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

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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 vaculolation 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
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 archival 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 diagnosed and underwent resection of cancer 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 archival 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 triplicate on Brucella 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 VacABF (forward) (5′-CGTTGGGT TTCTGGAGCCTG-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 preincubation 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 vaculating activity assay. The vacuolation activity of the different clinical isolates was assayed 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 archival 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 archival material, we aimed at designing a novel system that would allow vacA i-region
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 activity, 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...
194 (8.3%) of the cases. Eight (4.1%) cases could not be genotyped for the \( \text{vacA} \) i region. Of the 170 samples with a single genotype, 59 (34.7%) were genotyped as \( \text{vacA} \) i1, and 111 (65.3%) were genotyped as \( \text{vacA} \) 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 \( H. \text{pylori} \) \( \text{vacA} \) i-region genotypes and the \( \text{vacA} \) s and m regions and \( \text{cagA} \) status in Portuguese patients.** In order to evaluate the relationship between \( \text{vacA} \) i region and \( \text{vacA} \) s and m regions and the \( \text{cagA} \) status, we have used a subset of the samples (\( n = 189 \)) which had been previously characterized for the \( \text{vacA} \) s, \( \text{vacA} \) m, and \( \text{cagA} \) \( H. \text{pylori} \) genotypes (17). In this subset of cases, and excluding patients infected with multiple \( H. \text{pylori} \) strains (\( n = 70; 37.0\% \)), we observed the following \( \text{vacA} \) 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 \( \text{vacA} \) i1 strains were of the \( \text{vacA} \) s1 genotype (96.8%), while the \( \text{vacA} \) i2 strains were more frequently of the \( \text{vacA} \) s2 genotype (93.0%; \( P = 0.0001 \)) (Table 2). The \( \text{vacA} \) i1 strains were also more commonly of the \( \text{vacA} \) m1 genotype (82.3%), and \( \text{vacA} \) i2 strains were more frequently of the \( \text{vacA} \) m2 genotype (96.5%; \( P = 0.0001 \)). Regarding the \( \text{cagA} \) status, the i1 allele was more frequently found in \( \text{cagA} \)-positive (91.9%) strains, and conversely the i2 allele was more frequent in \( \text{cagA} \)-negative strains (84.2%; \( P = 0.0001 \)). These results reflect the strong association between \( \text{vacA} \) i and \( \text{vacA} \) s and m genotypes and \( \text{cagA} \) status.

**Evaluation of risk for atrophy and gastric carcinoma using \( H. \text{pylori} \) \( \text{vacA} \) i-region genotypes.** In order to evaluate the role of the \( \text{vacA} \) 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 \( H. \text{pylori} \) \( \text{vacA} \) 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 \( H. \text{pylori} \) \( \text{vacA} \) 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 \( H. \text{pylori} \) \( \text{vacA} \) i-region genotyping method can be used directly in DNA derived from archive material and reinforce that \( \text{vacA} \) i-region genotypes can be used as additional markers for disease risk estimation.

**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 \( H. \text{pylori} \) strains: 60190 (\( \text{vacA} \) s1/i1/m1), Tx30a (\( \text{vacA} \) s2/i2/m2), CI-11 (\( \text{vacA} \) s1/i2/m2), CI-12 (\( \text{vacA} \) s1/i1/m2), CI-13 (\( \text{vacA} \) s1/i1/m2), CI-14 (\( \text{vacA} \) s1/i2/m2), CI-15 (\( \text{vacA} \) s2/i2/m2), and CI-16 (\( \text{vacA} \) s1/i1/m1). Photos were obtained at a magnification of \( \times 400 \). Scale bar, 25 \( \mu \text{m} \).

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

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

\( ^a \) 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 \textit{H. pylori} genotypes of the vacA i region and the vacA s and m regions and \textit{cagA} status in Portuguese patients

<table>
<thead>
<tr>
<th>Strain</th>
<th>vacA s region</th>
<th>vacA m region</th>
<th>\textit{cagA}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>s1</td>
<td>s2</td>
<td>m1</td>
</tr>
<tr>
<td>vacA i1 strain</td>
<td>60 (96.8)</td>
<td>2 (3.2)</td>
<td>51 (83.6)</td>
</tr>
<tr>
<td>vacA i2 strain</td>
<td>4 (7.0)</td>
<td>53 (93.0)</td>
<td>2 (3.5)</td>
</tr>
</tbody>
</table>

\( P \text{value}\) = <0.0001

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

\section*{DISCUSSION}

\textit{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 \textit{H. pylori} virulence factors has clinical importance (17, 27, 36). \textit{vacA} and \textit{cagA} are two pathogenicity-associated \textit{H. pylori} genes that display variation among strains. Patients that are infected with the most virulent \textit{vacA} s1/m1 and \textit{cagA}-positive strains are at higher risk for gastric carcinoma development than those infected with the lower virulence \textit{vacA} s2/m2 and \textit{cagA}-negative strains (4, 17). A third polymorphic region of \textit{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 \textit{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 \textit{H. pylori} directly in archive materials.

The majority of the studies addressing the vacA i region have been performed in DNA derived from \textit{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 amplimers sizes yielded by the different methods. While the amplifiers 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 amplimers 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 \textit{H. pylori} detection, which has high interobserver variation, very much depending on the experience of the pathologist, and over \textit{H. pylori} culture, which

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Chronic superficial gastritis (no. of subjects [%])</th>
<th>Risk analysis for:</th>
<th>Gastric carcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chronic atrophic gastritis</td>
<td>No. of subjects (%)</td>
<td>OR (95% CI)(^a)</td>
</tr>
<tr>
<td>vacA i2 strain</td>
<td>64 (72.7)</td>
<td>4 (25.0)</td>
<td>1</td>
</tr>
<tr>
<td>vacA i1 strain</td>
<td>24 (27.3)</td>
<td>12 (75.0)</td>
<td>8.0 (2.3–27)(^*)</td>
</tr>
</tbody>
</table>

\(^a\) 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-PD/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

Correction for Ferreira et al., A Novel Method for Genotyping the Helicobacter pylori vacA Intermediate Region Directly in Gastric Biopsy Specimens

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Volume 50, no. 12, p. 3983–3989, 2012. Page 3988: The following sentence should be inserted after the first sentence of the Acknowledgments section. “This work was also supported by FEDER funds through Programa Operacional Factores de Competitividade—COMPETE (FCOMP-01-0124-FEDER 021251).”