Prevalence of non-*Helicobacter pylori* species in patients presenting with dyspepsia

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Abstract

**Background:** Helicobacter species associated with human infection include *Helicobacter pylori*, *Helicobacter heilmannii* and *Helicobacter felis* among others. In this study we determined the prevalence of *H. pylori* and non-*Helicobacter pylori* organisms *H. felis* and *H. heilmannii* and analyzed the association between coinfection with these organisms and gastric pathology in patients presenting with dyspepsia. Biopsy specimens were obtained from patients with dyspepsia on esophagogastroduodenoscopy (EGD) for rapid urease test, histology and PCR examination for Helicobacter genus specific 16S rDNA, *H. pylori* phosphoglucosamine mutase (*glmM*) and urease B (*ureB*) gene of *H. heilmannii* and *H. felis*. Sequencing of PCR products of *H. heilmannii* and *H. felis* was done.

**Results:** Two hundred-fifty patients with dyspepsia were enrolled in the study. The mean age was 39 ± 12 years with males 162(65%). Twenty-six percent (66 out of 250) were exposed to cats or dogs. PCR for Helicobacter genus specific 16S rDNA was positive in 167/250 (67%), *H. pylori* *glmM* in 142/250 (57%), *H. heilmannii* in 17/250 (6%) and *H. felis* in 10/250 (4%), respectively. All the *H. heilmannii* and *H. felis* PCR positive patients were also positive for *H. pylori* PCR amplification. The occurrence of coinfection of *H. pylori* and *H. heilmannii* was 17(6%) and with *H. felis* was 10(4%), respectively. Only one out of 66 exposed to pets were positive for *H. heilmannii* and two for *H. felis*. Histopathology was carried out in 160(64%) of 250 cases. Chronic active inflammation was observed in 53(56%) (p = 0.001) of the patients with *H. pylori* infection alone as compared to 3(37%) (p = 0.73) coinfected with *H. heilmannii* and two for *H. felis*. Intestinal metaplasia was observed in 3(3%) (p = 1.0) of the patients with *H. pylori* infection alone as compared to 2(25%) (p = 0.02) coinfected with *H. heilmannii* and *H. pylori* and 1(20%) coinfected with *H. felis* and *H. pylori* (p = 0.15).

**Conclusion:** The prevalence of *H. heilmannii* and *H. felis* was low in our patients with dyspepsia. Exposure to pets did not increase the risk of *H. heilmannii* or *H. felis* infection. The coinfection of *H. pylori* with *H. heilmannii* was seen associated with intestinal metaplasia, however this need further confirmation.

**Keywords:** Dyspepsia, gastric biopsies, *H. pylori*, *H. heilmannii*, *H. felis*, coinfection, cats, dogs

Background

*Helicobacter* species infect the gastrointestinal tracts of many animals from birds through humans. Some of these have been linked to a range of human diseases [1,2] including chronic gastritis, peptic ulcer disease, mucosa-associated lymphoid tissue lymphoma, and gastric adenocarcinoma [1,3]. The principal Helicobacter infection in humans is *Helicobacter pylori*, with infection rates in developing countries reaching 50% to 90% [2,4]. Human gastric biopsy samples, however, have shown to harbor bacteria which were morphologically different from *H. pylori* [5,6]. These include *Helicobacter heilmannii* and *Helicobacter felis* which are primarily pathogens of domestic animals and were later found to infect humans as well [7-9].

Gastric non-*Helicobacter pylori* helicobacters constitute a diverse group of bacterial species that are known to colonize the gastric mucosa of several animals [10]. These include morphologically distinct, typically long spiral shaped bacteria originally referred to as *Gastrospirillum hominis* and later as *H. heilmannii*. The latter was...
further subdivided in two taxa, types 1 and 2 [10]. *H. heilmannii* type 1 are identical to *H. suis* which colonizes the stomachs of pigs. The former *H. heilmannii* type 2 represent a group of species, known to colonize the gastric mucosa of dogs and cats and include *H. felis*, *H. bizzozeronii*, *H. salomonis*, *H. cynogastricus*, *H. baculiformis* and a bacterium provisionally named in 2004 as “Candidatus *H. heilmannii*” because at that time, it could not be cultured in vitro [10,11]. However, recently, in vitro cultures have been obtained resulting in description of *H. heilmannii*, as a novel Helicobacter species [12]. Sequencing of the 16S or 23S rRNA-encoding genes allows differentiation of *H. suis* from the other gastric non-*H. pylori* helicobacters species, but it cannot distinguish between *H. felis*, *H. bizzozeronii*, *H. salomonis*, *H. cynogastricus*, *H. baculiformis* and *Candidatus* *H. heilmannii* [10]. For differentiation between these species, sequencing of the heat shock protein 60 (hsp60) or gyrase B (gyrB) gene is used while sequencing of the urease A and B genes is considered to be the most suitable method since sequences of these genes are available [10,11,13,14].

Dyspepsia describes a variety of symptoms, including abdominal pain, bloating, nausea, and vomiting. In these patients, endoscopy is considered to rule out gastroesophageal reflux disease, peptic or duodenal ulcer and gastric cancer. The role of *H. pylori* infection in dyspepsia remains controversial. This study aims to identify the prevalence of *H. pylori* and non-*H. pylori* helicobacters, *H. felis* and *H. heilmannii* and to analyze the gastric pathology associated with co-infection of these organisms in patients presenting with dyspepsia.

**Results and discussion**

Majority of the patients with *H. pylori* infection were in the age range of 18-39 years, while *H. felis* and *H. heilmannii* positive patients did not show this distribution. (Table 1). There was no difference in the gender, ethnicity of patients, crowding index (CI) and source of water distribution among the patients with *H. pylori* and non-*H. pylori* infections (Table 1). All patients had abdominal pain with endoscopic gastritis as the predominant finding. The false positive and false negative results obtained with RUT were 15(36%) and 6(12%), respectively while with histology the false positive and false negative results obtained were 20(30%) and 10(11%), respectively (Table 1-2).

PCR for Helicobacter genus specific 16S rDNA was positive in 167/250 (67%), *glmM* (*H. pylori*) in 142/250 (57%), *H. heilmannii* in 17/250 (6%) and *H. felis* in 10/250 (4%), respectively (Table 2).

PCR was positive for both *H. pylori* and *H. heilmannii* in 17(6%) and for *H. pylori* and *H. felis* in 10(4%), respectively (Table 2). All the *H. heilmannii* and *H. felis* positive patients were also positive for *H. pylori glmM* PCR amplification (Table 2).

26% (66 out of 250) were exposed to pets either cats or dogs. Most *H. heilmannii* positive patients did not have pet contact. Only one out of 66 exposed to pets was positive for *H. heilmannii* and two for *H. felis* (Table 3).

A higher degree of bacterial density was associated with *H. pylori* infection alone (p < 0.001) (Table 1). Chronic active inflammation was observed in 53(56%) cases with *H. pylori* alone infection (p = 0.001) compared to 3(37%) in *H. heilmannii* (p = 0.73) and 3(60%) in *H. felis* positive patients coinfected with *H. pylori* (p = 0.66) (Table 1). Intestinal metaplasia (IM) was present in 3(3%) out of 94 cases with *H. pylori* infection alone compared to 2(25%) out of 8 cases of *H. heilmannii* and *H. pylori* coinfected, and 1(20%) out of 5 cases of *H. felis* and *H. pylori* coinfecion in which histology has been performed.

PCR product sequences were compared to the sequences of ureaseB of different *H. heilmannii* and *H. felis* strains. The *H. heilmannii* sequences had 100% similarity to ‘Candidatus Helicobacter heliannii’ strains GenBank: AF508012 and L25079; while it was 99% to GenBank:AY139170, AF507996, AY139172, AY139173 and 98% to GenBank: AY139171, respectively. The *H. felis* sequences had 100% similarity to *H. felis* strains GenBank: FQ670179 and X69080; while it was 99% to *H. felis* GenBank: AY368267, AY368261 and 98% to GenBank: DQ865138, respectively.

Among our patients, the cohort exposed to pet animals was limited to 26%. There were more patients with *H. pylori* infection who were in the 18-39 years age range. Such age distribution was not seen in cases with *H. felis* and *H. heilmannii* infection. There was no difference in the gender, ethnicity of patients, crowding index (CI) and source of water distribution among the patients with *H. pylori* and non-*H. pylori* helicobacters, *H. felis* and *H. heilmannii* and to analyze the gastric pathology associated with co-infection of these organisms in patients presenting with dyspepsia.
Table 1 Demography and clinical features of patients enrolled

|                      | PCR for H. pylori | PCR for H. heilmannii | PCR for H. felis |
|----------------------|-------------------|-----------------------|-----------------|
|                      | Positive n = 142  | Negative n = 108      | Positive n = 17  | Negative n = 233 | Positive n = 10 | Negative n = 240 |
| **Age**              |                   |                       |                 |                   |                 |                   |
| 18-39 years          | 81(57)            | 55(51)                | 8(47)           | 128(55)           | 6(60)           | 130(55)           |
| 40-55 years          | 53(37)            | 35(32)                | 0.02            | 74(41)            | 81(35)          | 0.82              |
| 56-75 years          | 8(6)              | 18(17)                | 0.02            | 2(12)             | 24(10)          | 0.02              |
| **Gender**           |                   |                       |                 |                   |                 |                   |
| Male                 | 98(69)            | 64(59)                | 0.11            | 11(65)            | 151(65)         | 0.99              |
| Female               | 44(31)            | 44(41)                | 6(35)           | 82(35)            | 5(50)           | 83(35)            |
| **Ethnicity**        |                   |                       |                 |                   |                 |                   |
| Karachiite           | 36(25)            | 36(33)                | 0.15            | 5(29)             | 73(31)          | 0.81              |
| Quetta resident      | 47(36)            | 31(25)                | 0.15            | 5(29)             | 73(31)          | 0.81              |
| Afghan               | 55(39)            | 45(42)                | 0.15            | 8(47)             | 92(40)          | 0.15              |
| **Crowding Index (CI)** |               |                       |                 |                   |                 |                   |
| 0-1 (low)            | 59(41)            | 39(36)                | 4(29)           | 93(40)            | 5(50)           | 93(40)            |
| 2-4 (moderate)       | 82(58)            | 62(57)                | 0.03            | 12(71)            | 132(57)         | 0.35              |
| > 4 (crowding)       | 1(1)              | 7(7)                  | 0.03            | 0(0)              | 8(3)            | 0.03              |
| **Water supply**     |                   |                       |                 |                   |                 |                   |
| Municipal            | 86(61)            | 59(55)                | 0.34            | 8(47)             | 137(59)         | 0.34              |
| Boring Water         | 56(39)            | 49(45)                | 0.34            | 9(53)             | 96(41)          | 0.34              |
| **EGD**              |                   |                       |                 |                   |                 |                   |
| Gastritis            | 136(96)           | 106(96)               | 0.47            | 17(100)           | 225(97)         | 1                 |
| Duodenal ulcer       | 6(4)              | 2(4)                  | 0.03            | 0(0)              | 8(3)            | 0.03              |
| **Rapid Urease test (n = 90)** |       |                       |                 |                   |                 |                   |
| Positive             | 42(88)            | 15(36)                | > 0.001         | 46(44)            | 53(63)          | 0.28              |
| Negative             | 6(12)             | 27(64)                | 0.01            | 5(56)             | 28(37)          | 0.01              |
| **Histopathology (n = 160)** |       |                       |                 |                   |                 |                   |
| Bacterial density    |                   |                       |                 |                   |                 |                   |
| Occasional           | 10(11)            | 40(61)                | 0.34            | 3(37)             | 47(31)          | 0.03              |
| Few in some fields   | 59(63)            | 21(32)                | > 0.001         | 2(25)             | 78(51)          | 0.27              |
| Only 1/2 small clusters | 25(27)           | 5(7)                  | 0.01            | 3(38)             | 27(18)          | 0.01              |
| Inflammation type    |                   |                       |                 |                   |                 |                   |
| Chronic              | 41(44)            | 47(71)                | 0.001           | 5(63)             | 83(55)          | 0.73              |
| Chronic active inflation | 53(56)         | 19(29)                | 0.01            | 3(37)             | 69(45)          | 0.35              |
| Lymphoid follicles   |                   |                       |                 |                   |                 |                   |
| Positive             | 14(15)            | 8(12)                 | 0.73            | 0(0)              | 22(14)          | 0.30              |
| Negative             | 80(85)            | 58(88)                | 0.73            | 10(100)           | 90(96)          | 0.73              |
| Intestinal metaplasia|                   |                       |                 |                   |                 |                   |
| Positive             | 3(3)              | 2(3)                  | 1.0             | 2(25)             | 3(2)            | 0.02              |
| Negative             | 91(97)            | 64(97)                | 1.0             | 67(75)            | 149(98)         | 1.0               |

Univariate analysis was performed by using the independent sample t-test, Pearson Chi-square or Fisher Exact test where appropriate. A p-value of <0.05 was considered as statistically significant. *All the H. heilmannii and H. felis PCR positive patients were also positive for H. pylori PCR amplification.
positive for *H. pylori* glmM PCR (Table 2). PCR product sequences of ureaseB gene of *H. heilmannii* had shown 100% similarity to ‘Candidatus *H. heilmannii* strains’ GenBank: AF508012 and L25079; while *H. felis* sequences had shown 100% similarity to strains GenBank: FQ670179 and X69080.

In this study, we used urease gene-based PCR method developed by Niegèr et al that detected only ‘Candidatus *H. heilmannii*’ DNA from pure in vitro cultures of other non-*H. pylori* helicobacter species [14]. This method was also used by other investigators to demonstrate the presence of *Candidatus *H. heilmannii* DNA in gastric biopsies from patients with dyspepsia [11,15,16]. The limitations of our study include the small number of patients who had non-*H. pylori* helicobacter infection and the presence of *H. pylori* co-infection which precluded assessment of the histological effect of these species under consideration. Also, the significance of coinfection in terms of disease development could not be determined. We could have identified few more cases of non-*H. pylori* helicobacter species by other reported methods used to study non-*H. pylori* helicobacter species including fluorescent in situ hybridization (FISH), transmission electron microscopy (TEM) and partial 16S ribosomal sequencing for analyses of the amplified products [12,17].

The implications of this study are that non-*H. pylori* helicobacter species are capable of producing complications similar to *H. pylori* so the identification of these species may be of importance in patients with dyspepsia. However, our study fails to show any increased risk of infection with these organisms on exposure to pet animals and any additional complications associated with co-infection in patients infected with *H. pylori*.

### Conclusion

As non-*H. pylori* Helicobacter species are capable of producing complications similar to *H. pylori* so the identification of these species may be of importance in patients with dyspepsia. However, our study fails to show any increased risk of infection with these organisms on exposure to pet animals and any additional complications associated with co-infection in patients infected with *H. pylori*.

### Methods

#### Study population

Between September 2009 and February 2011, a total of 250 patients with abdominal pain or discomfort who attended the gastroenterology outpatient clinic at a tertiary care hospital in Karachi were enrolled. The mean age of these patients was 39 ± 12 years, (range 18-75) with males 162(65%) and females 88(35%). Of these, 136 (54%) were in the age group of 18-39 years, 88(35%) in the group of 40-55 years and 26(10%) in the group of 56-75 years. Ethical approval for the study was obtained from the Aga Khan University Ethics Review Committee. Informed consent was taken for participation in the study. A complete socio-demographic questionnaire including determination of socio-economic status, educational level, ownership of the place of residence, number of rooms in the house, number of people living in the household beside siblings, source of water supply e.g. municipal water pipeline or bore water (ground water) and type of latrine in use, was obtained from the patients.

A history of exposure of enrolled patients to cats and dogs was determined and a physical examination was carried out. Inclusion criteria were i) ambulatory adult
Table 3 Association of Helicobacter species with pets

| Pets | PCR for H. pylori | PCR for H. helimannii | PCR for H. felis |
|------|------------------|----------------------|-----------------|
|      | Positive n = 142 | Negative n = 108     | P value         |
|      | Positive n = 17  | Negative n = 233     | P value         |
|      | Positive n = 10  | Negative n = 240     | P value         |
| Yes  | 42(30)           | 24(22)               | 0.19            |
|      | 1(6)             | 65(28)               | 0.05            |
|      | 2(20)            | 64(27)               | 1               |
| No   | 100(70)          | 84(78)               |                 |
|      | 16(94)           | 168(72)              |                 |
|      | 8(80)            | 176(73)              |                 |

Univariate analysis was performed by using the independent sample t-test, Pearson Chi-square test or Fisher Exact test where appropriate. A P-value of < 0.05 was considered as statistically significant. *All the H. helimannii and H. felis PCR positive patients were also positive for H. pylori PCR amplification.
males and non-pregnant females; ii) age 18 years or older; iii) patients with upper GI symptoms including abdominal/epigastric pain or discomfort, postprandial abdominal distension, postprandial nausea and vomiting. Exclusion criteria included i) receiving treatment for *H. pylori*, concurrent or recent antibiotic use such as metronidazole, clarithromycin, amoxicillin, tetracycline, doxycycline and other cephalosporin, ii) histamine-2 receptor blocker or proton pump inhibitor therapy and bismuth compounds in the last four weeks; iii) patients with regular use of NSAID; iii) patients with severe concomitant disease and iv) patients with upper GI surgery. A crowding index with three categories was constructed by dividing the number of individuals per household by the number of the rooms used as bedrooms [23]. A participant’s household crowding was defined as ‘low’ if they scored an index of 0-1.0, moderately-crowded were ‘2-4’ and > 4 were highly ‘crowded’.

On EGD, 242(97%) were found to have endoscopic gastritis (GS) alone while 8(3%) had duodenal ulcer (DU). Biopsy specimens from the gastric corpus and antrum were taken for rapid urease test (RUT) or histopathology for the diagnosis of *H. pylori* and DNA extraction for polymerase chain reaction (PCR) to amplify *H. pylori*, *H. felis* and *H. heilmannii* genes. Ninety patients (36%) out of 250 had a RUT done while 160(64%) out of 250 had histology and provided gastric biopsy specimen for the detection of Helicobacter species.

**Histopathology**

Biopsy specimens were stained with hematoxylin and eosin. Sections were examined by an experienced gastrointestinal pathologist blinded to the clinical details of the patients and graded according to the updated Sydney classification [24]. The bacterial density was graded from 0 to 3 (0, absent or occasional; 1 to 3, from few and isolated bacteria to colonies). The infiltration of gastric mucosa by mononuclear cells and polymorphonuclear leucocytes, atrophy, and intestinal metaplasia were graded as follows: 0, none; 1, mild; 2, moderate; 3, marked. Chronic inflammation was defined according to an increase in lymphocytes and plasma cells in the lamina propria graded into mild, moderate or marked increase in density. Chronic active gastritis indicated chronic inflammation with neutrophilic polymorph infiltration of the lamina propria, pits or surface epithelium graded as 0 = nil, mild = < 1/3 of pits and surface infiltrated; moderate = 1/3-2/3; and marked = > 2/3. Gastritis was scored by total sum of grade of gastritis (mild = 1, moderate = 2, marked = 3 infiltration with lymphocytes and plasma cells) and activity of gastritis (mild = 1, moderate = 2, marked = 3 infiltration with neutrophilic granulocytes) either in the antrum or in the corpus. Atrophy was defined as the loss of glandular tissue, with or without replacement by intestinal-type epithelium. Criteria for a true positive result was established with positive RUT or histology and 16S rDNA amplification.

**DNA Extraction**

DNA was extracted from biopsy samples by using a QIAamp DNA mini kit from QIAGEN (Hilden, Germany) according to the manufacturer’s protocol. Extracted DNA was stored at -70°C until required.

**Polymerase chain reaction**

PCR was performed using extracted DNA as the template to identify *H. pylori*, *H. heilmannii* and *H. felis*. Samples that were positive for Helicobacter genus 16S rDNA were subsequently analyzed with different sets of previously published primers (Table 4) which encode *H. pylori* phosphoglucomutase (*glmM*), *H. heilmannii* ureB and *H. felis* internal fragment of the *ureA* and *ureB* genes, respectively [14,21,25,26]. PCR amplification was carried out in a total volume of 25 μl containing 2 μl of 2 mM dNTPs, 1 μl of 50 pmol of each forward and reverse primer used before [14,25-27]. (synthesized by

| Table 4 Oligonucleotide primers used in this study to amplify Helicobacter spp. gene fragments |
|-----------------------------------------------|-----------------------------------------------|-----------------|-----------------|
| **Gene**                                      | **Sequence (5’ to 3’)**                         | **Amplicon size (bp)** | **Reference**   |
| Helicobacter 16S rRNA                         |                                               |                 |                 |
| C97                                          | GCT ATG ACG GGT ATC C                          | 400             | 18              |
| C98                                          | GAT TTT ACC CCT ACA CCA                        |                 |                 |
| *H. pylori* glmM                              |                                               |                 |                 |
| F                                            | GGAATAAGGTTTTAGGGAAGTTAGGGG                     | 294             | 19              |
| R                                            | GCCTATTTCTAACATACGCGC                         |                 |                 |
| *H. heilmannii* ureB                         |                                               |                 |                 |
| F                                            | GGGCGATAAAGTGCGCTG                             | 580             | 14              |
| R                                            | CTTGCTAAATGAGACGAG                            |                 |                 |
| *H. felis* ureA and B                         |                                               |                 |                 |
| F                                            | GTG AAG CGA CTA AAG ATA AAC AAT                | 241             | 20              |
| R                                            | GCA CCAAAT CTA ATT CAT AAG AGC                |                 |                 |
MWG Automatic synthesizer, Germany), 2.5 unit of Taq DNA polymerase (Promega, USA), 2.5 μl of 10 x PCR reaction buffer, 3 mM of MgCl₂, 2 μl of DNA template containing 0.5 ng of extracted DNA and total volume rounded to 25 μl by double distilled water. The reaction was carried out in a Perkin Elmer 9700 thermal cycler (Massachusetts, USA). The amplification cycles for the different Helicobacter spp. gene fragments were: 94°C for 5 min; 94°C for 1 min, 55°C-58°C for 1 min, 72°C for 60–90 sec (35 cycles); 72°C for 5-7 min. Positive and negative reagent control reactions were performed with each batch of amplifications. After PCR, the amplified PCR products were electrophoresed in 2% agarose gels containing 0.5 x Tris/acetate/ethylenediaminetetraacetic acid, stained with ethidium bromide, and visualized under a short wavelength ultraviolet light source. DNA from *H. pylori* strains ATCC 43504, *H. felis* ATCC 49179 and *H. heilmannii* JF804941.1 was used as a positive control and sterile deionized water as the negative control. Diagnosis of each of the *Helicobacter* species infection was established when Helicobacter genus PCR for 16S rDNA was positive along with a species specific PCR for *H. pylori, H. heilmannii* or *H. felis*. PCR product of *H. heilmannii* and *H. felis* were sequenced to further confirm individual infection. The specificity of *H. pylori* phosphoglucomutase (glmM) and segment of urease B primers for *H. heilmannii* and *H. felis* has been demonstrated previously [14,21,25-27].

**Sequencing of PCR product and BLAST Query**

The DNA fragments amplified by *H. felis* and *H. heilmannii* PCRs were purified by Qiaquick quick PCR purification kit (Qiagen, USA) and sequenced using both the forward and reverse primers (Table 4) to verify that they represented truly the *H. felis* and *H. heilmannii* ureB gene. Sequence analysis was performed by Macrogen (Seoul, South Korea). ClustalX was used to edit the sequences. The sequences were edited to a length of 488 bp for *H. heilmannii* and 210 bp for *H. felis*. Homology of the DNA sequences to published sequences was determined by using BLAST window on the National Center for Biotechnology Information (NCBI) site at http://www.ncbi.nlm.nih.gov/BLAST.

**Nucleotide sequence accession numbers**

The sequenced PCR products of *H. heilmannii* and *H. felis* obtained in this study have been deposited in GenBank under the following accession numbers: JF804941, JF804942, JF804943, JF804944, JF804945, JF815095, JF815096, JF815097, and JF815098. PCR product sequences were compared to the sequences of Urease B of *H. heilmannii* sequences GenBank: AF508012, L25079.1, AY139171.0, AY139171.1 and *H. felis* strains ref GenBank: FQ 6701792, AY368267.1 and AY368261.1.

**Statistical Method**

Using software EPI Info and using 10% prevalence in the study population [21] with 95% confidence level and a bound on error of ± 4% the estimated sample size was 217.

Results are expressed as mean ± standard deviation for continuous variables (e.g., age) and number (percentage) for categorical data (e.g., gender, etc.). Univariate analysis was performed by using the independent sample t-test, Pearson Chi-square test and Fisher Exact test whenever appropriate. A *P*-value of < 0.05 was considered as statistically significant. All *p* values were two sided. Statistical interpretation of data was performed by using the computerized software program SPSS version 19.

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**Authors' contributions**

JY conceived and designed the study, JY, ZAB, RK, WJ coordinated the study, JY, SN, FJ did the work, JY and ZA analyzed the data, ZAH analyzed the histopathology, JY, MI, SA performed the statistical analysis. JY wrote the manuscript. All authors read and approved the final manuscript.

**Competing interests**

The authors declare that they have no competing interests.

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