

A model of *Helicobacter pylori* persistence in a case of gastric cancer

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SUMMARY

The aim of this work was to analyze several clones of *Helicobacter pylori* isolated from a patient with gastric cancer, to evaluate i) genetic variability ii) virulence factors profile and iii) antimicrobial susceptibility against the drugs commonly used in the *H. pylori* therapy. A total of 32 *H. pylori* clones isolated from a biopsy sample coming from a patient with gastric cancer previously treated for *H. pylori* infection, were analyzed for: the genetic variability by amplified fragment polymorphism analysis; the *vacA*, *cagA* virulence status by PCR; the antimicrobial susceptibility by minimum inhibitory concentrations with the agar dilution method towards amoxicillin, clarithromycin, levofloxacin and tinidazole. The patient showed a mixed infection with the presence of at least 3 different strains. The clones isolated possessed the *vacA*, *cagA* virulence factors with a different allelic combination (*vacA* s1/i1/m1; s1/i1i2/m1; s2/i2/m2; s2/i1i2/m2) together with repeated *cagA* EPIYA motif pattern P1P2P3P3P3. Moreover, a pattern of multi-drug resistance was disclosed in the different clones. The presence of multiple *H. pylori* strains colonizing the same patient, with the main virulence factors displaying a different allelic combination and a different multi-drug resistance among isolates, point out the role of genetic variability generating, in time, more virulent and adapted strains.

KEY WORDS: *cagA* status, Drug resistance, *Helicobacter pylori*, Mixed infection

Received March 19, 2010

Accepted June 30, 2010

INTRODUCTION

Gastric cancer represents the second most common cancer in the world and its association with *Helicobacter pylori* infection was confirmed on the basis of several epidemiological studies (Ito *et al.*, 2009). *H. pylori* virulence genes and genomic plasticity play a key role in the onset of the infection (Cellini and Donelli 2000; Cellini *et al.*, 2006; Sgouras *et al.*, 2009). The *vacA* gene contains three variable parts corresponding to the s-region, the i-region and the m-region which encode the signal, intermediate and the middle peptides, respectively. For the signal region, two distinct isotypes, s1 and s2, were recognized, whereas, for the middle

region, m1 or m2 alleles were categorized. The *vacA* genotype is associated with *in vitro* cytotoxic activity: s1 and m1 have greater cytotoxin activity than s2 and m2, respectively (van Doorn *et al.*, 1998; Chisholm *et al.*, 2002; Sgouras *et al.*, 2009). Recently, a new polymorphism in the intermediate (i) region, located between the s and m regions, was identified and associated with gastric cancer in Iran (Rhead *et al.*, 2007). With regard to the *cagA* gene, *H. pylori* strains, possessing the *cag* pathogenicity island (PAI), are more associated with disease development than those lacking this region (Argent *et al.*, 2005). The existence of EPIYA motifs, repeated within the variable region of the protein, induces the phosphorylation of CagA protein by the Src kinases of the host, thereby producing a morphological modification of the epithelial cells (Argent *et al.*, 2005). *Helicobacter pylori* strains which encode CagA proteins containing a greater number of EPIYA motifs are mainly associated with gastric cancer development (Argent *et al.*, 2005).

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With regard to genetic variability, *H. pylori* strains, isolated from different patients, show a significantly high degree of variability either in size or in gene order because of the acquisition of new DNA sequences (Taylor *et al.*, 1992; Suerbaum, 2000): mixed *H. pylori* infection in the same patient is significantly related to strains more resistant to antibiotics with a more virulent genotype than strains responsible for single infection (Cellini *et al.*, 2006).

This study analyses several clones isolated from a patient with gastric cancer, previously treated for *H. pylori* infection, for the evaluation of: i) genetic variability, ii) virulence factors profile and iii) antimicrobial susceptibility against the drugs commonly used in the *H. pylori* therapy, to better understand the adaptation dynamic of the microorganism to the host.

MATERIAL AND METHODS

Patient and *H. pylori* culture

A 68-year-old female, selected from a previous study among a group of individuals subjected to upper gastrointestinal (GI) endoscopy for gastrointestinal complaints, was considered in this study (Cellini *et al.*, 2008b). Written informed consent was obtained from the patient.

The patient, previously treated for *H. pylori* infection with a 7-day standard treatment consisting of a proton pump inhibitor (PPI) (20 mg b.d.) combined with clarithromycin (500 mg b.d.) and amoxicillin (1 g b.d.) underwent an endoscopy because of persistent GI disturbances. A biopsy sample was collected from the antrum for culture in Portagerm-pylori (Bio-Merieux, Marcy L'Etoile, France) and processed microbiologically within 24 h as previously described (Cellini *et al.*, 2008b). *Helicobacter pylori* colonies were identified for colony morphology, Gram staining and positive reaction with urease, catalase and oxidase. From the isolated strain, named *H. pylori* 9L, 32 clones were picked up randomly from the primary culture, transferred on CA and incubated in a microaerophilic atmosphere for 3 days at 37°C.

Isolated clones were collected and stored until use at -80°C by Drumm and Sherman method (1989).

Amplified fragment length polymorphism (AFLP)

The chromosomal DNA was extracted from each clone by using Qiamp Tissue DNA isolation minikit (QIAGEN S.p.a, Milan, Italy) and the AFLP analysis was carried out following the methodology reported by Gibson *et al.*, (1998). The DNA fingerprintings were analyzed with GEL COMPAR Software, Windows version 4.1 (Applied Math, Kortrijk, Belgium). The similarity coefficient indicating the relationship between the strains was calculated by using band positions (Gerner-Smidt *et al.*, 1998) by the GEL COMPAR program as previously reported (Cellini *et al.*, 2006). A similarity coefficient <70% was considered significant for mixed infection. The experiments were performed in triplicate.

Virulence factors genotyping

PCR reactions of *vacA* s/m, *cagA* EPIYA motifs of the *H. pylori* isolated clones were carried out as described before (Chisholm *et al.*, 2002; Cellini *et al.*, 2008a), while, the amplification of *vacA* i-Region was performed by using the methodology reported by Rhead *et al.* (2007). The oligonucleotide primers used were listed in Table 1. The amplification was performed in a 2700 Thermocycler (PE-Applied Biosystems) and consisted of 5 min of denaturation followed by 30-35 cycles consisting of: 40 s at 94°C of denaturation, 1 min of annealing at 52°C and 1 min and 30 s of extension at 72°C for the analysis of *vacA* s/i/m regions; 1 min at 94°C, 1 min at 55°C and 1 min at 72°C for the amplification of *cagA* 3' variable region and EPIYA phosphorylation motifs. After the last cycle, the extension was continued for 5 min (Cellini *et al.*, 2008a). The PCR products were examined by electrophoresis in 2% (w/v) agarose gel at 100 V for 30 min. The experiments were performed in triplicate.

Analysis of a new *cagA*-P3 amplified product

The *cagA*-P3 PCR products were analyzed by agarose gel electrophoresis. A new band of about 300 bp was detected in all 32 samples. The band was cut from the gel and the amplified DNA fragment was eluted by using QIAquick Spin (QIAGEN), purified by spin column QIAquick (QIAGEN) and cyclesequenced (on both strands) using the ABI PRISM Big Dye Terminator Cycle Sequencing kit (Applied Biosystems). DNA se-

TABLE 1 - Oligonucleotides used for PCR-based typing.

Primer designation	Gene	Sequence (5'-3')	Expected size of PCR product (bp)
VA1-F VA1-R	vacA	ATGGAAATACAACAAACACAC CTGCTTGAATGCGCCAAAC	259 (s1) 286 (s2)
VAG-F VAG-R	vacA	CAATCTGTCCAATCAAGCGAG GCGTCAAATAATTCCAAGG	567 (m1) 642 (m2)
VacF1 C1R C2R	vacA	GTTGGGATTGGGGGAATGCCG TTAATTTAACGCTTGTTTGAAG GATCAACGCTCTGATTTGA	426 (i1) 432 (i2)
cag2a cag4a cagA28F cagA-P1C	cagA	GGAACCTAGTCGGTAATG ATCTTTGAGCTTGTCTATCG TCTCAAAGGAGCAATTGGC GTCTGCTTTCTTTTATTAACCTKAGC	550-800 264-291 (P1)
cagA-P2CG cagA-P2TA cagA-P3E	cagA cagA cagA	TTTAGCACTTGAGCGTAAATGGG TTTAGCACTTGAGTATAAATGGG ATCAATTGTAGCGTAAATGGG	309-336 (P2) 309-336 (P2) 465-498/672 (P3)
ADH1* ADH2* HI-A		ACGGTATGCGACAG AGCTCTGTGCGATACCGTGAG GGTATGCGACAGAGCTTA	

*Oligonucleotides used as adapters for ligation reaction in the AFLP method.

quences were analyzed on an automated sequencer, ABI PRISM 310, version 3.4.1 (Applied Biosystems). The band was isolated and sequenced in all samples in which was detected. The resulting nucleotide sequences of about 300 bp were aligned using the Sequence Navigator Software package (Applied Biosystems). Sequence comparisons were subsequently carried out using BLAST Search in the National Center of Biotechnology Information (NCBI). All detections were done in triplicate.

Antibiotic susceptibility tests

Amoxicillin (Sigma Chemical Co., St Louis, MO, USA), clarithromycin (Abbott Laboratories, Abbott Park, Ill., USA), levofloxacin (FLUKA-Biochemika, Buchs, Switzerland) and tinidazole (Sigma), commonly used in *H. pylori* therapy, were tested against the isolated clones. The Minimum Inhibitory Concentrations (MICs) were evaluated using the standard agar dilution method according to the Clinical and Laboratory Standards Institute (CLSI) (2005) guidelines, using Mueller-Hinton agar (Oxoid) with 7% of defibrinated horse blood. Two-fold dilutions of the antibiotics were

added to melted agar in order to obtain the following final concentrations: from 2 to 0.06 µg/ml for amoxicillin and clarithromycin, from 8 to 0.25 µg/ml for levofloxacin and from 20 to 0.6 µg/ml for tinidazole. Agar plates were inoculated using a Steers replicator delivering a bacterial suspension of approximately of 5x10⁴ colony-forming units (CFUs)/spot. Test plates were incubated as mentioned before (Cellini *et al.*, 2008b). MIC was defined as the lowest concentration of the antibiotics inhibiting the visible growth.

Bacteria were considered resistant when MIC was greater than 0.5 µg/ml for amoxicillin, 1 µg/ml for clarithromycin, 5 µg/ml for levofloxacin and tinidazole (Cellini *et al.*, 2008b). The reference strain *H. pylori* ATCC 43629 was inserted in the experiments as the control.

The experiments were performed in triplicate.

RESULTS

AFLP analysis

A total of 32 clones were isolated from a patient with gastric cancer which was previously treated

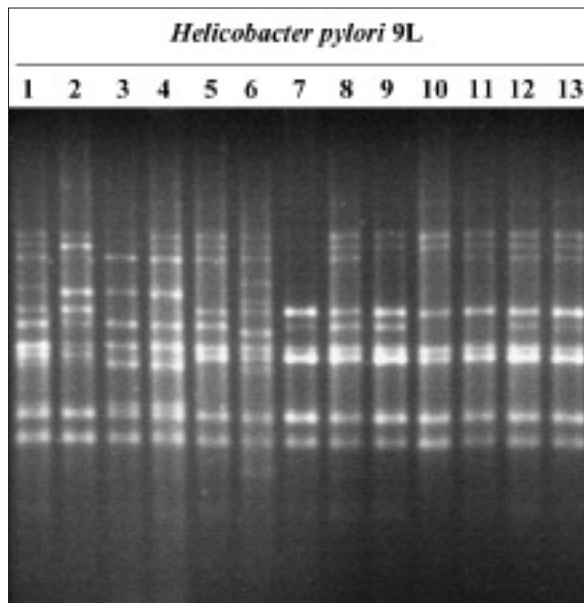


FIGURE 1 - Representative image of Amplified Fragment Length Polymorphism (AFLP) profiles of 13 *Helicobacter pylori* 9L clones isolated from a biopsy obtained from a patient with gastric cancer.

for *H. pylori* infection. The genetic analysis of the isolates, performed by AFLP technique, showed different DNA fingerprintings associated to the presence of a mixed infection in the host as shown in Figure 1. At least 3 different strains were detected, as confirmed by the Jaccard coefficients whose values were <70%. In particular, the comparison of the clone 9/4 with the clones 9/6 and 9/5 displayed a Jaccard coefficient of 29% and 46% respectively, while the Jaccard coefficient derived by the comparison of 9/5 with 9/6 was equal to 62%.

Virulence factors detection

The analysis of the main virulence factors in each clone showed a genetic plasticity associated to a different allelic combinations detected in the different analyzed colonies. All the isolates studied were *cagA* positive. A new amplified fragment size of about 300 bp was detected and sequenced in 32 clones. The 32 colonies displayed the *cagA* EPIYA motifs pattern corresponding to P1P2P3P3P3 as shown in Figure 2. In particular, the *cagA* EPIYA motifs pattern P1P2P3P3P3, displays a new fragment size of about 300 bp, which was sequenced and aligned by using BLAST Search with *H. pylori* J99 genomic sequence present in

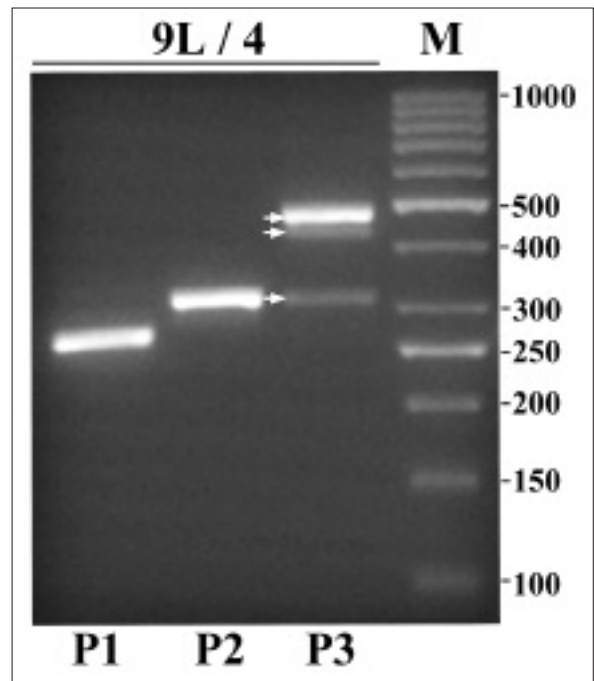


FIGURE 2 - Representative image of PCR amplification of the *cagA* EPIYA motifs pattern P1P2P3P3P3 from a clone isolated from a patient with gastric cancer. M, size marker in base pairs.

DATA BASE (NCBI), confirming that the sequences of 300 bp belong to *cagA* gene with an identity of about 83-85%. The sequence data, obtained by the 32 clones, had an identity of 93% when compared each other and 2 representative sequences of the 32 colonies were deposited in GenBank (accession numbers GQ855282 and GQ855283). With regard to *vacA* gene, the allelic combination s1/i1/m1 was detected in 25 out of 32, with a frequency of 78.13%; s1/i1i2/m1 in 2 out of 32 with a frequency of 6.25%; s2/i1i2/m2 in 4 out of 32 with a frequency of 12.5% and s2/i2/m2 in 1 out of 32 isolates with a frequency of 3.12%. A comparative analysis between the *vacA* isotypes and the *cagA* EPIYA combination pattern detected in the 32 colonies studied, showed a prevalence of isolates (71.87%) possessing both *vacA* s1/i1/m1 and *cagA* EPIYA P1P2P3P3P3.

Antibiotic susceptibility test

The antibiotic susceptibility test, performed on 32 clones displayed a different pattern of multi-drug resistances. A resistance against clar-

TABLE 2 - Comparison between *vacA* allelic combination and clarithromycin susceptibility of 32 *Helicobacter pylori* clones.

<i>vacA</i> allelic combination	Clarithromycin	
	Resistant clones (%)	Susceptible clones (%)
s1/i1/m1	1 (12.5)	24 (100)
s1/i1i2/m1	2 (25.0)	0
s2/i2/m2	1 (12.5)	0
s2/i1i2/m2	4 (50.0)	0

ithromycin and levofloxacin was found in different coinfectant clones. In particular, the resistance to clarithromycin was detected in 8 out of 32 (25%) analyzed clones, while the resistance to levofloxacin was detected in all isolated colonies. On the contrary, the amoxicillin and tinidazole were efficacious against all detected clones. Interestingly, no association was detected between the DNA profile or virulence marker pattern and the clarithromycin resistance. In fact, among the clones resistant to clarithromycin 4 out of 8 were s2/i1i2/m2; 2 out of 8 were s1/i1i2/m1; 1 out of 8 was s1/i1/m1 and 1 out of 8 was s2/i2/m2 (Table 2).

DISCUSSION

In the present work we describe a case of a patient colonized by *H. pylori* and affected by gastric cancer. Multiple *H. pylori* strains, possessing the main virulence factors and displaying a different antimicrobial susceptibility pattern were harboured in the patient studied. Several studies demonstrate that recombination events occurred frequently during chronic infection, producing multiple *H. pylori* mosaic genotypes (Kersulyte *et al.*, 1999; Falush *et al.*, 2001; Kraft *et al.*, 2006). The macroevolution of the strains, detected in the present study, might be associated either to a colonization of the host by multiple strains acquired in time or to an adaptation of a single strain to the host. The first hypothesis might be explained with the existence of a mixed infection, in which, one or more strains were present in a minor fraction or at a different site in the gastric mucosa so that

they escaped identification in the first isolation (Wang *et al.*, 1998).

The second hypothesis could be associated with a microbe adaptation to the stressing stimuli such as an ineffective antimicrobial therapy. Failure of the *H. pylori* treatment or sub-inhibitory concentrations of antibiotics might induce the evolution of more resistant strains, difficult to eradicate (Hung *et al.*, 2009; Wueppenhorst *et al.*, 2009). In the present work, the clones isolated from the same patient, displayed differences in the DNA fingerprintings, the antimicrobial susceptibility patterns and the allelic status of virulence genes. Interestingly, contrary to Miehle *et al.* (1999), who demonstrated that patients with gastric cancer were colonized by a single predominant strain, we found multiple strains in the same host. These differences might be explained by an increased consumption of antibiotics in patients during the last few years that presumably generated strains which developed a high genetic variability as well as a retention of the virulent factors associated with more severe gastric diseases. On the other hand (Suerbaum and Josenhans 2007) the persistence of *H. pylori* in the host is due to the continuous improvement of its genome, predominantly by inter-strain recombination, in the same. Therefore, the genetic variability of the microorganism contributes to the host adaptation also by evading the natural immune response favoring the bacterial survival and enabling the onset of the most dangerous complication of *H. pylori* infection: the gastric carcinoma. The analysis of the status of virulence markers in all studied clones confirmed the correlation between the severity of the disease and the presence of a greater number of the *cagA* EPIYA motifs (Basso *et al.*, 2008; Jones *et al.*, 2009) as well as the prevalence of the allelic combination s1i1m1 of the *vacA* gene. The wide genetic variability detected in the virulence marker status confirmed the genomic plasticity recorded in the DNA profiles, although no association was found between the same DNA fingerprintings and the same allelic combinations of the virulence markers. Sgouras *et al.* (2009) have recently demonstrated, in a study performed on 98 children, the presence of microevolving strains having identical RAPD profiles and producing CagA proteins possessing a variable number of EPIYA motifs. The

authors hypothesize that children may be colonized by multiple *H. pylori* variants which over time, under a selective pressure due to the host genetics and bacterial factors, generate different *H. pylori* genotypes that predominate in the adulthood (Sgouras *et al.*, 2009).

With regard to the antimicrobial susceptibility test, a resistance to clarithromycin and levofloxacin was detected in different co-infectant colonies. In particular, *H. pylori* cells were found to be either susceptible or resistant to clarithromycin suggesting that the selection of a single colony may not be representative of the *H. pylori* population to define the susceptibility profile in one host (Schwarz *et al.*, 2008). On the basis of the comments outlined above an antimicrobial susceptibility test of at least three different *H. pylori* colonies should be recommended to obtain a realistic situation of the colonizing strains, also considering that *H. pylori* heteroresistance has already been demonstrated (Toracchio *et al.*, 2005; Kim *et al.*, 2003). On the other hand, *H. pylori* resistance to antimicrobials is the leading cause of failure in eradication therapy (Toracchio *et al.*, 2005; Kim *et al.*, 2003). Moreover, our data emphasize the widespread variability of *vacA* allelic combination only within clarithromycin resistant clones.

In conclusion, the presence of multiple *H. pylori* strains colonizing the same patient, with the main virulence factors and different multi-drug resistance among isolates, disclose the role of genetic variability generating, in time, more virulent and adapted strains (Schwarz *et al.*, 2008; Wen and Moss 2008).

The intragastric distribution of *H. pylori* and severity of the chronic inflammatory process involves complex mechanisms such as characteristics of the colonizing strain, host genetics and immune response, diet, and the level of acid production (Figueiredo *et al.*, 2002; Lee *et al.*, 2003; Jarosz *et al.*, 2009). This suggests that other factors must play a role in disease pathogenesis. In fact, the bacterium-host interaction involves complex mechanisms that can balance or emphasize the effect of virulence factors together with environmental and dietary factors. Our data underline the wide genetic variability of *H. pylori* aimed at the survival and persistence in the host and emphasize the need for careful *H. pylori* surveillance to improve management of the infection.

ACKNOWLEDGMENTS

The Authors thank Stefania d'Aloisio, Nunzio Di Paolo and Lucinda Bessa for their technical assistance. This study was supported by a grant awarded by the "Ministero Università e Ricerca", PRIN 2007, Rome, Italy.

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