Original Article

Applications Of The Immune-Histochemical Technique For Diagnosis Of Helicobacter Pylori From Gastric Biopsy Samples

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Abstract

The Helicobacter pylori have ancestral relation with the family of Proteobacteria, order Campylobacterales, family Helicobacteraceae. It is the first and most important cause of gastric carcinoma along with peptic ulcer and dyspepsia carcinoma. H. pylori is also responsible for mucosa associated with lymphoid tissue (MALT) and Known Hodgkin Lymphoma. It is assessed that patients having H. pylori are a 30% to 40% possibility of evolving gastric ulcer and a 10% to 20% possibility of developing distal gastric cancer. It causes chronic inflammation and significantly increases the risk of duodenal, gastric ulcer, and cancer. It is the second leading cause of cancer-related deaths in the world. In the current study, a total of 210 gastric biopsy specimens were collected from the patient with a history of gastritis from Chughtai Lab in 10% NBF. All tissue size was measured and gross examined these samples were processed in an automated tissue processor Tissue-Tek VIP VI (Japan). After processing, the embedding of tissues was done in paraffin wax at Tissue-Tek TEC. 2- 3 µm sections were prepared using rotary microtome Leica 201 USA. A comparison was carried out between H&E and IHC. Helicobacter pylori were detected in 88 cases out of 200 while 09 samples were either poorly preserved or autolyzed that's why further processing and diagnosis were not possible. One case was diagnosed as poorly differentiated adenocarcinoma; the sample was collected from a female patient of 56 years. Out of 88 positive cases, 66 (75%) were initially screened as positive for H.pyloriby H&E staining due to the presence of mild or moderate colonization while the remaining 25% cases were negative by H&E staining but with suspicion of neutrophils and lymphoid aggregates. All 88 cases were further confirmed by IHC. Our findings confirmed that IHC is a batter reliable and more accurate method in the diagnosis of H.pylori and it should be considered the gold standard.

Keywords: Immunohistochemical, Helicobacter Pylori, Gastric Biopsy

Introduction

Helicobacter pylori are a Gram-negative pathogen that is the first and most important cause of gastric carcinoma along with peptic ulcer and dyspepsia carcinoma. H. pylori is also responsible for mucosa associated with lymphoid tissue

(MALT)and Known Hodgkin Lymphoma (Fox & G, 2012). Moreover, H. pylori are catalase, oxidase, and strong urease producers.

It is assessed that patients having H. pylori are a 30% to 40% possibility of evolving gastric ulcer and a 10% to 20% possibility of developing distal gastric cancer (Myint et al., 2015). There are various factors like environment, the strain of bacteria risk of development of these disorders in the presence of H. pylori infection depends on a variety of bacterial, host, and host types which favor the severity of infection and gastritis in the presence of H. pylori (Ali et al., 2011). Round about 70% of H. pylori is disseminated in developing countries like Pakistan while 30% – 40% is present in developed countries.

There are two major types of H. pylori; the first is gastric Helicobacter species and the second is enterohepatic Helicobacter (nongastric). Both of these Helicobacter species colonize their target organs (Solnick et al., 2007). Gastric Helicobacter species are always related to human infection or have significance for animal models of human Helicobacter infections. The exact mechanism of H. pylori is generally not clear. However, H. pylori can survive in a few hosts, most commonly in man and zoonotic primates ("Helicobacter pylori," 2016; Patel et al., 2014).

H. pylori are a demanding microbe and need supplemented growth media which are complemented with blood or serum for their reproduction. These additional sources of nutrition act as a supplement and also protect from the damaging effect of long-chain fatty acids. The protective mechanism can be achieved by well-defined growth medium supplements, such as IsoVitaleX, β-cyclodextrins, and activated charcoal (Taneera et al., 2008).

Phenotypic detection of H. pylori is still considering a challenge for the last two decades. There are several diagnostic tests including histopathology, rapid urease test, and culture are being used in diagnosing and treating the disease. However, immunohistochemistry (IHC) is now considered a gold standard method for the detection of H. pylori in gastric biopsies. The aim of the study is the application of the immunohistochemical technique for the diagnosis of H. pylori from the gastric biopsy. The objectives of the present study are to detect H. pylori in gastric biopsy using Hematoxylin and eosin stain, to identify the H. pylori in gastric biopsy by immunohistochemistry and to compare the H&E and IHC for H. pylori diagnosis.

Materials and methods

SpecimenCollection:

The study was conducted at the Department of Microbiology Government College University Faisalabad in collaboration with the Department of Histopathology Chughtai Lab (PVT) Lahore. A total of 200 gastric biopsies were collected from the patient with a history of gastritis from 10% neutral buffered formalin (NBF). Patient proforma was also collected with each case. Detailed gross examination of the specimens was carried out and recorded on the proforma. All tissue size was measured and grossly examined.

TissueProcessing:

The principle of tissue processing is based on the removal of extractable water from tissue, replacing it with a support medium that provides sufficient rigidity to enable the sectioning of tissue without damage or distortion. It consists of an exchange of fluids using a series of solutions for a predetermined length of time in a controlled environment(Celio, 2011). The clinical significance of this test is to process and prepare the tissue sections for embedding in paraffin wax. The biopsy samples were processed in an automated tissue processor (Tissue TEK VIP VI Japan). The temperature for processing was adjusted to 370C and Processing was done under variable pressure and vacuumed settings for overnight processing Table 1 explains the complete steps and chemicals involved in tissue processing in an automated tissue processor.

Steps	Reagent	Time Duration	Temperature(C)	P/V	Mix
1-	Formalin	50M	37	P/V	Slow
2-	Formalin	50M	37	P/V	Slow
3-	Isopropanol 70%	30M	37	P/V	Slow
4-	Isopropanol 80%	50M	37	P/V	Slow
5-	Isopropanol 90%	50M	37	P/V	Slow
6-	Isopropanol 100%	50M	37	P/V	Slow
7-	Isopropanol 100%	50M	37	P/V	Slow
8-	Xylene	50M	37	P/V	Slow
9-	Xylene	40M	37	P/V	Slow
10-	Xylene	40M	37	P/V	Slow
11-	Paraffin wax	30M	60	OFF	Slow
12-	Paraffin wax	30M	60	OFF	Slow
13-	Paraffin wax	25M	60	OFF	Slow
14-	Paraffin wax	25M	60	OFF	Slow

Tab 1: Tissue processing

Embedding:

Processed tissues were embedded in blocks with the labeled cassette used during processing as the base of the block containing the same tissue set. Embedding was done using Tissue (Tissue Tek tissue Embedding Console System Germany)

Microtomy:

The paraffin wax blocks with the embedded tissue were cut into sections using (LEICA RM 2125 RT Germany)which moved the blocks across a very sharp knife every 1 to 13 microns. The slices are thinner than the average cell and are floated on a warm water bath, picked up on a glass microscope slide, and dried in a warm oven.

Staining:

By The (Tissue-Tek® DRS™ Japan) automated system Slides were processed by chemical fixation were stained with a combination of hematoxylin and eosin. The DRS 2000 is an automated slide stainer designed for use in histology and cytology. Its operations include staining of tissue sections mounted on glass slides, frozen specimens, and cellular specimens.

Immunohistochemistry Staining:

Antigen retrieval, application of the primary antibody, and visualization system, ending with counterstaining. Antigen retrieval was performed in DAKO antigen retrieval system PT Link, to recover the antigens that may have been altered by fixation. Endogenous enzymes are blocked and a primary antibody is applied that specifically binds to the antigen of interest. The secondary antibody carries the label (enzyme); upon application, it binds to the primary antibody. Chromogen is applied to visualize the antibody/antigen complex. Counterstaining is performed to visualize nuclei and overall tissue architecture. All samples were examined by IHC, and the results were obtained using the rabbit anti-H. Pylori pAb (DAKO, Glostrup, Denmark). Automated IHC staining was performed with fully automated Auto Stainer-link 48Dako)

i- **Mounting Stained Tissue Sections Using DPX**

To preserve and support a stained section for light microscopy, it was mounted on a clear glass slide, and covered with a thin glass coverslip. The slide and coverslip were free of optical distortions, to avoid viewing artifacts. A mounting medium (DPX) is used to adhere the coverslip to the slide.

ii- Microscopy:

The prepared slides were examined using a CX 31 Olympus microscope at 40X by two observers and the results were noted in the Pro- forma. The results of H. pylori Immuno staining were based on the distinct brown staining. This study will give a beneficent outcome in the confirmed diagnosis of H. pylori by using IHC with 100% sensitivity.

Results

Samples sites:

Out of 210 samples, 140 were from Antrum, 36 were from the antrum and body, 13 were from the body,15 were gastric biopsies, 2 were from Antrum and fundus, one superficial biopsy from Antrum, one was from the duodenal and antral site, one from the pylorus and one from prepyloric and gastric body were collected.

Age and Gender

Different age groups of males and females were included in this study. Most of the female patients were from 41-50 years of age and Male patients from 21-30 years and the age ratio was 1.04:1 as shown in Table 2.

Identification of H.pylori by H&E

Helicobacter pylori were detected in 88 cases out of 200 while 09 samples were either poorly preserved or autolyzed that's why further processing and diagnosis were not possible. One case was diagnosed as poorly differentiated adenocarcinoma; the sample was collected from a female patient of 56 years. Out of 88 positive cases, 66 (75%) were initially screened as positive for H.pyloriby H&E staining due to the presence of mild or moderate colonization while the remaining 25% cases were negative by H&E staining but with suspicion of neutrophils and lymphoid aggregates. All 88 cases were further confirmed by IHC.

SITE	Number of Specimens	
Antrum	140	66.7%
Antrum and Body	36	17.1%
Antrum& Fundus	2	1.0%
Antrum (superficial biopsy)	1	0.5%
Body	13	6.2%
Duodenal and antral	1	0.5%
Gastric Biopsy	15	7.1%
Prepyloric& Gastric Body	1	0.5%
Pylorus	1	0.5%

Table 2 Distribution of samples based on the sampling site

Age Group	Female	Male
>10-20	11	5
21-30	15	39
31-40	22	21
41-50	27	19
51-60	17	9
>60	11	14
Total (n=210)	**49%**	**51%**

Table 3:Age Group

H&E Staining									
Age group	**Gender**	**MILD**	**MODERATE**	**NIL**	**Age group**	**GENDER**	**MILD**	**MODERATE**	**NIL**
(>10-20)	F	3	1	7	(>10-20)	M	1	0	1

(21-30)	F	3	3	9	(21-30)	M	20	2	15
(31-40)	F	6	1	13	(31-40)	M	5	2	14
(41-50)	F	6	5	14	(41-50)	M	6	2	9
(51-60)	F	4	1	12	(51-60)	M	4	1	4
(>60)	F	4	2	8	(>60)	M	2	4	6
Total	101	26	13	63		98	38	11	49

Table 4: H&E Staining

Table 4 shows that in female patients the age group (>10-20) showed 3 cases positive with mild colonization while only one case showed moderate colonization by H&E staining as shown in figures 11 and 12, in the age group (21-30) showed 3 cases positive with mild and 3 cases with moderate colonization, age group (31-40) showed 6 cases positive with mild colonization while only one case showed moderate colonization, age group (41-50) showed 6 cases positive with mild colonization and 5 cases showed moderate colonization, age group (51-60) showed 4 cases positive with mild colonization while only one case showed moderate colonization, age group (>60) showed 4 cases positive with mild colonization and two cases showed moderate colonization. 63 cases were negative among all age groups of female patients, 26 were positive with mild colonization and 13 were positive with moderate colonization

In male patients age group (>10-20) showed one case positive with mild colonization while no case showed moderate colonization by H&E staining as shown in figure 11, age group (21-30) showed 20 cases positive with mild colonization while two cases showed moderate colonization as shown in figure 12, age group (31-40) showed 5 cases positive with mild colonization while two cases showed moderate colonization, age group (41-50) showed 6 cases positive with mild colonization while two cases showed moderate colonization, age group (51-60) showed 4 cases positive with mild colonization while only one case showed moderate colonization, age group (>60) showed 2 cases positive with mild colonization while four cases showed moderate colonization. 49 cases were negative among all age groups of male patients. Normal Mucosa was seen as shown in figures 14 and 15. 38 cases were positive with mild colonization and 11 were positive with moderate colonization.

Diagnosis	**H&E and IHC**	
	Mild Colonization	**Moderate Colonization**
Mild Chronic Active Gastritis	25	0
Mild Active Gastritis	16	5
Moderate Active Gastritis	4	14

Chronic Active Gastritis	4	0
Granulomatous Gastritis	6	0
Mild Chronic Gastritis	4	0
Moderate Chronic Active Gastritis	2	0
Severe Active Gastritis	0	5
Mild Gastritis	3	0

Table 5: Confirm Diagnosis by IHC

Table 5 shows 25 cases had mild colonization as shown in figures 11, 12, 13, and 16, and were diagnosed as Mild chronic active gastritis by IHC and H&E, 16 cases showed Mild colonization and 5 cases showed moderate colonization as shown in figure 17, 18 and 19, all were diagnosed as Mild active gastritis, 4 cases had Mild colonization and 14 cases had Moderate colonization, all were diagnosed as Moderate active gastritis, chronic active gastritis was diagnosed in 4 cases with mild colonization, Granulomatous gastritis was diagnosed in 6 cases with mild colonization, four cases were diagnosed as Mild chronic gastritis with mild colonization, moderate chronic active gastritis was diagnosed in two cases with mild colonization while and Mild gastritis was diagnosed in only three cases with Mild colonization while 5 cases with moderate colonization were diagnosed as severe active gastritis.

Diagnostic Method	**IHC Positive**
HEMATOXYLIN AND EOSIN Positive	75%
HEMATOXYLIN AND EOSIN Negative	25%
Total	100%

Table6: IHC and H&E Comparison:

Immunohistochemistry was carried out with Helicobacter pylori immunostain, and all the 66 cases which were initially screened as positive by H&E were positive by IHC and the 22 cases which were negative on H&E were also positive by IHC (Table 1). Among our 210 cases, there were 51% males, and 49% females (Table 2). Age groups were formed and it was observed that mild colonization has a significantly higher percentage in both genders. Among 103 female samples, 39 were positive for H. pylori while out of 107 samples, 49 were positive in men Table 4.

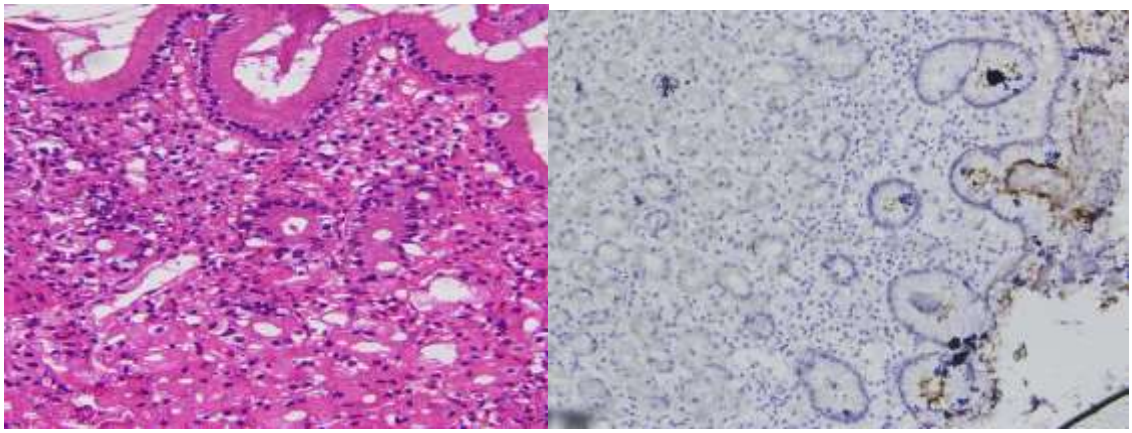
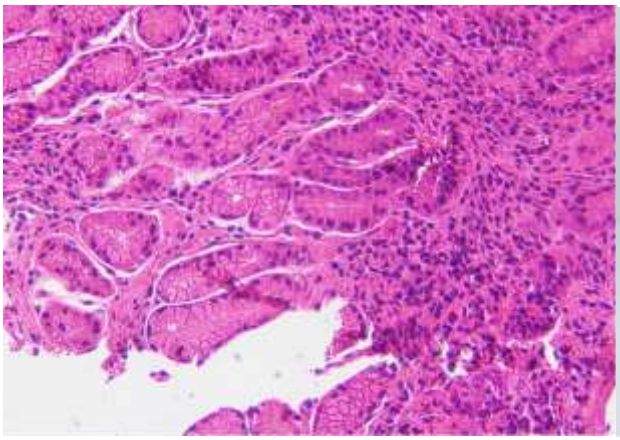


Fig 10: Mild Colonization H&E Staining

Fig 11: Mild Colonization IHC Staining



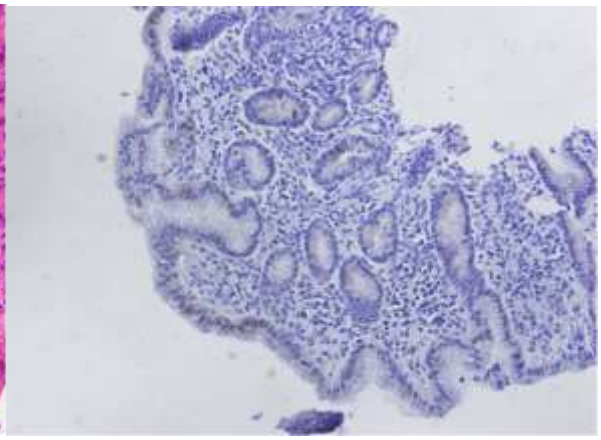
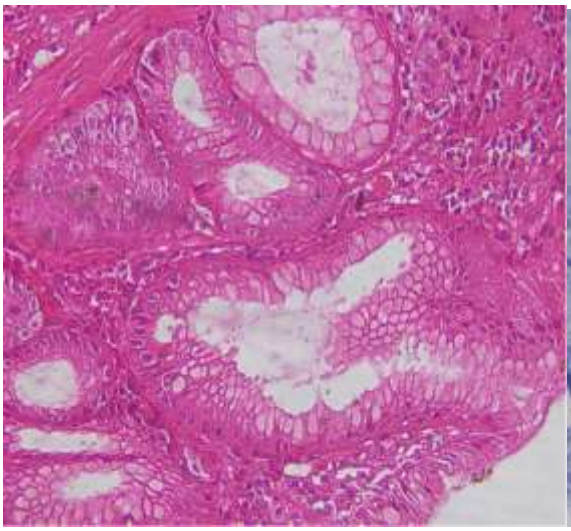


Fig 12: Mild Colonization H&E Staining

Fig 13: Mild Colonization IHC Staining



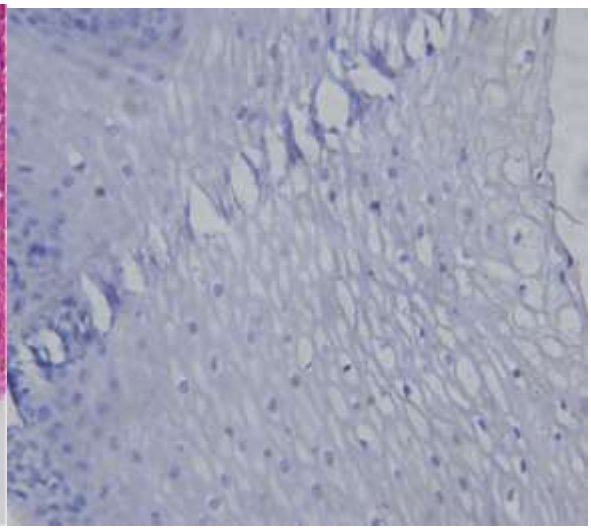


Fig 14: Normal Gastric Mucosa

Fig 15: Normal Gastric Mucosa

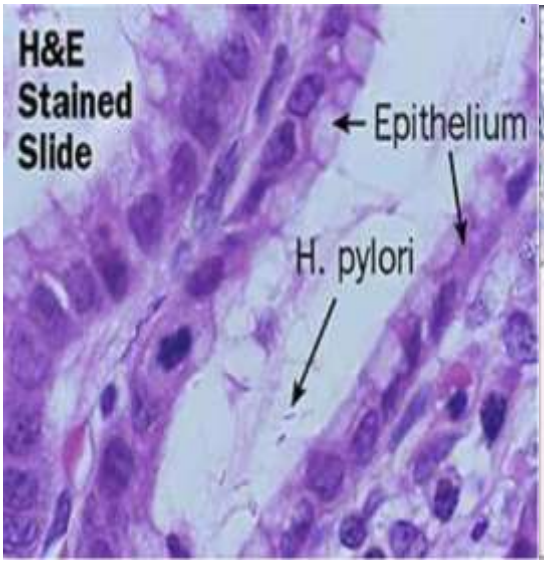


Fig 16: Mild Colonization H&E

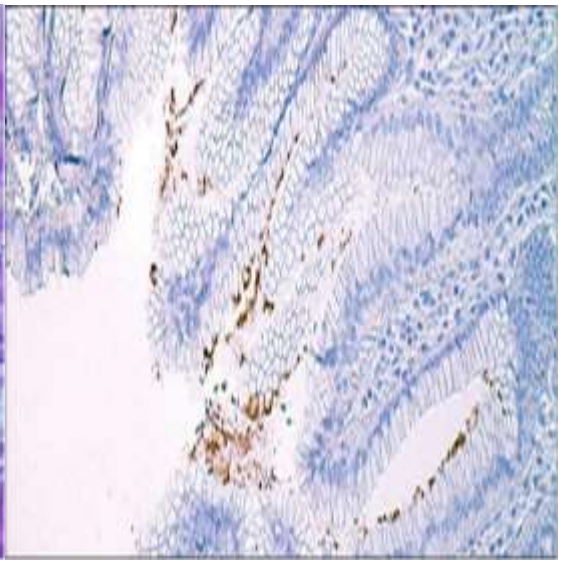


Fig 17: Mild Colonization IHC

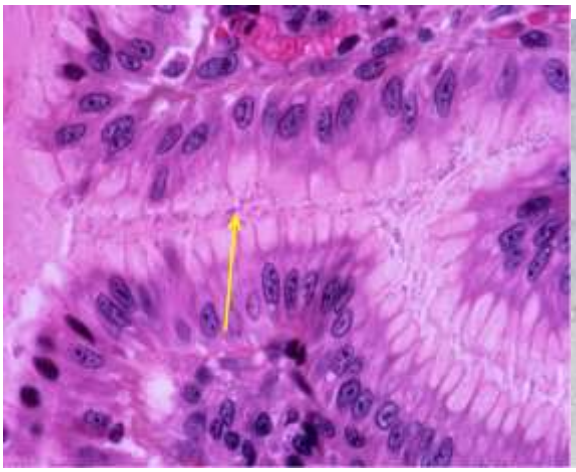


Fig 18: Moderate Colonization H&E

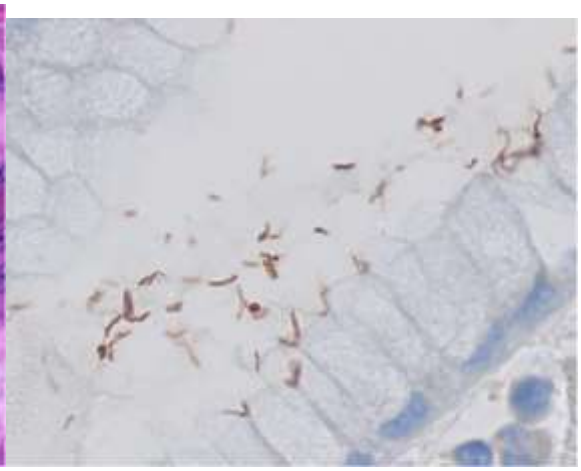


Fig 19: Moderate Colonization IHC

Discussion

In our study, most of the gastric biopsy samples have been collected from the antrum as this is the last part of the stomach with less acid-secreting so H. pylori can easily invade the musical surface by flagella and urease enzyme activity. Only 36 cases obtained from the body are positive due to some non-specific gastritis associated with H. pylori. These findings are also similar to the study conducted in Surabaya, Indonesia by Miftahussurur M. et al. (2015). Furthermore, patients are divided into different age groups including the highly-infected group of males (21-30) and females (31-40). Similar findings were reported in a study conducted in Lahore, Pakistan by Dogar and Qureshy (2012). Routinely H&E is considered the gold standard for the detection of H. pylori in gastric samples along with some special stains Byori (2012), but there are some limitations regarding the detection of H. pylori when the bacterial count is low (mild) H&E reports the moderate and severe cases of gastritis associated with H. pylori.

In our study, we used H&E and IHC for the diagnosis of H. pylori because IHC remains the gold standard for H .pylori detection as compared to other methods Tajalli et al. (2013). In various previous studies IHC has been

reported as the most reliable and accurate staining technique for the detection of H. pyloriSekine et al. (2012), Patel et al. (2014). There are various limitations regarding the reporting of gastritis by H&E such as background staining, pre, and post-treatment patients, and different shapes of bacterial (Cocci and Spiral shapes), similar limitations were also documented by Lee J. Y. and Kim (2015)&Malfertheiner et al. (2012). All the patients (with or without treatment) were reported with IHC, as it is an antigen/antibody-based reaction in which antibodies react with the somatic antigen of bacteria. The patients are diagnosed with active and chronic gastritis with mild and moderate colonization, most patients are reported as having mild active chronic gastritis and our results are supported by the study of Nurdin et al. (2016). The patients are grouped into different age groups, males within the most prominent group 21-30 and females 31-40), these two groups are severely affected, Dogar and Qureshy (2012) also concluded the same results in their study.

Kacar et al. (2011) reported that despite advanced diagnostic methods, immunohistochemical-based detection of H. pylori in gastric biopsy is the most reliable method and other techniques and methods can be compared against IHC. H. pylori can also be detected in good stained routine H&E section; however, detection becomes easier, more reliable, and more accurate using H. pylori immunohistochemical stain. The same techniques of IHC and H&E were utilized by Afzal et al. (2011) for the detection of H. pylori and they reported that the sensitivity and specificity of H. pylori detection by H&E was 75% whereas immunohistochemical staining specificity and sensitivity both became 100%.

In our study, Helicobacter pylori were detected in 88 cases out of 200. Out of 88 positive cases, 66 (75%) were initially screened as positive for H. pylori by H&E staining due to the presence of mild or moderate colonization while the remaining 25% cases were negative by H&E staining but with suspicion of neutrophils and lymphoid aggregates. All 88 cases were further confirmed by IHC. It was observed that in cases of a high bacterial count, only H&E was sufficient for confirmed diagnosis but for cases with lower bacterial load, IHC was necessary to make confirm the diagnosis. Similar findings were reported by Kacar et al. (2011) according to their observation When the bacterial colonization is high routine H&E staining is sufficient to demonstrate them. However, when colonization is mild, the special stain is compulsory for accurate diagnosis. Many studies have reported that immunohistochemistry using H. pylori immunostain is much better and more accurate than H&E as well as special stains for identifying these bacteria. We have reported 66 (38%) cases positive for H. pylori by H&E staining and 88 (45%) positive by immunostaining. These findings are supported by Afzal et al. (2011) who reported that it was found that H. pylori were detected in 38% of patients by H&E and in 58% of patients by immunohistochemistry. In the study of Shimizu et al. (2007)H. pylori were identified by H&E in 70% of biopsies and by immunohistochemistry in 78%. It was also seen that the bacteria were more prominent and much easier to identify in the immuno-stained sections. In the present study, H. pylori were reported to be 44% while Nwodiuko and Okafur (2007) reported positivity as 36.3% but their sample size was small as 80 samples were included in this study, various other studies also reported a low prevalence which might be due to smaller sample size. Our results have been supported by the study of El-Sayed et al. (2005), who reported the prevalence of H. pylori as 45%. In this study of 210 cases, the maximum number of patients fell in the age group 20 – 29 years and was 18 or 25.71%. However, Kacar et al. (2011) found the maximum number of cases in the age group 60 – 69 years. Our study showed that H. pylori-positive patients had a mean age of 21-30 years. This was greater than the mean age of H. pylori-negative patients who was 41.7 years reported by Dogar and Qureshy (2012). Our study however showed Helicobacter pylori detection to be 38% with H&E staining and 44% with immunostaining.

Hidaka et al. (2010) reported that a polyclonal IHC stain (DAKO, B471, Denmark) was highly specific and had a low inter-observer difference when compared to other special stains. However, the necessity for IHC stains has been emphasized in recent years. In this study, we evaluate the value of IHC for the detection of H. pylori in comparison with the H&E stain. The value of H&E stain compared with other special stains in the identification of H. pylori has been discussed by Koenig et al. (2009). The low clinical value of H&E has been reported by Pity et al. (2011). In our study, H&E had a sensitivity of 41.86%, and a specificity of 100%. Therefore, the H&E stain did not

possess the sensitivity needed for an adequate screening test. It was observed that H&E did not find an organism in 3 positive cases with moderate chronic active gastritis. This finding indicated that when H&E fails to detect H. pylori, IHC should be applied to prevent false negative results and validation of positive results.

This study concludes that among the 210 gastric biopsies, the histopathological examination of gastric biopsies is the best-known accurate reliable, and efficient method of diagnosis. Although it is an invasive method, its advantages are more beneficial than its panic procedure. In patients with chronic gastritis who are negative for H. pylori with routine H&E staining, the final diagnosis should be confirmed by IHC staining to avoid any false positivity or negativity in the diagnosis.

Conclusion

H. pylorus is a gastric pathogen that colonizes approximately 50% of the world's population. In the current study, 210 gastric biopsies were included and two staining methods IHC & H&E were evaluated. Out of 210 samples, 140 were from Antrum, 36 were from the antrum and body, 13 were from the body,15 were gastric biopsies, 2 were from Antrum and fundus, one superficial biopsy from Antrum, one was from the duodenal and antral site, one from the pylorus and one from prepyloric and gastric body were collected. Different age groups of males and females were included in this study. Most of the female patients were from 41-50 years of age and Male patients from 21-30 years and the age ratio was 1.04:1. Helicobacter pylori was detected in 88 cases out of 200 while 09 samples were either poorly preserved or autolyzed that's why further processing and diagnosis was not possible. One case was diagnosed as poorly differentiated adenocarcinoma; the sample was collected from a female patient of 56 years. Out of 88 positive cases, 66 (75%) were initially screened as positive for H. pylori by H&E staining due to the presence of mild or moderate colonization while the remaining 25% cases were negative by H&E staining but with suspicion of neutrophils and lymphoid aggregates. All 88 cases were further confirmed by IHC. Immunohistochemistry was carried out with Helicobacterpyloriimmunostain, and all the 66 cases which were initially screened as positive by H&E were positive by IHC and the 22 cases which were negative on H&E were also positive by IHC. Among our 210 cases, there were 51% males, and 49% females. Age groups were formed and it was observed that mild colonization has a significantly higher percentage in both genders. Among 103 female samples, 39 were positive for H. pylori while out of 107 samples, 49 were positive in men. In conclusion, H&E alone is not recommended as the diagnostic tool all gastric biopsies should be confirmed by IHC for the H. Pylori infection as a diagnosis.

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