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CLINICAL MICROBIOLOGY - RESEARCH PAPER

Helicobacter pylori virulence *dupA* gene: risk factor or protective factor?

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Abstract

Helicobacter pylori is the etiological agent of chronic gastritis, peptic ulcer, and gastric cancer. The duodenal ulcer-promoting gene *dupA*, which is located in the plasticity region of the *H. pylori* genome, is homologous to the *virB* gene which encodes a type IV secretion protein in *Agrobacterium tumefaciens*. Studies have shown associations between *H. pylori dupA*-positive strains and gastroduodenal diseases. However, whether *dupA* acts as a risk factor or protective factor in these diseases remains unclear. Therefore, in this study, we aimed to verify the presence of the *dupA* gene in infectious *H. pylori* strains in the Brazilian mid-west and to investigate its association with the clinical outcomes of patients with dyspepsia. Additionally, the phylogenetic origin of the strains was determined. Gastric biopsies from 117 patients with dyspepsia were analyzed using histological and molecular techniques. The *hpx* gene (16S rRNA) was used to screen for *H. pylori* infection, and positive samples were then subjected to *dupA* gene detection and sequencing. The estimated prevalence of *H. pylori* infection was 64.1%, with the *dupA* gene being detected in a high proportion of infectious strains (70.7%). Furthermore, a risk analysis revealed that for women, a *dupA*-positive *H. pylori* infection increased the chance of developing gastritis by twofold. The partial *dupA* sequences from isolated infectious strains in this work are similar to those of strains isolated in westerns countries. This study provides useful insights for understanding the role of the *H. pylori dupA* gene in disease development.

Keywords *H. pylori* · *dupA* · Gene · Gastritis · Molecular pathology · Factors virulence

Introduction

Helicobacter pylori is a gram-negative, microaerophilic, rod-shaped bacterium that infects the gastric mucosa in more than half of the human population worldwide [1, 2]. The prevalence of *H. pylori* is higher in developing countries owing to the generally lower basic sanitation and hygiene conditions brought on by low socioeconomic infrastructures [3, 4]. Infections with this bacterium can lead to the development of several gastropathies, such as chronic gastritis, peptic ulcer, atrophy, and metaplasia [5, 6]. Additionally, *H. pylori* infection is considered one of the main risk factors for the development of gastric adenocarcinoma. As a result, the International Agency for Research on Cancer, subsidized by the World Health Organization, has classified *H. pylori* as a type I carcinogen [7].

Helicobacter pylori infections have different clinical outcomes depending on the virulence of the bacterial strains, environmental factors, and the genetic characteristics and

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lifestyles of the hosts [8]. The *cagA* gene, located in the chromosomal region called the chromosomal pathogenicity island (*cag*PAI), encodes the CagA protein (cytotoxin-associated gene A), a highly immunogenic antigen that stimulates the production of chemotactic factors in the host gastric epithelium, inducing an intense inflammatory response. This gene is considered an important marker of virulence and is associated with an increased risk for the development of gastritis, peptic ulcer, atrophy, and gastric carcinoma [8, 9].

The *vacA* gene encodes vacuolating cytotoxin A (VacA), considered an important virulence factor in the pathogenesis of gastric cancer and peptic ulcer. VacA induces vacuoles in gastric epithelial cells. In addition, it induces the release of cytochrome C from mitochondria, leading to apoptosis, the inhibition of T cell proliferation, and the induction of pro-inflammatory responses [10–12].

In 2005, Lu et al. [13] described a gene located in the plasticity region of the *H. pylori* genome, which they named the duodenal ulcer-promoting gene (*dupA*). This gene was found to be homologous to the virulence gene *virB* which encodes a type IV secretion protein in *Agrobacterium tumefaciens*. The *dupA* gene is also related to neutrophil infiltration in the mucosa and the induction of elevated IL-8 levels by gastric epithelial cells [14].

According to Lu et al. [13], infection with *H. pylori dupA*-positive strains was associated with the development of duodenal ulcers, and the presence of the *dupA* gene in the infecting strain was considered a protective factor against gastric atrophy, intestinal metaplasia, and gastric cancer. As a result, this gene was characterized as the first disease-specific virulence gene of *H. pylori* [13]. However, similar to other *H. pylori* virulence genes, the role of this gene in the development of gastropathies varies according to geographical regions [15–17].

Although the pathogenic mechanism of the protein encoded by the *dupA* gene (i.e., DupA) is not yet well-understood, it may be involved in the induction of interleukin-8 (IL-8) production by the gastric epithelium [13]. Strains carrying the *dupA* gene also appear to be more resistant to stomach acid, which may partly explain the association of this virulence factor with the development of ulcers [18, 19].

Although several bacterial virulence factors have been suggested as markers for digestive diseases [20–24], there is still a lack of information on the biomarkers which are characteristic of the clinical conditions present in infected individuals, especially in South America. Some studies have indicated the usefulness of the *dupA* gene as a virulence biomarker [11, 19, 25–27]. However, the role of this gene in the etiology and pathogenesis of different gastropathies is not fully understood. Bearing in mind that identification of predictive biomarkers for a given clinical condition is a promising strategy for personalized medicine, the aim of this study was to verify the association between the presence of the *H. pylori dupA* gene and clinical outcomes in dyspeptic patients in the center-west of Brazil. In addition, a phylogenetic analysis was performed to assess the origin of circulating strains.

Materials and methods

Ethical Considerations and Participants

This study was approved by the Research Ethics Committee of Hospital das Clínicas of the Universidade Federal de Goiás (UFG) (Approval Numbers 2.519.032 and Certificate of Presentation of Ethical Appreciation 83,422,017.7.0000.5078). Patients with dyspepsia (age: > 18 years) who had undergone an upper gastrointestinal tract (GI) endoscopy at the Hospital das Clínicas of UFG were recruited. The exclusion criteria were applied: patients who had used proton pump inhibitors in the 2 weeks preceding sample collection, or blocking agents in the week preceding the procedure, patients who had used immunosuppressants or antibiotics in the past 8 weeks, patients with active gastrointestinal bleeding, and pregnant and lactating women. All participants provided signed informed consent.

Screening for *H. pylori* infection and detection of the *dupA* gene

Two samples of the gastric antrum and gastric body were collected from the 117 patients during the upper GI endoscopy. DNA extraction was performed using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The extracted DNA was used for the molecular screening of *H. pylori* infection by amplifying a fragment of the constitutive ribosomal 16S rRNA gene (*hpx*) using the polymerase chain reaction (PCR).

The *hpx*-positive samples were then subjected to *dupA* gene detection and sequencing. The PCRs were performed using primers previously described in the literature [28] (Table 1). The PCR mixture (final volume: 50 µL) comprised 33.5 µL of Milli-Q water, 5 µL of 10 × PCR buffer containing $MgCl_2$ (1.5 mM), 2 µL of dNTP (2.5 mM), 2 µL of each oligonucleotide (10 pmol each), 5 µL of DNA sample (50 ng), and 0.5 µL of *Taq* DNA polymerase (2.5 units). For each reaction, positive and negative controls were also tested.

After the PCR-amplified fragments had been electrophoresed on a 2% agarose gel, the fragment bands were visualized by nucleic acid staining with Blue Green Loading dye (Lac Biotecnologia, Sao Paulo, Brazil) and exposure under ultraviolet light. The size of the amplified product was

Table 1 Primer sequences, PCR amplification conditions, and gene fragment sizes

Gene	Primers	Sequence (5′–3′)	Amplification condition	Size (bp)
16S rRNA	*hpx1*	CTGGAGARACTAAGYCCTCC	94 °C, 1 min; 59 °C, 1 min; 72 °C, 1 min (40 cycles)	150
	hpx2	GAGGAATACTCATTGCGAAGGCGA		
dupA	*dupA1*	CGTGATCAATATGGATGCTT	94 °C, 45 s; 52 °C, 45 s; 72 °C, 45 s (35 cycles)	197
	dupA2	TCTTTCTAGCTTGAGCGA		

determined by comparison against a 100-bp DNA ladder molecular weight marker (Cellco, Sao Paulo, Brazil).

Diagnosis of gastroduodenal pathologies

The endoscopic examination of the patients was performed by the Department of Gastroenterology, while histopathological analyses of the biopsied gastric mucosal samples were done by the Clinical Pathology Laboratory of Hospital das Clínicas (UFG). Endoscopic examination was performed according to recommendations of the Brazilian Society of Digestive Endoscopy. The procedure was performed by a gastroenterologist using an Olympus CV endoscope (Olympus Medical Europe, Germany). Videoendoscopic images were viewed on a high-definition, flat-panel monitor. Alfentanil (50 mcg) and propofol (40 mg) were administered intravenously before the procedure [29].

For the histopathological examination, the gastric mucosal samples were fixed in 10% buffered formalin and stained with Giemsa and hematoxylin–eosin [30]. Grading of the inflammatory infiltrate was performed according to the Sidney system [31].

DNA sequencing and phylogenetic analysis

All *dupA* gene amplification products were purified with the Wizard SV Gel and PCR Clean-Up System (Promega, Madison WI, USA). DNA sequencing was performed using an ABI-PRISM BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Carlsbad, CA, USA) in an ABI-PRISM 3100 genetic analyzer (Applied Biosystems). Sequencing was performed at least twice, using the same forward and reverse primers used in the PCR assays. All the sequences were compared with those listed on the GenBank nucleotide and protein databases (National Center for Biotechnology Information, Bethesda, MD, USA), using the Blastn and Blastx algorithms, respectively.

To establish the phylogenetic origin of the Brazilian strains, a phylogenetic tree based on *dupA* was built using the gene sequences from the following 17 *H. pylori* strains from different parts of the world (available from GenBank): three sequences from Portugal (PORT: KT290607, KT290572, and KT290619); two from Brazil (BRA: HQ228198 and JF951131); three from Argentina (ARG: FJ763612, FJ763614, and AB649939); four from Japan (JAP: AB739514, AB739579, AB739507, and KC707841); two from China (CHI: KC707837 and KM245039); and three from India (IND: KC894690, KC894688, and KM245039). Additionally, three *dupA* sequences (NEHP04, NEHP05, and NEHP06) which belonged to *H. pylori*–infected patients in our study who had duodenitis, esophagitis, and gastritis, respectively, were included in the phyogenetic tree.

The sequences were first aligned with ClustalX v2.1.0.0 and then manually edited using BioEdit v7.1.7.0. Phylogenetic analysis was performed using Molecular Evolutionary Genetics Analysis (MEGA) v10.1. The tree was built using maximum-likelihood analysis, with 1000 bootstraps. The evolutionary model chosen by the algorithm for the set of sequences used was General Time Reversible + Invariant Sites (GTR + I) [32].

Statistical analysis

Descriptive statistics were performed to calculate the absolute frequencies and relative percentages for the categorical variables. Subsequently, inferential statistics were performed using Fisher's exact test to assess possible associations between the variables. Additionally, the relative risk for the development of gastric diseases in relation to the bacterial genetics was calculated. For the inferential statistics, a significance level of 5% ($p < 0.05$) was adopted. For statistical calculations, the BioEstat® 5.3 software was used.

Results

In this study, gastric biopsies were obtained from 117 patients with dyspepsia, of which 64.1% (75/117) were considered positive for *H. pylori* (mean age: 42 years; standard deviation: 10.8). Among the patients infected by the bacterium, 27 (36.0%) were men and 48 (64.0%) women. The *dupA* gene was detected in 70.7% (53/75) of *H. pylori*–positive samples.

In total, four gastropathies were diagnosed in the study population, with some patients having more than one gastric disorder. Of the individuals infected with *dupA*-positive strains, 67.9%, 15.1%, 11.3%, and 5.7% had a diagnosis of gastritis, duodenitis, oesophagitis, and atrophy respectively,

and 18.9% had a gastric mucosa without changes (normal). Of the individuals infected with *dupA*-negative strains, 68.2%, 13.6%, 22.7%, and 4.5% had gastritis, duodenitis, oesophagitis, and atrophy respectively, and 22.7% had a normal mucosa (Fig. 1). These data suggested that the presence of the *dupA* gene in infectious *H. pylori* strains was not associated with the development of these gastropathies in the population studied (p-value = 0.8059).

The most prevalent gastropathy among individuals infected with a *dupA*-positive strain was gastritis. Stratification by sex revealed that 88.6% of the women infected with *dupA*-positive strains had gastritis, whereas only 44.4% of the men likewise infected had this disease, with the difference between the two groups being statistically significant (p = 0.0020) (Table 2). Additionally, the relative risk analysis showed that for women, a *dupA*-positive *H. pylori* infection increased the chance of developing gastritis by twofold.

To evaluate the phylogenetic relationships of the *H. pylori* strains analyzed in the present work with *H. pylori* strains from other geographical regions, partial sequences (~ 200 bp) of the *dupA* gene from the Brazilian strains were used for the construction of the phylogenetic tree. These partial sequences amplified from the infectious strains showed 100% homology with one another (data not shown).

The phylogenetic tree consisted of two main clades, one of which grouped the strains from the eastern countries (except for the KT290619 sequence) and the other of which grouped the strains from the western countries. The sequences obtained in this study were grouped with those from the western countries (Fig. 2). The KT290619 sequence was positioned in an intermediate zone between the two main clades and was therefore used to root the phylogenetic tree.

Table 2 *Helicobacter pylori dupA* positivity in patients with gastritis

dupA +	Female		Male		p-value
	n	*f (%)*	*n*	*f (%)*	
With gastritis	31	88.6	8	44.4	0.002
Without gastritis	4	11.4	10	55.6	
Total	35	100	18	100	

Discussion

Helicobacter pylori plays a critical role in development of various digestive diseases. However, there is no consensus regarding the main virulence factor involved in the etiology and pathogenesis of each disease [33]. Several studies have been carried out to elucidate the role of *dupA* in the development of gastric disorders [14].

In this study, carried out in the mid-western region of Brazil, the *dupA* gene was detected in 70.7% of the infectious *H. pylori* strains but was not associated with gastropathies. Similarly, in another study carried out in the southeastern region of Brazil, the *dupA* gene was found in 92.3% of infectious *H. pylori* strains but was also not associated with the development of gastric diseases, despite its high frequency in the region [34]. Similar results were found in the study by Pacheco et al. [35], carried out on patients with dyspepsia in the state of São Paulo, Brazil, where the *dupA* gene was detected in 92.4% of samples positive for *H. pylori* and was not associated with gastric diseases. Other studies in Belgium, China, South Africa, and the USA have also found no association between the gene and gastric diseases [36].

Talebi et al. [19] demonstrated that infection by *dupA*-positive strains in fact protected against the development of gastric ulcers. Additionally, Lu et al. [13] proposed the use of the *dupA* gene as a protective biomarker for gastric atrophy, intestinal metaplasia, and gastric cancer. By contrast, that same study revealed a positive association between the presence of the *dupA* gene in the infecting strain and the development of duodenal ulcer. In our study, it was not possible to assess the association of the *dupA* gene with ulcers, atrophy, and gastric cancer owing to the low sample size of infected individuals with these diseases.

In a study by Schmidt et al. [37], the presence of the *dupA* gene was found to be associated with chronic gastritis. This result corroborates our findings, where the presence of *dupA* in the infecting strain showed a positive correlation with the development of gastritis in women. In 2017, Paredes-Osses et al. [11] demonstrated that the

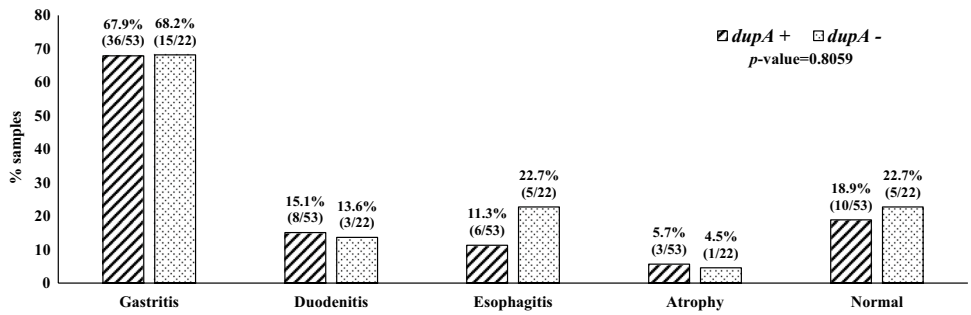

Fig. 1 Gastric disease profile of patients infected with *dupA*-positive and *dupA*-negative *Helicobacter pylori* strains

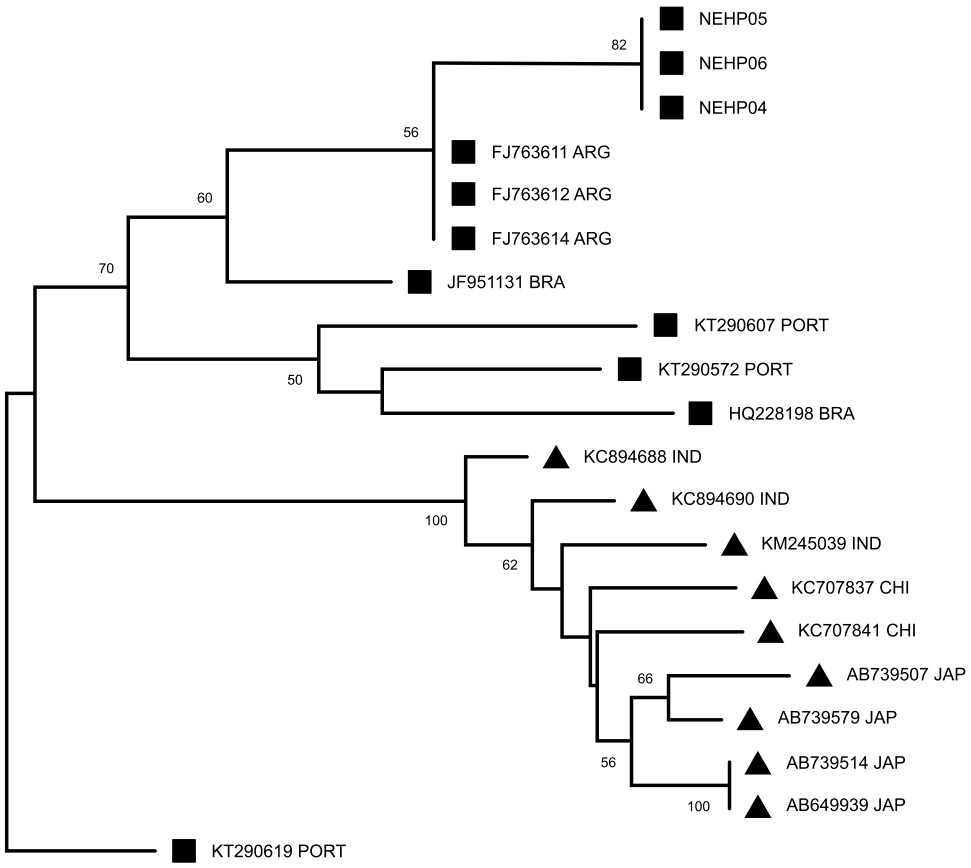

Fig. 2 Phylogenetic tree based on the partial sequences of the *dupA* gene of *Helicobacter pylori* strains from Brazil (local strains) and sequences from strains reported in different parts of the world (available in public databases). Triangles: strains from eastern countries; squares: strains from western countries

severity of gastric diseases was significantly decreased in women infected with *dupA*-positive *H. pylori* strains. By contrast, *dupA* did not have any significant effect on the disease severity in male patients.

The pathogenic mechanisms of DupA in women have not yet been clarified and deserve further investigation. Given that the establishment of gastric diseases is due to virulence factors of the pathogen as well as the genetic characteristics of the host and environmental factors, it would be necessary to validate the host factors that predispose patients to gastroduodenal diseases. A study by Yeh et al. [38] provides evidence that polymorphisms in the matrix metalloproteinase-3 promoter contribute to the increased susceptibility of *H. pylori*–infected women to duodenal ulcers.

The phylogenetic analysis showed that the *dupA* sequences from this study were grouped in the same clade as sequences from strains isolated from patients residing in western countries (Argentina, Brazil, and Portugal). This grouping may have been influenced by geographical proximity or by the migration or transit history of the peoples of those countries. It is important to consider that Brazil was once colonized by Portugal and for many years was dependent on this country, and the intense movement of people between the two countries could have increased the chances of transmission of *H. pylori* strains.

The grouping of the sequences from the eastern countries (China, India, and Japan) had strong statistical support, indicating their total separation from the sequences from the western countries. This genetic separation between western and eastern countries has also been described by Chiurillo et al. [21], who used *H. pylori iceA1* sequences (encoding a virulence factor) from Venezuelan strains. Although the number of *dupA* sequences from western strains is limited in the GenBank database, our phylogenetic tree was similar to that reported for other *H. pylori* virulence genes [39].

The *dupA* sequences isolated in this study revealed a high rate of homology, suggesting that the gene is highly conserved in circulating strains in mid-western Brazil. This is likely related to the fact that isolates from a single individual, a family member, and a specific geographical region are often clonal over short periods of time, despite that the general population structure of *H. pylori* is panmictic [40].

This study was limited by the small number of samples screened. Therefore, a more comprehensive phylogeographic analysis with a larger number of samples is needed to elucidate the impact of the genetic heterogeneity of *H. pylori* on patients with dyspepsia in Brazil. Notwithstanding, this is the first study that has used partial sequences of *dupA* from *H. pylori* strains in Brazil to determine their phylogenetic relationships and origin.

Conclusion

Presence of *dupA* in the infecting strain cannot be considered a biomarker of gastropathies in the study population. However, in women, infection with *dupA*-positive *H. pylori* increases the risk of developing gastritis. The *dupA* gene is highly conserved among circulating strains in mid-western Brazil. Our data provide perspectives for future studies aiming to evaluate parasite-host relationship factors, which can influence development of gastropathies.

Author contribution LLLS and AKSO performed data collection, data analysis, and interpretation and wrote the scientific work; ARG and JDGV helped in scientific writing and data collection; AMTCS, AJVB, and LTR data analysis; LLC and MSB conceived the study, participated in its design and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript.

Data availability All data generated or analyzed during this study are included in this published article.

Code availability Not applicable.

Declarations

Ethical approval The study was conducted according to the ethical standards of the Research Ethics Committee of the University Hospital of the Federal University of Goias (HC/UFG), under the opinion number 2.519.032, according to Resolution CNS/196/96.

Competing interests The authors declare no competing interests.

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