.* (2021).

Helicobacter pylori poses a challenge for cultivation due to its fastidious nature, characterized by intricate nutritional requirements and a preference for microaerophilic conditions (5% O2, 10% CO2 and

85% N2) to support optimal growth (Rojas-Rengifo et al., 2019). As observed in the majority of studies, Columbia blood agar is the most commonly used culture medium for isolating *H. pylori*, despite its limited culture sensitivity (44.1%) as provided by Al-Sulami *et al.* (2008). Furthermore, Ragab *et al.* (2022) found that the sensitivity of the culture method for detecting *H. pylori* was comparatively low at 16.5%, as opposed to histopathology.

Numerous researchers have explored the nutritional requirements of bacteria, Sasidharan and Uyub (2009) discovered optimal conditions for the yield and growth of *H. pylori*. Their findings indicated that a medium comprising 1.5% (w/v) pepticase, 0.5% (w/v) peptone, 0.4% (w/v) sodium chloride, 0.03% (w/v) L-cysteine, 0.55% (w/v) dextrose, and 0.25% (w/v) K2HPO4 provided the greatest yield, highlighting the essential role of L-cysteine for *H. pylori* growth, while sodium sulfite exhibited inhibitory effects on bacterial growth.

The sensitivity of *H. pylori* culturing displays variability, influenced by clinical and practical factors. These factors encompass the selection of the biopsy site, conditions during transportation, and intricacies in the incubation environment (Cuchi et al., 2002). This variability emphasizes the need for meticulous methodologies and optimized culture conditions to ensure reliable and consistent outcomes.

This research endeavors to address the limitations of conventional culture methods by designing and optimizing a novel culture medium tailored specifically for the efficient isolation and growth of *H. pylori*. Through a methodical and comprehensive approach, the study aims to enhance the sensitivity, specificity, and overall performance of *H. pylori* cultivation. The ultimate goal is to contribute the microbiological techniques and enhance diagnostic capabilities for diseases associated with *H. pylori* in the gastric system.

Materials and Methods

Multi-media were meticulously designed based on the identified nutritional requirements. The composition, containing a variety of amino acids, salts, and other components as mentioned in Table 1,2 and 3, aimed to create an environment conducive to *H. pylori* growth. The methodology involved precise preparation steps, the components were combined with 250 ml of distilled water, filtered using a 0.22-micron pore filter and pH was adjusted to 7.2 ± 2. Subsequently, 18 grams of agar were added to 750 ml, autoclaved, and allowed to cool to 45°C. The dissolved mixtures were then added to the cooled agar. X1-media was added along with 6% human blood, while X2-media mixture was added with 10% human blood and 1% Cysteine Solution. In comparison, the reference culture medium used for evaluation is Columbia blood agar (HiMedia), prepared following the manufacturer's instructions.

Table 1: Components of X1-media.

Components		g/L
1.	Peptone	5
2.	Yeast Extract	5
3.	Casein Peptone	10

4.	Leucine	1.5
5.	Valine	1.5
6.	Methionine	1.5
7.	Arginine	1.5
8.	Histidine	1.5
9.	Phenylalanine	1.5
10.	Isoleucine	1.5
11.	Glutamine	1.5
12.	Alanine	1.5
13.	Thiamine	1.5
14.	Purine	1.5
15.	Adenine	1.5
16.	K2HPO4	2.5
17.	NaCl	4
18.	Dextrose	5.5
19.	Sodium Pyruvate	0.5
20.	Ferrous Sulphate	0.5
21.	Cysteine	0.3

Table 2: Components of X2-media

Components		g/L
1.	Peptone	5
2.	Casein Peptone	5
3.	Proteose Peptone	5
4.	Yeast Extract	5
5.	Histidine	0.2
6.	Methionine	0.2
7.	Phenylalanine	0.2
8.	Arginine	0.2
9.	Ferrous Sulphate	0.1
10.	Sodium Pyruvate	0.1
11.	Sodium thiosulphate	0.1
12.	Nacl	0.5

Table 3: Components of X3-media

Components		g/L
1.	Yeast Extract	5
2.	Peptone	5
3.	Histidine	1
4.	Arginine	1
5.	Phenylalanine	1
6.	Methionine	1
7.	Cysteine	1
8.	Sodium Pyruvate	0.1
9.	Ferrous Sulphate	0.1
10.	Phenyl Red	0.2
11.	Cholestrol	1
12.	Urea	1
13.	Dextrose	1

Gastric biopsy specimens were gathered from patients experiencing symptoms such as gastric pain, burning, dyspepsia, weight loss, or dysphagia. Three biopsies were collected from each patient for the rapid urease test (RUT), and culturing process. The collected samples were promptly transported to the laboratory within an 8-hour timeframe, utilizing Stuart transport media under cooling conditions.

The confirmed RUT-positive were manually homogenized with 500µl of normal saline and subsequently cultured on previously prepared media and Columbia blood agar under both anaerobic conditions. The cultures were then incubated at 37°C for a period ranging from 3 to 7 days for confirming the culture results. All positive cultures underwent Gram staining, biochemical tests and molecular analysis by Polymerase Chain Reaction (PCR) using specific 16s rRNA gene primer designed by Salman *et al.* (2021), included the forward primer 5'- TTGGAGGGCTTAGTCTCT-3' and the reverse primer 5'-AAGATTGGCTCCACTTCACA -3'.

Results and Discussion

The outcomes of the detection and culturing procedure offer significant revelations regarding the diagnostic of *Helicobacter pylori*. Utilizing the rapid urease test as the primary diagnostic measure, 43/102 was identified as positive, indicating a prevalence of 42.15% within the study population, whereas 21/43 (48.8%) of the positive samples successfully progressed to the culturing step.

Regarding to culture, no cultures were acquired from X2-media and X3-media; however, successful culturing was observed on Columbia blood agar and X1-media. They showed growth 48.8% on both of these media, notably, the colonies appeared as small, pinhead, white formations, lacking mucoid appearance. Remarkably, the colonies observed on X1-media (Figure 2) exhibited larger and distinct appearance within the first 24 hours, a stage that was reached on Columbia blood agar (Figure 3) after 48 hours. The morphological characterization appeared as Gram negative curved bacilli aligns with typical characteristics of *Helicobacter pylori*. Furthermore, the positive results for urease, catalase, and oxidase

activities biochemically confirm the identity of the cultured bacteria, whereas the PCR test confirmed *Helicobacter pylori* molecularly (Figure 1), Table (4) compared growth on new media and Columbia blood agar.

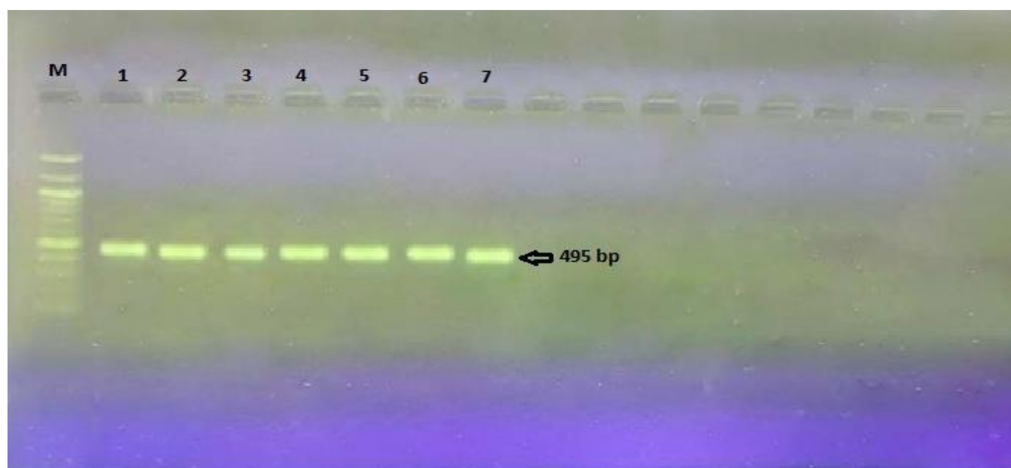

Figure (1): PCR result for 16s rRNA for confirmation of positive cultured samples. The product was 80V electrophoresis on 1.5% agarose at. 1x TAE buffer and stained by RedSafe® for 1:30 hours . M: DNA ladder (100).

Table 4: Comparison of growth on new media with Columbia Blood Agar.

Culture media	Incubation Time		Culture samples Success %
	After 24 hours	After 48 hours	
Columbia Blood Agar	Indistinguishable grey colonies	Distinct pin-head shape, white colonies	48.8%
X1- media	Distinct pin-head shape, white colonies	Larger in size	48.8%
X2- media	-	-	0
X3- media	-	-	0

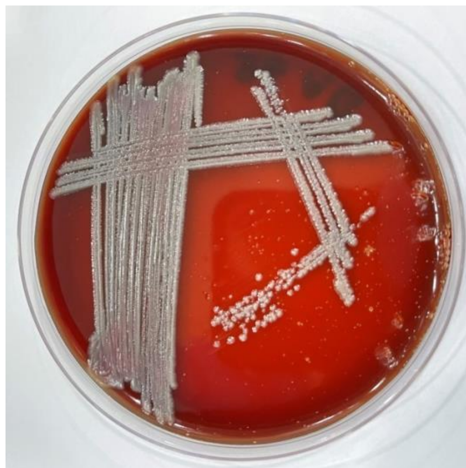

Figure 2: *Helicobacter pylori* growth on X1-media within 24 hours showed distinct white colonies.

Figure 3: *Helicobacter pylori* growth on Columbia blood agar within 24 hours showed small colonies and lesser concentration than growth on X1-media.

The literature review provides a comprehensive overview of the challenges associated with cultivating *Helicobacter pylori* and highlights the familiar use of Columbia blood agar in *H. pylori* isolation, despite its limited sensitivity (44.1%) reported by Al-Sulami *et al.* (2008), the low sensitivity of the culture method also noted by many researchers as Ragab *et al.* (2022) and Metwally *et al.* (2022). It is clearly appeared a crucial need for alternative or optimized cultivation approaches.

According to result of Table (4), the X1-media which consist of amino acids, peptones, yeast extract and other components have been given the best growth with clear characteristic features; it has been named as AA-media. Studies on the nutritional requirements of *H. pylori*, conducted by Nedenskov (1994), Jiang and Doyle (2000), and Sasidharan and Uyub (2009), contribute valuable insights. Sasidharan and Uyub identified optimal conditions for *H. pylori* growth, mentioned that K2HPO4 improved the growth of *H. pylori* and revealing inhibitory effects of sodium sulfite. Jiang and Doyle (2000) recommended that 0.05%

ferrous sulfate and sodium pyruvate enhance the growth and aerotolerance by neutralizing the toxic effect of oxygen. While Nedenskov investigated essential, stimulatory, and inhibitory amino acids for *H. pylori* growth. Their study revealed that all strains had an absolute requirement for leucine, valine, methionine, arginine, histidine, thiamine, purine, alanine and phenylalanine. Isoleucine was either necessary for growth or stimulatory for larger colonies at concentrations ranging from 5 to 10 mg/100 ml, while a few strains showed larger colonies in the presence of adenine.

Other study by Davami *et al.* (2015), reported that although the molecular mechanisms of growth promoting effects of peptones are not fully understood, the positive effects observed could be considered as a consequence of the diverse amino acid composition of the peptones. Furthermore, a study by Hakobyan *et al.* (2012) said that yeast extract was indicated to be an effective nitrogen source for bacterial cell growth stimulation and enhanced H2 production (compared to glutamate).

This research, integrating literature insights with practical experimentation, provides a comprehensive understanding of *H. pylori* cultivation challenges and potential solutions. The novel AA-media, designed based on nutritional requirements, shows promise in improving culturing outcomes. The study's clinical relevance and systematic approach contribute to advancing diagnostic methodologies for *H. pylori*-associated diseases. Future research should explore the broader applicability of AA-media and its implications for clinical diagnostics and treatment strategies.

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