Original Research Article

Comparison of Stool Antigen and PCR-Based Diagnostics for *Helicobacter pylori* Infection and Associated Gut Dysbiosis in Wad-Medani City, Sudan.

ABSTRACT

Background: The development of stomach illnesses has been linked, in part, to *Helicobacter pylori* (*H. pylori*) infection; in clinical practice, accurate identification of *Helicobacter pylori* infection is crucial, H. pylori infection was associated with a decrease in microbial diversity and an increase in pathogenic taxa, indicating its role in gut dysbiosis.

Objectives: We tested a noninvasive method using immunochromatography to detect the presence of the *H. pylori* antigen in stool specimens. PCR was used to validate the presence of *H. pylori* by identifying the bacteria's ureC gene in the DNA extracted from stool specimens. Additionally, gut microbiome changes were assessed, understanding the link between H. pylori infection and gut dysbiosis is vital for public health interventions.

Method: A total of 100 stool samples obtained from dyspeptic patients (46 men and 54 women, average age 46.1) were assessed using these methods. The results were then compared between the PCR-based technique and the SAT technique.

Results: The results showed that 35 (35%) out of 100 samples were positive by the PCR-based technique, compared to 33 (33%) positive by the SAT technique and The PCR method demonstrated a sensitivity of 95% and specificity of 92% in detecting H. pylori. Beta diversity was assessed using Bray-Curtis dissimilarity, revealing significant variations in microbial composition among samples.

Conclusion: These data suggest that the techniques used in this study are valuable for studying the molecular epidemiology of *H. pylori* infection in dyspeptic patients. Stool, as a non-invasive sample, has the potential to be a good replacement for the detection of *H. pylori*. Additionally, we found that infection with this bacterium contributes to gastric microbial dysbiosis, while no statistically significant differences were observed.

Keywords: *Helicobacter pylori*, dyspeptic, stool antigen test, microbiome, polymerase chain reaction, dysbiosis.

INTRODUCTION:

Helicobacter pylori (*H. pylori)* is a human pathogen that considers gram-negative bacteria with a microaerophilic spiral structure that can colonize the stomach□[1]□. The primary disorder associated with its infection is chronic active gastritis□[2]□. Invasive and non-invasive techniques are used to diagnose *H. pylori* infection, and a number of variables affect the selection of a particular testing approach, such as test cost-effectiveness, clinical conditions, sensitivity, and specificity□[3]□. Notably, each of these methods has specific restrictions□□□. Since *H. pylori* is not an intestinal pathogen, it might be found in trace amounts in stool. The stool specimens can be used to test for the *H. pylori* stool antigen, perform PCR, or even culture the bacteria□□.□□.

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As demonstrated by recent studies, the stool antigen test has proven to be very helpful in diagnosing H. pylori infection□□.□□, however a recent study revealed that although the test has excellent sensitivity and specificity, the results of currently available ICA-based tests are less trustworthy□□□, therefore it is important to remember that a negative SAT test does not always mean that there is no *H. pylori* infection because a low level of bacterial colonization in the stomach lowers the amount of *H. pylori* antigen in the sample□[1]□□. Although the patient does not need to be prepared in advance due to the method's simplicity, according to the most recent SAT standards, in order to prevent a false-positive result, it is advised that the patient refrain from using proton pump inhibitors (PPIs) for two weeks and from using antibiotics and bismuth compounds for four weeks before testing□[11].[12]□. Also, we need to exercise caution because there are some situations in which the sensitivity of SAT may decline, such as in patients experiencing gastrointestinal bleeding or receiving bismuth-based medication□[13]□.
"Although reported success rates for the identification of *H. pylo*ri DNA in feces range from 25% to 100%, stool-PCR could also be a highly effective method for detecting*H. pylori* infection"□[1]□.[1]□□, this fluctuation is most likely caused by the gastrointestinal tract's *H. pylori* breakdown and/or the presence of inhibitors like complex polysaccharides□[1]□.[1]□□. "A PCR-based assay has not been accepted for routine testing. Since PCR targets the DNA of active bacteria; it may also detect DNA from dead bacteria, which can result in false-positive outcomes. Furthermore, challenges with both false negative and false positive results persist. PCR-based methods also necessitate specialized equipment and skilled workers" [33].

The human microbiota is made up of up to 10-100 trillion commensal microbial cells that live in everyone's digestive system□[1]□□. The gut microbiota can be strongly impacted by *H. pylori* infection, and an altered microbiota creates advantageous conditions for *H. pylori* colonization□[1]□□. The purposes of this study were to evaluate molecular detection of *H. pylori* in stool compared tostool antigen test, and potential dysbiosis arising from *H. pylori* infection.

2. MATERIALS AND METHODS:

2.1 Clinical Samples: The study was conducted from July to October 2022 in Wad-Medani city, Sudan. Informed consent was given by all participants. Stool specimens were collected from 100 dysbiosis individuals (46 men and 54 women) aged from 20 to 75 years old. Positive controls were selected based on previous confirmed cases of H. pylori infection, ensuring reliable amplification of the ureC gene

The research protocols were approved by the Ethics Committee of the University of Gezira and the Ministry of Health (Gezira State, Sudan).

2.2 Detection of *H. pylori* antigen and extraction of the bacterial DNA from stool specimens:"A commercially available rapid-test kit (*Right Sign Rapid Pylori Antigen, China*) was used for the detection of the *H. pylori* antigen in stool specimens. The detection was performed by following the manufacturer's instruction manual. Bacterial DNA was extracted from all stool specimens using the QIAamp Fast DNA Stool Mini kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions. In brief, 200 gram samples were lysed in 1 ml of inhibit EX buffer and vortex for 1 min, centrifuge samples for 1 min, 25 µl of proteinase K and 200 µl supernatant samples, and 200 µl of buffer AL added and vortex for 15 seconds, then incubated for 10 min at 70 °C. Two hundred microliters of ethanol (96%) were added to the

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lysate and mixed by vortex. The lysates were purified over a QIAamp column as specified by the manufacturer. The column was washed stepwisely with 500 µl buffer AW1 and buffer AW2, after which an ultra-pure DNA product was eluted for PCR assay" [33].

2.3 PCR Analysis: PCR was performed on extracted DNA from stool samples using primers specific for *H. pylori* ureC under conditions as in (Table 1). "The PCR amplification was performed using a thermocycler system (GeneAmp® PCR System 9700). Each 25 µl PCR reaction mixture contained 5 µl PCR master mix (Maxime PCR premix, iNtRON® Korea), 1 µl each of primer (0.2 µM) (Macrogen, Europe), 3 µl of template DNA, and 15 µl of PCR-grade water. For each PCR experiment, appropriate positive and negative controls were included" [33].

2.4 16s rRNA Gene Amplification: For the analysis of microbial composition, the hypervariable region V3-V4 of the microbial 16s rRNA gene was amplified using the universal primers and the PCR reaction as in Table 1. PCR reactions were performed in a 25 µL mixture containing 5 µl PCR master mix (Maxime PCR premix, iNtRON® Korea), 1 µl each of primer (Macrogen, Europe), 2 µl of template DNA, and 16 µl of PCR-grade water. The amplicons were then extracted from 1.5% agarose gels. The samples are prepared according to NGS library preparation and sequenced using the Illumina platform.

Table 1: Primers used and PCR conditions.

Primers	Sequences	Product size (bp)	PCR conditions
ureC	F:AAGCTTTTAGGGGTGTTAGGGGTTT R:AAGCTTACTTTCTAACACTAACGC	294	95°C for 5 min (1 cycle); 94°C for 1 min; 55°C for 1 min; 72°C for 2 min (39 cycles); 72°C for 7 min.
Universal 16s rRNA	F:GTGCCAGCMGCCGCGGTAA R:GGACTACHVGGGTWTCTAAT-3	300	95°C for 5 min (1 cycle); 94°C for 1 min; 50°C for 1 min; 72°C for 1 min (39 cycles); 72°C for 7 min.

2.5 Microbial Statistical Analysis: Statistical analysis was performed using Chi-square tests to compare prevalence rates among different age and gender groups, with p-values calculated to assess significance. Raw sequencing data was analyzed with FASTQC and processed with BBDUK in order to remove low-quality portions of the reads. The software Kraken2 was used together with a database of 16S (Silva SSUNR99) to perform the classification of the trimmed reads and quantify the organisms in the samples. The read count matrix was imported into the software R and analyzed with the phyloseq package. The analysis was performed at the genus level, which is the lowest taxonomic level that can be reached with 16S. Alpha diversity was assessed with the Shannon index on raw OTU abundance tables; we also tested beta diversity.

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3. RESULTS:

The demographic characteristics of the 100 patients regarding age and gender are shown in Table 2. The clinical presentation as follows: "Recurrent abdominal pain was the most common presentation, followed by heartburn, dyspepsia, bloating, acidity, nausea, and vomiting (82%, 79%, 67%, 62%, 54%, and 23%), respectively" [33]. The prevalence of *H. pylori* in five age subgroups shows that prevalence varies with age up to 55 years and then slightly increases, but without reaching statistical significance (*P = 0.153*), despite the age-related increases in the incidence of *H. pylori* infection being well-established, and larger prevalences are observed in anyone above 40 years of age□²□□.

Table 2: Demographic characteristics of 100 patients, including p-values for gender and age group prevalence [33]

Variable	Total of Patients	Prevalence
Male	46	16 (34.7%)
Female	54	19 (35.1%)
Group 1 (18-27)	13	2
Group 2 (28-37)	18	2
Group 3 (38-47)	24	6
Group 4 (48-57)	21	9
Group 5 (58-67)	13	7
Group 6 (68-77)	11	9

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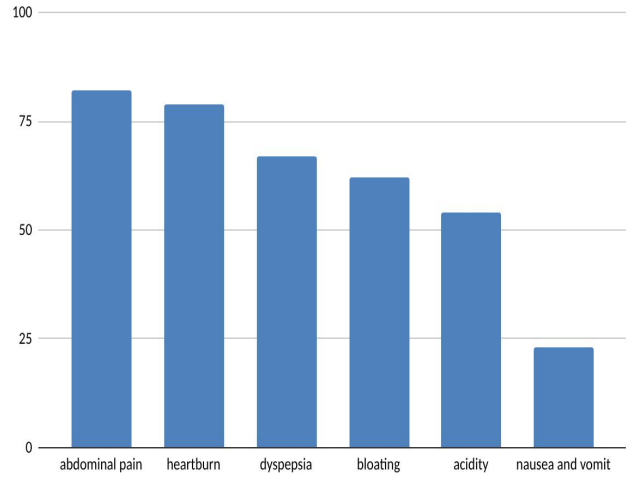
Figure 1: patient's presentation in%.



The *H. pylori* antigen was detected in 33 (33%) of the 100 specimens collected from dysbiosis individuals; the existence of *H. pylori* in the extracted DNA was confirmed by PCR specific for *H. pylori* ureC gene, where 35 samples (35%) were positive for*H. pylori*ureC gene. Lage and colleagues demonstrated that *H. pylori* was the only urease-positive or related bacterium that produced the anticipated amplified DNA products using ureC amplifications[21]. There isn't yet a thorough study that utilizes a variety of techniques to assess the prevalence of *H. pylori* infection in patients from Sudan, and the results of SAT compared to the PCR technique (positive predictive value (PPV) is 100% and the negative predictive value (NPV) is approximately 95.59%.); this result differs from the result of a recent study report by Galal *et al.* who stated a 64.6% occurrence rate using SAT method[22], and closely similar to Ussein RA. *et al* who showed that SAT methods have 95.0% sensitivity and 91.2% specificity[23]. These differences may be the consequence of differences in the social standing, level of education, food habits, and sanitary conditions of the studied locations.

Figure 2: PCR amplification of UreC for *H. pylori* detection: 100-bp DNA ladder, lane 1 positive control (294 bp), lane 2 negative control, lane 3 positive sample, lane 4,5 negative samples.

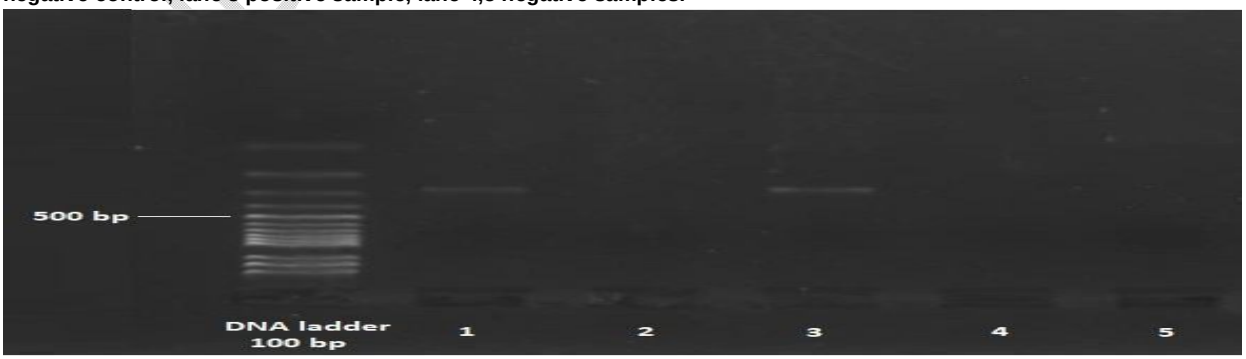


Figure 3: PCR amplification of universal 16s rRNA gene, lane 1: 100 bp ladder, lane 2, 3, 4, 5, and 6 positive samples (300 bp).

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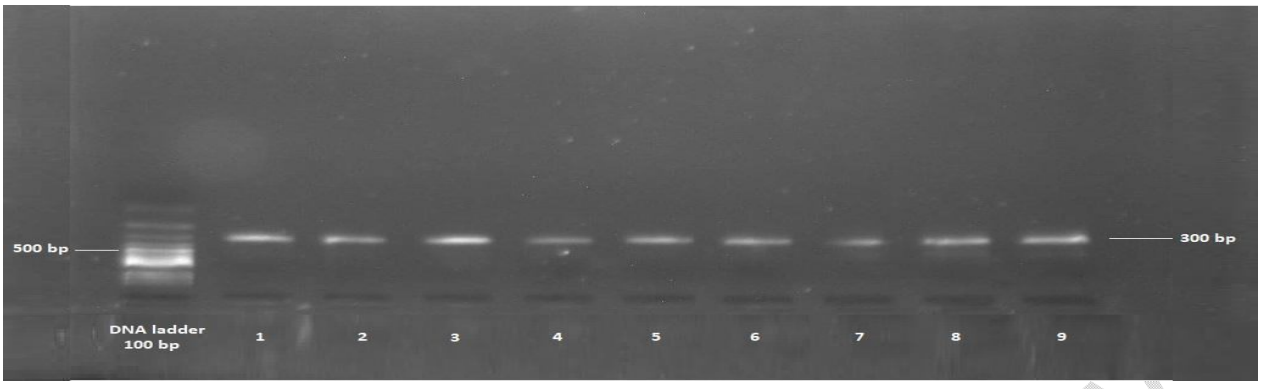

Bacterial Sequence and Taxonomic Classification and Quantification: MiSeq-generated Fastq files were quality-filtered and grouped into 97% similarity operational taxonomic units (OTUs). The analysis included the quality control and trimming of the data, followed by the taxonomic classification and quantification from the reads using a database of 16 sequences. At the genus level, the fecal microbiota was dominated by Yersiniacea, Streptomycetaceae, Clostridiaceae, and Clostridiaceae, with average relative abundances of 37.73, 28.25, 17.92, and 8.42%, respectively.

Table 3: Alpha diversity indices calculated for each sample (Genus's level).

Sample	Observed	Chao1	Shannon	Simpson
1	154	201.53	2.99	0.85
2	149	231.65	3.68	0.94
3	157	193.43	3.97	0.96
4	185	210.64	4.04	0.96
5	152	219.03	4.15	0.96

Figure 4: Top 10 abundant genera per sample. Relative abundance (%) is shown on the Y axis.

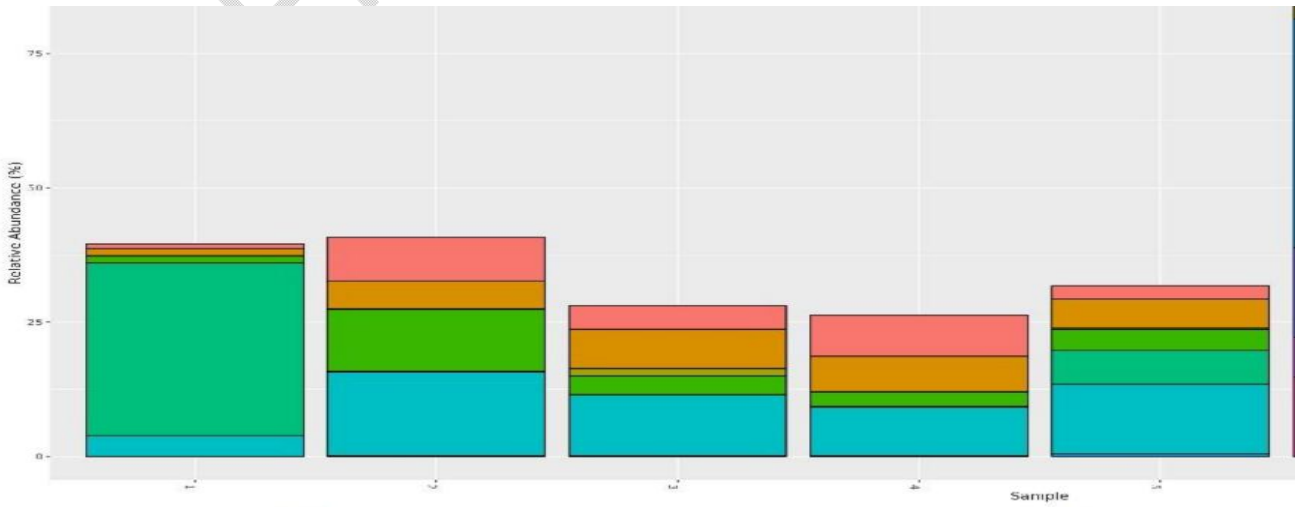

Figure 5: Alpha diversity indices calculated in each sample at genus level.

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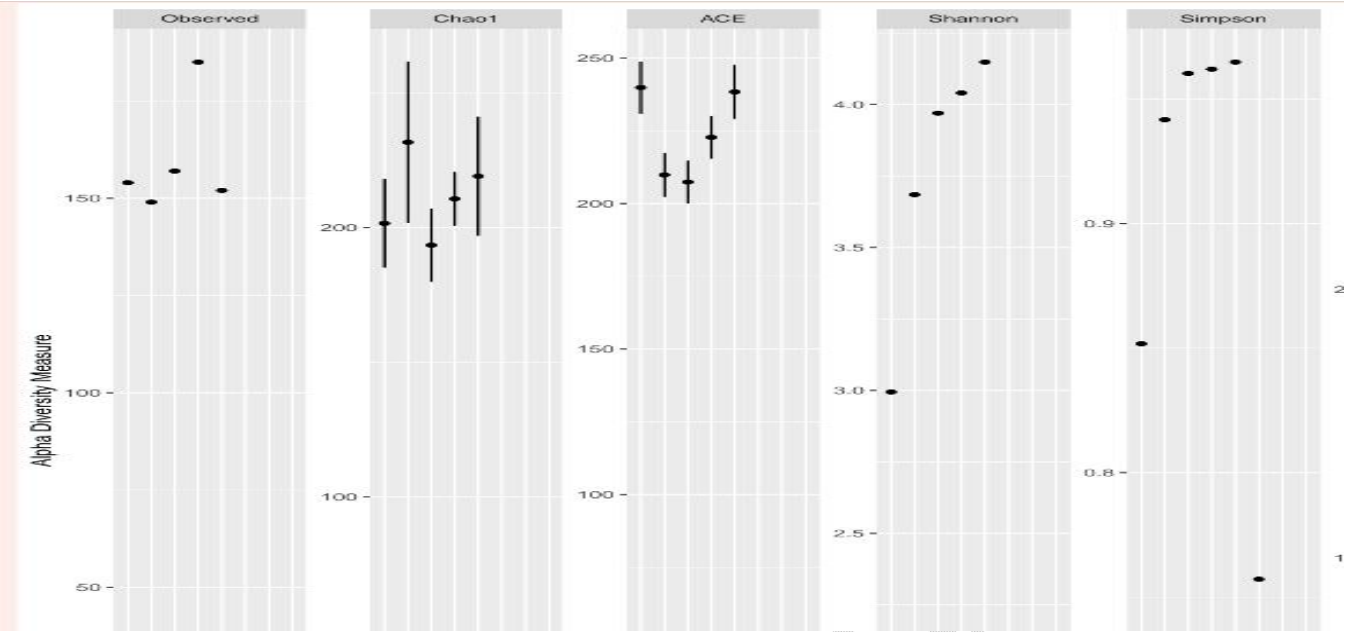

We utilized the widely-used Bray-Curtis dissimilarity, which is determined by taking the minimal shared abundance of every taxon into account; the analysis of the community structure revealed that the typical composition of the stomach had no significant microbial diversity (*p = 0.137)*.

Table 4: Beta diversity (Bray-Curtis) matrix of the samples

sample	1	2	3	4	5
1	0.00	0.76	0.73	0.74	0.63
2	0.76	0.00	0.41	0.54	0.50
3	0.73	0.41	0.00	0.53	0.49
4	0.74	0.54	0.53	0.00	0.54
5	0.63 0	0.50	0.49	0.54	0.00

4. DISCUSION: There is proof that infected people expel *H. pylori* in their feces□²□□. Various diagnostic methods have been presented to identify *H. pylori;* the choice is frequently based on the resources that are available, the population being sampled, the patients' health status, and the credentials or experience of the investigator. This study takes advantage to compare two techniques for detection of *H. pylori:* PCR assay and stool antigen test (SAT).

With an accuracy rate of more than 90%, the SAT is a valuable diagnostic tool. This rapid test is helpful for both diagnosis and verifying the continued presence of germs following therapy. Despite the need to be mindful of the stool's form and the time between sample collection and detection, watery feces can lead to false-negative results due to dilution of antigens, and a false-negative result could arise from the reduced *H. pylori* colonization□²□□.

When it comes to PCR-based techniques, we should be concerned about the quantity and quality of DNA collected, the target sequence's design, and the choice of amplification process. A PCR-based assay can be applied in the detection of antibiotic resistance and virulence factors, and it is crucial for clinicians to

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assess the infection. Since *H. pylori* can shift to the coccoid form when it becomes difficult for them to thrive in the environment, this feature may make it more challenging for PCR-based techniques to detect *H. pylori* accurately[2].[2]. Low *H. pylori* loads may have been below the stool antigen assay's detection limit in the three samples that tested positive for *H. pylori* PCR but negative for *H. pylori* stool antigen. The *H. pylori* PCR test findings were confirmed to be positive in one of these three individuals, whose medical records also revealed a prior diagnosis of *H. pylori* infection.

There isn't yet a thorough study that utilizes a variety of techniques to assess the prevalence of *H. pylori* infection in patients from Sudan. The results of SAT compared to the PCR technique as follow: Positive 33%, negative 77%, false positive 0 and false negative 3%; this result was closely similar to the recent report by Galal *et al.* who stated a 64.6% occurrence rate using SAT method[22], and Ussein RA. *et al.* who showed that SAT methods have 95.0% sensitivity and 91.2% specificity[23] . These differences may be the consequence of differences in the social standing, level of education, food habits, and sanitary conditions of the studied locations. Although there are currently barriers to the clinical use of PCR-based testing, such as high costs, because of its good diagnostic performance in the pre- and post-treatment scenario and the added advantage of finding strains resistant to clarithromycin, this diagnostic approach remains great for the foreseeable future[2], and since *H. pylori* is becoming increasingly resistant to the majority of widely used therapies, it is imperative to set up a fast and accurate test for identifying the genes responsible for antibiotic resistance in the bacteria[2].

"Comparing both results from two techniques, we observed a higher prevalence rate (35%) in the PCR method than SAT (32%). The difference between the two methodologies' finding might have resulted from low colonization of bacteria in the stomach leading to low-concentration of *H. pylori* antigen in the sample, and in some situations, the sensitivity of SAT may decrease, such as those for patients with gastrointestinal bleeding. The current study's total prevalence of 35% is rather low in comparison to several other earlier studies conducted in poor countries where higher prevalence figures were found"[3].[31],33[].

Within an individual, the gut microbiota differs greatly, although between individuals it is mostly constant. Numerous factors, such as food and the use of antibiotics, have been shown to affect the gut microbiota[32]. We examined the fecal microbiota of individuals with *H. pylori* infection; in comparison to *H. pylori*-negativecontrols, diversity analysis revealed somewhat higher microbial richness and evenness in individuals exhibiting symptoms of *H. pylori* infection.

According to the results of the analysis, Alpha diversity indices showed no significant differences between *H. pylori*-positive and negative groups ($p = 0.137$), indicating similar microbial diversity, there were no statistically significant variations, indicating that the *H. pylori* infection just promotes species richness, despite there being no effect on the total variability. It's remarkable to note which microbial populations are more prevalent as a result of *H. pylori* infection and to further investigate and recognize their importance in host immunological interactions and the pathophysiology of *H. pylori.*

5. CONCLUSION:

This study investigated the prevalence of *Helicobacter pylori* (*H. pylori*) infection among patients with upper gastrointestinal disorders in Gezira State, Sudan, aiming to evaluate the association between *H.*

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pylori infection, the presence of virulence genes *cagA* and *vacA*, clinical outcomes, and alterations in gut microbiome composition. Key findings revealed that patients diagnosed with gastritis exhibited a significantly higher rate of *H. pylori* positivity compared to other upper gastrointestinal disorders. Multiplex PCR analysis identified the presence of *vacA* and *cagA* genotypes in 82.8% and 48.5% of the 35 isolated *H. pylori* strains, respectively. Additionally, 16S rRNA-based microbial profiling of stool samples from *H. pylori*-infected individuals demonstrated an increased abundance of the Yersiniaceae and Victivallaceae families, while no statistically significant differences in alpha diversity indices (Shannon index) were observed between *H. pylori*-positive and negative groups.

The findings underscore the need for optimized diagnostic protocols and public health initiatives targeting *H. pylori* in the studied population. Further research is warranted to elucidate the complex interplay between *H. pylori*, its virulence determinants, and the gut microbiome and their collective impact on gastrointestinal health and disease pathogenesis. To enhance diagnostic performance, it is recommended to combine multiple target genes (ureA, glmM, and vacA) in testing. Future studies should include histological examinations and focus on *H. pylori* virulence factors to better understand disease pathogenesis in Sudan.

6. STUDY LIMITATION:

Small sample size, the need for more sophisticated functional analysis, and the use of direct gastric biopsy to clarify the pathophysiology of the disease and any alterations to the mucosa, especially true for situations where antibiotics were used before sampling.

ETHICAL APPROVAL:

The Ministry of Health of Gezira State and the Faculty of Medical Laboratory Sciences, University of Gezira, ethically approved the study.

CONSENT:

Informed consent was collected from each participant.

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Disclaimer (Artificial intelligence)

Option 1:

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

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Author(s) hereby declare that generative AI technologies such as Large Language Models, etc have been used during writing or editing of manuscripts. This explanation will include the name, version, model, and source of the generative AI technology and as well as all input prompts provided to the generative AI technology

Details of the AI usage are given below:

1.
2.
3.

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