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Diagnostic Performance of glmM Gene and Histological Stains for Detection of *Helicobacter pylori* in Gastric Biopsy from Patients Admitted to Wad Madani Teaching Hospital, Sudan

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ABSTRACT

Helicobacter pylori is the microbial agent most responsible for gastro-duodenal ulcer and chronic gastritis, which can develop into carcinoma of the stomach. This study was performed in Wad Medani Teaching Hospital, Sudan to detect _Helicobacter pylori_ in stomach samples, and evaluate the performance of the tests used, which were histological stains and PCR. Gastric biopsies were obtained from 105 referred patients during endoscopy, and fixed specimens examined by haematoxylin-eosin and Warthin-Starry silver stains, while DNA was extracted for glmM gene amplification. Epigastric pain was the most common symptom at 78% (82/105) and chronic gastritis recorded with 71% (68/105) of endoscopy results. Warthin-Starry silver stain gave 31% (33/105) as positive for _Helicobacter pylori_ followed by glmM gene 27% (28/105) and haematoxylin-eosin 24% (25/105). The study indicated good performance of histological staining and high specificity of glmM gene in detection of _Helicobacter pylori_ from gastric biopsies.

1. INTRODUCTION

Helicobacter pylori (*H. pylori*) is the microbial agent most responsible for gastro-duodenal ulcer and chronic gastritis, which can develop into carcinoma of the stomach [1]. As in mammals, the human sto-

mach consists of four layers, whoever the pathological changes occur in mucosa, sub-mucosa [2]. The infection occurs in over 50% of the world population and the distribution has increased in developing countries with varying prevalence according to the different ages, socioeconomic status and geographical regions [3]. About 10 years ago, most of the studies reported a worldwide increasing prevalence of *H. pylori* infection with age, for example 40% - 60% prevalence rate founded in asymptomatic elderly individuals and >70% in elderly patients with gastroduodenal disease [4]. The highest rates of infection are associated with low socio-economic status and the prevalence is declined where the socioeconomic improved [5]. Data on the prevalence of *H. pylori* infection in Sudan is limited, although it was positively detected in 80% of gastro-oesophageal reflux cases [6, 7]. A majority of infected cases with *H. pylori* are asymptomatic [8]. Accurate detection of *H. pylori* is the first step in eliminating the disease. There are many approaches for diagnosis and detecting *H. pylori,* varying in their sensitivity and specificity including serological tests [9], which can be distinguished crudely into invasive and non-invasive methods [10]. Although there are many invasive techniques, physicians prefer the non-invasive approaches for several reasons, including safety considerations, time and cost [11]. Invasive tests usually require endoscopic intervention to collect samples and monitor pathological changes caused by *H. pylori* along the stomach wall and also for histological and immuno-histological tests [12]. Endoscopic specimens can be further confirmed by culture, molecular and histological tests. Non- invasive methods include serology, urea breath test and stool antigen testing [13]. In histopathological practice, there are many stains available to detect bacteria in the stomach sample [14, 15]. Haematoxylin-eosin staining is routinely used for gastric biopsy examination in Sudan [16]. Its reliability has been demonstrated in detecting bacterial agents even within biofilm-coated proteins in surgical specimens [17]. Furthermore, other stains such as Warthin-Starry and Gram's stains are extensively used to differentiate and identify the bacteria in tissues [18, 19]. Molecular biology techniques are more accurate and sensitive for diagnosis of *H. pylori*; for example, the phosphoglucosamine-mutase gene (gmlM) essential for the development of the cell wall in bacteria) has been widely used for identification of *H. pylori* and this is attributed to its conservatism and efficiency when compared to other genes [20, 21].

In Sudan, the use of stomach samples in endoscopy departments was not common; however, the frequency of gastric biopsy collection for histopathology examination in hospitals such as Wad Medani Teaching Hospital is about 10 - 12 per week (Source: personal communication). The glmM gene, molecular test, is not currently used for diagnosis of *H. pylori*; rather the diagnosis is based solely on the reliability of histopathological tests after endoscopy to confirm the presence of bacteria, to describe the histological changes on the affected stomach mucosa. Therefore, the present study attempts to assess the diagnostic performance of this molecular assay for identifying *H. pylori* in gastric biopsy.

2. MATERIAL AND METHODS

2.1. Study Design and Setting

Cross-sectional laboratory based study methods were followed. The ethical approval was obtained from the Ministry of Health in Gezira State and Faculty of Medical Laboratory Sciences of the University of Gezira, Sudan. A total of 105 patients suspected to have gastritis and those suffering from epigastricupset who were referred for diagnostic upper endoscopy operation in Wad Medani Teaching Hospital in the period from 2019 to 2020 were enrolled. Patients treated with antibiotics (e.g. metronidazole, amoxicillin and clarithromycin, omeprazole or bismuth compounds) less than three weeks prior to endoscopy were excluded from the study. Also, exclusion criteria involved alcohol intake of patients and patients under non-steroidal anti-inflammatory drugs and aspirin. Demographics and clinical data of gastric symptoms and signs were collected after verbal consent from each participant.

2.2. Gastric Biopsies Collection and Processing

During upper endoscopy, two gastric biopsies from each patient were taken, preferably from the antrum region. This process was performed by expert physician by inserting sterile forceps through the en-

doscopy device. The first biopsy sample was immediately fixed in 10% v/v buffered formalin, whilst the second was transferred to eppendorf tube with TE buffer and maintain at 20 C until bacterial DNA isolation. The fixed biopsy was processed in an automated tissue processor (model: KD-TS2, China). Formalin-fixed paraffin embedded (FFPE) gastric biopsy was sectioned in thickness of 3 - 5 μm.

2.3. Staining of Sections

Prepared sections were staining with hematoxylin-eosin and Warthin-starry stains. For hematoxylin-eosin [22] after dehydration unwanted fixation pigments were removed. Alum (Harris) hematoxylin was applied for 10 minutes. Bluing was done in a tap water for 3 minutes. Differentiation was achieved in 1% HCl in 70% alcohol for 10 Seconds. Then sections were washed in a tap water for 10 minutes and stained with 1% eosin Y for 5 minutes. after washing in running tap water for 3 minutes, final dehydration was done. Sections were cleared and amounted with DPX. Microscopic examination was carried out at 10× and 40×.

Warthin-Starry staining was performed using ab150688 Warthin-Starry, Microorganism Stain [23], where the, reducing solution was prepared containing acidulated gelatin 12.5 mL, acidulated Silver nitrate solution (2%) 20 - 30 drops and acidulated hydroquinone solution 7.5 ml.

The procedure was as followed; sections were de-paraffinised and hydrated to distilled water, then slides were placed in 1% warmed acidulated silver nitrate and the staining jar placed at room temp for 3 - 5 minutes. Slides were transferred into previously prepared Reducing Solution and agitated. Staining jar containing slides were placed in a water bath at 65˚C - 70˚C with frequent agitation until tissue section is brown (approximately 1 - 5 minutes). Slides were washed in tap water and rinsed in distilled water. Dehydration, clearing and mounting were performed as before and sections were examined using 40X objective.

2.4. Genomic DNA Extraction and PCR of glmM Gene

The innu PREP DNA Mini Kit DNA extraction protocol (analyticjena, Germany, LOT: 023-17, REF: 845-KS-1040050) was followed for extraction of DNA from gastric biopsy. Extracted genomic DNA were amplified using glmM Helicobacter pylori specific primers (Ligo, India) [24] (**Table 1**).

Primers were prepared by adding 300 μl deionized sterile water, 10 μl 10 m of each stock primer was added to 90 μl deionized sterile water in 0.5 ml PCR polypropylene tube.

The reactions were performed using Gene Amp PCR system 9700 (Applied Biosystems), on 13 μl reaction volume containing 5 μl genomic DNA, 1.5 ul forward primer (10 μm), 1.5 ul reverse primer (10 μm), and 5 μl master mix. Cycling for primers was as follows: primary denaturation (94˚C for 5 mints) then 30 cycles of alternating denaturation (94˚C for 45 secs), annealing of primers (60˚C for 45 secs) and extension by the thermo-stable polymerase (72˚C for 1 min) with final extension of 10 mints. The resulted amplicon was visualized by UV in a 2% agarose gel.

2.5. Statistics

Obtained data were analyzed and displayed using Descriptive SPSS v20 (Statistical Package for Social Sciences) and medical calculator (version 16) Sensitivity, specificity, positive predictive values and negative predictive values were calculated.

3. RESULTS AND DISCUSSION

3.1. Demographics and Patient's Data

During this study, a total of 105 patients suspected to have *H. pylori* were included. All patients presented with epigastric upset. The frequency of males and females was 47% (49/105) and 53% (56/105) respectively. Age groups were closed; from 20 - 35 years appeared 40% (4/105). Family history to gastritis was recorded in 43% (45/105) of participants and rural housing predominated in 62% (66/105). Homemade job among participated women represented 38% (40/105) while farmers equal 36% (38/105) (**Table 2**).

H. pylori infection is associated with various clinical complications, including chronic atrophic gastritis, duodenal and gastric ulcers, adenocarcinoma and gastric MALT (mucosa-associated lymphoid tissue lymphoma) [25, 26]. Since the isolation of *H. pylori* in 1983 by Warren and Marshall, several approaches have been developed toward diagnosis [27]. In diagnostic methods, histology plays an important part in detection of H. pylori bacteria, as it provides more information regarding the degree of inflammation of infected tissues, associated diseases and amount of bacteria present, than a molecular PCR-based approach.

Table 1. Primers used for glmM gene detection.

Target (reference), nucleotide (nt) positions amplified, and size of PCR products	Primers sequence
glmM gene (1), nt 784 - 1077, 294 bp	*Forward primer*, 59-*AAGCTTTTAGGGGTGTTAGGGGTTT*-39 *Reverse primer*, 59-*AAGCTTACTTTCTAACACTAACGC*-39

Table 2. Demographic data of study participants.

Demographics		% (Frequency/total)
Gender	Male	47% (49/105)
	Female	53% (56/105)
Age group/years	20 - 35	40% (42/105)
	36 - 50	28% (29/105)
	51 - 65	22% (23/105)
	66 - 80	11% (11/105)
Special food habits	Lipid	37% (39/105)
	Milk	8% (8/105)
	Spices	2% (2/105)
	Rice	2% (2/105)
	No	54% (54/105)
Family history	Yes	43% (45/105)
	No	57% (60/105)
Location	Urban	37% (39/105)
	Rural	62% (66/105)
Education levels	Uneducated	20% (21/105)
	Primary	60% (63/105)
	Secondary	8% (9/105)
	University	12% (13/105)
Jobs	Farmer	36% (38/105)
	Teacher	8% (8/105)
	Homemade	38% (40/105)
	Free business	18% (19/105)

At the local level in Sudan, several factors make the diagnosis of *H. pylori* difficult; most physicians use serological tests for diagnosis, which may lead to over-diagnosis (false positives) of infections. Irrational use of antibiotics and proton pump inhibitors limits the accuracy of a faecal antigen test, which is another diagnostic option [28]. In addition, the urea breath test, the most useful non-invasive test, is not normally implemented in Sudan, especially in peripheral and rural areas either because it requires laboratory infrastructures and or it is unavailable.

In the present study, for the age group from 20 to 50 years representing 68% from all studied cases, similar and near-identical findings were reported by other authors from Nepal [29], Cameroon [30] and Bosnia and Herzegovina [31]. We also noted that, a proportion 37% (39/105) of studied participants have high unsaturated fat diet, and this increases the chances of infection with *H. pylori* as documented by previous studies [32].

3.2. Symptoms and Signs

Clinically, 78% (82/105) of study population had epigastric pain followed by vomiting in 48% (50/105) and gastric reflex in 24% (25/105) (**Figure 1**).

Although all patients included in the study were not undergoing chemotherapy, a significant proportion of them 48% (50/105) suffer from vomiting (**Figure 1**), which suggests acute stomach upsets, and possibly a relationship has been demonstrated between *H. pylori* and chemotherapy-induced nausea and vomiting [33] and even hyperemesis gravidarum [34].

3.3. Endoscopy Results

The endoscopy findings diagnosed 68% (72/105) of suspected cases as chronic gastritis, 13% (14/105) esophagitis, 5% (5/105) stomach cancer, 7% (7/105) duodenal ulcer and normal endoscopy result was in 7% (7/105) (**Table 3**).

The endoscopic results indicated that, chronic gastritis is the predominant diagnosis among studied group at 68% (72/105), and this agrees with other studied population such as from Iran [27].

3.4. Detection of *H. pylori* by Hematoxylin-Eosin and Warthin-Starry Stains

Presence of *H. pylori* in gastric biopsies (positivity) by hematoxylin-eosin showed the bacteria in groups with a pink color and spiral shape, while Warthin-Starry stains gave black/silver stain of the bacteria on the gastric mucosal surface against brown and yellow/dark yellow on the tissue background (**Figure 2**). Thirty one percent (33/105) of examined specimens gave positive results for *H. pylori* with Warthin-Starry stains and 24% (25/105) were positive by hematoxylin-eosin.

Histologically, *H. pylori* are present within the surface mucus of the gastric mucosa staining with hematoxylin-eosin (a), Warthin-Starry silver stains (b).

Table 3. Endoscopy results of H. pylori positive and negative participants.

Endoscopy finding	Positive *H. pylori*	Negative *H. pylori*	Frequency (%)
Normal	2	5	7 (7)
Chronic gastritis	23	49	72 (68)
Esophagitis	3	11	14 (13)
Duodenal ulcer	1	6	7 (7)
Stomach cancer	4	1	5 (5)
Total	33	72	105 (100)

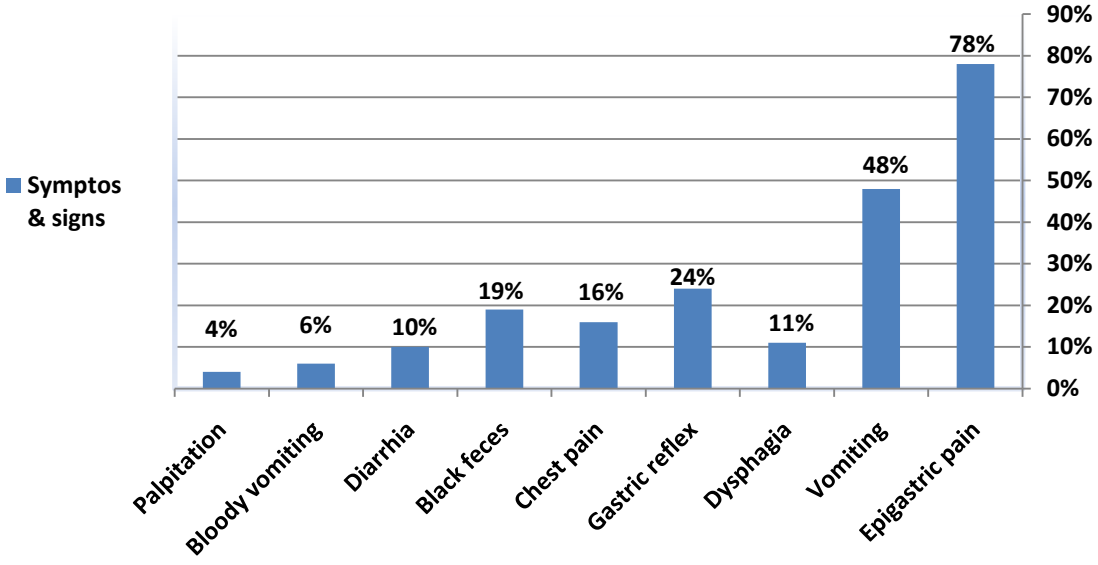

Figure 1. Frequency of symptoms and signs in study participants.

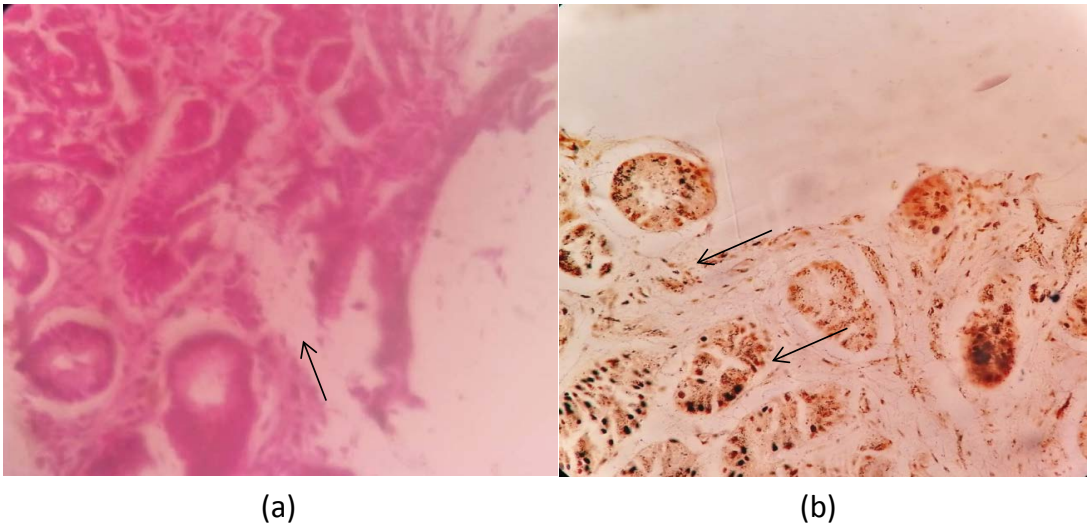

Figure 2. Detection of *H pylori* by hematoxylin-eosin and Warthin-Starry stains.

The results of *H. pylori* detection is in consistent with Ashton *et al.* study, who in their results showed that *H. pylori* was found in 23 (61%) of gastric biopsy specimens [27]. Moreover, Farah *et al.* examined Warthin-starry silver techniques for detecting *H. pylori,* their sensitivity was 72% [35]. Looking to the result of the hematoxylin-eosin for staining gastric biopsies, detection of *H. pylori* has several advantages for its use in routine work, ease of procedure, and lower cost. Two studies with similar results for diagnosis of *H. pylori* by hematoxylin-eosin were observed [33, 35] and, another study preferred the dye after comparing the results with other methods including Giemsa stain, Toluidine blue stain and imprint cytology [36]. However, Boldt and coworkers preferred Giemsa stain for gastric biopsies staining when compared to hematoxylin-eosin [14].

3.5. Detection of *H. pylori* by glmM Gene

During the study 105 collected gastric biopsies were subjected to genomic DNA extraction, 27% (28/105) of them showed characteristic band size of 294 bp (**Figure 3**).

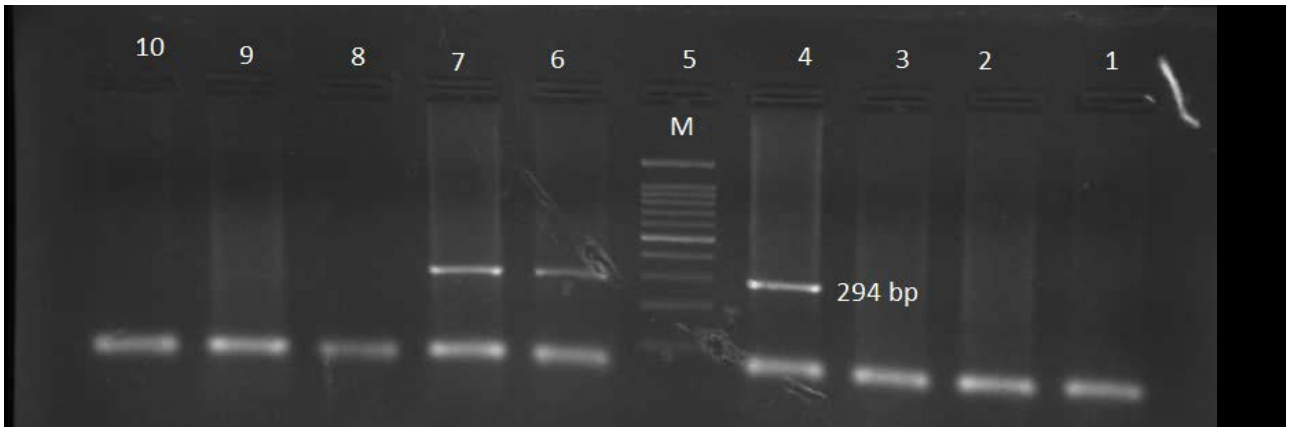

Figure 3. **Gel electrophoresis of *H. pylori* glmM gene. Lane 5 (M): 100 bp DNA ladder. Lanes: 4, 6 and 7 are positive. Lanes: 1, 2, 3, 8, 9, and 10 are negative.**

PCR-based methods have been developed to detect the organism directly in gastric biopsy specimens, the gmlM encodes for a *phosphoglucosamine mutase* ("a housekeeping" gene) and it participates directly in cell wall synthesis. The *glmM* gene has been extensively used for confirming the presence of *H. pylori* due to its enhanced sensitivity (high ability in detecting negative results) [34]. In this study, the validity of Warthin starry silver stain in detection of *H. pylori* was determined using PCR method as a reference method. The sensitivity and the specificity of the Warthin starry silver stain was 69.7%, 93.1%, respectively. This result supported by Farouk *et al.* study who revealed that Warthin Starry was sensitive method for confirming the presence of *H. pylori*; sensitivity and specificity was 100% and 84% respectively [11].

Sensitivity, specificity, positive predictive value, negative predictive value and accuracy of Warthin starry silver stain compared to the glmM gene as the molecular standard method were 69.7%, 93.1%, 82%, 87% and 85.7% respectively indicating good performance of glmM gene as the molecular standard method.

4. CONCLUSION

The study found that histological staining performed good and that the glmM gene had high specificity in detection of *Helicobacter pylori* from gastric biopsies.

AVAILABILITY OF DATA AND MATERIALS

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest regarding the publication of this paper.

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