The Journal of Microbiology, December 2006, p. 660-664 Vol. 44, No. 6
Copyright © 2006, The Microbiological Society of Korea

Claritromycin Resistance and *Helicobacter pylori* Genotypes in Italy

Vincenzo De Francesco[1], Marcella Margiotta[2], Angelo Zullo[3], Cesare Hassan[3], Nicola Della Valle[4], Osvaldo Burattini[4], Roberto D'Angelo[4], Giuseppe Stoppino[4], Ugo Cea[4], Floriana Giorgio[2], Rosa Monno[5], Sergio Morini[3], Carmine Panella[4], and Enzo Ierardi[4,*]

[1]*Gastroenterology Unit, "Riuniti" Hospital, Foggia, Italy*
[2]*Section of Gastroenterology, Department of Emergency and Organ Transplantation, University of Bari, Italy*
[3]*Gastroenterology and Digestive Endoscopy, "Nuovo Regina Margherita" Hospital, Rome, Italy*
[4]*Section of Gastroenterology, Department of Medical Sciences, University of Foggia, Italy*
[5] *Section of microbiology, Department of Hygiene and Public Health, University of Bari, Italy*

(Received July, 15, 2006 / Accepted November 2, 2006)

The relationship between *H. pylori* clarithromycin resistance and genetic pattern distribution has been differently explained from different geographic areas. Therefore, we aimed to assess the clarithromycin resistance rate, to evaluate the bacterial genetic pattern, and to search for a possible association between clarithromycin resistance and *cagA* or *vacA* genes. This prospective study enrolled 62 consecutive *H. pylori* infected patients. The infection was established by histology and rapid urease test. Clarithromycin resistance, *cagA* and *vacA* status, including *s/m* subtypes, were assessed on paraffin-embedded antral biopsy specimens by TaqMan real time polymerase chain reaction (PCR). Primary clarithromycin resistance was detected in 24.1% of cases. The prevalence of *cagA* was 69.3%, and a single *vacA* mosaicism was observed in 95.1% cases. In detail, the *s1m1* was observed in 23 (38.9%) patients, the *s1m2* in 22 (37.2%), and the *s2m2* in 14 (23.7%), whereas the *s2m1* combination was never found. The prevalence of *cagA* and the *vacA* alleles distribution did not significantly differ between susceptible and resistant strains. Primary clarithromycin resistance is high in our area. The *s1m1* and *s1m2* are the most frequent *vacA* mosaicisms. There is no a relationship between clarithromycin resistance and bacterial genotypic pattern and/or *cagA* positivity.

Keywords: *H. pylori*, primary clarithromycin resistance, *cagA*, *vacA* alleles

Helicobacter pylori (H. pylori) genoma is characterized by two main genes: *cagA* and *vacA*. The *cagA* pathogenicity-island is a marker of enhanced virulence, associated with development of major gastroduodenal diseases, such as peptic ulcer and gastric cancer (Atherton *et al.*, 1995; van Doorn *et al.*, 1998), whilst *vacA* gene shows a complex structure. It is composed by two variable regions - *s*-region (signal region) and *m*-region (middle region) - each with two different alleles (*s1, s2, m1, m2*). The production of vacuolating toxin occurs in the presence of the mosaic combination of the alleles of the two regions. In detail, either *s1m1* and *s1m2* mosaicism has been associated with severe gastric disease. *H. pylori* infection could be sustained by single or multiple strains (i.e. co-infection), as demonstrated by the heterogeneity for both *cagA* gene and *vacA* (Han *et al.*, 1998; Kauser *et al.*, 2005).

Since clarithromycin resistance diffusion worldwide represents, at the moment, a problem of paramount relevance, some attempts have been performed in order to identify its relationship with bacterial genetic factors. The possibility of co-infection with multiple strains seems to be higher when *H. pylori* colonization is sustained by clarithromycin susceptible than to resistant strains (van Doorn *et al.*, 2001). On the other hand, it has been recently reported a strong association between clarithromycin resistant bacteria and the presence of both *cagA* and *s1m2 vacA* allele combination (100% resistant and less than 50% susceptible strains) (*Elviss et al.*, 2004) thus suggesting that this genetic pattern could provide a selective advantage during bacterial replication. Moreover, a transformation of *cagA/s1* negative into positive strains simultaneously to the acquisition of antibiotic resistance has been demonstrated "*in*

* To whom correspondence should be addressed.
(Tel) 39-0881-733848; (Fax) 39-0881-733849
(E-mail) e.ierardi@virgilio.it

vitro" of Yakoob *et al.* (2004). In overall contrast with these findings, a high probability of the co-infection with multiple *vacA* mosaicisms in the presence of clarithromycin resistance has been described (Cellini *et al.*, 2006). Despite all these evidences, none of these explanations appears to be supported by unambiguously convincing data of a preferential association between macrolide resistance and a single genotypic pattern, since no theoretical basis may be put forward to predict an association between antibiotic resistance and *cagA*/*vacA* genotype, as such loci appear to be neither physically nor functionally linked by genomic organization (Alm *et al.*, 1999; Godoy *et al.*, 2003).

On the bases of these controversial data, we performed this study in order to assess the clarithromycin resistance rate, to evaluate the distribution of *H. pylori* genetic pattern in our geographic area, and to further verify a possible association between clarithromycin resistance and *cagA* or *vacA* genes.

Materials and Methods

Patients

Two or more antral biopsy specimens (anterior and posterior wall) of *H. pylori* positive dyspeptic patients consecutively observed in the two participating centres (Foggia, Southern; Roma, Central; Italy) were collected. In these areas clarithromycin resistance rate as well as the race of participating patients did not differ (De Francesco *et al.*, 2006b). *H. pylori* infection was considered present when bacteria were detected at both histology (Giemsa staining), jointly with an active chronic gastritis (Haematoxylin/Eosin), and rapid urease test. The study enrolled only patients never previously treated for *H. pylori* infection. Patients having taken proton pump inhibitors or antibiotics during the previous 8 weeks before endoscopy were excluded. Informed consent was obtained from each patient.

Reagents and Instruments

Nucleo-SpinnTissue (Macherey-Nagel GmbH and Co, Germany), E-Test (AB Biodisks, Sweden), primers Hp23-F Wizard PCR preps (Promega, USA), Dye Terminator 3.1 Ready Reaction Kit (Applied Biosystems Division, USA), automated DNA sequencer ABI Prism 377 (Applied Biosystems, USA), Sequence Navigator software package (Applied Biosystems, USA), Express program and Genotyping Assay Service (Applied Biosystems, USA), Universal PCR Master Mix (Applied Biosystems, USA), ABI Prism 7900HT instrument (Applied Biosystems, USA), Sequence Detector software (Applied Biosystems, USA)

Clarithromycin resistance assessment

Clarithromycin resistance in *H. pylori* is mainly sustained by 3 point mutations in the 23S rRNA (A2142C, A2142G, and A2143G) (Megraud, 2004). For molecular analysis, we used a novel method (TaqMan real time PCR) which has been firstly used for *Mycobacterium tuberculosis* detection (Wada *et al.*, 2004) and successively for *H. pylori* DNA sequencing on paraffin-embedded samples (Lascols *et al.*, 2003), as we have previously reported (De Francesco *et al.*, 2004).

The investigators (M. M. and F. G.) who performed the real-time PCR for genotyping resistance were blinded for all demographic and clinical characteristics of patients. The methods were performed in one only Centre (Bari) where 10 μ sections of paraffin embedded samples from each Centre were collected.

Our methods were validated by comparing real-time TaqMan PCR detection of bacterial DNA with rapid urease test, urea breath test and histology with the 100% sensitivity and specificity. Moreover, in order to evaluate the lower limit of detection of real-time PCR protocol, serial dilutions of *H. pylori* DNA extracted from paraffin-embedded sections and bacterial culture were processed. Serial dilutions ranging from 100 ng to 100 fg of DNA were performed before real-time PCR and a linearity of amplification was obtained until 500 fg. The amplification efficiency of DNA from paraffin-embedded sections was identical to that obtained of DNA samples from bacterial colonies.

Assessment and amplification of cagA and vacA

The extracted DNA was subjected to PCR for detection of *cagA H. pylori* gene, using the primers described by *Tummuru et al.* which amplified a region of 400 bp (Tummuru *et al.*, 1993), and the *vacA* gene, using primers described by Atherton *et al.* (1995), which evaluated the midregion (*m*) and the region encoding for the signal peptide (*s*) of the gene. Four different PCR products were obtained: *s1* (259 bp) or *s2* (286 bp) from the *s*-region, and *m1* (290 bp) or *m2* (352 bp) from the *m*-region. The details of the method have been previously reported by us (De Francesco *et al.*, 2004). Even in this case, the amplification efficiency of DNA from paraffin-embedded sections was identical to that obtained of DNA samples from bacterial colonies in detecting bacterial genomic monoclonality and polyclonality.

Statistical analysis

Differences between groups were statistically evaluated by using the Student's t-test for unpaired data, Chi-square test with Yate's correction for small numbers, and Fisher's exact probability test, as appropriate. Differences were considered significant at 5% probability level. Statistical analysis was performed using a specific software (Statsoft 6.0 program for Windows 98.00).

Results

cagA gene and vacA mosaicism distribution

The *cagA* and *vacA* (*s* and *m* region) genotypes were assessed in 62 *H. pylori* infected patients (Mean age: 48.8 ± 13.7 years; 32 males). The *cagA* gene was detected in 43 (69.3%) cases. As far as *vacA* gene is concerned, the *s1* allele was found in 43 (72.8%) strains, and the *m1* allele in 22 (37.2%) cases. A restrict bacterial clonality i.e. a single mosaic combination of *s/m* region was observed in 59 (95.1%) patients, whilst a colonization with a double *vacA* mosaic combination was detected only in 3 patients, which were excluded from further statistical analysis. In detail, the *s1m1* combination was observed in 23 (38.9%) patients, the *s1m2* in 22 (37.2%), and the *s2m2* in 14 (23.7%), whilst the remaining *s2m1* combination was never found in our series. The distribution of *vacA* mosaicism according to *cagA* gene is provided in the Table 1. As shown, the *s2m2* combination was detected more frequently in the *cagA* negative than in cagA positive strains (52.7% vs 10%; p = 0.0002).

Clarithromycin resistance relationship with cagA and vacA

Clarithromycin resistance assessment showed that 47 patients (75.8%) were infected with susceptible strains and 15 (24.2%) with resistant bacteria, including 4 purely resistant and 11 heteroresistant strains. In detail, the A2143G point mutation was detected in 8 cases, the A2142C in 6, whilst a double point mutation (A2143G plus A2142C) was found in the remaining case.

Table 1. *vac*A mosaicism distribution between cagA-positive and negative strains

	cagA positive (40 patients)	*cagA* negative (19 patients)	*P*
S1m1	18 (45%)	5 (26.3%)	NS
S1m2	18 (45%)	4 (21.0%)	NS
S2m2	4 (10%)	10 (52.7%)	0.0002

Table 2. *vac*A mosaicism distribution between cagA-positive and negative strains

	Single strain (59 pts)			Multiple strains (3 pts)	
Claritromycin	*s1m1*	*s1m2*	*s2m2*	*s1m2* plus *s2m2*	*s1m1* plus *s1m2*
Sensible 47 (%)	19 (41)	16 (34)	10 (21)	1 (2)	1 (2)
Resistant 15 (%)	4 (27)	6 (40)	4 (27)	0	1 (6)

No statistically significant difference was detected for each comparison.

The *cagA* gene was similarly distributed between susceptible and resistant strains (70.2% *vs.* 66.6%; p = 0.9). Similarly, the prevalence of both s1 *vacA* allele (75.5% vs. 64.2%; p = 0.4) and the m1 allele (42.2% vs. 21.4%; p = 0.2) did not significantly differ between susceptible and resistant strains, respectively.

As above reported, the *H. pylori* infection was due to a single strain in 45 (95.7%) patients harbouring clarithromycin susceptible strains, in 4 (100%) with purely resistant strains and in 10 (90.9%) with hetero-resistant infection. Therefore, *H. pylori* infection was due to a single strain in 45 (95.7%) patients harbouring clarithromycin susceptible strains as well as in 14 (93.3%) with resistant strains (p = 0.9). The distribution of each mosaic combination in clarithromycin susceptible and resistant strains is reported in Table 2. As shown, the prevalence of different *vacA* mosaicism did not significantly differ between clarithromycin susceptible and resistant strains.

Discussion

The first relevant finding of the present study is that primary *H. pylori* clarithromycin resistance is high (24.1%) in our geographic area (Central and Southern Italy), and it would appear higher than that computed in previous Italian studies (range 1.8-14%) (Pilotto *et al.*, 2000; Savarino *et al.*, 2000; Perri *et al.*, 2001), and in agreement with the 23% rate detected in another recent study (Toracchio and Marzio, 2003). Such an observation is important for *H. pylori* management in clinical practice, bacterial clarithromycin resistance being proved to be the main factor hampering the efficacy of standard therapies (Megraud, 2004; De Francesco *et al.*, 2006a).

H. pylori is a worldwide spread infection, and several epidemiological studies have shown a geographical variation of its virulence factors, such as the *cagA* locus and the mosaic combination of the *vacA* gene alleles (Covacci *et al.*, 1999). Nevertheless, we assessed in the present study the prevalence of both *cagA* gene and *vacA* allele mosaicism in our geographic area, being this district never been examined for these factors. The prevalence of *cagA*, *s1* and *m1 vacA* alleles found in our series was similar to that observed in other European countries (Crabtree *et al.*, 1992; Weel *et al.*, 1996; Elviss *et al.*, 2004). The assessment of *vacA* gene mosaicism found only three out of the four possible combinations, the *s2m1* mosaicism being never detected in our series. This finding is in agreement with other previous studies, and probably depends on a selective disadvantage of this mosaicism which undermines bacterial viability (Atherton *et al.*, 1995). We also observed that *s1m1* and *s1m2* mosaicisms

were equally frequent, and they were more prevalent as compared to the *s2m2* combination. This distribution differs from that observed in other countries, such as US where *s1m1* and *s2m2* are equally prevalent (Atherton *et al.*, 1995), and Germany or UK in which *s1m1* and *s1m2* are respectively the most frequent combinations (Han *et al.*, 1998; Kauser *et al.*, 2005). However, in agreement with a previous study (Elviss *et al.*, 2004), the *s2m2 vacA* mosaicism was significantly associated with the *cagA* negative status.

As reported in other countries (Han *et al.*, 1998; Kauser *et al.*, 2005), we found that *H. pylori* infection is sustained by a single *vacA* mosaicism (one strain for each patient) in more than 90% of cases. This finding conflicts with what reported in Mexico and in China, where a co-infection with multiple *vacA* mosaicisms is common (Morales-Espinosa *et al.*, 1999; Yakoob *et al.*, 2001). Most likely, the very high prevalence of *H. pylori* in these areas plays a role in increasing the risk of infection with multiple strains. On the other hand, previous reports have shown that the probability of a co-infection with more than one strain is higher in the presence of clarithromycin susceptible than resistant bacteria (Kim *et al.*, 2003). Our data failed to confirm such a finding. Indeed, we found a restrict clonality (i.e. a single bacterial genotype for each patient) in more than 90% of infected subjects with either clarithromycin susceptible or resistant strains. In particular, the prevalent monoclonality observed even in the group of heteroresistant organisms (10 out of 11 of cases) could suggest that resistance development is likely due to a point mutation from a pre-existing susceptible *H. pylori* strain (Kim *et al.*, 2003) rather than the exchange of bacterial genetic material from a strain to another (Yakoob *et al.*, 2004). Indeed, differently form previous studies (Yakoob *et al.*, 2001; Kauser *et al.*, 2005), our data failed to confirm the association of bacterial resistance to clarithromycin with a peculiar *cagA* and/or *vacA* status, suggesting an independent segregation between *cagA* locus/*vacA* gene and clarithromycin resistant genotypes.

In conclusion, the prevalence of primary *H. pylori* clarithromycin resistance is high in our area, whilst the prevalence of the *cagA* appears to be comparable to that reported in other countries. Moreover, the *s1m1* and *s1m2 vacA* are the most frequent mosaicisms, and a bacterial co-infection appears to be rare. Finally, we did not found any relationship between clarithromycin resistance and bacterial genotypic pattern and/or *cagA* posititvity.

Conflcit of interest
None declared.

References

Alm, R.A., L.S. Ling, D.T. Moir, B.L. King, E.D. Brown, P.C. Doig, D.R. Smith, B. Noonan, B.C. Guild, B.L. de Jonge, G. Carmel, P.J. Tummino, A. Caruso, M. Uria-Nickelsen, D.M. Mills, C. Ives, R. Gibson, D. Merberg, S.D. Mills, Q. Jiang, *et al.* 1999. Genomic sequence comparison of two unrelated isolated of the human gastric pathogen *Helicobacter pylori*. *Nature* 397, 176-180.

Atherton, J.C., P. Cao, R.M. Peek, Jr, M.K. Tummuru, M.J. Blaser, and T.L. Cover. 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.

Cellini, L., R. Grande, E. Di Campli, S. Di Bartolomeo, S. Capodocasa, and L. Marzio. 2006. Analysis of genetic variability, antimicrobial susceptibility and virulence markers in *Helicobacter pylori* identified in Central Italy. *Scand. J. Gastroenterol.* 41. 280-287.

Covacci, A., J.L. Telford, G. Del Giudice, J. Parsonnet, and R. Rappuoli. 1999. *Helicobacter pylori* virulence and genetic geography. *Science* 284, 1328-1333.

Crabtree, J.E., N. Figura, J.D. Taylor, M. Bugnoli, D. Armellini, and D.S. Tompkins. 1992. Expression of 120 kilodalton protein and cytotoxicity in *Helicobacter pylori*. *J. Clin. Pathol.* 45, 733-734.

De Francesco, V., A. Zullo, M. Margiotta, S. Marangi, O. Burattini, P. Berloco, F. Russo, M. Barone, A. Di Loe, M.F. Minenna, V. Stoppino, S. Morini, C. Panella, A. Francavilla, and E. Ierardi. 2004. Sequential treatment for *Helicobacter pylori* does not share the risk factors of triple therapy failure. *Aliment Pharmacol. Ther.* 19, 407-414.

De Francesco, V., M. Margiotta, A. Zullo, C. Hassan, N. Della Valle, O. Burattini, U. Cea, G. Stoppino, A. Amoruso, F. Stella, S. Morini, C. Panella, and E. Ierardi. 2006a. Primary clarithromycin resistance in Italy assessed on *Helicobacter pylori* DNA sequences by TaqMan real time polymerase chain reaction. *Aliment Pharmacol. Ther.* 23, 429-435.

De Francesco, V., M. Margiotta, A. Zullo, C. Hassan, L. Troiani, O. Burattini,

F. Stella, A. Di leo, F. Russo, S. Marangi, R. Monno, V. Stoppino, S. Morini, C. Panella, and E. Ierardi. 2006b. Clarithromycin-resistant genotypes and eradication of *Helicobacter pylori*. *Ann. Intern. Med.* 144, 94-100.

Elviss, N.C., R.J. Owen, J. Xerry, A.M. Walker, and K. Davies. 2004 *Helicobacter pylori* antibiotic resistance patterns and genotypes in adult dyspeptic patients from a regional population in North Wales. *J. Antimicrob. Chemother.* 54, 435-440.

Godoy, A.P., M.L. Ribeiro, Benvengo, L. Vitiello, C. Miranda Mde, S. Mendonca, J. Pedrazzoli, Jr. 2003. Analysis of antimicrobial susceptibility and virulence factors in *Helicobacter pylori* clinical isolates. *BMC Gastroenterol.* 3, 1-6.

Han, S.R., H.J. Schreiber, S. Bhakdi, M. Loos, and M.J. Maeurer. 1998. *vacA* genotypes and genetic diversity in clinical isolates of *Helicobacter pylori*. *Clin. Diagn. Lab. Immunol.* 5, 139-145.

Kauser, F., M.A. Hussain, I. Ahmed, S. Srinivas, S.M. Devi,

A.A. Majeed, K.R. Rao, A.A. Khan, L.A. Sechi and N. Ahmed. 2005. Comparative genomics of *Helicobacter pylori* isolates recovered from ulcer disease patients in England. *BMC Microbiol.* 5, 32-35.

Kim, J.J., J.G. Kim, and D.H. Kwon. 2003. Mixed-infection of antibiotic susceptible and resistant *Helicobacter pylori* isolates in a single patient and underestimation of antimicrobial susceptibility testing. *Helicobacter* 8, 202-206.

Lascols, C., D. Lamarque, J.M. Costa, C. Copie-Bergman, J.M. Le Glaunec, L. Deforges, C.J. Soussy, J.C. Petit, J.C. Delchier, and J. Tankovic. 2003. Fast and accurate quantitative detection of *Helicobater pylori*and identification of clarithromycin resistance mutations in *H. pylori* isolates from gastric biopsy specimens by real-time PCR. *J. Clin. Microbiol.* 41, 4573-4577.

Megraud, F. 2004. *H. pylori*antibiotic resistance: prevalence, importance, and advances in testing. *Gut* 53, 1374-1384.

Morales-Espinosa, R., G. Castillo-Rojas, G. Gonzalez-Valencia, G. Gonzalez-Valencia, S. P. de Leon, A. Cravioto, J.C. Atherton, and Y. Lopez-Vidal. 1999. Colonization of Mexican patients by multiple *Helicobacter pylori* strains with different *vacA* and *cagA* genotypes. *J. Clin. Microbiol.* 37, 3001-3004.

Perri, F., M.R. Villani, V. Festa, M. Quitadamo, and A. Andriulli. 2001. Predictors of failure of *Helicobacter pylori* eradication with the standard 'Maastricht triple therapy'. *Aliment. Pharmacol. Ther.* 15, 1023-1029.

Pilotto, A., M. Rassu, G. Leandro, M. Franceschi, and F. Di Mario. 2000. Prevalence of *Helicobacter pylori*resistance to antibiotics in Northeast Italy: a multicentre study. *Dig. Liver Dis.* 32, 763-768.

Savarino, V., P. Zentilin, M. Pivari, G. Bisso, R. Mele, N. Pandolfo, V. Pugliese, and S. Vigneri. 2000. The impact of antibiotic resistance on the efficacy of three 7-day regimens against *Helicobacter pylori. Aliment Pharmacol. Ther.* 14, 893-900.

Toracchio, S. and L. Marzio. 2003. Primary and secondary antibiotic resistance of *Helicobacter pylori* strains isolated in central Italy during the years 1998-2002. *Dig. Liver Dis.* 35, 541-545.

Tummuru, M.K.R., T.L. Cover, and M.J. Blaser. 1993. Cloning and expression of a high molecular mass major antigen of *Helicobacter pylori. Infect. Immun.* 61, 1799-1809.

van Doorn, L.J., C. Figueiredo, R. Sanna, A. Plaisier, P. Schneeberger, W. de Boer, and W. Quint. 1998. Clinical relevance of the *cagA, vacA*, and *iceA* status of *Helicobacter pylori. Gastroenterology* 115, 58-66.

van Doorn, L.J., Y. Glupczynski, J.G. Kusters, F. Megraud, P. Midolo, N. Maggi-Solca, D.M. Queiroz, N. Nouhan, E. Stet, and W.G. Quint. 2001. Accurate prediction of macrolide resistance in *Helicobater pylori* by a PCR line probe assay for detection of mutations in the 23S rRNA gene: multicenter validation study. *Antimicrob Agents Chemother* 45, 1500-1504.

Wada, T., S. Maeda, A. Tamaru, S. Imai, A. Hase, and K. Kobayashi. 2004. Dual-probe assay for rapid detection of drug resistant *Mycobacterium tuberculosis* by Real-Time PCR. *J. Clin. Microbiol.* 42, 5277-5278.

Weel, J.F., R.W. van der Hulst, Y. Gerrits, P. Roorda, M. Feller, J. Dankert, G.N. Tytgat, and A. van der Ende. 1996. The interrelationship between cytotoxin-associated gene A, vacuolating cytotoxin, and *Helicobacter pylori*-related diseases. *J. Infect Dis.* 173, 1171-1175.

Yakoob, J., X.G. Fan, G.L. Hu, and Z. Zhang. 2004. Genetic and phenotype changes following in vitro interactions between *Helicobacter pylori* strains. *J. Gastroenterol. Hepatol.* 19, 626-631.

Yakoob, J., X.G. Fan, G.L. Hu, H.X. Yang, L. Liu, S.H. Liu, D.M. Tan, T.G. Li, and Z. Zhang. 2001. Polycolonization of *Helicobacter pylori* among Chinese subjects. *Clin. Microbiol. Infect.* 7, 187-192.