

Full Length Research Paper

Helicobacter pylori cytotoxin-associated gene A protein among adult dyspeptic patients in South-western Nigeria

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cagA gene, a marker for the **cag** pathogenicity island (CagPAI) and a virulent factor in *Helicobacter pylori* infection codes for 120 to 145 kDa protein that is associated with cytotoxin production and more severe clinical outcomes. The aim of this study was to determine whether any correlation exists between *H. pylori* CagA protein and the endoscopic findings among dyspeptic patients from South-western, Nigeria and also to investigate the evolutionary relationships among *H. pylori* cagA gene with the GenBank strains. A total of one hundred and twenty four *H. pylori* positive isolates were amplified for the detection of **cagA** by polymerase chain reaction (PCR) and *H. pylori* isolates positive for **cagA** were further evaluated for protein expression using western blotting analysis. Also, DNA sequencing, blasting and phylogenetic analysis were performed on twelve selected isolates positive for **cagA**. **cagA** gene was detected in all the 124 samples (100%) and protein expressions of **cagA** by western blotting analysis was 88.7% (110/124). There was a high prevalence (91.7%) of expressed **cagA** strains in patients with positive endoscopic lesions than those with normal endoscopic findings (85.7%); however, association was not statistically significant ($P>0.05$). Also, the expressed **cagA** had no statistically significant association with all the positive endoscopic findings ($P>0.05$). Phylogenetic analysis of the selected *H. pylori* cagA gene showed high similarity with some GenBank strains of western **cagA** *H. pylori*. This study shows that CagA is high among dyspeptic patients in Nigeria but with no statistically significant association with the endoscopic findings.

Key words: Protein expression, CagA, protein, GenBank, dyspeptic.

INTRODUCTION

Helicobacter pylori is a bacterial pathogen that colonizes exclusively the mucous layer of the human stomach and classified as carcinogenic to humans by the International

Agency for Research on Cancers (IARC, 1994). However, *H. pylori* organisms vary in their carcinogenic potential and the common type of gastric cancers is

Table 1. PCR primers used in this study.

Region amplified	Primer sequence (5' to 3')	Size of PCR products (bp)	References
16S rRNA- forward	GCGCAATCAGCGTCAGGTAATG	502	Mona et al. (2015)
16S rRNA – Reverse	GCTAAGAGAGCAGCCTATGTCC		
CagA- Forward	TTGACCAACAACCACAAACCGAAG	183	Smith et al. (2002)
CagA- Reverse	CTTCCCTTAATTGCGAGATTCC		

adenocarcinoma which occurs most commonly in East Asian countries than western countries (Higashi et al., 2002; Suzuki and Mori, 2016). Since the discovery of *H. pylori* by Marshall and Warren (1984), it has been confirmed that *H. pylori* infection results in chronic active gastritis and can lead to gastric ulcer, duodenal ulcer and gastric cancer (Malfertheiner et al., 2007; Yamaoka, 2010). Infection with *H. pylori* is more common and acquired at an earlier age (30 to 50%) in children and attain over 90% during adulthood in developing countries (Salih, 2009). In South-west, Nigeria, studies reported high prevalence rates (60.5 to 73%) of *H. pylori* among dyspeptic patients (Ndububa et al., 2001; Adesanya et al., 2002; Ola et al., 2006). Cytotoxin associated gene A (*cagA*) is one of the important virulence factors of *H. pylori*. The CagPAI contains 31 genes, including *CagA* gene, which is one of the markers for the cag pathogenicity island (CagPAI). *CagA* codes for 120 to 145 kDa protein that is associated with cytotoxin production and more severe clinical outcomes such as peptic ulcer and gastric cancer (Arents et al., 2001; Hatakeyama, 2006).

Previous studies have reported the prevalence of *H. pylori* and the virulence genes in relation to disease outcome among dyspeptic patients in Nigeria (Smith et al., 2002; Olokoba et al., 2013). However, data relating to the information on the association of the *cagA* gene with the disease outcome is still controversial and there is dearth of information on protein expression of putative *cagA* genes in relationship to clinical outcomes. This information is important as CagA protein is one of the major virulence factors of *H. pylori*. Hence, this study was aimed at confirming whether any correlation exists between the CagA protein of these strains and the endoscopic findings among dyspeptic patients from South-western, Nigeria and also to investigate the evolutionary relationships among *H. pylori* *cagA* gene with the GenBank strains.

MATERIALS AND METHODS

A total number of one hundred and twenty-four isolates of *H. pylori*

from dyspeptic patients were obtained from the laboratory of Dr. S. I. Smith of the Nigeria Institute of Medical Research, Yaba. Medical history and the endoscopic findings of the patients were obtained from four different hospitals based on the records provided by the gastroenterologist between April 2012 - December 2015, by which approval to conduct the study was obtained from Ethics Committee of the Nigerian Institute of Medical Research Yaba, Lagos.

Culturing of *H. pylori*

The isolates were sub-cultured on Columbia blood agar base (Oxoid), containing laked horse serum (7%) and Dent's antibiotic supplement (Oxoid) in a microaerophilic atmosphere conditions at 37°C as described previously (Toledo and Lopez-Solis, 2010). Colonies were identified by Gram staining and biochemical tests such as oxidase, catalase and urease reactions.

DNA extraction and PCR amplification

Genomic DNA of the isolates were extracted with the QIAamp® DNA kit (Qiagen, Germany) according to the manufacturer's instructions. The extracted DNA was stored at -20°C until use. The PCR amplification of *H. pylori* 16S rRNA was carried out in 25 µl reaction mixture and PCR conditions were: 35 cycles at 94°C for 1 min, annealing 60°C for 1 min, 72°C for 1 min, and a final elongation at 72°C for 5 min. The DNAs of J99 and P12 were used as positive controls. *H. pylori* 16S rRNA gene-positive samples were subjected to PCR to detect *cagA* gene using the primers and PCR conditions as described previously by Smith et al. (2002) and the PCR primers are listed in Table 1.

Protein expression of *cagA* gene

Antigens were prepared from lysates of strains of *H. pylori* positive for *cagA* genes. Single gel system for protein separation was prepared according to a protocol of Ahn et al. (2001) and the procedures for Western blotting were performed following the methods of Figueroa et al. (2002) with some modifications. Briefly, the proteins from the lysates were electrophoretically separated in polyacrylamide gel electrophoresis (SDS-PAGE) according to molecular weight (MW) of the proteins. Proteins were then blotted to nitrocellulose membrane (PVDF). For the development of immunoblots, polyvinylidene difluoride (PVDF) filters were blocked in TBS containing 3% (w/v) bovine serum albumin. *CagA* protein expression was analyzed using a 1:2,000 dilution of *CagA* specific polyclonal antibodies. Anti-rabbit antibody was used as a secondary

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Table 2. Association between expressed *cagA* and the endoscopic findings.

Disease	CagA+	CagA-	X ²	P-value
NUD	24	4	0.878	0.349
GERD	16	0	1.690	0.194
Erosive gastritis	66	10	0.684	0.408
Ulcer	4	0	0.103	0.748

Table 3. Association between expressed *cagA* and positive endoscopic lesions.

cagA status	Positive endoscopic lesions		Normal endoscopic findings	
	n	%	n	%
cagA+	88	91.7	24	85.7
cagA-	8	8.3	4	14.3
Total	96	100	28	100
P-value	0.3488			

antibody and immunoreactive bands were visualized using staining solution.

Sequencing and bioinformatic analysis

The amplified PCR products of *cagA* gene from twelve selected strains based on each centre used were purified and subjected to sequencing reactions by inqaba™ biotech, South Africa (BigDye® Terminator v3.1 Cycle Sequencing). Blasting and Genebee (http://www.genebee.msu.su/services/phtree_reduced.html) were used for phylogenetic prediction.

Statistical analysis

The data obtained were subjected to descriptive statistical analysis using EPI info 7. Chi squared test was used to determine associations between the protein status of the isolates and the endoscopic findings and the differences were considered to be statistically significant when the p value obtained was less than 0.05.

RESULTS

One hundred and twenty four *H. pylori* isolates were amplified for the detection of *H. pylori* 16S rRNA and *cagA* gene by polymerase chain reaction (PCR) and all were 100% positive for both *H. pylori* and *cagA* gene. CagA protein was expressed in 88.7% (110/124) and the positive rate of CagA protein was 85.71% (24/28) in the isolates from patients with non ulcer dyspepsia (NUD), 100% (16/16) from those with GERD, 86.84% (66/76) from those with erosive gastritis, and 100% (4/4) from ulcer patients (Table 2). However, no statistical relation was found between CagA positivity and the endoscopic findings (p >0.05 by Chi-square test) (Tables 2 and 3).

Immunoreactive bands for CagA protein expression is shown in Figure 1.

The phylogenetic relationships among the 12 twelve selected strains and four *cagA* genes of *H. pylori* strains (PHL 121, LSU 2003-1, CAM23 and 01-1086) obtained from GenBank based on high sequence similarity with blasting, revealed that *cagA* genes were divided into two major groups: Strains LU 14 to 01-1086 and strains LA2 to LA4 (Figure 2), having four sub groups: LU 14 – IF9; LA1-IB6; CAM23-10-1086 and LA2-LA4 (Figure 2)

DISCUSSION

From these findings, CagA gene was found in all the isolates (100%) studied and the result is in agreement with previous studies in Africa and East Asia countries where *cagA* genes were present in more than 90% of cases (Tanih et al., 2010; Yamaoka et al., 1999). However, this result is higher than those observed from western populations where *cagA* were present in about 60 to 70% of *H. pylori* causing infections (Podzorski et al., 2003; Ribeiro et al., 2003). This indicates that *cagA* prevalence varies in every part of the world (Ozbey and Aygun, 2012). Several studies have demonstrated that the genotype varies among *H. pylori* strains isolated from different geographic regions (Kersulyte et al., 2000; Faundez et al., 2002), indicating that there are important geographic differences. In this study, CagA proteins was expressed in 88.7% (110/124) isolates and expression was observed with CagA specific antibodies by SDS-PAGE and Western blot (WB) analysis. This study showed that most *H. pylori* strains isolated were positive for Cytotoxin associated gene (CagA) proteins. Also, Hatakeyama (2004) reported that many strains of *H.*

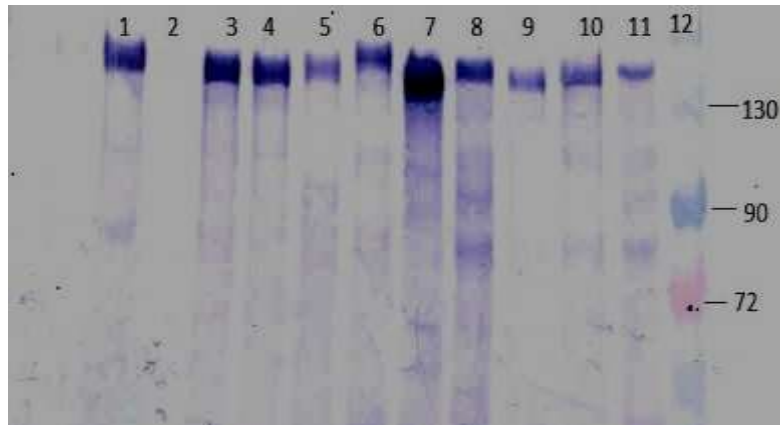


Figure 1. *cagA* protein expression of 130-140kD. Lane 1, positive control; lane 2, negative control; lanes 3 to 11, samples; lane 12, pre stained ruler.

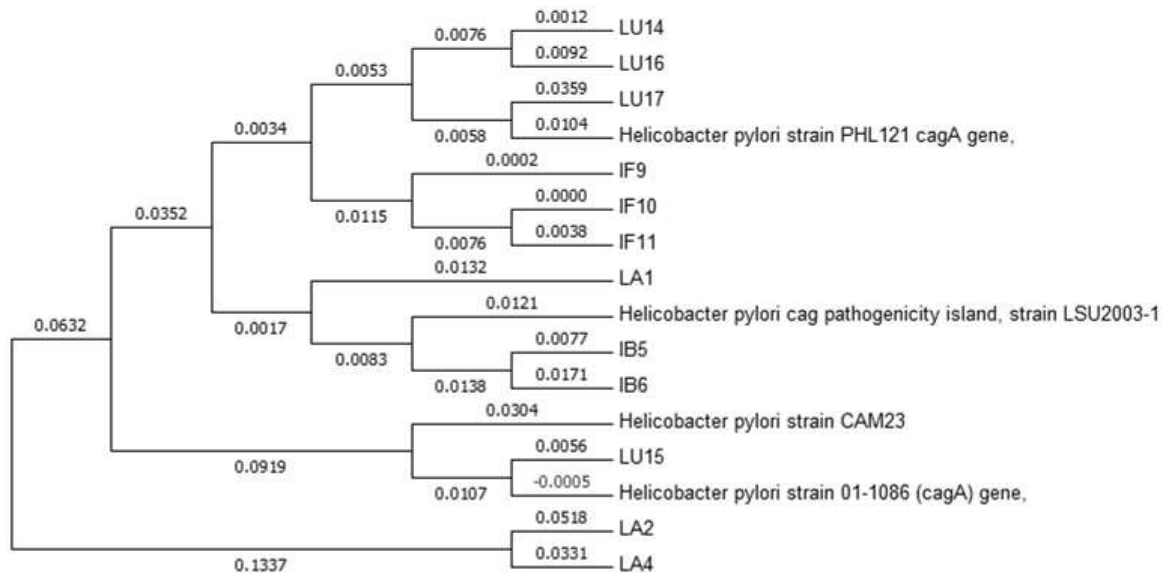


Figure 2. Phylogenetic tree of *cagA* genes of twelve strains from southwest Nigeria and four strains from the GenBank.

pylori produce *cagA* protein, which is a 120 to 145 kDa hydrophilic protein that induces several alterations in the signalling pathways. From these findings, there was no significant association between expressed *cagA* and the clinical outcomes and this is in agreement with earlier reports on *cagA* genotypes (Smith et al., 2002; Ozbey et al., 2013). Whereas in Western countries, the occurrence of *vacAs1* and *cagA* genotypes have been reported to be significantly associated with peptic ulcers (Ribeiro et al., 2003). However, the reported studies were based on the association of *cagA* genes with the endoscopic findings, but this present study focuses on the association of CagA protein with the endoscopic findings.

From these findings, none of the patients studied were

diagnosed with gastric cancer, more so, with the presence of *cagA* positive isolates. Abdulkareem et al. (2015) reported that, there is usually low incidence of gastric cancer in Africa (Africa enigma) despite a high prevalence of *H. pylori* with most strains positive for the virulent genes such as *cagA* gene and *vacA* gene. A lot of factors may contribute to this, and one of which could be mixed infection of *cagA* negative and *cagA* positive strains which could lower disease burden, and diets rich in anti-oxidants which reduces risk of gastric cancer (Abdulkareem et al., 2015). A study from Soweto, South Africa indicated that host response to *H. pylori* among Africans may be protective against virulent form of *H. pylori* (Segal et al., 2001). Also, the ability of CagA

secreted by different strains of *H. pylori* to perturb host-cell functions and to trigger gastric cancer is determined by the binding potential of Src homology 2-containing protein-tyrosine phosphatase-2 (SHP-2) which is high in *H. pylori* strains from East Asian countries than those from Western countries (Higashi et al., 2002).

The phylogenetic tree of *cagA* genes revealed that the strains were clearly delineated into 2 major phylogenetic clades with strains LA2 and LA4 distantly separated from others. However, blasting of the edited sequences of strains LA2 and LA4 in NCBI platform, revealed that they were 89 and 87% respectively, most similar to the western type of cytotoxin associated protein A (*cagA*) gene, partial cds of *H. pylori* strain 01-1086 from Colombia (Accession no: JQ318034.1). The other strains clustered in the second clade but with significant genetic variability. This clade further subdivided into 3 minor clades with strain LU15 most distant and stands as a bridge linking the clade with strains LA 2 and LA4. Strains LA1, IB5 and IB6 clustered to form a minor clade with IB 5 and IB6 more related. Also, the results from blasting indicated that IB 5 and IB6 were 95 and 96%, respectively, most similar to *H. pylori* cag Pathogenicity Island, strain LSU2003-1 originated from African with Accession number of FR666843.1 (Olbermann et al., 2010). Strains from Ife (IF 9, IF 10 and IF 11) clustered forming a minor clade and were all related (96, 95 and 95%). While cluster from LU 14, LU 16 and LU 17 were related to Western *cagA* gene of *H. pylori* strain PHL121 (accession no: GU173869) obtained primarily from gastritis patients in philippines (Cortes et al., 2010).

Conclusion

This study demonstrated a high prevalence of *H. pylori* infection of the *cagA* genotype and the phylogenetic analysis revealed that *cagA* gene was related to the western *cagA*. However, there was no significant association between CagA protein and the clinical outcomes.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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