Helicobacter Pylori in Children: Molecular Characterization, Antibiotics Resistance, and MLST of Isolated Strains in an Algerian Hospital

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Abstract

Background: Helicobacter pylori infection is generally acquired in childhood. Algeria is a country with a high prevalence of H. pylori infection. The aim of this work was to take stock of H. pylori infection in Algerian children.

Materials and Methods: About 31 antral biopsies were cultured, and then antibiotic susceptibility testing was performed. The statuses of cagPAI and vacA s, m, l, and d regions were determined as well as geographical typing was done by Multi Locus Sequence Typing (MLST) method.

Results: Culture was H. pylori positive in 12 children. Only one resistance to clarithromycin and one to metronidazole were detected. Four out of six strains possessed cagPAI, and five out of six strains were identified as vacA s2m2l2d2. The five strains tested by MLST were of the hpEurope type.

Conclusion: This study revealed high prevalence of H. pylori infection and low resistance to antibiotics and reported for the first time in Algeria a genetic typing of H. pylori strains isolated from Pediatrics.

Keywords: Culture, Gastric biopsies, Antibiotics resistance, cagPAI, vacA

1. Background

Helicobacter pylori infection is usually acquired in childhood and can remain as asymptomatic for several years(1). In children, the prevalence rate of H. pylori infection is low in industrialized countries and high in developing countries(2). Chronic gastritis associated with H. pylori infection may evolve into peptic ulcer, MALT lymphoma, or gastric cancer over time. The expression of the virulence factors and the geographical origin of the strains are among the factors most influencing the evolution towards the most severe pathologies (3). The choice of an H. pylori eradication therapy is based primarily on the rate of clarithromycin resistance in the region. In cases with more than 15% resistance, triple therapy based on clarithromycin is not recommended (3).

2. Objective

The aim of this study was to take stock of H. pylori infection in children and to study its antibiotic resistance, the proportion of its major virulence factors, and its phylogeographic typing by MLST method in the strains isolated at a hospital in Algiers, a country with a high prevalence of H. pylori infection.

3. Materials and Methods

This study included patients who were referred to the pediatric department of Ibn Ziri Bologhine hospital (Algiers, Algeria) for a digestive endoscopy from January 2013 to March 2016. An antral biopsy was sampled and placed into a brain heart infusion (BHI) broth (Institut Pasteur d’Algérie, Algiers, Algeria) at 4°C, and accompanied with patient’s information sheet transported to the clinical laboratory laboratory of the Bologhine hospital at the same day.

The biopsy was grounded in 1 mL of BHI then cultured on Colombia agar medium supplemented with 10% of human blood and selective supplement (H. pylori Selective Supplement, Oxoid, England). The cultures were incubated at 37°C in microaerophilic conditions (CampyGen, Oxoid, Basingstoke, UK) for 3 to 10 days. The identification of suspected colonies was based on the specific form of Gram staining and the production of oxidase, catalase, and urease. The identified strains were stored at -80°C in BHI supplemented with 20% glycerol in order to be used in molecular biology tests at the French National Reference Center for Campylobacter and Helicobacter (Bordaux, France). An antibiogram was identified on Mueller-Hinton medium supplemented with 10% human blood by a bacterial suspension of 3 McFarland. Amoxicillin (10µg), tetracycline (30µg), rifampicin (5µg), levofloxacin (5µg), and clarithromycin (15µg) were tested using ATB disks (bioMerieux, Marcy-l’Etoile, France). E-test was used for metronidazole and to confirm resistance to clarithromycin. Critical concentrations were interpreted according to EUCAST guidelines (http://www.eucast.org/). Critical diameters used for interpretation were as follows: clarithromycin: resistant < 17 mm, sensible > 22 mm; tetracycline: resistant < 17 mm, sensible > 19 mm; rifampicin: resistant < 14 mm, sensible > 19 mm, levofloxacin: resistant < 17 mm, sensible > 20 mm; amoxicillin: resistant < 17, sensible > 20 mm.

The DNA extraction was performed with DNA extraction kit (QIAamp DNA mini-kit, Qiagen, France) according to the manufacturer’s instructions.

The molecular identification of H. pylori isolates and the determination of the mutation points of 23S rRNA gene associated with clarithromycin resistance were carried out by real-time PCR method using the fluorescence resonance energy transfer (FRET) principle for the detection of H. pylori and an amplicon fusion curve for the detection of clarithromycin resistance, as previously described (4).
The cagPAI and the vacA allelic status (s, m, i, and d regions) were evaluated by PCR (Table 1). PCR amplifications of the cagPAI empty site was carried out in a 25 µL volume containing 2.5 µL of 10X PCR buffer, 1.5 mM MgCl2, 200 µM (each) of dNTPs, 2 U of Taq DNA polymerase, 1 µM (each) of primers, and 10 ng of H. pylori DNA. After 2 min of denaturation at 95°C, reaction mixture was amplified for 40 cycles as follows: 30 s at 95°C, 30 s of annealing at 58°C; and 30 s at 72°C. After the last cycle, extension was continued for another 5 min at 72°C. PCR amplifications of the vacA allelic status were carried out in a 25 µL volume containing 2.5 µL of 10X PCR buffer, 1.5 mM MgCl2, 400 µM of the dNTPs each, 1.2 U of Taq DNA polymerase, 1.75µM of primers each, and 10 ng of H. pylori DNA. After 2 min of denaturation at 94°C, each reaction mixture was amplified for 40 cycles (35 cycles for i1 and i2) as follows: 30 s at 94°C, 30 s of annealing at 60°C (58°C for i1 and 27°C for i2); and 30 s (45 s for i2) at 72°C. After the last cycle, extension was continued for another 5 min at 72°C.

Phylogeographic typing was performed by MLST. PCR amplification and sequencing of 7 H. pylori housekeeping genes (aptA, efd, trpC, ppa, mutC, yphC and ureI) were performed, as previously described (5). The sequences obtained were aligned and compared to 25 reference strains of the PubMLST database (https://pubmlst.org/helicobacter/). Phylogenetic tree was reconstructed based on the sequences obtained and those available in the PubMLST database, using the Neighbor-Joining algorithm implemented in MEGA 6.0 software.

4. Results
Thirty-one patients included in this study aged from 5 to 16 years (median age was 12 years) with a boy/girl ratio of 0.47. Digestive endoscopy revealed that 26 patients (84%) had gastritis, and 5 cases (16%) had normal gastric mucosa. Nine patients (29%) had already received an eradication treatment against H. pylori.

Culture was H. pylori positive in 12 patients (38.7%). Eight of whom had not received eradication treatment, and four had already been treated against H. pylori.

No resistance was detected to amoxicillin, tetracycline, rifampicin, and levofloxacin by antibiogram. One strain was resistant to metronidazole with MIC >256 µg/mL (primary resistance). A single strain was resistant to clarithromycin with MIC >256 µg/mL (secondary resistance); this strain belonged to a patient identified with gastritis; the antibiogram of this patient revealed a strain sensitive to clarithromycin before the eradication treatment. Real-time PCR performed on 7 of out 12 isolated strains confirmed the identification and strains clarithromycin susceptibility.

The distribution of the virulence factors of 6 tested strains is shown in Table 2.

The phylogeographic typing by MLST, which was performed on 6 strains shows that all the strains were of hpEurope type (Fig.1).

5. Discussion
The prevalence of H. pylori infection in Algeria is high (6-7). H. pylori infection is usually acquired during childhood (1) and highly dependent on socioeconomic conditions (2). The prevalence rate of pediatric H. pylori infection varies considerably from one country to another. Its low in industrialized countries, for example, 15% in Spain (8), 10% in Sweden (9), and 1.8% in Japan (10) and high in developing countries, for example, 30% in Tunisia (11) and 82% in Iran (12). These rates also vary according to the diagnostic techniques used (13). There is no published study investigating the current prevalence rate of H. pylori infection in Algeria. In this study, 38.7% of the children had H. pylori positive culture. As the only H. pylori diagnostic technique available in our hospital, culturing produces very specific results. The identification of the isolates was confirmed by PCR which is not the most sensitive technique due to the fragility of the bacterium. Although culturing makes it possible to obtain the results of antibiotic resistance, it remains as an invasive test requiring a digestive endoscopy which is poorly tolerated by children. Clinicians use these tests only when necessary, indicating the low number of patients.

Antibiotic resistance in this study was low. Few large-scale studies conducted on antibiotic resistance in children are available. An European multi-center pediatric study reported primary and secondary clarithromycin resistance as 20 and 42%, respectively (14). In our case, no primary clarithromycin resistance was detected; there was only one secondary resistance. The use of clarithromycin in the eradication treatment depends on the resistance level in the region (3). The rate of clarithromycin resistance needs to be monitored in pediatric by conducting more studies with more sample size because resistance in adults seems to be increasing in Algeria (7). A single strain was found to be resistant to metronidazole. In contrast to clarithromycin, resistance to metronidazole in vitro has little impact on the efficacy of in vivo eradication therapy (14). Although the strains were susceptible to antibiotics, it was found that 4 children were still infected with H. pylori after eradication treatment. Studies show that in addition to antibiotic resistance, the non-adherence to eradication therapy, common in pediatrics, is an important factor in eradication failure (15).

One of the factors influencing the evolution of the disease in the long term is the presence of certain bacterial virulence factors. Thus, the presence of cagPAI pathogenicity island in the bacterial genome, which expresses the cagA protein, increases the risk of developing duodenal ulcers and gastric carcinomas (16). In contrast to the non-cytotoxic s2m2 genotype, the slm1 genotype of vacA is associated with the most severe pathologies (17). In our study, 4 out of 6 strains possessed the pathogenicity island (cagPAI), which can potentially lead to serious lesions, and 5 out of 6 strains expressed non-cytotoxic vacA, and one strain had a combination of cagPAI and the vacAs1b allele.

Phylogenetic analysis of 6 strains by MLST revealed that they were all of hpEurope type, this finding is not unexpected due to the location of Algeria in North African and the human migrations since the Palaeolithic period, as illustrated by Falush and Moodley (18-19). Population genetic studies based on MLST analysis help predict prehistoric human migration accompanied by H. pylori. Also, the relationships between the phylogeny of housekeeping genes and cagPAI or VacA phylogeny were reported (20-21). The incidence of different gastric cancers can be partly attributed to the different genotypes of H. pylori circulating in different geographical areas (22).

6. Conclusions
This study reported a high prevalence rate of H. pylori infection in Algerian children and H. pylori low resistance to antibiotics. It also reports for the first time in Algeria a genetic typing of H. pylori strains isolated from pediatrics. These results must be supplemented by the results of other studies involving more patients.
Helicobacter pylori in Algerian children

Conflict of Interest
The authors have no competing interests.

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Authors’ Contributions
All of authors contribute to this study.

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Table 1. Primers used for the amplification of cagPAI, and vacA.

<table>
<thead>
<tr>
<th>Gene / Region amplified</th>
<th>Primer designation</th>
<th>Primer sequence (5' to 3')</th>
<th>PCR Product size</th>
<th>References</th>
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<td>F1-468-HP519</td>
<td>GCTTGGTGTATGGCCCTTG</td>
<td>324</td>
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<td></td>
<td>R1-496-HP549</td>
<td>GCAATGCAATTTCCCTAAAGTG</td>
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<td>vacA s1/s2</td>
<td>VA1F</td>
<td>ATGGAAATACAACAAACACCAAC</td>
<td>s1: 259</td>
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<td></td>
<td>VA1R</td>
<td>CTGCTTGAATCGCCAAAAC</td>
<td>s2: 286</td>
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<td>vacAs1/a</td>
<td>Forward</td>
<td>GTCAGCATAACCGCAAGAG</td>
<td>190</td>
<td>(25)</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
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<td></td>
</tr>
<tr>
<td>vacAs1/b</td>
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<td>187</td>
<td>(25)</td>
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<tr>
<td>vacAs1/c</td>
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<td></td>
<td>Reverse</td>
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<td>VAGF</td>
<td>CAACTGTGCCAAATCAACCGAG</td>
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<td>vacAi</td>
<td>VacF1</td>
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<td>vacAd</td>
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<td>VAGFR</td>
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<td>d2: 298</td>
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Table 2. Characteristics of 12 H. pylori strains.

<table>
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<tr>
<th>Patients</th>
<th>Age</th>
<th>Pathology</th>
<th>CLR</th>
<th>MZ</th>
<th>AMX</th>
<th>TE</th>
<th>RA</th>
<th>LVX</th>
<th>cagPAI</th>
<th>vacA</th>
<th>MLST</th>
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<tr>
<td>1</td>
<td>14</td>
<td>Gastritis</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>2</td>
<td>14</td>
<td>Gastritis</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>3</td>
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<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>4</td>
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<td>Gastritis</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
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<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>NT</td>
<td>NT</td>
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<td>S</td>
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<td>NT</td>
<td>NT</td>
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<td>S</td>
<td>S</td>
<td>Pos</td>
<td>s2m2i2d2</td>
<td>hpEurope</td>
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<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>Pos</td>
<td>S1bm2i2d2</td>
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<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>Neg</td>
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<tr>
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<td>S</td>
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<td>Pos</td>
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<td>S</td>
<td>S</td>
<td>Pos</td>
<td>s2m2i2d2</td>
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Figure 1. MLST analysis of 6 Algerian strains of *H. pylori* (ALG) with 25 reference strains. Phylogenetic tree constructed using neighbor-joining-tree with MEGA v6.

References


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