Application of PCR amplicon sequencing using a single primer pair in PCR amplification to assess variations in *Helicobacter pylori* CagA EPIYA tyrosine phosphorylation motifs

Hans-Jürg Monstein¹,²³*, Anneli Karlsson³, Anna Ryberg¹, Kurt Borch²³

**Abstract**

**Background:** The presence of various EPIYA tyrosine phosphorylation motifs in the CagA protein of *Helicobacter pylori* has been suggested to contribute to pathogenesis in adults. In this study, a unique PCR assay and sequencing strategy was developed to establish the number and variation of cagA EPIYA motifs.

**Findings:** MDA-DNA derived from gastric biopsy specimens from eleven subjects with gastritis was used with M13- and T7-sequence-tagged primers for amplification of the cagA EPIYA motif region. Automated capillary electrophoresis using a high resolution kit and amplicon sequencing confirmed variations in the cagA EPIYA motif region. In nine cases, sequencing revealed the presence of AB, ABC, or ABCC (Western type) cagA EPIYA motif, respectively. In two cases, double cagA EPIYA motifs were detected (ABC/ABCC or ABC/AB), indicating the presence of two *H. pylori* strains in the same biopsy.

**Conclusion:** Automated capillary electrophoresis and Amplicon sequencing using a single, M13- and T7-sequence-tagged primer pair in PCR amplification enabled a rapid molecular typing of cagA EPIYA motifs. Moreover, the techniques described allowed for a rapid detection of mixed *H. pylori* strains present in the same biopsy specimen.

**Background**

*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 [1], but also with peptic ulcer [2] and gastric cancer [3]. A commonly used molecular marker of *H. pylori* virulence is the cagA gene (cytotoxin-associated gene) [4], which is a part of the 40 kb Cag-Pathogenicity Island (cag-PAI) [5]. The CagA cytotoxin is directly injected into epithelial cells by a type IV secretion system, encoded by genes located in the cag-PAI [6-8]. 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 [9,10]. The C-terminal part, which contains the EPIYA motifs, has been shown to be highly variable, as opposed to the highly conserved N-terminal part [7,11-13]. CagA EPIYA motifs are defined as EPIYA-A, -B, -C, and -D, according to the amino acid sequences that surround the EPIYA sequence [10,13,14]. CagA proteins nearly always possess EPIYA-A and EPIYA-B sites, followed by one to three repeats of EPIYA-C in Western-type [13] or EPIYA-D sites in East Asian-type of *H. pylori* clinical isolates [14]. It has been suggested that the 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 [10,15]. It has also been shown that the number of CagA EPIYA-C motifs is an important factor for cancer risk among Western strains [16].

Numerous PCR assays have been reported for the identification of CagA EPIYA phosphorylation motifs [12-14,17,18]. To simplify the determination of the number and types of cagA EPIYA motifs present, Argent

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*Correspondence: hans-jurg.monstein@liu.se

¹Clinical Microbiology, Molecular Biology Laboratory, University Hospital, S-581 85 Linköping, Sweden

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and co-workers [17] developed an elegant PCR-based approach for identification of individual EPIYA motifs, using a single forward primer and multiple reverse primers. In most studies, cagA EPIYA amplicons have been visualised by agarose gel electrophoresis and sequenced using various region specific primers [12-14,17,18].

In this study, we report on the analysis of amplicons derived from a single primer pair by automated capillary electrophoresis combined with direct sequencing using universal sequencing primers to assess variations in the H. pylori cagA EPIYA motifs. The technique also works in the presence of multiple H. pylori strains in the same biopsy specimen.

Methods

Study subjects and tissue collection

Eleven individual archival frozen H. pylori positive gastritis tissue samples were used in this study. Preparation of multiple displacement amplified DNA (MDA-DNA) derived from DNA isolations and the detection limit of Helicobacter pylori MDA-DNA have been described previously [19,20].

PCR amplification

The CagA gene EPIYA repeat regions were amplified using 10 pmol of each primer M13-CagA-EPIYA.SE (5'-TGT AAA ACG ACG GCC AGT CCC TAG TCG G TA ATG GRT TRT CT-3') and T7-CagA-EPIYA.AS (5'-TAA TAC GAC TCA CTA TAG GGT GTG GCT GTT AGT AGC GTA ATT GTC-3'). In seven of the eleven cases, an AB or ABC gene in nine of eleven MDA-DNA extracts from H. pylori positive gastritis biopsy specimens using a single PCR amplification, did not yield any empty-sites (Table 1), suggesting that there were no PAI empty sites in these cases.

Results and discussion

We successfully amplified the variable 3’-region of the cagA gene in nine of eleven MDA-DNA extracts from H. pylori positive gastritis biopsy specimens using a single PCR amplification, followed by automated capillary electrophoresis and universal primer-tagged amplicon sequencing. Electrophoretic analysis of the eleven cases revealed the presence of a single band in seven cases, multiple bands in two cases, while two cases were PCR negative (Figure 1; table 1). The amplicons ranged in size between ~600 and ~900 bp, indicating the presence of varying numbers of cagA EPIYA motifs in the different biopsies. Amplicons derived from H. pylori 26695 and H. pylori J99 revealed bands of similar sizes, whereas H. pylori ATCC 43509 generated a larger amplicon of ~1000 bp (Figure 1).

To assess the presence or loss of cag-PAI, cagE and cag-PAI empty-site PCR assay was carried out. CagE was detected in nine of eleven cases corresponding to the results of cagA genotyping (Table 1). Amplification of cag-PAI empty-site yielded a fragment of ~380 bp in biopsy specimen No. 21, revealing loss of cag-PAI. Thus, the result confirms the absence of cagA EPIYA motif and cagE amplicon in this biopsy specimen. H. pylori DNA derived from biopsy specimen No. 28, negative in cagA and cagE amplification, did not yield any empty-site amplicon of the expected size (Table 1), suggesting the presence of a deviating cag-PAI.

To confirm the cagA EPIYA motif genotype results obtained by fragment length analysis, we sequenced the amplicons using universal M13- and T7-sequencing primers. In seven of the eleven cases, an AB or ABC...
In two additional cases, double \textit{cagA} EPIYA motifs (ABC+ABCC or ABC+AB) were detected. Presumably, this indicates the presence of two individual strains in the same biopsy specimen (Table 1). The analysis of \textit{cagA} EPIYA motifs from mixed \textit{H. pylori} strain infection was possible by a combination of capillary electrophoresis and sequencing. The presence of \textit{cagA} EPIYA-A and EPIYA-B motifs could be determined from the sequencing chromatograms, but the region of the repeating C-motifs contained double peaks caused by amplicons of different sizes and nucleotide compositions. Instead, the high resolution capillary electrophoreses analysis enabled us to determine the number of EPIYA-C motifs by the size of the amplicons.

DNA sequencing of reference strains revealed the presence of a \textit{cagA} EPIYA-ABC motif in \textit{H. pylori} 26695, a \textit{cagA} EPIYA-BC motif in \textit{H. pylori} J99, and a \textit{cagA} EPIYA-ABCCC motif in \textit{H. pylori} ATCC 43509\textsuperscript{T} (Table 1).

In previous reports, the 3’-end of the \textit{cagA} gene encoding the EPIYA repeats were analysed by single or multiplex PCR assays and visualisation of amplicons by agarose gel electrophoresis. In most studies, amplicons are sequenced using a battery of gene specific primers (often the PCR primers). DNA sequence analysis of...
cloned amplicons with universal sequence primers (such as M13 uni -21), targeting sequences flanking cloned inserts [14,16,17], has also been described. The present study describes a unique PCR assay that detects all of the cagA phosphorylation sites, including the Asian EPIYA-D type. Tagging of the PCR primers enables rapid sequencing for revealing individual differences in the samples. Moreover, many laboratory workers are also concerned about the use of ethidium-bromide stained agarose gels, which is a health-risk factor. In agreement with a previous study from our laboratory we show that the use of automated capillary electrophoresis, which is a rapid technique that also minimizes the health risk during electrophoresis, overcomes these obstacles [19].

Commonly, work identifying cagA genotypes as potential virulence factors has been performed on bacterial isolates cultured from gastric biopsy specimens. However, bacterial culture methods are often time-consuming. In this view, the present and a previous study have shown that direct PCR on MDA-DNA derived from biopsy DNA provides a reliable source for multiple molecular analyses [19]. Using random amplified polymorphic DNA (RAPD) fingerprint analysis, it was found that ~60% of the patients were infected by two or more different H. pylori strains [23]. Using the methodological approaches described herein, we were able to detect multiple DNA fragments, indicating that the method indeed is suitable for analyzing mixed H. pylori infection in two gastric biopsy specimens (Table 1).

Due to the limited number of biopsies analysed here, we were not able to draw any conclusions regarding a possible correlation between the gastritis classification and cagA genotypes. However, the primary goal of the present study was not to perform a clinical study at large but rather to establish a new and simple methodological approach to assess variations in H. pylori cagA EPIYA motifs.

Altogether, the single PCR reaction with MDA-DNA as template, in combination with the automated capillary electrophoresis and direct sequencing of universal primer-tagged amplicons, offers a rapid means of genotyping H. pylori DNA isolated from biopsy specimens. Moreover, the technique described allowed for a rapid detection of mixed H. pylori strains present in the same biopsy specimen.

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Author details
1Clinical Microbiology, Molecular Biology Laboratory, University Hospital, S-581 85 Linköping, Sweden. 2Division of Surgery, University Hospital, S-581 85 Linköping, Sweden. 3Department of Clinical and Experimental Medicine, Faculty of Health Sciences, Linköping University, S-581 85 Linköping, Sweden.

Authors’ contributions
HJM, AK, AR and KB participated in the conception, design, drafting of the manuscript, and final approval of the version to be published. HJM, AK and AR were responsible for the acquisition, analysis and interpretation of data.

Table 1 H. pylori genotyping

| Subject No. | Gastritis classificationa | 16S rDNA type | cag-PAI PCR analysis | CagA EPIYA types |
|-------------|--------------------------|---------------|---------------------|-----------------|
|             |                          |               | ES                  | cagE            |
| 6           | P-2-na                   | "Strain A"    | -                   | +               | AB              |
| 9           | A-I-a                    | J99           | -                   | +               | ABC             |
| 12          | C-3-a                    | 26695         | -                   | +               | ABC + ABCC      |
| 14          | A-I-a                    | 26695         | -                   | +               | ABC             |
| 18          | P-I-na                   | 26695         | -                   | +               | ABC             |
| 21          | P-I-na                   | J99           | +                   | -               | -               |
| 22          | C-I-a                    | J99           | -                   | +               | AB              |
| 23          | P-2-na                   | 26695         | -                   | +               | ABC             |
| 25          | P-2-na                   | 26695         | -                   | +               | ABC             |
| 27          | A-I-a                    | 26695         | -                   | +               | ABC + AB        |
| 28          | A-I-na                   | 26695/J99    | -                   | -               | -               |

reference strain HP 26695 - + ABC  
reference strain HP J99 - + BC  
reference strain ATCC 43509T - + ABCC

a) Gastritis classification according to the revised Sydney system [24]. A: antrum predominant gastritis; P: pangastritis; C: corpus dominant gastritis; t: mild degree; 2: moderate degree; 3: severe degree; a: atrophy; na: no atrophy
b) 16S rDNA variable V3 region motifs established by pyrosequencing analysis [19].
c) ES: cag-PAI empty-site, + presence of a 380 bp amplicon, indicating loss of cag-PAI; - presence of cag-PAI [6].
KB collected and selected the biopsy specimens in the study. All authors have read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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