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Multicolonisation and mixed *Helicobacter pylori* infections: situation in Tunisian patients

(Original Article)

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Abstract:

H .pylori is known as one of the bacterial species having greatest genetic polymorphism in the world. It is also known that this genetic diversity could be due to mixed infections followed by genetic exchanges between several strains. Our study aimed to determine the presence of both multiple and mixed infections, for that goal, 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. Antral and fundic biopsies were taken and studied separately. The multicolonisation was defined as simultaneous presence of several strains in the same patient, whether it is antral, fundic or both. Antibiotic susceptibility of the *H.pylori* strains was tested by disk diffusion method and E-test. The Tunisian strains were more polymorphic. Exchanges of genetic material between the various strains were probable. The Tunisian subjects seemed to be colonized by different strains or different clones from the same strain. Several hypotheses might be proposed: initial infection by at least two different strains, infections by different strains at different times, recolonisations are probable.

Key words: *Helicobacter pylori ;* Multicolonisations ; Tunisia

Introduction:

Since its discovery in 1982 by Warren and Marshall (1) on the surface of gastric mucous membrane of patients carrying a chronic gastritis, *Helicobacter pylori* (*H. pylori*) became an uncontested pathogenic in the field of the gastroenterology in adults and children (2). *H. pylori* is now known as one of the bacterial species having greatest

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genetic polymorphism in the world (3, 4). It is also known that this genetic diversity could be due to mixed infections (5, 6) followed by genetic exchanges between several strains (7). This diversity was explained by various factors: either by the presence of specific changes, or by the mosaïcism phenomenon of some genes (gene *vac* A) or by the presence or the absence of blocks of genes (small island of pathogenicity *cag*).

Our previous study aimed to identify the presence of allelic mixtures found at several virulence factors genes in the isolated strains of the Tunisian subjects infected by *H. pylori* (8). Indeed, we noted the coexistence of the allele's *iceA* 1 and *iceA* 2 of the gene *iceA*, and allele's *m* 1 and *m* 2 of the gene *vacA* at one subject. Moreover, we observed molecular profiles discordance at some subjects between the fundic and antral strains. These observations let us think that a Tunisian subject could be colonized by a mixture of several strains. Thus, the aim of this study was primarily to determine the existence of a genetic polymorphism in strains of the same subjects of a Tunisian population.

Material and methods:

Material

This study was concerned by 21 strains of adults having consulted in the A and B gastroenterology wards of the Rabta hospital in Tunis, Tunisia for peptic disorders.

Methods

1.2.1 H. pylori isolation from antral and fundic biopsies:

The biopsies were taken away from all the subjects then collected in physiological serum. Two antral and fundic fragments were taken and were studied separately. The biopsy specimens were cut into small pieces, homogenized in a Petri dish with a sterile scalpel and were smeared on the surface of Columbia agar plates containing 10% horse blood and Skirrow (Oxoid, England) supplement. Incubation was performed in microaerophilic conditions at 37°C for a maximum of 6 days. The *H. pylori* colonies were smooth, translucent and small (2 mm of diameter). Colonies that exhibited the described characteristic morphologies were identified as *H. pylori* if they were Gram negative and shaped bacilli, and urease, catalase and oxidase positive.

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Study of the double populations:

From the primary growth, eight to twenty colonies were isolated, from antral and fundic localization and were mended separately for each individual, so 291 colonies were studied on the whole. The multicolonisation was defined by the simultaneous presence of several strains in the stomach of the same patient, whether it is antral, fundic or both. Genomic DNA was extracted with the QIAamp DNA mini kit (Qiagen, Germany) according to the manufacturer's instructions. The isolated DNA was eluted in 200 µl of 1× TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) and stored at -20°C until use.

Standard Antibiogram:

Antibiotic susceptibility of the *H. pylori* strains was tested by disk diffusion method and E-test on a Columbia medium added with 10% of horse blood without Skirrow mixture and results were interpreted according to the standards of the French Antibiogram Committee (CA-SFM 2010) (http://www.sfm.asso.fr) for the following antibiotics : Erythromycin, clarithromycin, metronidazole, ciprofloxacin, tetracyclin and rifampicin. Minimal inhibitory concentration cut-offs to define resistance were 0.25/0.50mg/L for clarithromycin, 1mg/L for tetracycline, rifampicin and ciprofloxacin, and 8mg/L for metronidazole. Before carrying out the antibiogram, a direct examination (Gram coloration) was done from the culture in order to highlight the predominance of bacillar forms (more than 90%) compared to coccoides forms.

PCR-Random Amplified Polymorphic DNA (PCR-RAPD)

To compare the genotypic profiles of the antral and fundic strains at the same individual, polymerase chain reactions (PCR) were performed as described in our previous study (8). Random amplified polymorphism DNA reaction (RAPD PCR) was carried out as previously described (9). RAPD-PCR was performed in a Perkin-Elmer Gene Amp PCR system 2400 thermal cycler (Perkin-Elmer Cetus, USA) in 100 µL containing 1µL of chromosomal DNA(~20ng), 3mM MgCl2, each primer at a con centration of 0.2 µM, 2.5 U of Eurotaq DNA polymerase (Eurogentec, France), each dinucleotide triphosphate (Eurogentec, France) at a con centration of 0.2 µM, 10 mM Tris–HCl (pH 8.3), and 50 mM KCl. Two arbitrary primers were used: primer 1254 (5′-CCGCAGCCAA-3′) and 1247 (5′-AAGAGCCCGT-3′). 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

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for 2 min, 37 °C for 3min, and 72°C for 7min. After PCR, 20µL of. The amplified PCR products were resolved in 1.5% agarose gels stained with ethidium bromide and visualized under a short wave length ultraviolet light source.

Results:

Study of genetic polymorphism:

The results are given by the following profiles:

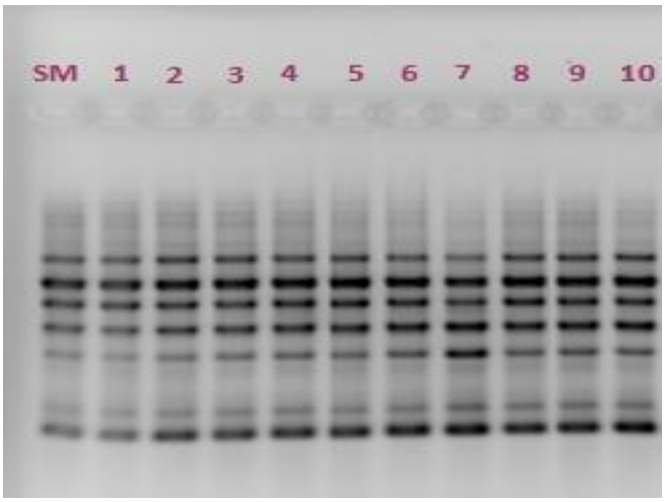

Fig.1. Profile A showing a perfect identity between antral (1-5) and fundic (6-10) colonies (UFC); SM: Ancestral strain

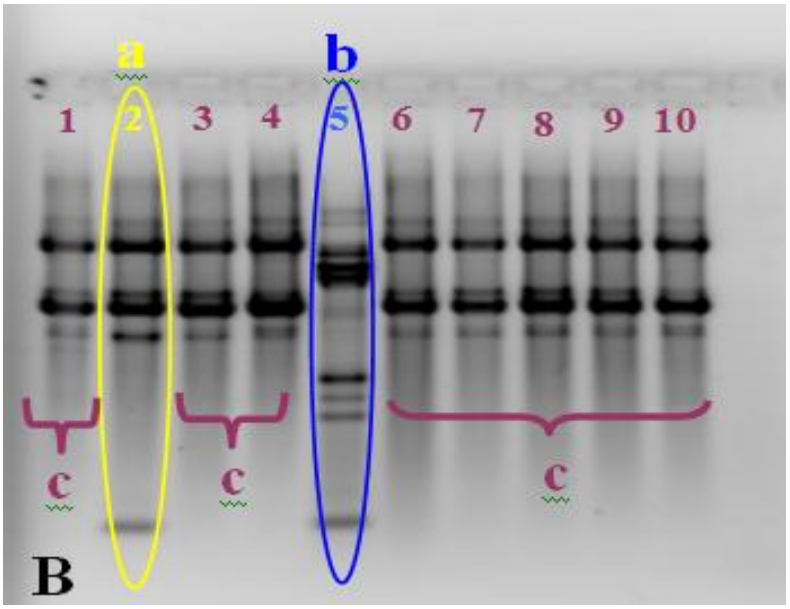

Fig. 2 Profile B showing a discordance between fundic colonies (UFC)

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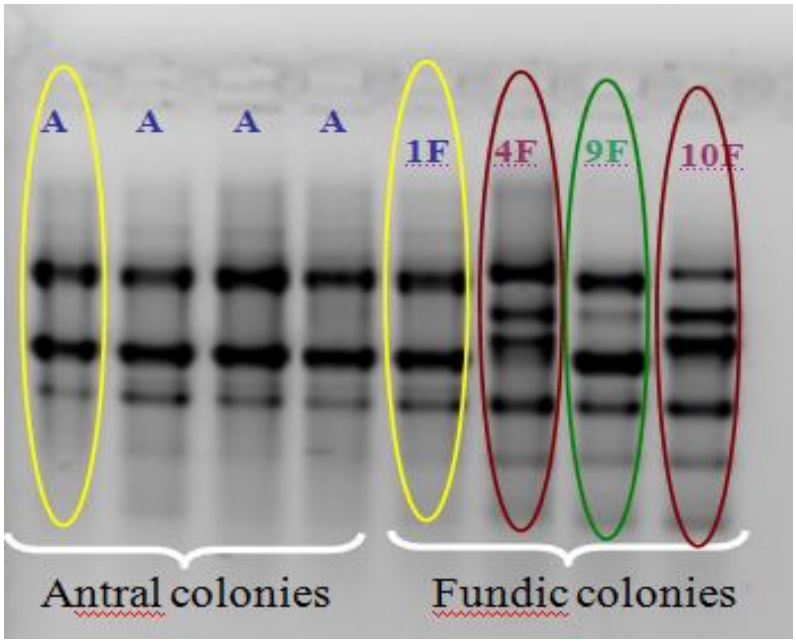

Fig.3. Profile C: Electrophoretic profiles obtained at the patient X

Table. 1. Genetic polymorphism of the various strains

Profile	Strains numbers (N=21)
Profile A and/or F identical	12 (57,1 %)
Profile A and/or F different	9 (42,9%)

The antibiotics resistance study

Results of antibiotics resistance study in the Tunisian population

Principal antibiotics resistances of the Tunisian strains are summarized in table 2.

Table 2: Results of the study of antibiotic resistance in the Tunisian population

			Sensitivity to antibiotics					
Bacterial strain (Hp)	**Number of colonies**	**Profile of migration RAPD (vil3 and vil5)**	**Cla**	**Mtz**	**E**	**Rif**	**Cip**	**Tet**
1	8	> 1	S	R (9) Other S	S	S	S	S

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7	10	> 1	S	R (5/6) Other S	S	S	S	S
9	13	1	S	S	S	S	S	S
10	18	> 1	S	S	S	S	S	S
11	8	1	S	S	S	S	S	S
13	19	> 1	S	S	S	S	S	S
21	17	1	S	S	S	S	S	S
27	20	1	S	S	S	S	S	S
28	20	> 1	S	S	S	S	S	S
29	10	1	S (10) Other R	S (3/4/8/9/10) Other R	S	S	S	S
35	20	> 1	S	S	S	S	S	S
36	20	> 1	S	S	S	S	S	S
37	10	1	S	S	S	S	S	S
38	10	> 1	S	S	S	S	S	S
40	20	> 1	S	S	S	S	S	S
41	10	1	S	S	S	S	S	S
41	10	1	S	S	S	S	S	S
42	9	> 1	S	S	S	S	S	S
43	19	1	S	S	S	S	S	S
45	10	> 1	R	R	R	S	S	S
140	10	1	R (10) Other S	S	R (10) Other S	S	S	S
21 Strains	**291**	**10** identical profiles **11 different profiles**	**4 strains having different sensitivities**					

Claclarithromycin, E: erythromycin Mtz: mtronidazole Cipciprofloxacin Tet: tetracyclin Rifrifampicin S: sensitive R: resistant

Discussion

We distinguish 3 various profiles from migration : a, b and c. These various profiles corresponded to different strains although isolated at the same patient. For the patient X, we noticed that all antral clones (A) were identical (the same profile and the same number of bands), thus, only one common clone to all the colonies. However, the fundic colonies of this patient showed the existence of a genetic polymorphism. Indeed, adding to the antral profile found on the fundic clone (clone 1F), two other fundic profiles (4F, 10F and 9F) were highlighted. These results are in accordance of a mixture of several *H. pylori* strains at the same patient.

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Our strains showed a genetic variability by RAPD. Indeed, we obtained 10 identical and 11 different profiles. Four strains showed discordance in antibiotic resistance. In final, the presence of several genotypes at the same patient favorizes the presence of multiple strains. This will be able to be explained by the acquisition of the infection by one or more strains of *H. pylori* in the childhood especially in the countries with strong infection prevalence by *H. pylori*; but it is not yet precise how several strains could colonize the stomach simultaneously (in case of the co-infection), or a progressive contamination by several strains (it is the case of the super-infection). Moreover, the majority of studies showed that the co-infection and the super-infection are two common processes (10, 11).

The coexistence of more than only one strain in the stomach of the same patient could be explained by the fact that *H. pylori* can acquire genetic variations during the beginning of colonization as in childhood (12); thus, the dynamic colonization by several strains at the same patient was studied on animal's models (13).

One of the more striking *H. pylori* characteristics resides in its genetic diversity. Indeed, it is one of the bacterial species having greatest polymorphism. This bacterium was isolated from various individuals who has a high level of polymorphism. But we also observed variations between the isolates at the one host. Several studies reported that the strains polycolonisation distinct from *H. pylori* was possible (14). At least, it is still difficult to say which is the frequency of the mixed infections and of the genetic exchanges which occur between the various strains.

We point out that on 21 biopsies coming each one from only one individual, the study from 8 to 20 isolated colonies from the antral and/or the fundic area showed a genetic polymorphism. These results corroborate those found in a study carried out on twelve Mexicain patients among whom the probability of infection by several strains at the same individual is very high and a genetic rate of polymorphism of 8,33% was found (15). The differences observed between the strains would result from the accumulation of changes, recombination and also of horizontal exchanges of genes.

Two major types of genetic diversity were described in the clinical isolates of *H. pylori*: the macrodiversity (from significant areas of chromosomes vary from one strain to another) and the microdiversity (a specific diversity in the particular gene sequence).

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The Tunisian strains showed a polymorphism. Exchanges of genetic material between the various strains were probable. The Tunisian subjects seemed to be colonized by different strains or different clones from the same strain. Several hypotheses might be proposed: initial infection by at least two different strains, infections by different strains at different times and the high frequency of recolonisations are probable, and the role of the socio-economic factors in the Tunisian area especially culinary traditions.

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