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Research paper

Multiple and mixed *Helicobacter pylori* infections: Comparison of two epidemiological situations in Tunisia and France

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ABSTRACT

Individuals can be infected by either a single or multiple strains of *Helicobacter pylori*. Multiple infection with genetically different isolates and particularly mixed infection with both antibiotic-susceptible and resistant isolates are difficult to detect and should impact the effectiveness of eradication treatment. It is largely assumed that multiple infections are more frequent in developing countries but an actual comparison developing/developed using a single methodology has never been reported. To compare the prevalence of multiple and mixed *H. pylori* infection in Tunisia and France, we conducted a prospective study including 42 *H. pylori*-culture positive infected patients (21 Tunisian and 21 French) never previously treated for *H. pylori* infection. One gastric biopsy was collected from antrum. Three to eleven (mean = 9) colonies were isolated from each biopsy. A total of 375 different isolates were genotyped using RAPD fingerprinting and antimicrobial susceptibility testing was performed on amoxicillin, clarithromycin, ciprofloxacin, rifampicin, tetracycline and metronidazole with E-tests. Multiple infection was defined by different RAPD fingerprintings among the different isolates from a single patient. Mixed infection was defined by different resistance profiles among the different isolates from a single patient. Multiple *H. pylori* infection is more prevalent in Tunisia than in France. It occurred in ten (48%) Tunisian patients and in one (5%) French patient ($p < 0.001$). Mixed infection is common (24%), it occurred in 4 (19%) Tunisian patients and in 6 (29%) French patients ($p = 0.46$) and was mainly (8/10) due to genetically related clones in single infection.

1. Introduction

Helicobacter pylori infection is one of the most common chronic bacterial infections in the world and has been established as a major cause of gastritis, peptic ulcer disease, and gastric cancer (Suerbaum and Michetti, 2002). *H. pylori* is one of the most genetically diverse bacterial species (Suerbaum et al., 1998). It chronically infects the gastric mucosa. Infection is acquired in early childhood and persists over decades, if not lifelong, in the absence of specific treatment. About 50% of the world's population is colonized with a prevalence of over 80% in many developing countries and a rapid decline in developed countries, where prevalence is now estimated at around 20–30% (Frenck and Clemens, 2003; Ben Mansour et al., 2010a). In Tunis, prevalence of *H. pylori* infection was estimated in a multicenter serological study performed in 2006–2007 (Ben Mansour et al., 2010a). The seroprevalence in blood donors was 64% and 99% in patients referred for gastroduodenal endoscopy but since this study was published we have observed a progressive decrease in the prevalence of *H. pylori* infection diagnosed by culture of biopsy specimens now reaching 50% in Tunis (personal data). In France, where there has been no recently published study, we can estimate the prevalence of *H. pylori* infection by the prevalence of *H. pylori* infection diagnosed by culture of French patients' biopsies, which is now only 22% in Poitiers (personal data). This global decrease of *H. pylori* infection has already been described and is believed to be due to improved hygiene, and active elimination by antibiotics during childhood, both of which have contributed to declining transmission risk (den Hollander et al., 2015).

Individuals can be infected by either a single or multiple strains of *H. pylori*. A multiple infection is defined as the infection of a single patient by two or more genetically distinct isolates. The genetic distinction of isolates can be performed using genotyping of virulence genes (*cagA*, *vacA*, *iceA*) (Audibert et al., 2000; Ben Mansour et al., 2010b; Morales-Espinosa et al., 1999; Patra et al., 2012), random amplified polymorphism DNA (RAPD) (Patra et al., 2012; Wong et al., 2001; Yakoob et al., 2001; Ren et al., 2012; Sheu et al., 2009; Hua et al., 1999; Norazah et al., 2009; Kao et al., 2014; Selgrad et al., 2014; Van Der Ende et al., 2001; Toita et al., 2013; Enroth et al., 1999; Marshall

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et al., 1995), Multilocus Sequence Typing (MLST) (Suerbaum et al., 1998) or whole genome sequencing (Kenneman et al., 2011; Krebes et al., 2014).

The prevalence of such multiple infections has been reported to vary (0–100%) (Patra et al., 2012; Wong et al., 2001; Yakoob et al., 2001; Ren et al., 2012; Sheu et al., 2009; Hua et al., 1999; Norazah et al., 2009; Kao et al., 2014; Selgrad et al., 2014; Van Der Ende et al., 2001; Toita et al., 2013; Enroth et al., 1999; Marshall et al., 1995; Dore et al., 1998) depending on geographical region, whether in a developed or developing country (low and high overall infection risk, respectively), but probably also according to the methods used to sample isolates from the stomach or on account of the discriminatory power of the strain typing method used in different studies. It is largely assumed that multiple infections are more frequent in developing countries but an actual comparison developing/developed using a single methodology has never been reported.

In addition to multiple infection, which is characterized by genetic diversity, an individual can be infected by both antibiotic-susceptible and resistant *H. pylori* strains (Wong et al., 2001; Yakoob et al., 2001; Norazah et al., 2009; Kao et al., 2014; Selgrad et al., 2014; Van Der Ende et al., 2001; Raymond et al., 2010; Ben Mansour et al., 2010c). These strains can be either derived from the same genetic isolate or genetically totally different (multiple infection). In the first case it is assumed that the resistant clone emerges from the susceptible one under selective pressure due to antibiotic consumption. In the second case, the patient has been infected by two different strains, one susceptible and the other resistant. Mixed infection is defined by the infection of a single patient with two or more isolates presenting different antibiotic susceptibility profiles. Mixed infection is usually detected by the presence of a subpopulation of resistant colonies in the inhibitory area of a disk or E-test diffusion test or by discordant results of susceptibility testing in two biopsies from the same patient (Wong et al., 2001; Kao et al., 2014). Mixed infection is difficult to detect and should impact the effectiveness of eradication treatment if the resistant strain is not detected.

For precise determination of both multiple and mixed infections it is important to work on pure isolates obtained from isolated colonies and not from the mixture of different colonies harvested together on the first culture plate (Wong et al., 2001). Inworks on mixtures of different colonies or by PCR directly on DNA extracted from a single biopsy, multiple infection can be suspected when *vacA* genotyping yields a mixture of the different alleles m1 and m2, s1 and s2 (Audibert et al., 2000; Ben Mansour et al., 2010b). In Tunisia, in a previous study, the prevalence of multiple infections was estimated at 31.4% for the *vacA* genotyping performed directly on DNA extracted from biopsy (Ben Mansour et al., 2010b). In France, in a previous study performed in Poitiers, the prevalence of multiple infections was estimated at 2.6% for *vacA* genotyping performed directly on DNA extracted from biopsy (Audibert et al., 2000). Virulence gene genotyping is not sensitive enough to detect multiple infections and certainly underestimates this phenomenon. RAPD has been proven to achieve an excellent discriminatory power allowing precisely distinguishing *H. pylori* isolates (Burucoa et al., 1999) and has been widely used to identify different genotypes among and within individual patients (Patra et al., 2012; Wong et al., 2001; Yakoob et al., 2001; Ren et al., 2012; Sheu et al., 2009; Hua et al., 1999; Norazah et al., 2009; Kao et al., 2014; Selgrad et al., 2014; Van Der Ende et al., 2001; Toita et al., 2013; Enroth et al., 1999; Marshall et al., 1995).

The aim of this study was to compare the prevalence of multiple and mixed *H. pylori* infection between two locations in different epidemiological situations: Tunis in a developing country with high prevalence of infection and Poitiers in a developed country with low prevalence of infection. With this in mind we chose to work on multiple isolated colonies from 21 Tunisian and 21 French *H. pylori* infected patients in view of genotyping isolates using RAPD analysis and of determining antibiotic susceptibility on isolated colonies.

2. Material and methods

2.1. Patients

This prospective study was conducted from June to October 2009 in two University Hospitals: CHU La Rabta in Tunis, Tunisia and CHU La Milétrie in Poitiers, France. Inclusion criteria were, for each center, the first 21 consecutive adult patients of both genders referred for gastroduodenal endoscopy, detected *H. pylori*-infected by a positive *H. pylori* culture from the antrum biopsy specimen and never previously treated for *H. pylori* infection. One gastric biopsy was routinely collected for bacteriological diagnosis of *H. pylori* infection in the antrum during upper gastrointestinal endoscopy. The demographic characteristics, including age, gender, results of endoscopic examination and histology were collected for each patient.

2.2. Strains

Biopsy was crushed and culture was performed in the same manner in the two centers following the same recommendations (Burucoa and Mégraud, 2011). Cultures were carried out on Columbia agar plates supplemented with 10% horse blood and Skirrow's supplement (trimethoprim 5 mg/L, vancomycin 10 mg/L, polymyxin B 2500 IU/L, Oxoid, France) at 37 °C in micro-aerophilic conditions for 3–7 days (CampyGen*, Oxoid, France). Depending on the number of colonies obtained in primary culture, three to eleven single colonies per patient (mean = 9) were picked from the primary culture plates and were subcultured so as to obtain pure isolates. *H. pylori* isolates were identified as positive for urease activity and spiral shape morphology on Gram stain.

2.3. DNA extraction and RAPD genotyping

Genomic DNA was extracted from each isolate of *H. pylori* using QIAamp DNA mini-kit (Qiagen, France) according to the manufacturer's instructions. Random amplified polymorphism DNA reaction (RAPD-PCR) was carried out as previously described (Burucoa et al., 1999). RAPD-PCR was performed in a Perkin-Elmer GeneAmp PCR system 2400 thermal cycler (Perkin-Elmer Cetus, USA) in 100 μL containing 1 μL of chromosomal DNA (~20 ng), 3 mM $MgCl_2$, each primer at a concentration of 0.2 μM, 2.5 U of Eurotaq DNA polymerase (Eurogentec, France), each dinucleotide triphosphate (Eurogentec, France) at a concentration of 0.2 μM, 10 mM Tris–HCl (pH 8.3), and 50 mM KCl (Burucoa et al., 1999). Two arbitrary primers were used: primer 1254 (5′-CCGCAGCCAA-3′) and 1247 (5′-AAGAGCCCGT-3′) (Burucoa et al., 1999). The cycling program was 1 cycle of 94 °C for 2 min, 37 °C for 1 min, and 72 °C for 4 min and 29 cycles of 94 °C for 2 min, 37 °C for 3 min, and 72 °C for 7 min. After PCR, 20 μL of the amplification products was electrophoretically separated in 2% agarose gels.

2.4. Definition of multiple infection using RAPD genotyping

For each patient, genomic diversity among the different colonies (3 to 11, mean = 9) of *H. pylori* isolates was analyzed by RAPD genotyping. Criteria defined by Tenover et al. were used for interpreting RAPD banding patterns (Tenover et al., 1995). Indistinguishable and closely related RAPD banding patterns in the isolates of one patient were defined as single-strain infection. Bacterial infection with more than one RAPD banding pattern found in the isolates of one patient was defined as multiple infection.

2.5. Antimicrobial susceptibility testing

Susceptibility testing for amoxicillin, clarithromycin, tetracycline, metronidazole, ciprofloxacin and rifampicin was performed with the E-test method (bioMérieux, France) on Columbia agar plates