Serum antibodies to selected *Helicobacter pylori* antigens are associated with active gastritis in patients seen at the University Teaching Hospital in Lusaka, Zambia

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Abstract

**Introduction**

Little is known about specific bacterial characteristics of *Helicobacter pylori* (H. pylori) infection influencing gastric carcinogenesis in Zambia. The aim of this study was to evaluate the associations between pre-selected *H. pylori* antibodies with gastric cancer, premalignant lesions and active gastritis.

**Methods**

This was cross-sectional study with multiple comparisons of patients with gastric cancer (GC), gastric premalignant (GP) lesions and active or chronic gastritis. A fluorescent bead-based antibody multiplex serology assay was used to quantify antibodies to thirteen immunogenic *H. pylori* antigens. Logistic regression models were used to examine the associations.

**Results**

Included were 295 patients with: 59 GC, 27 GP lesions, 48 active and 161 chronic gastritis. Overall, 257/295 (87%) were *H. pylori* positive. *H. pylori* seropositivity was not associated with sex, age, body mass index, socio-economic status, HIV infection, alcohol consumption or cigarette smoking (p-values all above 0.05). When compared to the patients with chronic gastritis, the presence of catalase and cinnamyl alcohol dehydrogenase (Cad) antibodies was positively associated with GP lesions (OR 3.53; 95% CI 1.52-8.17 and OR 2.47; 95% CI 1.08-5.67 respectively). However, seropositivity to Cad antibodies was significantly lower in GC patients (OR 0.28; 95% CI 0.09-0.83). Compared to chronic, active gastritis was significantly associated with (p<0.05) *H. pylori* sero-positivity (OR 4.96; 95% CI 1.25-17.52) and specific antibodies including cytotoxin-associated gene A, vacuolating cytotoxin A, *Helicobacter cysteine-rich* protein C, hypothetical protein HP0305 and outer membrane protein HP1564.

**Conclusions**

Among Zambian patients seen at a single center, antibodies to *H. pylori* (CagA, VacA, Omp, HcpC, HP0305 and HpaA) were associated with active gastritis.

**Key words:** Gastric cancer; *Helicobacter pylori*, Zambia

**Introduction**

Gastric cancer is the fifth most common malignancy in the world, but due to lack of population-based cancer registries the exact incidence in sub-Saharan Africa (sSA) is unknown. In Zambia, a disconcerting trend of increased occurrence of gastric cancer particularly among adults below the age of 60 years has been observed in hospital records, with up to 25% of the patients being below the age of 45 years.

*Helicobacter pylori* (H. pylori) is the most important cause for non-cardia gastric cancer. *H. pylori* is a gram negative, helical, *microaerophillic bacterium* believed to infect almost half of the world’s population. It is usually acquired during childhood and if untreated, persists throughout the lifetime of the host, causing chronic gastritis. However, most infected individuals do not develop clinical symptoms, perhaps as a result of the co-evolution between *H. pylori* and Homo sapiens. There are great variations in the prevalence of *H. pylori* globally, with Africa having the highest prevalence. *H. pylori* genetic signatures of African strains are uncommon in non-African populations. There are also variations in predominant genes
such as cytotoxin-associated gene A (CagA) or vacuolating cytotoxin A (VacA) positivity. These variations are thought to contribute towards differences in H. pylori-related disease manifestation. For example, gastric cancer is not necessarily more common in regions with the highest H. pylori prevalence.

In addition, varying host responses are believed to influence the development of H. pylori-associated disease including genetics, immune responses and the relationship of the host to specific bacterial virulence factors. With these large variations in H. pylori-associated gastric cancer risk, characterization of bacterial diversity is crucial for identification of high-risk populations and informing decisions about management of H. pylori infection both at individual and population levels.

The H. pylori multiplex serology assay that we used measures antibody responses to 13 immunogenic H. pylori proteins, including above-mentioned virulence factors CagA and VacA. The assay was previously applied in various gastric cancer case-control and prospective studies identifying serological markers associated with increased gastric cancer risk. To our knowledge, this assay has not yet been used on samples from SSA, despite the high H. pylori prevalence in this region. In Zambia, the prevalence of H. pylori (determined using commercially available antibody kits), among healthy community volunteers is 81% (n=221) and we previously failed to demonstrate any association of CagA expression and gastric cancer.

The aim of this study was to evaluate associations of seropositivity to specific H. pylori antigens with gastric cancer or gastric premalignant lesions using the above mentioned multiplex assay. In addition, we evaluated the association with active gastritis. The University of Zambia Biomedical Research Ethics Committee (reference number 003-03-16) and the Zambia National Health Research Authority approved this study.

Methods

Study population and collection of samples

This was a cross-sectional study conducted at the University Teaching Hospital (UTH) in Lusaka, Zambia. UTH is the largest referral hospital in Zambia, attending to patients from all ten provinces of the country. The study was conducted between August 2016 and April 2018. All consenting patients referred for oesophagogastroduodenoscopy (OGD) were considered for enrolment. Written and fully informed consent was obtained from adults above the age of 18 years before enrolment. We excluded patients with a history of caustic ingestion and those with a prior diagnosis and/or treatment for cancer. During the OGD, six biopsies were taken from any gastric lesions (both malignant and benign). To assess for gastric premalignant lesions, two biopsies each were taken from the antrum, incisura and body. Biopsies were then fixed in formalin for histopathological analysis.

After the OGD, 10 ml of blood was also collected from each patient and serum was extracted and kept frozen at -80°C prior to testing as indicated below.

Group classification

Patient groups were classified as follows:

i. Gastric cancer cases: Patients with histologically confirmed gastric cancer which was either cardia or non-cardia. Also included in this group were (n=4) patients with clearly visible gastric tumours whose only available biopsies showed high-grade dysplasia. For the present study, we only analysed non-cardia gastric cancers, as this is the subtype with the strongest link to H. pylori.

ii. Gastric premalignancy cases: Patients with chronic atrophic gastritis without intestinal metaplasia, gastric intestinal metaplasia (GIM) or low-grade dysplasia were grouped together as having premalignant lesions.

iii. Active gastritis cases: Included in this group were patients with inflammation showing polymorphonuclear neutrophils.

iv. Chronic gastritis controls: These patients were employed as the comparison group. They had non-atrophic gastritis (NAG) without any evidence of active inflammation.

For each patient, a global diagnosis was made based on the most severe histological diagnosis. 12 The Operative Link for Gastritis Assessment (OLGA) and Operative Link on Gastritis/Intestinal-Metaplasia Assessment (OLGIM) staging systems were used to stratify GP patients for potential gastric cancer risk. An experienced histopathologist (AS) evaluated all the tissue sections and a second pathologist with specific expertise in gastric premalignant lesions (MBP) provided the final classification of premalignant lesions. Testing for Human Immunodeficiency Virus (HIV) was done using Uni-Gold™ rapid diagnostic kits (Trinity Biotech, Wicklow, Ireland).

H. pylori antibody, urease and histology testing

H. pylori multiplex serology testing was performed as described previously. Briefly, thirteen H. pylori proteins were recombinantly expressed as glutathione-S-transferase-tag fusion proteins and affinity-purified on fluorescently coupled polystyrene beads (Luminex Corp., Austin, Tx, USA). The thirteen H. pylori proteins evaluated included chaperonin HS60 (GroEL), urease alpha subunit (UreA), hypothetical proteins HP0231 and HP0305, neutrophil activating protein A (NapA), neuraminilactose-binding hemagglutinin homolog (HpaA), cytotoxin associated gene A (CagA), hydantoin utilization protein A (HyuA), catalase, vacuolating cytotoxin A (VacA), Helicobacter cysteine-rich protein C (HcpC), cinnamyl alcohol dehydrogenase (Cad) and outer membrane protein or Hypothetical protein HP1564 (Omp). A mixture of the antigen-loaded beads allowed the simultaneous detection of antibodies (IgG, IgA, IgM) against the selected antigens in one reaction. A Luminex flow cytometer (Luminex Corp., Austin, Tx, USA) quantified the amount of bound serum antibodies by detection of a fluorescent reporter (Streptavidin-R-phycocerythrin) on each bead set and the output was the median fluorescence intensity detected on at least 100 beads per type. Cut-offs were defined by visual inspection of percentile plots at the approximate inflection point as described for other antigens. Overall H. pylori sero-positivity was defined as being positive to four or more of the evaluated H. pylori antigens. Previously, in an H. pylori gastric cancer study from an Asian cohort consortium, double sero-positivity to H. pylori antibodies HP0305 and HP1564 was identified as a potential gastric cancer risk biomarker.

Questionnaires

Interviewer-administered questionnaires were used to collect basic demographic data including age, sex, family history of gastric cancer, and socio-economic status estimated by location of permanent residence, occupation, educational level attained cigarette smoking and alcohol use. In addition,
Figure 1: A flow chart showing the enrolment of patients into the study. Patients without histology reports, unconfirmed or cancer other than gastric adenocarcinoma were excluded from the analysis.

Figure 2: Helicobacter pylori seropositivity stratified by five-year age bands. The prevalence ranged from 73% to 100% without any evidence of an age cohort variation.
the weight and height of each patient was taken to calculate the body mass index (BMI).

**Statistical analysis and sample size calculation**

Differences in study characteristics between participants with active inflammation, premalignant lesions or gastric cancer compared to controls (NAG with chronic inflammation) as well as factors associated with *H. pylori* sero-positivity was assessed using Chi-square test, Fisher’s exact test or the Kruskal–Wallis test.

We applied logistic regression models to estimate the odds ratios (OR) and 95% confidence intervals (CI) for the associations of overall *H. pylori* sero-positivity, individual antigen positivity, and previously established gastric cancer sero-marker HP0305/ HP1564 with active gastritis, premalignant lesions and gastric cancer. None of the a priori defined potential confounders (age, sex, BMI, socio-economic status, family history of gastric cancer, history of smoking and alcohol intake) were simultaneously associated with the exposure and outcome, so they were not included in the final model for adjustment. Probability value of 0.05 with a confidence interval set at 95% was employed to determine statistical significance. Data were analysed in STATA 15 (Stata Corp, College Station TX).

**Results**

**Demographic and clinical characteristics of patients enrolled**

During the study period, 388 consecutive patients were enrolled (Figure 1).

Of those with gastric tumours, eight were excluded due to lack of confirmatory histopathology reports. Twelve had other types of gastric cancer, including eight with squamous cell or unclassified carcinomas, two with gastric stromal tumours, one with non-Hodgkin’s lymphoma and one with a “haematolymphoid tumour”. Of the remaining 72 patients, 68 had adenocarcinoma (GA) and four patients had high-grade dysplasia (carcinoma in situ), which for
Table 2: Risk factors for *H. pylori* (HP) sero-positivity (≥4 antigens positive in multiplex serology) among patients with chronic gastritis

Table 3: Risk factors for *H. pylori* (HP) sero-positivity (≥4 antigens positive in multiplex serology) among patients with chronic gastritis

Table 4: Association of sero-positivity to *H. pylori* (HP) multiplex serology antigens with gastric cancer and premalignant lesions

Variable | Total (n=161) | HP negative (n=27) | HP positive (n=134) | p-value*
--- | --- | --- | --- | ---
Sex, n (%) | | | | |
Female | 82 (51) | 12 (44) | 70 (52) | |
Male | 79 (49) | 15 (56) | 64 (48) | 0.460
Age group, n (%) | | | | |
<45 years | 66 (41) | 11 (41) | 55 (41) | |
45-59 years | 56 (35) | 8 (30) | 48 (36) | |
≥60 years | 39 (24) | 8 (30) | 31 (23) | 0.726
BMI [kg/m²], n (%) | | | | |
<25 | 79 (54) | 15 (60) | 64 (53) | |
25-29.9 | 40 (28) | 4 (16) | 36 (30) | |
≥30 | 26 (18) | 6 (24) | 20 (17) | 0.323
Missing | 16 | 2 | 14 | |
Resident in rural area, n (%) | | | | |
No | 133 (83) | 21 (78) | 112 (84) | 0.468
Yes | 28 (17) | 6 (22) | 22 (16) | |
Occupation, n (%) | | | | |
No | 40 (25) | 4 (15) | 36 (27) | |
Yes | 121 (75) | 23 (85) | 98 (73) | 0.229
Education, n (%) | | | | |
None | 7 (4) | 0 (0) | 7 (5) | |
Primary | 34 (21) | 4 (15) | 30 (22) | |
Secondary | 65 (40) | 10 (37) | 55 (41) | |
Tertiary | 55 (34) | 13 (48) | 42 (31) | 0.265
Family history of gastric cancer, n (%) | | | | |
No | 158 (96) | 28 (96) | 132 (99) | |
Yes | 3 (2) | 1 (4) | 2 (1) | 0.426
Smoking history, n (%) | | | | |
Never | 124 (77) | 20 (67) | 104 (78) | |
Former | 9 (6) | 0 (0) | 9 (8) | |
Current | 9 (6) | 3 (13) | 6 (5) | 0.158
Missing | 19 | 4 | 15 | |

*Logistic regression models using patients with chronic gastritis used as the comparison group; significant associations highlighted in bold font.

clinical classification and the purposes of this study were classified hereafter as gastric cancer cases. Among those without gastric tumours, eight had chronic atrophic gastritis (CAG) and 27 had gastric intestinal metaplasia (GIM). These were hereafter grouped to as gastric premalignant (GP) lesions. The OLGA staging classification was available for 26 GP patients: 8 (31%) had stage 1, 8 (31%) had stage 2, 9 (34%) had stage 3, and 1 (4%) stage 4. OLGIM classification showed that 7 patients (27%) had stage 0, 11 (42%) had stage 1, 7 (27%) had stage 2 and 1 (4%) had stage 3. Patients with chronic gastritis were analysed as the comparison group (n=161).

Overall, only 6/295 (2%) of the patients had family history of GC. GC was not positively associated with cigarette smoking, alcohol consumption or HIV infection. GC and not GP lesions were significantly associated with lower levels of body mass index (BMI). Gastric cancer patients were less educated and they mostly came from rural areas (Table 1).

Antibody response to *H. pylori* antigens by group status and detection of chronic and active gastritis

*H. pylori* serology results were available for 59 GC, 27 GP, 48 active gastritis and 161 chronic gastritis (Figure 1). Overall, 257/295 (87%) were *H. pylori* positive. Patients with GC or GP lesions were significantly older (Table 1). However, further analysis showed that age had no influence on overall *H. pylori* seropositivity; prevalence ranged between 73% and 100% when stratified in five-year age bands.
The number of positive antibodies were also similar across all age groups, p=0.48. We divided the comparison groups based on the presence of acute gastric inflammatory cells. The levels of H. pylori antibodies were compared between the following four groups: active gastritis (n=48), chronic gastritis (n=161), non-cardia gastric adenocarcinoma (NCGA; n=42) and GP (n=27) using a logistic regression model. Among patients who were H. pylori multiplex serology positive, those with active gastritis had significantly higher levels of CagA, VacA, Omp, HepC, HP0305 and HpaA than those with chronic gastritis (Table 2). A comparison of NCGA and chronic gastritis showed no significant difference apart from Cad which was significantly higher in the latter group. Cad and catalase were significantly higher in patients with GP than the comparison group (Table 2).

Risk factors for H. pylori sero-positivity determined the presence of four or more positive multiplex serology

We then analysed lifestyle risk factors for H. pylori infection exclusively in the patients with gastritis. Factors analysed included age, sex, BMI, area of residence, occupation, educational attainment, family history of gastric cancer, HIV infection, cigarette smoking and alcohol intake. None of these factors was significantly associated with H. pylori seropositivity (Table 3).

Discussion

We evaluated links between seropositivity to specific H. pylori proteins and GC or GP in Zambian patients seen at the University Teaching Hospital, in Lusaka, Zambia. We found no association between GC and these antibody responses. However, patients with GP were more frequently sero-positive to Cad and catalase than those with chronic gastritis. As this was not a population-based study, the results cannot necessarily be taken to be representative of the whole country.

H. pylori infection is very common in Zambia; more than 80% of the population carries H. pylori antibodies. The exact prevalence of H. pylori-associated gastric disease is however, unknown and available data underestimate the burden, as diagnostic facilities are fragmented and only available in a few centres. In our previous work, we found that the prevalence of active gastritis was 23% while that of chronic gastritis was 68%. There is currently no reliable and affordable strategy for identification of H. pylori infected individuals likely to develop complications of the infection such as peptic ulceration and GC. The H. pylori multiplex serology assay was developed to quantitate antibodies against carefully selected immunogenic antigens and has shown promising results for GC risk stratification. Other investigators reported associations between GC and some H. pylori antibodies such as GroEL, CagA, HyuA. Similarly, Omp and HP0305 were suggested as new serum biomarkers of GC risk as they were associated with GP in a Chinese population.

CagA is generally accepted as a virulence factor increasing GC risk in infected individuals. However, previous work in Zambia did not show any association with GC or peptic ulceration risk. Differences in African strains could explain the inability to demonstrate the influence of CagA and VacA on gastric carcinogenesis. Results from this study showed no significant association between GC and the tested H. pylori proteins. Of note, H. pylori proteins included in the multiplex serology assay were expressed from the genome of strains 26695 and G27 (GroEL), which are strains not originating from Africa. Development of strain-specific serological assays could help clarify the reason for the observed absence of an association of H. pylori serology and GC in this study.

Conversely, antibody responses to Cad, known to catalyse dismutation of benz aldehyde to benz alcohol and benzoic acid showed significantly lower levels among GC cases. Cad is one of the antibodies that reported to have an association with GC in cohorts form China, Japan and Korea. Some study groups have also found inverse relationships between antibodies to GroEL or NapA 25 and GC while others found higher GC risk associated with these and more antigens. In a study by Camargo et al., with data from three Latin American countries, it was concluded that humoral responses to H. pylori were insufficient to distinguish high and low GC risk populations.

The presence of GP was positively associated with catalase but due to the small number of patients with this diagnosis included in the study, caution needs to be employed when considering this result. In addition, we did not separately analyse for associations with each individual premalignant lesion. A population-based study from Germany showed significant associations between chronic atrophic gastritis and all thirteen antibodies tested in this study with an additional two, CagM and Cag. This study also suggested a dose effect of antibody levels of CagA, GroEL, VacA and HepC. Therefore, to fully understand the association of these antibodies with the development of premalignant lesions in Africa, there is need to conduct larger population-based studies. We acknowledge that with the development of GC, H. pylori organisms might be lost after having influenced the process of carcinogenesis. This could reduce the antibody levels in patients with GC or GP and prevent detection of differences. The high prevalence of H. pylori in this population could also explain the observed absence of an association with GC or GP. Epplein et al. found over 4-fold increased odds for premalignant lesions in a Chinese population with H. pylori sero-positivity, however, the sero-prevalence among controls was only 54% as opposed to 83% in our study.

The distinction between active and chronic gastritis requires histopathological evaluation of gastric biopsies, which are obtained invasively. In a population such as this one, where the proportion of individuals with H. pylori is high, there is
need for a non-invasive way of identifying those with active gastritis. Intensity of the active component of gastritis largely depends on the cytotoxicity of the H. pylori strain and therefore increased activity is linked to more aggressive gastritis\textsuperscript{30}. The high sero-prevalence in individuals with active gastritis shown in this study could potentially be used as biomarkers signifying active gastritis, and therefore help guide the treatment options.

In many western populations, the prevalence of H. pylori infection has been reducing with subsequent generations, a change influenced by improving environmental conditions\textsuperscript{31}. Our data did not show any age cohort effect, as the prevalence of H. pylori was consistent throughout the five-year age bands. These data suggest that factors influencing H. pylori acquisition are not changing in this population. H. pylori infection is generally known to be most prevalent in low-resource communities, with evidence of differential prevalence within individual countries along socio-economic strata\textsuperscript{32}. In this study, area of residence, educational level or occupation had no influence on H. pylori infection.

The numbers included in this study were small, suggesting the possibility of a missed effect. Patients included in this study were drawn from a tertiary hospital in Lusaka, and therefore only those either residing within the city or those with resources to travel were included. Preparation of the multiplex assay used did not included African strains and we did not have H. pylori breath test results to confirm current active infection. However, with these limitations do not invalidate our findings. We have provided good preliminary data and raised important questions that would justify further studies.

Conclusions
Antibody responses to H. pylori antigens (CagA, VacA, Omp, HecP, HP0305 and HpaA) have potential for use in identifying individuals likely to have active gastritis.

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Author contributions
VK, DCH, AM and PK were involved in designing the study; patient enrolment and sample collection was done by VK, KZ, SM and CM. Histopathological analyses were done by JB under the supervision of TW. All authors contributed toward manuscript preparation.

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Authors have no conflict of interest to declare

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