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Analysis of antimicrobial susceptibility and virulence factors in *Helicobacter pylori* clinical isolates

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Abstract

Background: In this study, we evaluated the prevalence of primary resistance of Brazilian *H. pylori* isolates to metronidazole, clarithromycin, amoxicillin, tetracycline, and furazolidone. In addition, the *vac*A, *ice*A, *cag*A and *cag*E genotypes of strains isolated from Brazilian patients were determined and associated with clinical data in an effort to correlate these four virulence markers and antibiotic resistance.

Methods: *H. pylori* was cultured in 155 *H. pylori*-positive patients and MICs for metronidazole, clarithromycin, amoxicillin, tetracycline, and furazolidone were determined by the agar dilution method. Genomic DNA was extracted, and allelic variants of *vacA*, *iceA*, *cagA* and *cagE* were identified by the polymerase chain reaction.

Results: There was a strong association between the *vacA* s1/*cagA* -positive genotype and peptic ulcer disease (OR = 5.42, 95% CI 2.6–11.3, p = 0.0006). Additionally, infection by more virulent strains may protect against GERD, since logistic regression showed a negative association between the more virulent strain, *vac*A s1/*cag*A-positive genotype and GERD (OR = 0.26, 95% CI 0.08–0.8, p = 0.03). Resistance to metronidazole was detected in 75 patients (55%), to amoxicillin in 54 individuals (38%), to clarithromycin in 23 patients (16%), to tetracycline in 13 patients (9%), and to furazolidone in 19 individuals (13%). No significant correlation between pathogenicity and resistance or susceptibility was detected when MIC values for each antibiotic were compared with different *vacA*, *iceA*, *cagA* and *cagE* genotypes.

Conclusion: The analysis of virulence genes revealed a specific association between *H. pylori* strains and clinical outcome, furthermore, no significant association was detected among pathogenicity and resistance or susceptibility.

Background

Helicobacter pylori is a gastric pathogen that chronically infects more than half of the world's population, with a prevalence ranging from 25% in developed countries to more than 90% in developing areas [1]. Infection with *H. pylori* is associated with chronic superficial gastritis, peptic ulceration and gastric cancer. The reason for such a clinically diverse outcome of infection remains uncertain, but may include host and environmental factors as well as differences in the prevalence or expression of bacterial virulence factors [1].

The *cagA* gene (cytotoxin-associated gene) is considered to be a marker for the presence of a pathogenicity island (*cag* PAI), and individuals infected with *cagA*-positive *H. pylori* strains have a higher risk of developing peptic ulcers and gastric cancer [2–4]. Several studies have reported that some strains may contain a partially-deleted *cag* PAI [5]. Thus, the presence of the *cagA* gene does not guarantee that critical genes within the *cag* PAI are intact [6], however the *cagE* gene appears to be a better marker of intact *cag* PAI than the *cagA* gene [6,7].

The *vacA* gene encodes a vacuolating toxin excreted by *H. pylori*, which has been associated with epithelial cell damage [8]. This gene is present in all strains and comprises two variable parts [2,9], the s-region (signal) and the m-region (middle) [3]. The production of vacuolating cytotoxin is related to the mosaic combination of s and m allelic types. The mosaic combination of the *vacA* gene has been associated with specific genotypes and different clinical outcomes [3,4,10].

The gene designated, *iceA* (induced by contact with epithelium), has two main allelic variants, *iceA*1 and *iceA* 2. Although *iceA*1 has been associated with peptic ulcer disease [3], other studies have failed to confirm this correlation, and some groups have suggested an inverse relationship [5,11].

H. pylori infection can be cured by antibiotics, however the ideal anti-*H. pylori* treatment has yet to be found [12,13]. Many factors have been implicated in treatment failure, including ineffective penetration of antibiotics into the gastric mucosa, antibiotic inactivation by low gastric pH, lack of compliance, and emergence of acquired antibiotic resistance by *H. pylori* [3,14]. Despite the success of the current anti-Helicobacter therapies, it is suggested that eradication rates among patients with gastritis are lower than among patients with peptic ulcer disease, with the causes of this phenomenon still being the subject of speculation [15].

In this study, we aimed to correlate primary resistance of Brazilian *H. pylori* isolates to metronidazole, clarithromycin, amoxicillin, tetracycline, and furazolidone with *vacA*, *iceA*, *cagA* and *cagE* status and clinical outcome.

Methods

Patients

Helicobacter pylori isolates were obtained from 155 patients (86 males and 69 females; age 43.6 ± 14.9 years) living in southeastern Brazil. Eighty patients with peptic ulcer disease (PUD), 54 with non-ulcer dyspepsia (NUD) and 21 with gastroesophageal reflux disease (GERD) were included. The study was approved by the Ethics Committee of the São Francisco University and performed in accordance with the Declaration of Helsinki. Each patient signed a written informed consent prior to entering the study.

H. pylori *culture and MIC determination*

H. pylori isolates were obtained by inoculating the specimens into selective media followed by incubation for 3–5 days at 37°C under microaerophilic conditions, as previously described [16]. The colonies were identified by Gram staining and by oxidase, catalase and urease production. *H. pylori* strains were stored at -70°C in BHI broth containing glycerol 30%. The MIC for amoxicillin, furazolidone, metronidazole, tetracycline (Sigma Chemical Co., St Louis, MO) and clarithromycin (Abbot Laboratories, North Chicago, Ill) were determined by the agar dilution method, using twofold increments (0.125 to 256 μg ml^{-1}) on Mueller-Hinton agar (Merck, Darmstadt, Germany) supplemented with 10% sheep blood, and incubated at 37°C under microaerophilic conditions for 72 h. All tests were performed in duplicate. Isolates were considered to be resistant when the MIC was greater than 8 μg ml^{-1} for amoxicillin or metronidazole, and greater than 2 μg ml^{-1} for clarithromycin, furazolidone or tetracycline [16].

Molecular Biology Methods

Genomic DNA was extracted with DNAzol™ reagent (Gibco BRL, Cincinnati, OH, USA), and the integrity of the DNA was assessed by electrophoresis in 0.8% agarose gels stained with ethidium bromide.

Polymerase chain reactions (PCR) were performed in a total volume of 50 μl containing 50 pmol of primer, 100 ng of genomic DNA, 1.0 mmol L^{-1} of each of four dNTPs (Invitrogen™ Life Technologies, Alemeda, CA, USA) and 2.5 U of *Taq* DNA polymerase (Invitrogen™ Life Technologies). The reaction mixtures were cycled in an automated GeneAmp® PCR System 9700 thermal cycler (PE Applied Biosystems, Foster City, CA, USA) under the following conditions: initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 1 min, annealing ranging from 45°C to 60°C for 1 min and

Table 1: The *vacA*, *iceA*, *cagA* and *cagE* status of *H. pylori* strains and clinical outcome

	Clinical Outcome			
Genotype	**PUD n (%)**	**NUD n (%)**	**GERD n (%)**	**Total n (%)**
vacA				
s1	57 (78)*	25 (54)	4 (21)	86 (62)
s2	16 (22)	21 (46)	15 (79)**	52 (38)
m1	27 (37)	11 (24)	3 (16)	41 (30)
m2	46 (63)	35 (76)	16 (84)	97 (70)
s1m1	27 (37)	11 (24)	3 (16)	41 (30)
s1m2	30 (41)*	14 (30)	1 (5)	45 (32)
s2m2	16 (22)	21 (46)	15 (79)**	52 (38)
iceA				
*iceA*1	12 (16)	6 (13)	3 (16)	21 (15)
*iceA*2	55 (75)	32 (70)	15 (79)	102 (74)
iceA -	6 (9)	8 (17)	1 (5)	15 (11)
cagA				
cagA +	55 (75)*	25 (54)	9 (47)	89 (65)
cagA -	18 (25)	21 (46)	10 (53)	49 (35)
cagE				
cagE +	60 (82)	39 (85)	17 (90)	116 (84)
cagE -	13 (13)	6 (15)	2 (10)	22 (16)

* *vacA* s1 (χ^2 = 14.3; p = 0.0002) s1m2 (χ^2 = 15.67; *p* = 0.0004) *cagA* (χ^2 = 7.954; *p* = 0.0048). ** s2m2 (χ^2 = 16.89; *p* = 0.0002)

72 °C for 1 min. The final cycle included a 7 min extension step to ensure full extension of the PCR products.

The presence of *H. pylori* was confirmed by PCR of the 16S rRNA [17] and *glmM* [18] genes. The *cagA* gene was analyzed using the primers D008 and R008 [19]. The *cagE* gene was analyzed using the primers described by Fallone *et al.* [10]. For analysis of the *vacA* m region, primers VA3-F and VA3-R were used, whereas primers VA4-F and VA4-R were used to amplify the m1 and m2 subtypes, respectively [5]. The *vacA* s region was analyzed using the primers, VA1-F and VA1-R [5]. For *iceA* genotype analysis, primers iceA1-F, iceA1-R, iceA2-F and iceA2-R were used. The primers, iceA2-F and iceA2-R, yielded a fragment of 124, 229 or 334 bp depending on the existence of repeated sequences of 105 nucleotides [3].

Statistical analysis

The association between H. pylori genotypes and clinical disease, as well as among the virulence markers and antibiotic resistance was evaluated using either the χ^2 test with Yates continuity correction or Fisher's exact test. Only cases containing single genotypes were included. Logistic regression analysis was used to evaulate the relationship between virulence markers and antibiotic resistance of H. pylori and clinical outcome. A logistic regression model was constructed using variables such as cagA, vacA and iceA status, and antibiotic resistance. The association of each variable with PUD or NUD or GERD (dependent variables) was tested by univariated analysis. All variables with p values of 0.25 or less were included in the full model of logistic regression and variables with p values <0.05 were remained in the model. The odds ratio (OR) and the 95% confidence interval (95% CI) were used as estimates of the risk.

Results and Discussion

Virulence factors

The presence of the genes vacA, cagA, cagE and iceA were investigated in all 155 clinical isolates positive for 16S rRNA and for glmM. Based on the vacA and iceA genotypes, 138 (89%) specimens were colonized by a single H. pylori strain. More than one strain was detected in 17 isolates and none of these patients were included in either the analysis of the relationship between clinical disease with the virulence factors or with antibiotic resistance. Since the prevalence of H. pylori infection, in our geographic region, reaches 80%, such prevalence of multiple infection would be expected [20].

The *vacA* s and m genotype could be identified in all cases studied. Most of the isolates had the s1 signal sequence allele (62%). The prevalence of *vacA* s1 was significantly higher in *H. pylori* isolates from PUD patients (χ^2 = 14.3 p = 0.0002). The *vacA* m1 allele was found in 41 (30%) and the m2 allele in 97 (70%) of the samples. There was no relationship between *vacA* m and clinical outcome. Considering the *H. pylori* isolates with only one single geno-

type (138), three mosaic combinations were found: s1m1 (30%), s1m2 (32%), and s2m2 (38%). Statistical analysis showed a significant association between PUD and s1m2 (χ^2 = 15.67; p = 0.0004), and between GERD and the s2m2 genotype (χ^2 = 16.89; p = 0.0002, Table 1).

The *iceA*1 genotype was detected in 21 (15%) of the 138 isolates analyzed, while *iceA*2 was found in 102 cases (74%). Fifteen isolates (11%) did not yield any PCR product for *iceA* (Table 1). There was no relationship between the size of the *iceA2* amplicon and the patient's disease. In contrast to results obtained by others, *iceA*2 was the most frequent genotype detected in our population [21,22]. In this study, the *iceA* type was not associated with the clinical outcome, as previously described [11,21].

With regard to the *cag* PAI, the *cagA* gene was present in 89 (65%) of the 138 *H. pylori* isolates, whereas the c*agE* gene was present in 116 (84%) isolates (Table 1). Both genes were absent in 11% (15/138) of the isolates and, in these cases, the *cag* PAI could also be absent. There was an association between PUD and the presence of the *cagA* (χ^2 = 7.954; p = 0.0048). Our data are supported by previous reports and suggest that individuals colonized with *cagA*-positive *H. pylori* strains are at increased risk of developing peptic ulceration [3,4]. Conversely, no association was detected between *cagE* genotypes and clinical outcome.

The combination of the *cagA* and *vacA* status was also investigated. An association was observed between the genotype, *cagA*-positive *vacA* s1 and PUD (χ^2 = 11.871; p = 0.0006) and between GERD and the *cagA*-negative *vacA* s2 genotype (p < 0.0001). Although our data showed a trend towards an association between the *cagA*-positive, *vacA* s1m2 genotype and PUD (χ^2 = 13.849; p = 0.001), logistic regression analysis suggested that this could be explained by a higher prevalence of the *cagA* and *vacA* s1 alleles (OR = 5.42, 95% CI 2.6–11.3, p = 0.0006) This finding agrees with those of others reports and suggests a possible role for these factors in the pathogenesis of *H. pylori*-related peptic ulceration [3,4]. Gastroesophageal reflux disease (GERD) results from exposure of the esophageal mucosa to acid [23]. There is no apparent causal relationship between *H. pylori* colonization and the presence of GERD or its complications, although GERD patients are infected with less virulent *H. pylori* strains [24]. Infection by *H. pylori* may protect against GERD, thus these patients may have a lower frequency of *vacA* s1 genotype and a higher frequency of the *vacA* s2 and m2 genotypes [4,10,25]. Our data support this hypothesis and logistic regression showed a negative association between the more virulent strain, *cag*A-positive *vac*A s1 genotype, and GERD (OR = 0.26, 95% CI 0.08–0.8, p = 0.03). Although the protective effect of infection with *cag*A-positive *H. pylori* strains against GERD could be explained by the lower gastric output due to the more intense gastric lesions induced by these strains [26]. Further studies are required to determine the putative protective role of the more virulent strains.

Antimicrobial Susceptibility

H. pylori resistance to antimicrobial is of particular concern because it is a major determinant of eradication regimen failure. We tested the primary resistance to amoxicillin, clarithromycin, furazolidone, metronidazole and tetracycline in 138 *H. pylori* isolates.

Until recently, resistance to amoxicillin was considered to be absent or very rare, however, amoxicillin-resistant *H. pylori* strains have now been identified in USA, Italy and Brazil [16,27,28]. In this study, the amoxicillin resistance was found in 39% of isolates (54/138). The reason for these high rates of amoxicillin resistance remains unclear, however, since no pharmaco-epidemiological data regarding amoxicillin use in Brazil exists, it may be speculated that this drug is used in a disproportionate manner.

The increased consumption of macrolides is expected to influence the prevalence of *H. pylori* resistance to clarithromycin. Resistance to clarithromycin was detected in 16% of strains (23/138). Our results agree with previous reports from Brazil, being lower those from Peru [29] and higher than rates in Europe [30]. Resistance to clarithromycin has a serious clinical implication, since it impairs the efficacy effect of anti-*H. pylori* treatment [31].

The occurrence of metronidazole-resistant strains may be the consequence of increased consumption of this antibiotic in the community. In this study, the resistance to metronidazol was observed in 54% (75/138) of strains. Metronidazole has been widely prescribed for other infections such as parasitic or genital infections, and the use or abuse of this inexpensive drug may contribute to the increase in metronidazole resistance, with rates ranging from 5% to 90% [32].

Tetracyclines are a family of broad-spectrum antibiotics that have been widely used for the treatment of bacterial infections since the 1950s. Until the end of the last century, only a few cases of spontaneous tetracycline resistance in *H. pylori* had been reported [33]. During the last 3 years, however, an increase in the incidence of tetracycline resistance in *H. pylori* has been reported. In this study, we found 9% (13/138) of tetracycline resistance in strains. Similar data were published in previous reports [16,34].

Furazolidone has been used as an alternative to overcome metronidazole resistance. In our study, resistance to furazolidone was 13% (19/138), higher than rates detected by others [22]. The reason for the high rate of furazolidone

resistance and factors leading to it are still unknown and deserve further investigation.

Thirty one strains were resistant to two agents, 19 isolates to three drugs, and 3 strains to four antimicrobials. *H. pylori* resistance to these antimicrobials may be partially explained by the high prevalence of this bacteria in our population. Eradication failure has been associated with several reasons, the most important being primary *H. pylori* resistance.

Additionally, the primary *H. pylori* resistance was related to the clinical outcome, as well as to virulence factors. We were unable to detect an association between outcome and bacterial resistance, as previously reported [35,36]. With regard to virulence factors, no association was found when the MIC values for metronidazole, amoxicillin, clarithromycin, tetracycline, and furazolidone were compared with the different *vac*A, *ice*A, *cag*A and *cag*E genotypes, as described by several authors [35,37]. Thus, it seems that different virulence markers and antibiotic resistance in *H. pylori* strains are not interrelated.

Conclusions

In conclusion, the analysis of virulence genes revealed a specific association between *H. pylori* strains and clinical outcome. Our data suggest an association between *cag*A-positive, *vac*A s1 genotype, and PUD and also support the hypothesis that virulent strains may protect against GERD development. Furthermore, no significant correlation was detected among pathogenicity and resistance or susceptibility.

List of abbreviations

PUD – peptic ulcer disease; NUD – non-ulcer dyspepsia; GERD – gastroesophageal reflux disease; PCR – Polymerase chain reaction.

Competing interests

None declared.

Author's contributions

APOG, MLR and MCBM carried out the molecular genetic studies; APOG, YHBB, LV and SM participated in the antimicrobial susceptibility analysis; APOG and MLR drafted the manuscript; MLR and APOG performed the statistical analysis; MLR, SM and JPJ participated in the design of the study and coordination; All authors read and approved the final manuscript.

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