A Novel Method for Genotyping the *Helicobacter pylori* *vacA* Intermediate Region Directly in Gastric Biopsy Specimens

Rui M. Ferreira,^{a,b} Jose C. Machado,^{a,b} Darren Letley,^c John C. Atherton,^c Maria L. Pardo,^d Carlos A. Gonzalez,^e Fatima Carneiro,^{a,b,f} and Ceu Figueiredo^{a,b}

IPATIMUP, Institute of Molecular Pathology and Immunology of the University of Porto, Porto, Portugal^a; Medical Faculty of the University of Porto, Porto, Portugal^b; NIHR Biomedical Research Unit, Nottingham Digestive Diseases Centre, University of Nottingham, Nottingham, United Kingdom^c; Department of Pathology, Complejo Hospitalario de Soria, Soria, Spain^d; Unit of Nutrition, Environment and Cancer, Catalan Institute of Oncology, Barcelona, Spain^e; and Department of Pathology, Centro Hospitalar de Sao João, Porto, Portugal^f

The present report describes a novel method for genotyping the virulence-associated *vacA* intermediate (i) region of *Helicobacter pylori* in archive material. *vacA* i-region genotypes as determined by the novel method were completely concordant with those of sequence analysis and with those of functional vacuolation activity. The method was further validated directly in gastric biopsy specimens of 386 *H. pylori*-positive cases, and effective characterization of the *vacA* i region was obtained in 191 of 192 (99.5%) frozen and in 186 of 194 (95.9%) formalin-fixed paraffin-embedded gastric biopsy specimens, respectively. The genotyping method was next used to address the relationship between the *vacA* genotypes and the *cagA* status. The *vacA* i1 genotype was associated with *vacA* s1 (where s indicates signal region), *vacA* m1 (where m indicates middle region), and *cagA*-positive genotypes ($P < 0.0001$), while the *vacA* i2 genotype was closely related with *vacA* s2, *vacA* m2, and *cagA*-negative genotypes ($P < 0.0001$). The relationship between *H. pylori* *vacA* i-region genotypes and gastric disease development was subsequently evaluated in the Portuguese population. Patients infected with *vacA* i1 strains showed an increased risk for gastric atrophy and for gastric carcinoma, with odds ratios of 8.0 (95% confidence interval [CI], 2.3 to 27) and of 22 (95% CI, 7.9 to 63), respectively. Taken together, the results show that this novel *H. pylori* *vacA* i-region genotyping method can be applied directly to archive material, providing a fast evaluation of strain virulence determinants without the need of culture. The results further emphasize that the characterization of the *vacA* i region may be useful to identify patients at higher risk of gastric carcinoma development.

Helicobacter pylori persistently infects the gastric mucosa of more than half of the world's population (5). The infection is associated with several diseases, including peptic ulcer disease and gastric carcinoma. Gastric carcinoma is a result of a cascade of events that starts with chronic superficial gastritis induced by *H. pylori*, which may progress to chronic atrophic gastritis, intestinal metaplasia, and dysplasia that ultimately leads to gastric carcinoma (9). Disease development is likely to depend on the combination of host susceptibility, environmental factors, and bacterial virulence (15, 17, 27, 28).

One of the most important virulence factors of *H. pylori* is CagA, which is encoded by the *cagA* gene and is present in about 60 to 70% of the Western strains. *cagA* has been considered a marker for the presence of the *cag* pathogenicity island (PAI), a portion of the *H. pylori* genome that encodes a type IV secretion system and through which CagA is translocated into the host cells (3, 7, 30). It has been shown that patients infected with *cagA*-positive strains have an increased risk for gastric atrophy and gastric carcinoma (17, 23).

VacA is another important *H. pylori* virulence factor which has been associated with disease. The VacA toxin has multiple cellular activities, including the formation of vacuoles in epithelial cells (1, 10, 11). The gene encoding VacA is present in all *H. pylori* strains and displays allelic diversity in three main regions: the s (signal), the i (intermediate), and the m (middle) regions. Different combinations of two major alleles of each region (e.g., s1 and s2 in the s region) may exist, which result in VacA toxins with distinct capability of inducing vacuolation in epithelial cells (2, 5, 35). While *vacA* s1/m1 strains are consistently vacuolating and *vacA* s2/m2

strains are nonvacuolating, only some *vacA* s1/m2 strains are able to induce the formation of vacuoles in cells (25, 26). s1/m2 strains that have an i1 allele are vacuolating, whereas s1/m2 strains that have an i2 allele are nonvacuolating (32).

Allelic variation in the *vacA* i region was defined on the basis of sequence analysis, which revealed polymorphisms in three amino acid clusters (A, B, and C) (32). Further functional analysis of this region showed that only clusters B and C were determinant of the VacA cytotoxicity (32). Moreover, it was also shown that there is an insertion/deletion in cluster C that impacts cell vacuolation (32). In addition to the i1 and i2 alleles, the rare i3 allele has been described and was defined as a discordant pair of clusters B and C, in which the amino acid sequence at one cluster is i1-like and i2-like at the other cluster (8, 32). The i3 allele is phylogenetically associated with the i2 allele, which encodes nonvacuolating type VacA (8).

It has been shown that *H. pylori* *vacA* s1 and m1 strains are associated with high levels of inflammation in the gastric mucosa and increased risk for gastric atrophy and carcinoma compared with the less virulent *vacA* s2 and m2 strains (17, 18, 29). After the

Received 6 August 2012 Returned for modification 31 August 2012

Accepted 22 September 2012

Published ahead of print 3 October 2012

Address correspondence to Ceu Figueiredo, cfigueiredo@ipatimup.pt.

Copyright © 2012, American Society for Microbiology. All Rights Reserved.

doi:10.1128/JCM.02087-12

description of the *vacA* i region, it was also shown that the determinant of cytotoxicity, the i1 allele, is associated with gastric carcinoma (4, 32). Therefore, there has been an increased interest to evaluate if the characterization of this region could be used as a determinant of the clinical outcome of *H. pylori* infection.

To the best of our knowledge, the only *vacA* i-region genotyping method available is the original (32). However, this method has only been applied to DNA samples derived from *H. pylori* clinical isolates (32). Therefore, we have developed a novel PCR-based genotyping method for characterizing the *vacA* i region, which can be used directly in archive material, avoiding the need for *H. pylori* culture. Furthermore, we have used this new method to investigate the relationship between *H. pylori vacA* i-region genotypes and gastric atrophy and carcinoma development in Portuguese patients.

MATERIALS AND METHODS

Patients and study population. A total of 192 *H. pylori*-infected patients from the north of Portugal were analyzed; the group included 114 patients with chronic superficial gastritis (mean age, 42.0 ± 7.1 years; male/female ratio of 37:1), 22 with chronic atrophic gastritis (mean age, 44.5 ± 8.5 years; male/female ratio of 22:0), and 56 with gastric carcinoma (mean age, 59.4 ± 13.5 years; male/female ratio of 1.9:1). Subjects with chronic gastritis were recruited from a group of shipyard workers and underwent standard gastroscopy in 1998 at the Hospital S. João, Porto, Portugal, during a screening program for premalignant lesions of the gastric mucosa. Only individuals without evidence of past or present peptic ulcer disease were included. Patients with gastric carcinoma were diagnosed and underwent resection of cancer at the Hospital S. João and the Institute of Molecular Pathology and Immunology of the University of Porto (IPATIMUP). All procedures followed in the study were in accordance with the institutional ethical standards. Samples were delinked and unidentified from their donors, and written informed consent was obtained from all donors.

An additional group of 194 formalin-fixed and paraffin-embedded (FFPE) gastric biopsy specimens of *H. pylori*-infected patients that underwent gastroscopy with gastric biopsy between 1988 and 1994 and that were part of a follow-up study of preneoplastic lesions in Soria, Spain (18, 19), were used for comparison of the method between this type of archive material and frozen biopsy specimens.

Histopathology. Two gastric biopsy specimens from the antrum (one from the greater curvature and one from the incisura angularis) and one from the corpus were immersed in 10% formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin, Alcian blue-periodic acid-Schiff, and modified Giemsa. Only cases with adequately sized biopsy specimens of both antral and corpus mucosa were accepted for histological assessment by an experienced pathologist, who was blinded with respect to the clinical information of each patient. The histopathological parameters were classified according to the criteria described in the updated Sydney classification system (13). Gastric carcinoma cases were categorized according to Lauren's classification (24).

***H. pylori* strains and growth conditions.** *H. pylori* strains 60190 (ATCC 49503; *vacA* s1/m1 and *cagA* positive), Tx30a (ATCC 51932; *vacA* s2/m2 and *cagA* negative), and 34 clinical isolates from patients undergoing gastroscopy for the investigation of dyspeptic complaints were used. Strains were cultured for 48 h in tryptic soy agar supplemented with 5% sheep blood (BD, Germany) and incubated at 37°C under microaerobic conditions using a GENbox microaer (bioMérieux).

DNA isolation and *vacA* s- and m-region and *cagA* genotyping. DNA was extracted from each clinical isolate using a Genomic DNA Purification Kit (Fermentas), following the manufacturer's instructions. DNA was isolated from paraffin-embedded gastric tissue after digestion in a solution containing 10 mM Tris-HCl (pH 8.0), 5 mM EDTA, 0.1% sodium dodecyl sulfate, and 0.1 mg/ml proteinase K for at least 12 h at 55°C.

Proteinase K was then inactivated by incubation for 10 min at 95°C. In the frozen biopsy specimens, DNA was obtained using the method described by Boom et al. (6). Briefly, biopsy specimens were homogenized in guanidinium isothiocyanate with a sterile micropestle. DNA was captured onto silica particles, washed, and then eluted in 100 µl of 10 mM Tris-HCl (pH 8.3). *vacA* and *cagA* genotyping was performed by PCR, followed by reverse hybridization on a line probe assay (LiPA), as previously described (35, 36).

***H. pylori vacA* i-region genotyping.** The *vacA* i region was amplified using primers VacF1 (forward), C1R (reverse), C2R (reverse), previously reported by Rhead et al. (32), and VacIABF (forward) (5'-CGTGTGGGT TCTGGAGCCG-3') developed in this study. PCR mixtures were prepared in a volume of 25 µl, containing 1× PCR buffer (Applied Biosystems), 2.5 mM MgCl₂, 1 mM concentrations of deoxynucleotide triphosphates (dNTP), 0.5 U of AmpliTaq Gold (Applied Biosystems), and 0.5 µM concentrations of forward and reverse primers. PCR was performed with a 9-min predenaturation at 95°C, followed by 45 cycles of 30 s at 95°C, 45 s at 50°C, and 45 s at 72°C. Final extension was performed for 10 min at 72°C. PCR products were electrophoresed on 2% agarose gels and examined under UV light, according to standard procedures. The accurate i-region genotyping was confirmed by PCR/sequencing using primers VacR9 (reverse) and DL3 (forward) (32) with a BigDye Terminator, version 3.1, Cycle Sequencing Kit and run in an ABI Prism 3100 DNA automated sequencer (Applied Biosystems).

Sequence analysis. Nucleotide sequences were aligned by using Clustal W2 at the EMBL-EBI website and Genedoc (version 2.2.000).

***VacA* vacuolating activity assay.** The vacuolation activity of the different clinical isolates was assessed as described previously (12, 26, 32). Briefly, water extracts were prepared by harvesting a 48-h growth plate in 1 ml of sterile distilled water and incubating the culture at room temperature for 20 min. Cells were removed by microcentrifugation, and the supernatant containing VacA was filter sterilized (0.20-µm pore size). A total of 10⁴ AGS cells were adhered to a 96-well plate in each well with RPMI medium (Invitrogen, United Kingdom) supplemented with 10% fetal bovine serum (FBS) (HyClone, United Kingdom) for 18 h. The medium was then replaced with fresh medium containing 10 mM ammonium chloride, and a 5-fold dilution of water extract and cells was incubated for 24 h. Vacuolating activity was evaluated under a light microscope (Nikon Eclipse TS100-F; China), and the strains were considered vacuolating when at least 50% of the cells exhibited vacuoles. This assay was performed in triplicates, and photos were taken at a magnification of ×400.

Statistical analysis. Comparison of genotype frequencies and the clinical outcome was performed by Fisher's exact test. Odds ratios (ORs) with 95% confidence intervals (CIs) and logistic regression models were computed with the software Statview for Windows (version 5; SAS Institute Inc., Cary, NC). Differences were considered statistically significant at a *P* value of <0.05.

Nucleotide sequence accession numbers. *vacA* i-region nucleotide sequences shown in Fig. 1 were submitted to the GenBank under accession numbers JX666364 to JX666373.

RESULTS

Development of a novel genotyping system for characterization of the *H. pylori vacA* i region. The currently available genotyping method for the characterization of the *H. pylori vacA* i region has been used in DNA samples derived from *H. pylori* clinical isolates but has never been tested in archive material (4, 8, 20, 32). The method generates amplicons containing the clusters A, B, and C, using the VacAF1 forward primer and the C1R or C2R reverse primer, yielding amplicons of 426 bp and 432 bp for i1 and i2 alleles, respectively (32). Since amplicons with these sizes are difficult to obtain from DNA derived from archive material, we aimed at designing a novel system that would allow *vacA* i-region

FIG 1 Schematic representation of the proposed genotyping method for *H. pylori vacA* i-region characterization in archive material. The combination VacIABF and C1R was used to amplify the i1 allele, yielding an amplicon of 145 bp, and the combination of VacIABF and C2R was used to amplify the i2 allele, yielding an amplicon of 151 bp. The alignment shown was produced using 10 sequences from our clinical isolates (GenBank accession numbers JX666364 to JX666373) and *H. pylori* strains 60190 (i1) and Tx30a (i2) as controls. In the alignment, a dash indicates the absence of a nucleotide, and identical nucleotides are indicated by dots.

genotyping directly in frozen and in formalin-fixed paraffin-embedded (FFPE) gastric biopsy specimens.

Based on the alignment of sequences of the full *vacA* gene deposited in the GenBank, knowing that clusters B and C are determinant for VacA toxin's activity and that sequence differences within these clusters determine the i1 or i2 type (4), we designed a new forward primer. This primer (VacIABF) is located upstream of cluster B (nucleotides [nt] 1412 to 1430 of *vacA* from strain 60190; GenBank accession number U05676) and was combined with the original reverse primers C1R and C2R (Fig. 1). This novel system yields amplicons of 145 bp for the i1 allele (deduced from strain 60190) and 151 bp for the i2 allele (deduced from strain Tx30a). These smaller amplicons are expected to be easily amplified in DNA obtained from archive material.

We first tested and optimized the PCR conditions on DNA extracted from our collection of *H. pylori* strains ($n = 34$). In these isolates, we were able to amplify the *vacA* i region in all samples. From the 34 strains, we found that 20 (58.2%) were *vacA* i1 and 14 (41.2%) were *vacA* i2 (data not shown), and these results were all confirmed by DNA sequencing. Sequence analysis did not show the presence of the *vacA* i3 allele in any of the strains.

To further confirm the accuracy of the proposed *vacA* i-region genotyping method in terms of prediction of VacA cytotoxic ac-

tivity, we performed vacuolation assays using the human gastric AGS cell line and 12 *H. pylori* isolates (Fig. 2). All strains that were genotyped as *vacA* i1 were cytotoxic to AGS cells, whereas strains that were genotyped as *vacA* i2 were noncytotoxic. These results demonstrate that amplification of clusters B and C is sufficient for the characterization of the *vacA* i region and for the prediction of VacA vacuolation activity.

Validation of the genotyping method for *H. pylori vacA* i region in archive material. In order to further evaluate the novel genotyping method, a total of 386 (192 frozen and 194 FFPE) *H. pylori*-positive gastric biopsy specimens were used. In frozen gastric biopsy specimens, 90.1% and 99.5% of the *H. pylori*-positive cases could be amplified with the original and with the novel primer sets, respectively (Table 1). Using the novel primer combination, both *vacA* i-region genotypes were observed in 40 of 192 (20.8%) of the samples. One (0.5%) specimen repeatedly failed to yield a PCR product. Of the 151 samples with a single genotype, 78 (51.7%) contained *vacA* i1, and 73 (48.3%) contained *vacA* i2.

In FFPE gastric biopsy specimens, only 37.1% of the *H. pylori*-positive cases could be amplified with the original primers, whereas the novel primer combinations allowed effective amplification of 95.9% of the cases (Table 1). Using the novel primer combination, both *vacA* i-region genotypes were observed in 16 of

FIG 2 Vacuolating activity exerted by different clinical isolates of strains 60190 and Tx30a in AGS cells. (A) AGS cells were grown in 96-well plates, which were then incubated with water extracts from the following *H. pylori* strains: 60190 (*vacA* s1/i1/m1), Tx30a (*vacA* s2/i2/m2), CI-11 (*vacA* s1/i2/m2), CI-12 (*vacA* s1/i1/m2), CI-13 (*vacA* s1/i1/m2), CI-14 (*vacA* s1/i1/m2), CI-15 (*vacA* s2/i2/m2), and CI-16 (*vacA* s1/i1/m1). Photos were obtained at a magnification of $\times 400$. Scale bar, 25 μ m.

194 (8.3%) of the cases. Eight (4.1%) cases could not be genotyped for the *vacA* i region. Of the 170 samples with a single genotype, 59 (34.7%) were genotyped as *vacA* i1, and 111 (65.3%) were genotyped as *vacA* i2. These results indicate that the novel genotyping method is more suitable for archive material than the original, especially for FFPE gastric biopsy specimens.

Relationship between the *H. pylori vacA* i-region genotypes and the *vacA* s and m regions and *cagA* status in Portuguese patients. In order to evaluate the relationship between *vacA* i region and *vacA* s and m regions and the *cagA* status, we have used a subset of the samples ($n = 189$) which had been previously characterized for the *vacA* s, *vacA* m, and *cagA* *H. pylori* genotypes (17). In this subset of cases, and excluding patients infected with multiple *H. pylori* strains ($n = 70$; 37.0%), we observed the following *vacA* gene structures: s1/i1/m1, 42.9%; s1/i1/m2, 7.2%; s1/i2/m2,

1.7%; s2/i2/m2, 44.5%. The rare s1/i2/m1 and s2/i1/m2 combinations were each found in two cases (1.7%).

The majority of *vacA* i1 strains were of the *vacA* s1 genotype (96.8%), while the *vacA* i2 strains were more frequently of the *vacA* s2 genotype (93.0%; $P < 0.0001$) (Table 2). The *vacA* i1 strains were also more commonly of the *vacA* m1 genotype (82.3%), and *vacA* i2 strains were more frequently of the *vacA* m2 genotype (96.5%; $P < 0.0001$). Regarding the *cagA* status, the i1 allele was more frequently found in *cagA*-positive (91.9%) strains, and conversely the i2 allele was more frequent in *cagA*-negative strains (84.2%; $P < 0.0001$). These results reflect the strong association between *vacA* i and *vacA* s and m genotypes and *cagA* status.

Evaluation of risk for atrophy and gastric carcinoma using *H. pylori vacA* i-region genotypes. In order to evaluate the role of the *vacA* i region in the development of gastric atrophy and gastric carcinoma in the Portuguese population, we performed a logistic regression analysis, presented in Table 3. Infection with *H. pylori vacA* i1 strains increased the risk for chronic atrophic gastritis, with an odds ratio of 8.0 (95% CI, 2.3 to 27). Moreover, infection with *H. pylori vacA* i1 strains also increased the risk of gastric carcinoma with an OR of 22 (95% CI, 7.9 to 63). These associations remained significant even after adjustment for age and sex.

Taken together, our results show that this novel *H. pylori vacA* i-region genotyping method can be used directly in DNA derived from archive material and reinforce that *vacA* i-region genotypes can be used as additional markers for disease risk estimation.

TABLE 1 Comparison of the original *H. pylori vacA* i-region genotyping method and the one proposed in this study in 192 frozen and in 194 FFPE gastric biopsy specimens

Type of specimen	No. of specimens successfully typed/no. of specimens tested (% successfully typed) by primer type	
	Original primers ^a	Novel primers ^b
Frozen gastric biopsy specimen	173/192 (90.1%)	191/192 (99.5%)
FFPE gastric biopsy specimen	72/194 (37.1%)	186/194 (95.9%)

^a Primers are from Rhead et al. (32). PCR product length: i1, 426 bp; i2, 432 bp.

^b Primers are from the present study. PCR product length: i1, 145 bp; i2, 151 bp.

TABLE 2 Relationship between *H. pylori* genotypes of the *vacA* i region and the *vacA* s and m regions and *cagA* status in Portuguese patients

Strain	Frequency of allele by region (no. of strains [%])					
	<i>vacA</i> s region		<i>vacA</i> m region		<i>cagA</i>	
	s1	s2	m1	m2	Positive	Negative
<i>vacA</i> i1 strain	60 (96.8)	2 (3.2)	51 (83.6)	11 (16.4)	57 (91.9)	5 (8.1)
<i>vacA</i> i2 strain	4 (7.0)	53 (93.0)	2 (3.5)	55 (96.5)	9 (15.8)	48 (84.2)
<i>P</i> value ^a	<0.0001		<0.0001		<0.0001	

^a Fisher-exact test. Only the single genotypes for the *vacA* s, m, and i regions were included in this analysis.

DISCUSSION

H. pylori causes chronic gastritis in all infected individuals and is associated with development of several clinically important diseases, such as atrophic gastritis, duodenal and gastric ulcers, and gastric carcinoma (5, 9). Thus, it is important that accurate diagnostic methods are available. In addition to the contribution of host susceptibility and environmental factors for disease development, there is mounting evidence that genetic variability of *H. pylori* virulence factors has clinical importance (17, 27, 36). *vacA* and *cagA* are two pathogenicity-associated *H. pylori* genes that display variation among strains. Patients that are infected with the most virulent *vacA* s1/m1 and *cagA*-positive strains are at higher risk for gastric carcinoma development than those infected with the lower virulence *vacA* s2/m2 and *cagA*-negative strains (4, 17). A third polymorphic region of *vacA* has been described, the i region, which can occur as an i1, i2, or i3 genotype (8, 32). The i1 genotype has been associated with peptic ulcer disease and gastric carcinoma development (4, 32). Therefore, the characterization of the *H. pylori vacA* i region has become important to evaluate disease risk. This study describes the development of a method for analysis of the *vacA* i region of *H. pylori* directly in archive materials.

The majority of the studies addressing the *vacA* i region have been performed in DNA derived from *H. pylori* clinical isolates (4, 8, 21, 22). In fact, the only study that has characterized *vacA* i region in archive materials, and more specifically in FFPE gastric biopsy specimens, has been performed by our group using the method we describe herein (16).

To deduce a novel primer combination that results in amplification of small fragments, we used sequences that were deposited in the GenBank. We designed a novel forward primer in a conserved region upstream of cluster B, which was combined with the two original reverse primers at cluster C (32). We have achieved complete concordance between genotyping with the novel primer combinations and sequencing analysis of clinical isolates, confirming the high degree of specificity of the method. Furthermore, we confirmed the accuracy of the genotyping method in terms of prediction of VacA cytotoxic activity by showing in functional

assays that all of the *vacA* i1 and none of the *vacA* i2 strains induced epithelial cell vacuolation.

The method was then tested directly in archive materials, comprising frozen and FFPE gastric biopsy samples. We obtained efficient characterization of the *vacA* i region in more than 95% of the cases. Importantly, in FFPE gastric biopsy specimens a significant improvement was observed in *vacA* i-region amplification (95.9%) in comparison with the original method (37.1%). These results may be explained by the amplicon sizes yielded by the different methods. While the amplicons obtained with the original *vacA* i-region genotyping method were bigger than 400 bp, fragments obtained with the novel method were about 150 bp. Indeed, the size of the amplicons is critical for the effectiveness of PCR in archive materials and should not be longer than 200 bp (33, 34).

The overall distribution of the *vacA* i1 and i2 genotypes appeared to be different in frozen and FFPE biopsy specimens. However, and while FFPE biopsy specimens comprised only samples from patients with chronic gastritis, frozen gastric biopsy specimens comprised samples from patients with chronic gastritis as well as from patients with gastric carcinoma. Therefore, if one compares the distribution of genotypes in FFPE (i1, 34.7%; i2, 65.3%) with that in frozen biopsy specimens of the chronic (superficial and atrophic) gastritis patients (i1, 36/104, or 34.6%; i2, 68/104, or 65.4%) (Table 3), there is perfect concordance in the prevalence of genotypes.

The prevalence of infection with both *vacA* i-region genotypes in the same biopsy specimen (interpreted as multiple strains) was different in FFPE and in frozen biopsy specimens. While this may represent a difference in prevalence of multiples in the two populations, we cannot exclude that the processing and conservation of FFPE biopsy specimens during an 18- to 24-year period may have led to technical PCR problems and to an underestimation of the number of multiple infections in this type of samples.

The method we present here offers advantages over routine diagnostic tools such as histological *H. pylori* detection, which has high interobserver variation, very much depending on the experience of the pathologist, and over *H. pylori* culture, which

TABLE 3 *H. pylori vacA* i-region genotyping and risk of chronic atrophic gastritis and gastric carcinoma in Portuguese patients

Strain	Chronic superficial gastritis (no. of subjects [%])	Risk analysis for:			
		Chronic atrophic gastritis		Gastric carcinoma	
		No. of subjects (%)	OR (95% CI) ^a	No. of subjects (%)	OR (95% CI) ^a
<i>vacA</i> i2 strain	64 (72.7)	4 (25.0)	1	5 (10.7)	1
<i>vacA</i> i1 strain	24 (27.3)	12 (75.0)	8.0 (2.3–27)*	42 (89.4)	22 (7.9–63)*

^a OR, odds ratio (unadjusted) for developing chronic atrophic gastritis and gastric carcinoma using chronic superficial gastritis subjects as controls. Only the single genotypes of the *vacA* i regions were included in the analysis. *, *P* < 0.001.

is more time-consuming, requires expertise, and is not always successful. Overall, PCR-based techniques show high sensitivity for *H. pylori* detection and also permit further characterization of bacteria virulence-associated genotypes. In archive materials, the genotyping of the *vacA* i region allows the analysis of the *H. pylori* strains in the same biopsy specimen that can also be used for histopathological evaluation, conferring a more reliable measurement of the effects of local infecting strains. Another advantage of the use of archive materials is that it permits retrospective studies to be performed without the need of *H. pylori* isolation from fresh biopsy specimens. The development of a multiplex PCR comprising the primers described here in combination with primers for the *vacA* s and m regions (35, 36) could constitute a powerful technique that would allow full *vacA* genotyping in a single step.

Using the proposed genotyping method, the *H. pylori vacA* i region was characterized in samples from a Portuguese population comprising patients with chronic gastritis and gastric carcinoma. We confirmed the clinical relevance of *H. pylori* i-region genotyping by showing that *vacA* i1 strains conferred an increased risk for gastric atrophy 8-fold higher than that of *vacA* i2 strains. Additionally, the risk for gastric carcinoma was 22-fold higher in patients infected with *vacA* i1 strains than in those infected with *vacA* i2 strains. Accordingly, along with the results of other investigators, our results confirmed the strong association between the *vacA* i1 allele and gastric carcinoma (4, 14, 31, 32). *vacA* i1 strains were associated not only with gastric carcinoma but also with the development of premalignant lesions of the stomach, namely, atrophy. The results obtained here are in agreement with those of a follow-up study conducted in a region of high risk for gastric carcinoma in Spain in which we have used this genotyping method. In that study, we have shown that progression of gastric preneoplastic lesions (after a mean follow-up of 12.8 years) occurred more frequently in patients infected with *vacA* i1 strains than in patients infected with *vacA* i2 strains, with an adjusted OR of 3.4 (16). Our results also support the observations of Basso et al. that indicated an association between *vacA* i1 strains and pangastritis, which is the topographic pattern of gastritis in which gastric carcinoma develops (4).

In this population, we observed that the *vacA* i1 genotype was strongly associated with *vacA* s1, *vacA* m1, and *cagA*-positive genotypes, while the *vacA* i2 genotype was closely associated with *vacA* s2, *vacA* m2, and *cagA*-negative genotypes, in accordance with previously published data (4, 8, 20). These results emphasize the fact that association studies can hardly evaluate which *H. pylori* genotype better predicts disease outcome since all *H. pylori* genotypes correctly mark disease-causing strains. Nonetheless, the accurate *vacA* i-region genotyping may provide an additional tool to predict gastric carcinoma development.

The present study describes for the first time a robust genotyping method that can be extensively used for *H. pylori vacA* i-region characterization in archive materials, providing a fast evaluation of strain determinants without the need of *H. pylori* culture. Moreover, we have confirmed the strong relationship between *vacA* i region and the development of gastric carcinoma. Together, our results suggest that the accurate characterization of the *vacA* i region may be useful to identify patients at higher risk of severe disease who could be offered eradication treatment and more intensive surveillance.

ACKNOWLEDGMENTS

This study was supported by the ERA-NET Pathogenomics (HELDIVPAT-ERA-PTG/0001/2010) and by the Portuguese Foundation for Science and Technology (FCT-PTDC/SAU-SAP/120024/2010). R.M.F. is supported by an FCT fellowship (SFRH/BD/45841/2008). IPATIMUP is an Associate Laboratory of the Portuguese Ministry of Science, Technology and Higher Education and is partially supported by FCT. J.C.A. and D.L. were funded by the UK National Institute for Health Research through their Biomedical Research Unit in the Nottingham Digestive Diseases Centre.

REFERENCES

- Atherton JC. 2006. The pathogenesis of *Helicobacter pylori*-induced gastro-duodenal diseases. *Annu. Rev. Pathol.* 1:63–96.
- Atherton JC, et al. 1995. Mosaicism in vacuolating cytotoxin alleles of *Helicobacter pylori*. Association of specific *vacA* types with cytotoxin production and peptic ulceration. *J. Biol. Chem.* 270:17771–17777.
- Backert S, Tegtmeyer N, Selbach M. 2010. The versatility of *Helicobacter pylori* CagA effector protein functions: The master key hypothesis. *Helicobacter* 15:163–176.
- Basso D, et al. 2008. Clinical relevance of *Helicobacter pylori* *cagA* and *vacA* gene polymorphisms. *Gastroenterology* 135:91–99.
- Blaser MJ, Atherton JC. 2004. *Helicobacter pylori* persistence: biology and disease. *J. Clin. Invest.* 113:321–333.
- Boom R, et al. 1990. Rapid and simple method for purification of nucleic acids. *J. Clin. Microbiol.* 28:495–503.
- Censini S, et al. 1996. *cag*, a pathogenicity island of *Helicobacter pylori*, encodes type I-specific and disease-associated virulence factors. *Proc. Natl. Acad. Sci. U. S. A.* 93:14648–14653.
- Chung C, et al. 2010. Diversity of *VacA* intermediate region among *Helicobacter pylori* strains from several regions of the world. *J. Clin. Microbiol.* 48:690–696.
- Correa P. 1992. Human gastric carcinogenesis: a multistep and multifactorial process—First American Cancer Society Award Lecture on Cancer Epidemiology and Prevention. *Cancer Res.* 52:6735–6740.
- Cover TL, Blanke SR. 2005. *Helicobacter pylori* *VacA*, a paradigm for toxin multifunctionality. *Nat. Rev. Microbiol.* 3:320–332.
- Cover TL, Blaser MJ. 1992. Purification and characterization of the vacuolating toxin from *Helicobacter pylori*. *J. Biol. Chem.* 267:10570–10575.
- Cover TL, Puryear W, Perez-Perez GI, Blaser MJ. 1991. Effect of urease on HeLa cell vacuolation induced by *Helicobacter pylori* cytotoxin. *Infect. Immun.* 59:1264–1270.
- Dixon MF, Genta RM, Yardley JH, Correa P. 1996. Classification and grading of gastritis. The updated Sydney System. International Workshop on the Histopathology of Gastritis, Houston 1994. *Am. J. Surg. Pathol.* 20:1161–1181.
- Douraghi M, et al. 2009. Multiple gene status in *Helicobacter pylori* strains and risk of gastric cancer development. *Digestion* 80:200–207.
- El-Omar EM, et al. 2001. The role of interleukin-1 polymorphisms in the pathogenesis of gastric cancer. *Nature* 412:99.
- Ferreira RM, et al. 2012. *Helicobacter pylori vacA* intermediate region genotyping and progression of gastric preneoplastic lesions. *Am. J. Gastroenterol.* 107:145–146.
- Figueiredo C, et al. 2002. *Helicobacter pylori* and interleukin 1 genotyping: an opportunity to identify high-risk individuals for gastric carcinoma. *J. Natl. Cancer Inst.* 94:1680–1687.
- Gonzalez CA, et al. 2011. *Helicobacter pylori cagA* and *vacA* genotypes as predictors of progression of gastric preneoplastic lesions: a long-term follow-up in a high-risk area in Spain. *Am. J. Gastroenterol.* 106:867–874.
- Gonzalez CA, et al. 2010. Gastric cancer occurrence in preneoplastic lesions: a long-term follow-up in a high-risk area in Spain. *Int. J. Cancer* 127:2654–2660.
- Hussein NR, et al. 2008. Differences in virulence markers between *Helicobacter pylori* strains from Iraq and those from Iran: potential importance of regional differences in *H. pylori*-associated disease. *J. Clin. Microbiol.* 46:1774–1779.
- Jang S, et al. 2010. Epidemiological link between gastric disease and polymorphisms in *VacA* and *CagA*. *J. Clin. Microbiol.* 48:559–567.
- Jones KR, et al. 2011. Polymorphisms in the intermediate region of *VacA* impact *Helicobacter pylori*-induced disease development. *J. Clin. Microbiol.* 49:101–110.

23. Kuipers EJ, Perez-Perez GI, Meuwissen SG, Blaser MJ. 1995. *Helicobacter pylori* and atrophic gastritis: importance of the *cagA* status. J. Natl. Cancer Inst. 87:1777–1780.
24. Lauren P. 1965. The two histological main types of gastric carcinoma: diffuse and so-called intestinal-type carcinoma. An attempt at a histo-clinical classification. Acta Pathol. Microbiol. Scand. 64:31–49.
25. Letley DP, Atherton JC. 2000. Natural diversity in the N terminus of the mature vacuolating cytotoxin of *Helicobacter pylori* determines cytotoxin activity. J. Bacteriol. 182:3278–3280.
26. Letley DP, Rhead JL, Twells RJ, Dove B, Atherton JC. 2003. Determinants of non-toxicity in the gastric pathogen *Helicobacter pylori*. J. Biol. Chem. 278:26734–26741.
27. Machado JC, et al. 2003. A proinflammatory genetic profile increases the risk for chronic atrophic gastritis and gastric carcinoma. Gastroenterology 125:364–371.
28. Machado JC, et al. 2001. Interleukin 1B and interleukin 1RN polymorphisms are associated with increased risk of gastric carcinoma. Gastroenterology 121:823–829.
29. Nogueira C, et al. 2001. *Helicobacter pylori* genotypes may determine gastric histopathology. Am. J. Pathol. 158:647–654.
30. Odenbreit S, et al. 2000. Translocation of *Helicobacter pylori* CagA into gastric epithelial cells by type IV secretion. Science 287:1497–1500.
31. Ogiwara H, et al. 2009. Role of deletion located between the intermediate and middle regions of the *Helicobacter pylori* *vacA* gene in cases of gastro-duodenal diseases. J. Clin. Microbiol. 47:3493–3500.
32. Rhead JL, et al. 2007. A new *Helicobacter pylori* vacuolating cytotoxin determinant, the intermediate region, is associated with gastric cancer. Gastroenterology 133:926–936.
33. Scholte GH, et al. 2002. Genotyping of *Helicobacter pylori* in paraffin-embedded gastric biopsy specimens: relation to histological parameters and effects on therapy. Am. J. Gastroenterol. 97:1687–1695.
34. Scholte GH, van Doorn LJ, Quint WG, Linderman J. 2001. Genotyping of *Helicobacter pylori* strains in formalin-fixed or formaldehyde-sublimate-fixed paraffin-embedded gastric biopsy specimens. Diagn. Mol. Pathol. 10:166–170.
35. van Doorn LJ, et al. 1998. Typing of *Helicobacter pylori* *vacA* gene and detection of *cagA* gene by PCR and reverse hybridization. J. Clin. Microbiol. 36:1271–1276.
36. van Doorn LJ, et al. 1998. Clinical relevance of the *cagA*, *vacA*, and *iceA* status of *Helicobacter pylori*. Gastroenterology 115:58–66.