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Original article

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 31antral biopsies were cultured, and then antibiotic susceptibility testing was performed. The statuses of *cag*PAI and *vac*A *s*, *m*, *I*, 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 *cag*PAI, and five out of six strains were identified as *vac*A *s2m2i2d2*. 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, *cag*PAI, *vac*A

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 Zir iBologhine 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 biology 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 clarithoromycin. Critical concentrations were interpreted according to EUCAST guidelines (http://www.eucast.org/). Critical diameters used for interpretation were as follows: clarithromycin: resistant < 17mm, sensible > 22mm; tetracycline: resistant < 17mm, sensible >19mm; rifampicin: resistant < 14mm, sensible > 19mm, levofloxacin: resistant < 17mm, sensible > 20mm; amoxicillin: resistant < 17, sensible >20mm.

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 *cag*PAI and the *vac*A allelic status (*s*, *m*, *i*, and *d* regions) were evaluated by PCR (Table 1). PCR amplifications of the *cag*PAI 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 *vac*A 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 (*atpA, efp, trpC, ppa, mutY, yphC* and *urel*) 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 (medium 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 clarithromycine 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 out of 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. Itis 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 pediatrics 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 *cag*PAI pathogenicity island in the bacterial genome, which expresses the *cag*A protein, increases the risk of developing duodenal ulcers and gastric carcinomas (16). In contrast to the non-cytotoxic *s2m2* genotype, the *s1m1* genotype of *vac*A is associated with the most severe pathologies (17). In our study, 4 out of 6 strains possessed the pathogenicity island (*cag*PAI), which can potentially lead to serious lesions, and 5 out of 6 strains expressed non-cytotoxic *vac*A, and one strain had a combination of *cag*PAI and the *vac*A*s1b* 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 Faluch 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 *cag*PAI 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.

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 *cag*PAI, and *vac*A.

Gene / Region amplified	Primer designation	Primer sequence (5' to 3')	PCR Product size	References
*cag*PAI	F1-468-HP519 R1-496-HP549	GCTTGCTTGTATTGGCCTTG GCATGCACATTCCCTAAAGTG	324	(23)
*vac*A s1/s2	VA1F VA1R	ATGGAAATACAACAAACACAC CTGCTTGAATGCGCCAAAC	s1: 259 s2: 286	(24)
vacAs1a	Forward Reverse	GTCAGCATCACACCGCAAC CTGCTTGAATGCGCCAAAC	190	(25)
vacAs1b	Forward Reverse	AGCGCCATACCGCAAGAG CTGCTTGAATGCGCCAAAC	187	(25)
vacAs1c	Forward Reverse	TTAGTTTCTCTCGCTTTAGTRGGGYT CTGCTTGAATGCGCCAAAC	220	(26)
vacAm1/m2	VAGF VAGR	CAATCTGTCCAATCAAGCGAG GCGTCAAAATAATTCCAAGG	m1: 567 m2: 642	(27)
vacAi1	VacF1 C1R	GTTGGGATTGGGGGAATGCCG TTAATTTAACGCTGTTTGAAG	426	(28)
vacAi2	VacF1 C2R	GTTGGGATTGGGGGAATGCCG GATCAACGCTCTGATTTGA	432	(28)
vacA*d*	VAS5F VAGFR	ACTAATATTGGCACACTGGATTTG CTCGCTTGATTGGACAGATTG	d1: 367 to 379 d2: 298	(29)

Table 2. Characteristics of 12 *H. pylori* strains.

Patients	Age	Pathology	Antibiotics resistance						*cag*PAI	*vac*A	MLST
			CLR	MZ	AMX	TE	RA	LVX			
1	14	Gastritis	S	S	S	S	S	S	NT	NT	NT
2	14	Gastritis	R	S	S	S	S	S	NT	NT	NT
3	14	Gastritis	S	S	S	S	S	S	NT	NT	NT
4	11	Gastritis	S	R	S	S	S	S	NT	NT	NT
5	16	Gastritis	S	S	S	S	S	S	NT	NT	NT
6	13	Gastritis	S	S	S	S	S	S	NT	NT	NT
7	14	Gastritis	S	S	S	S	S	S	Pos	s2m2i2d2	hpEurope
8	10	Gastritis	S	S	S	S	S	S	Pos	S1bm2i2d2	hpEurope
9	13	Gastritis	S	S	S	S	S	S	Neg	s2m2i2d2	hpEurope
10	13	Gastritis	S	S	S	S	S	S	Pos	s2m2i2d2	hpEurope
11	9	Gastritis	S	S	S	S	S	S	Neg	s2m2i2d2	hpEurope
12	10	Gastritis	S	S	S	S	S	S	Pos	s2m2i2d2	NT

CLR: Clarithromycin, MZ: Metronidazole, AMX: Amoxicillin, TE: Tetracycline, RA: rifampicin, LVX :levofloxacin, S: Sensible, R: Resistant, Pos: Positive, Neg : Negative, NT: No tested.

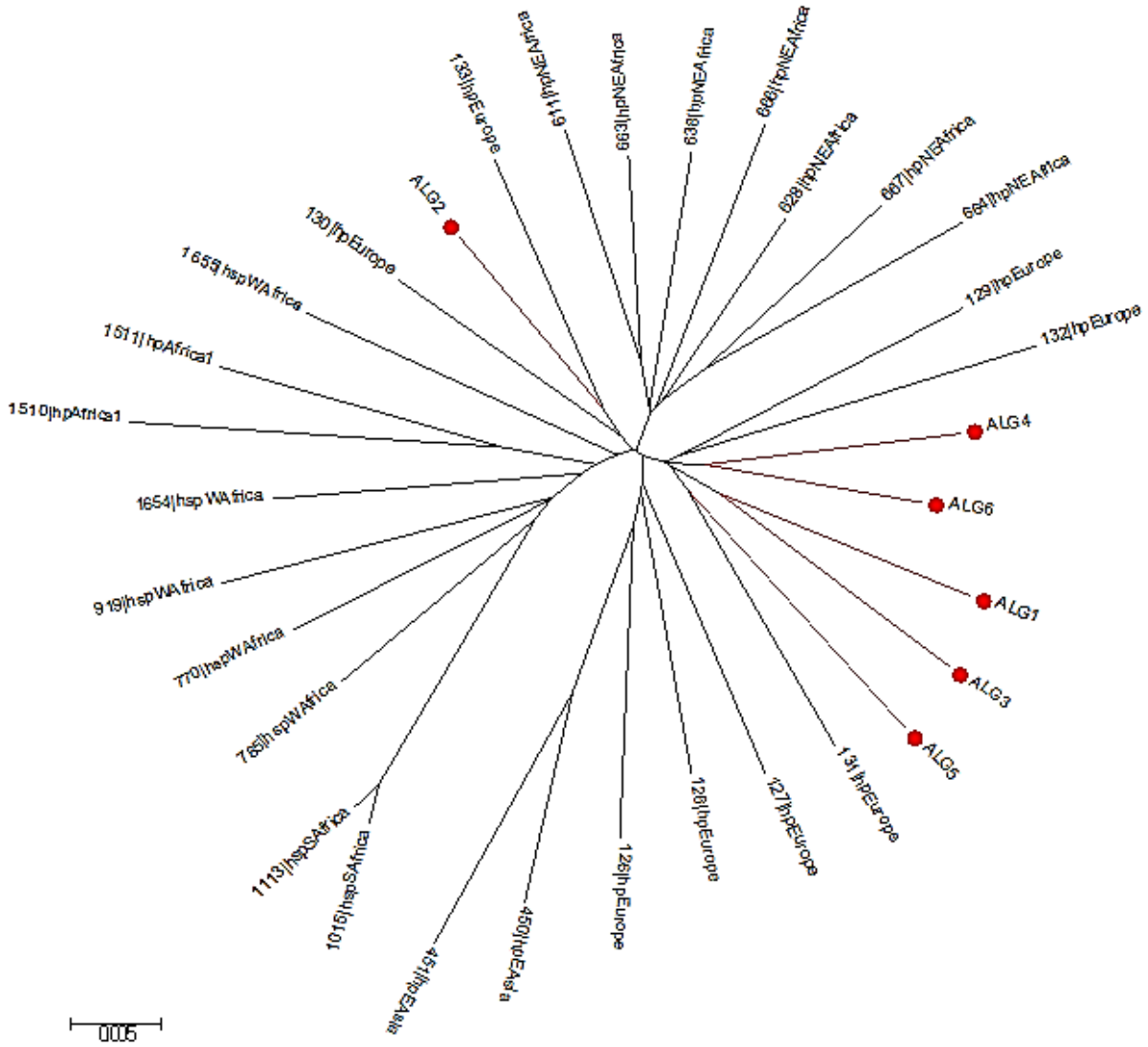

Figure 1. MLST analysis of 6 Algerian strains of *H. pylori* (ALG) with 25 reference strains. Phylogenetic tree constructed using neighbor-joining-tree with MEGA v6.

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