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Variation in number of *cagA* EPIYA-C phosphorylation motifs between cultured *Helicobacter pylori* and biopsy strain DNA

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Abstract

The *Helicobacter pylori* *cagA* gene encodes a cytotoxin which is activated by phosphorylation after entering the host epithelial cell. Phosphorylation occurs on specific tyrosine residues within EPIYA motifs in the variable 3'-region. Four different *cagA* EPIYA motifs have been defined according to the surrounding amino acid sequence; EPIYA-A, -B, -C and -D. Commonly, EPIYA-A and -B are followed by one or more EPIYA-C or -D motif. Due to observed discrepancies in *cagA* genotypes in cultured *H. pylori* and the corresponding DNA extracts it has been suggested that genotyping assays preferentially should be performed directly on DNA isolated from biopsy specimens. Gastric biopsies randomly selected from a Swedish cohort were homogenised and used for both direct DNA isolation and for *H. pylori* specific culturing and subsequent DNA isolation. In 123 of 153 biopsy specimens, the *cagA* EPIYA genotypes were in agreement with the corresponding cultured *H. pylori* strains. A higher proportion of mixed *cagA* EPIYA genotypes were found in the remaining 30 biopsy specimens. Cloning and sequencing of selected *cagA* EPIYA amplicons revealed variations in number of *cagA* EPIYA-C motifs in the mixed amplicons. The study demonstrates that culturing of *H. pylori* introduces a bias in the number of EPIYA-C motif. Consistent with other *H. pylori* virulence genotyping studies, we suggest that *cagA* EPIYA analysis should be performed using total DNA isolated from biopsy specimens.

1. Introduction

Helicobacter pylori is a microaerophilic Gram-negative bacterium that chronically infects the gastric mucosa. It is recognised as a human pathogen associated not only with chronic gastritis (Marshall and Warren, 1984), but also with peptic ulcer (Cover and Blaser, 1992) and gastric cancer (Parsonnet et al., 1991). Initially, classification of *H. pylori* was based on the combination of morphological and biochemical characteristics and growth requirements (Marshall and Warren, 1984). Genetic criteria have become increasingly important in the identification and characterisation of *H. pylori*. The *cagA* gene is a commonly used molecular marker of *H. pylori* virulence (Oleastro et al., 2009; van Doorn et al., 1998). The CagA cytotoxin is directly injected into epithelial cells via a type IV secretion system (Akopyants et al., 1998; Covacci et al., 1993; Yamazaki et al., 2003). In the host cell, CagA localises to the plasma membrane and undergoes phosphorylation on specific tyrosine residues within repeating penta amino acid Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs, present at the C-terminus of the protein (Backert et al., 2001; Hatakeyama, 2003; Higashi et al., 2002). The 3'-end region of *cagA* where the tyrosine phosphorylation sites are located are highly polymorphic (Covacci et al., 1993; Tummuru et al., 1993; Yamaoka et al., 1998; Yamazaki et al., 2005). Four different CagA EPIYA motifs, EPIYA-A, -B, -C, and -D, have been defined based on the amino acid sequences surrounding the EPIYA residue (Higashi et al., 2002; Jones et al., 2009; Panayotopoulou et al., 2007; Sgouras et al., 2009; Yamazaki et al., 2005). CagA proteins nearly always possess an EPIYA-A and an EPIYA-B, followed by various number of EPIYA-C repeats in Western-type (Yamazaki et al., 2005) or EPIYA-D motifs in East Asian type strains (Panayotopoulou et al., 2007; Sgouras et al., 2009). It has been suggested that the considerable variation in number of repeating EPIYA-C or -D motifs determines the biological activity of CagA in phosphorylation-dependent as well as phosphorylation-independent ways (Costa et al., 2009; Higashi et al., 2002). Furthermore, it has been shown

that the number of CagA EPIYA-C motifs is an important risk factor for cancer among Western strains (Basso et al., 2008; Batista et al., 2011). A high number of *H. pylori* CagA EPIYA-C phosphorylation sites increase the risk of gastric cancer, but not duodenal ulcer (Basso et al., 2008; Batista et al., 2011; [Chuang et al., 2011](#)), and Batista and co-workers further showed that mixed strain infection was significantly more frequent in patients with gastric cancer than in those with gastritis.

Most studies on the *H. pylori cagA* gene have been carried out on DNA isolated from cultured *H. pylori* isolates or from mucosal biopsy specimens (Fujimoto et al., 1994; Gunn et al., 1998; Lopez-Vidal et al., 2008; Morales-Espinosa et al., 1999; Yamaoka et al., 1998). Different PCR-based assays have been described for molecular typing of EPIYA phosphorylation motifs both in gastric biopsy specimens (Gunn et al., 1998; Monstein et al., 2010; Rota et al., 2001) and in co-cultured *H. pylori* isolates (Argent et al., 2005). Some studies established a correlation between genotypes and disease outcome, while other studies [did](#) not (Acosta et al., 2010; Ahmad et al., 2009; Sgouras et al., 2009; Shokrzadeh et al., 2009). The question arises whether the choice of different PCR-based assays used in the various studies contributes to the inconsistent results, or if other factors may contribute to the result outcome. One such factor may be the occurrence of mutations, selection of a single strain from a sample containing mixed strains, or both, when culturing *H. pylori* strains (Kraft and Suerbaum, 2005; Marshall et al., 1998). It is still debated whether or not molecular genotyping of *cagA* should be performed on cultured *H. pylori* [strains](#) or biopsy DNA (Gunn et al., 1998; Kim et al., 2009; Park et al., 2003).

Herein, we compare the number of *cagA* EPIYA genotypes between 153 biopsy total DNA and the corresponding DNA isolated from cultured *H. pylori* strains using a recently described improved PCR-based strategy (Monstein et al., 2010; Ryberg et al., 2008).

2. Materials and methods

2.1 Study subjects and tissue collection

Frozen (-80° C) gastric biopsy specimens from a gastroscopic screening study in a randomly selected cohort of the population of Linköping, Sweden (Borch et al., 2000), were used. The study was approved by the local ethical committee in Linköping, Sweden (Dnr. 98007) and conducted in accordance with the Helsinki declaration. From this cohort, 71 individuals with *H. pylori* infection were selected and gastroscopic biopsies from antrum, corpus or bulbus duodeni were analysed. A total of 153 gastric biopsy specimens from 71 individuals (59 corpus, 57 antrum, 37 bulbus duodeni) were homogenized by grinding. For 51 of the individuals, biopsies from more than one location were included. The homogenates were then divided into two parts. Approximately one part was used for direct automated DNA isolation and whole genome amplification by means of multiple displacement amplification (MDA), generating total MDA-DNA (cellular and bacterial DNA), using a Illustra GenomiPhi V2 DNA kit (GE-Healthcare, Uppsala, Sweden) according to the manufacturer's instruction. The other part of the homogenate was used for bacterial culturing using established clinical routine procedures (Redeen et al., 2011). Subsequent, bacterial DNA was extracted, followed by multiple displacement amplification generating *H. pylori* MDA-DNA (providing equal genotyping conditions for biopsy and cultured *H. pylori* strain derived DNA). In both cases, DNA was isolated using the BioRobot M48 and MagAttract DNA Mini M48 kit following the manufacturer's instruction (Qiagen, Hilden, Germany).

2.2 *cagA* EPIYA motif sequence analysis.

The 3'-end of the *cagA* gene encoding the EPIYA motifs, was amplified using MDA-DNA derived from biopsy specimens and cultured *H. pylori* strains. Primers used were M13-*cagA.epiya*.SE (TGT AAA ACG ACG GCC AGT CCC TAG TCG GTA ATG GRT TRT CT)

and T7-cagA.epiya.AS (*TAA TAC GAC TCA CTA TAG GGT GTG GCT GTT AGT AGC GTA ATT GTC*) (Monstein et al., 2010), tagged with a universal M13 uni (-21) or T7 sequence, respectively (in italics). PCR was performed in a final reaction volume of 20 µl, including 10 pmol of each primer, 1 µl of MDA-DNA, and 1x HotStarTaq Master mix (Qiagen, Hilden, Germany) using PCR conditions as follows: 95° C for 15 min; 30 cycles of 95°C for 20 s, 55°C for 20 s, 72° C for 40 s; and final extension at 72° C for 10 min. Prior to DNA sequence analysis, amplicons were analysed by capillary gel electrophoresis (CGE) using a QIAxcel system and a QIAxcel DNA Screening kit (Qiagen, Hilden, Germany). The *cagA* EPIYA amplicons were sequenced using a M13 uni (-21) sequencing primer at a customer sequencing service (Eurofins MWG Operon, Ebersberg, Germany). The obtained DNA sequences were analysed using the CLC Bioinformatics DNA Workbench version 5.5 (CLC-Bio, <http://www.clcbio.com>). CagA empty site was verified as described previously (Monstein et al., 2010).

2.3 Cloning and sequence analysis of *cagA* amplicons

Amplicons derived from MDA-DNA of five biopsies (Nos. 125C, 242C, 310C, 346A, 346C) (Table 3) were selected and cloned using a TOPO-TA cloning kit (pCR 2.1-TOPO vector) according to the protocol (Invitrogen, Carlsbad, USA). One to ten white colonies of each isolate were picked and used directly in a confirmatory *cagA* EPIYA PCR amplification assay as described above. The amplicons were sequenced using M13-cagA.epiya.SE (described in section 2.2) as sequencing primer at a custom sequencing service (Eurofins MWG Operon).

2.4 16S rDNA Pyrosequencing analysis

For detection and verification of the presence of *H. pylori* DNA, the 16S rDNA variable

V3 region was amplified using primers bHJ.HP.JBS.V3.SE (Biotin-CCT AGG CTT GAC ATT GAN AGA A) and B-V3.AS (ACG ACA GCC ATG CAG CAC CT). PCR amplification was performed in the same concentrations and conditions as described in section 2.2. Prior to sequencing, amplicons were analyzed by CGE using QIAxcel DNA High Resolution kit (Qiagen, Hilden, Germany). Pyrosequencing was carried out using a PyroMark Gold Q24 kit following the manufacturer's instruction (Qiagen, Hilden, Germany). Obtained DNA sequences were aligned and compared with catalogued *H. pylori* 26695 [GenBank:[NC000915](#)], *H. pylori* J99 [GenBank:[AE001439](#)], *H. pylori* Shi470 [GeneBank:[CP001072](#)], and *H. pylori* P12 [GeneBank:[CP001217](#)] sequences using the CLC Bioinformatics DNA workbench version 5.5 (CLC-Bio, <http://www.clcbio.com>).

3. Results

3.1 Overall comparison between biopsy DNA and cultured *H. pylori* results

A total of 153 gastric biopsy specimens from 71 individuals were investigated for *cagA* EPIYA genotypes. 123 of the samples revealed equal *cagA* genotypes between biopsy MDA-DNA and the corresponding cultured *H. pylori* MDA-DNA. Multiple (two or more) *cagA* EPIYA amplicons of different sizes were detected in 16 of these 123 biopsies (Table 1; Figure 1). DNA sequencing of the single amplicons revealed the presence of different *cagA* EPIYA motifs; EPIYA-ABC in 52, -ABCC in 23, -ABCCC in one, -AB in two, -AC in one, -ACC in one, and -AABC in one of the 123 samples. In 26 biopsies, no *cagA* amplicons were generated, which was verified by *cagA* empty site PCR (Table 1).

3.2 Variations between biopsy DNA and cultured *H. pylori*

CGE and sequencing of amplicons derived from biopsy MDA-DNA and the corresponding

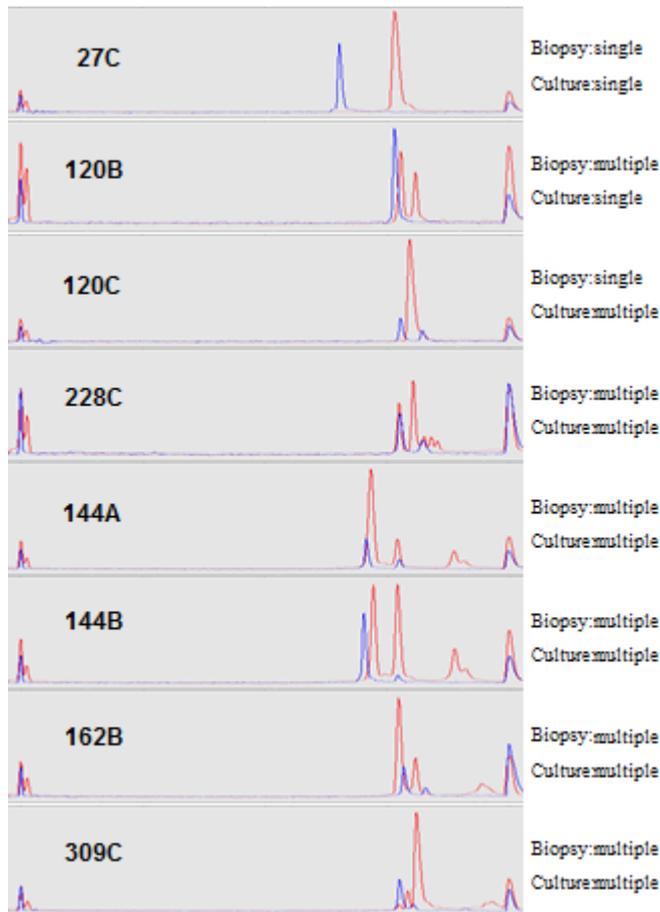


Figure 1

Superimposed electropherograms of *cagA* EPIYA amplicons with diverging amplicon patterns derived from DNA isolated from eight selected gastric biopsy samples (red), and from DNA isolated from the corresponding *H. pylori* cultures (blue). First and last peak in each electropherogram indicates internal alignment markers. Each peak between the alignment markers indicates the presence of one *cagA* EPIYA genotype. Although multiple *cagA* EPIYA amplicons were detected in biopsy total DNA and the corresponding DNA isolated from cultured *H. pylori* strains, in five of the eight samples (228C, 144A, 144B, 162B, 309C) the size pattern for each amplicon mix was unique. Single = one amplicon; multiple = two or more amplicons.

cultured *H. pylori* MDA-DNA revealed different *cagA* EPIYA genotypes in 30 of 153 biopsies. In these 30 biopsies, multiple *cagA* EPIYA amplicons were observed in 21 of the biopsy MDA-DNA, whereas the corresponding cultured *H. pylori* MDA-DNA revealed single amplicons of *cagA* EPIYA -AB, -ABC or -ABCC genotypes (Table 2). Two of the 30 biopsy MDA-DNA samples (Nos. 120C and 290C) yielded single amplicons of *cagA* EPIYA-ABCC

genotype (Table 2), whereas the corresponding cultured *H. pylori* MDA-DNA yielded multiple amplicons. In one sample (No. 152A), multiple amplicons were generated using biopsy MDA-DNA, however no amplicon was generated using MDA-DNA derived from the corresponding cultured *H. pylori* MDA-DNA. In five biopsies (144A, 144B, 162B, 228C and 309C), both biopsy MDA-DNA and the corresponding cultured *H. pylori* MDA-DNA displayed multiple amplicons with different size patterns (Figure 1; Table 2).

3.3 Cloning and sequence analysis of selected mixed amplicons derived from biopsy DNA

Cloning of five selected samples with multiple amplicons (gastric biopsy DNA Nos. 125C, 242A, 310C, 346A, 346C) and subsequent sequencing confirmed considerable variations in the number of EPIYA-C motifs within each sample (Table 3). In one case (sample no. 310C), five different *cagA* EPIYA-C genotypes (ABCC, ABCCC, ABCCCC, ABCCCCC and ABCCCCC) were identified (Table 3). Similar variations in the number of EPIYA-C motifs were observed in the other cloned amplicons. Only one *cagA* EPIYA-ABCC genotype could be established from cultured *H. pylori* isolate No. 242A, since cloning of the amplicon yielded only one colony (Table 3).

3.4 16S rDNA Pyrosequencing

16S rDNA pyrosequencing revealed the presence of *H. pylori* DNA in all biopsy specimens. DNA sequence comparison with catalogued sequences revealed the presence of 16S rDNA V3 sequences corresponding to *H. pylori* 26695 in 80 of 153, *H. pylori* J99 in 28 of 153, *H. pylori* 26695/J99 in 34 of 153, and *H. pylori* strain A in 9 of 153 biopsy specimens. In one biopsy each (Nos. 71C and 75C), the pyrogram revealed the presence of two 16S rDNA V3 motifs corresponding to *H. pylori* 26695 and 26695/J99, and 26695 and J99, respectively.

4. Discussion

Mutation and recombination occurring in the *H. pylori* genome are considered to be responsible for generating strain diversity (Kraft and Suerbaum, 2005). In this view, it is assumed that founder strains of *H. pylori*, which initially colonize the gastric mucosa, undergo microevolution of their genome structure over a relative short period of time, generating *H. pylori* strains with highly similar genomes that display minor genetic differences (Carroll et al., 2004; Marshall et al., 1998). The general view is that microevolution occurs in most, if not all *H. pylori* strains. Therefore, it is conceivable that adaptation over time of individual *H. pylori* strains to different environmental conditions (biopsy specimen vs. cultured strains) may **in part be responsible** for the observed discrepancies reported in associating bacterial genotypes to diseases. Furthermore, a recent study has revealed that adaptive evolution may occur especially in host interaction genes, such as the *cagA*, resulting in proteome diversification (Kawai et al., 2011).

It has been discussed that PCR-based genotyping directly from biopsy specimens tend to underestimate the prevalence of *H. pylori* specific virulence genes (Park et al., 2003; Secka et al., 2011). This may be due to limited access of *H. pylori* DNA, inhibition of PCR amplification due to high level of cellular genomic DNA, other PCR inhibitors or potent nucleases in gastric biopsy specimens (Monstein et al., 2005; Park et al., 2003; Thoreson et al., 1999). Whole genome amplification by multiple displacement amplification (MDA) can be used as a pre-PCR amplification step under conditions where PCR amplifications normally are hampered due to presence of inhibitors (Gonzalez et al., 2005) or where the amount of DNA is not sufficient for analysis (Ryberg et al., 2008). In this view, our previous and present studies have shown that PCR using MDA-DNA derived from biopsy DNA provides a reliable source for multiple molecular genotyping analysis (Monstein et al., 2010; Ryberg et al., 2008)

In this study, the majority of the cultured *H. pylori cagA* EPIYA-C genotypes corresponded with the biopsy genotypes, but discrepancies were observed in 30 of the 153 biopsies (20 %; table 2). Similarly, Kim and co-workers showed that the inconsistent *cagA* genotyping results between cultured *H. pylori* strain DNA and biopsy DNA were 16 % (Kim et al., 2009).

Different methodological approaches using either biopsy DNA or cultured strains to verify the presence of mixed *H. pylori* strains have shown conflicting results (Batista et al., 2011). Secka and co-workers have suggested that both biopsy DNA and cultured *H. pylori* should be analysed concomitantly (Secka et al., 2011). Park and co-workers have suggested that studies identifying associations between virulence factors and disease outcome should be restricted to sites with rare mixed *H. pylori* strain infection. However, this might lead to false perception of the actual relationship of bacterial strains and disease outcome (Park et al., 2003). Furthermore, they observed a higher proportion of mixed *H. pylori* strain infection in biopsy specimens (27%) compared to cultured *H. pylori* strains (9%) (Park et al., 2003). Similarly, based on *cagA* EPIYA genotyping we detected a higher proportion of mixed *H. pylori* strains in biopsy specimens (24%) compared to cultured *H. pylori* strains (11%). Cloning of *cagA* amplicons and subsequent sequence analysis was able to provide further information concerning the variation of *cagA* EPIYA genotypes. None of the methods described provided information whether or not the genotype variations were due to mixed *H. pylori* strain infection or arise within the stomach from an ancestor *H. pylori* strain as suggested in an early study by Yamaoka and co-workers (Yamaoka et al., 1999).

In view of a recent study (Sheu et al., 2009) where it was suggested that *H. pylori* infection at different sites of the stomach in the same patient could change the histological features in the antrum and the corpus, establishing of a correct number of *cagA* EPIYA-C motifs appears

to be crucial for assessing links between *H. pylori* strains and gastroduodenal diseases. However, it is still not known whether or not certain threshold concentrations of individual *H. pylori* strains (quantitation) present in biopsy specimens have an impact on the disease outcome. Consequently, we believe that it is important to genotype all *H. pylori* strain variations present in a biopsy specimen. So far, molecular biology based methods do not allow for an unequivocal discrimination between mixed *H. pylori* strain infection or infection with an *H. pylori* founder strain undergoing microevolution (Carroll et al., 2004; Kraft and Suerbaum, 2005; Marshall et al., 1998). Consistently with other studies, we recommend that molecular typing of total DNA (human and bacterial DNA) isolated directly from biopsy specimens should be performed. Moreover, the improved PCR-based strategy provides a promising tool for high throughput molecular typing of *H. pylori* strains in a clinical routine microbiology laboratory.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AK, AR, MND, KB, HJM participated in the conception, design, data interpretation and drafting of the manuscript. AK, AR, MND performed molecular genotyping. KB collected and selected the biopsy specimens. All authors have read and approved to the manuscript.

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Tables

Table 1. *CagA* EPIYA genotypes revealed in biopsies.

CagA EPIYA	No. of biopsy specimens	Results (compared to culture)	
		equal	not equal
mixed strains	37	16	21
ABC	55	52	3
ABCC	28	23	5
ABCCC	1	1	
AB	2	2	
AABC	1	1	
AABCC	1		1
AC	1	1	
ACC	1	1	
empty site	26	26	

Table 2. *CagA* EPIYA genotype differences between biopsy and culture *H. pylori* DNA.

Biopsy no.	CagA EPIYA genotype	
	biopsy	culture
346A	mixed strains	AB
1A	mixed strains	ABC
110C	mixed strains	ABC
120B	mixed strains	ABC
121A	mixed strains	ABC
121C	mixed strains	ABC
154C	mixed strains	ABC
201C	mixed strains	ABC
242A	mixed strains	ABC
273C	mixed strains	ABC
275C	mixed strains	ABC
281A	mixed strains	ABC
346C	mixed strains	ABC
26C	mixed strains	ABCC
125A	mixed strains	ABCC
125C	mixed strains	ABCC
193C	mixed strains	ABCC
273B	mixed strains	ABCC
310C	mixed strains	ABCC
352C	mixed strains	ABCC
372C	mixed strains	ABCC
152A	mixed strains	empty site
144A	mixed strains ^b	mixed strains ^b
144B	mixed strains ^b	mixed strains ^b
162B	mixed strains ^b	mixed strains ^b
228C	mixed strains ^b	mixed strains ^b
309C	mixed strains ^b	mixed strains ^b
120C	ABCC	mixed strains
290C	ABCC	mixed strains
27C	ABC	AB

^aA, antrum; B, duodenum; C, corpus

^bboth biopsy and culture contained multiple amplicons, however not identical size patterns

(Figure 1).

Table 3. *CagA* EPIYA phenotypes deduced from sequencing of cloned amplicons.

Biopsy no..	Cloning of biopsy DNA	Number of amplicons disclosed by CGE^b
125C ^a	ABCC ABCCCC	2
242A ^a	ABC	2
310C ^a	ABCC ABCCC ABCCCC ABCCCCC ABCCCCC	5
346A ^a	AB ABC	2
346C ^a	ABC ABCC	2

^aA, antrum; C, corpus

^bCGE, capillary gel electrophoresis