Available online at www.derpharmachemica.com

ISSN 0975-413X
CODEN (USA): PCHHAX

Der Pharma Chemica, 2017, 9(15):42-45
(http://www.derpharmachemica.com/archive.html)

Helicobacter pylori in Children: Molecular Characterization and MLST of Isolated Strains in an Algerian Hospital

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ABTRACT

Introduction: *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 is to take stock of H. pylori infection in Algerian children.*
Materials and Methods: *The culture of 31 biopsies was performed and the sensitivity to antibiotics tested. The statuses of cagPAI and vacA were determined as well as geographical typing by Multilocus Sequence Typing (MLST).*
Results: *Culture was positive in 12 children. One resistance to clarithromycin and one metronidazole resistance were detected. Four out of six strains possessed cagPAI and five out of six strains were vacA s2m2i2d2. The five strains tested by MLST are of the hpEurope type.*
Conclusion: *The study reveals a high infection rate and low resistance to antibiotics and reports for the first time in Algeria a genetic typing of strains isolated in Pediatrics.*

Keywords: *Helicobacter pylori*, Children, Virulence factors, Antibiotics resistance, Algeria

INTRDUCTION

Helicobacter pylori infection is usually acquired in childhood and can remain asymptomatic for years [1]. Chronic gastritis associated with infection may evolve over time in peptic ulcer, Multilocus Sequence Typing (MLST) lymphoma or gastric cancer [2]. The prevalence of infection in children is low in industrialized countries and high in developing countries [3]. The aim of this study is to take stock of *H. pylori* infection in children and study their antibiotic resistance, the proportion of major virulence factors and phylogeographic typing by MLST of strains isolated at a hospital in Algiers, a country with a high prevalence of infection.

MATERIALS AND METHODS

This study included patients who were referred for a digestive endoscopy at the pediatric department of Ibn Ziri Bologhine Hospital (Algiers, Algeria) Between January 2013 and March 2016. An Antral Biopsy is sampled and transported immediately to heart-brain broth (BHI) at the hospital's clinical biology laboratory together with a patient information sheet.

The biopsy is ground 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). The cultures are incubated at 37°C in micro-aerophilic conditions (CampyGen, Oxoid) for 3-10 days. The identification of suspect colonies is based on the specific form of Gram staining and the production of oxidase, catalase and urease. The identified strains are stored at -80°C in BHI supplemented with 20% of glycerol in order to carry out molecular biology tests at the French National Reference Center for Campylobacter and Helicobacter (Bordaux, France). An antibiogram is realized on Muller-Hinton medium supplemented with 10% human blood with a bacterial suspension of 3 McFarland. Sensitivity to amoxicillin, tetracycline, rifampicin, levofloxacin and clarithromycin is tested with the agar diffusion method (ATB disks, Biomerieux), E-test are used for metronidazole and to confirm resistance to clarithromycin. Critical concentrations are interpreted according to The European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines. Critical diameters used for interpretation are: 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.

The extraction of the DNA is performed with DNA extraction kit QIAamp DNAmini-kit (Qiagen, France) according to the manufacturer's instructions. The molecular identification of the *H. pylori* isolates coupled to the determination of the mutation points of the 23S rRNA gene associated with clarithromycin resistance is carried out by real-time Polymerase Chain Reaction (PCR) as previously described [4].

The *cag*PAI and the *vac*A allelic status (s, m, i and d regions) are evaluated by PCR (Table 1). PCR amplifications of the *cag*PAI empty site is carried out in a 25 µl volume containing 2.5 µl of 10X PCR buffer, 1.5 mM $MgCl_2$, 200 µM (each) of the Deoxynucleotides (dNTPs), 2 U of Taq DNA polymerase, 1 µM (each) of the primers and 10 ng of *H. pylori* DNA. After 2 min of denaturation at 95°C, reaction mixture is amplified for 40 cycles as follows: 30 s at 95°C, 30 sec 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 are carried out in a 25 µl volume containing 2.5 µl of 10X PCR buffer, 1.5 mM $MgCl_2$, 400 µM (each) of the dNTPs, 1.2 U of Taq DNA polymerase, 1.75 µM (each) of the primers and 10 ng of *H. pylori* DNA. After 2 min of denaturation at 94°C, each reaction mixture are amplified for 40 cycles (35 cycles for i1 and i2) as follows: 30 sec at 94°C, 30 sec of annealing at 60°C (58°C for i1 and 27°C for i2) and 30 sec (45 sec for i2) at 72°C. After the last cycle, extension was continued for another 5 min at 72°C.

Table 1: Primers used for the amplification of *cag*PAI and *vac*A

Gene/Région amplified	Primer désignation	Primer sequence (5' to 3')	PCR product size
cag PAI [5]	F1-468-HP519 R1-496-HP549	GCTTGCTTGTATTGGCCTTG GCATGCACATTCCCTAAAGTG	324
*vac*A s1/s2 [6]	VA1F VA1R	ATGGAAATACAACAAACACAC CTGCTTGAATGCGCCAAAC	s1: 259 s2: 286
*vac*A *s1a* [7]	Forward Reverse	GTCAGCATCACACCGCAAC CTGCTTGAATGCGCCAAAC	190
*vac*A *s1b* [8]	Forward Reverse	AGCGCCATACCGCAAGAG CTGCTTGAATGCGCCAAAC	187
*vac*A *s1c* [8,9]	Forward Reverse	TTAGTTTCTCTCGCTTTAGTRGGGYT CTGCTTGAATGCGCCAAAC	220
*vac*A *m1/m2* [10]	VAGF VAGR	CAATCTGTCCAATCAAGCGAG GCGTCAAAATAATTCCAAGG	m1: 567 m2: 642
*vac*A *i1* [10]	VacF1 C1R	GTTGGGATTGGGGGAATGCCG TTAATTTAACGCTGTTTGAAG	426
*vac*A *i2* [10]	VacF1 C2R	GTTGGGATTGGGGGAATGCCG GATCAACGCTCTGATTTGA	432
vacA *d* [11]	VAS5F VAGFR	ACTAATATTGGCACACTGGATTTG CTCGCTTGATTGGACAGATTG	d1: 367to 379 d2: 298

Phylogeographic typing was performed by MLST. PCR amplification and sequencing of 7 *H. pylori* housekeeping gene (*atpA, efp, trpC, ppa, mutY, yphC* and *urel*) was performed as previously described [12]. The sequences obtained are aligned and compared to 25 reference strains of the PubMLST database (https://pubmlst.org/helicobacter/). Phylogenetic tree reconstitutions were made based on the sequences obtained and those available in the PubMLST database, using the Neighbor-Joining algorithm implemented in MEGA 6.0 software.

RESULTS

Thirty-one patients were included in this study aged between 5 and 16 years (medium age 12 years) with a boy/girl ratio of 0.47. Digestive endoscopy revealed that 26 patients had gastritis, and 5 had normal gastric mucosa. Nine (29%) patients had received an eradication treatment against *Helicobacter pylori*. Culture was positive in 12 patients (38.7%). Eight of them had not received eradication treatment and four had already been treated for *H. pylori*.

No resistance to amoxicillin, tetracycline, rifampicin and levofloxacin was detected by antibiogram. One strain was resistant to metronidazole with Minimum Inhibitory Concentration (MIC) > 256 µg/ml (primary resistance). A single strain was resistant to clarithromycin with MIC > 256 µg/ml (secondary resistance), this strain belongs to of a patient with gastritis; the antibiogram of this patient before the eradication treatment revealed a strain sensitive to clarithromycin. Real-time PCR performed on 7 of the 12 strains isolated confirmed the identification and susceptibility of strains to clarithromycin. The distribution of the virulence factors of the 6 strains tested is shown in Table 2.

Table 2: Characteristics of 12 *Helicobacter 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; Négative; NT: No tested

The phylogeographic typing by MLST was performed on 6 strains shows that all strains are of hpEurope type (Figure 1).

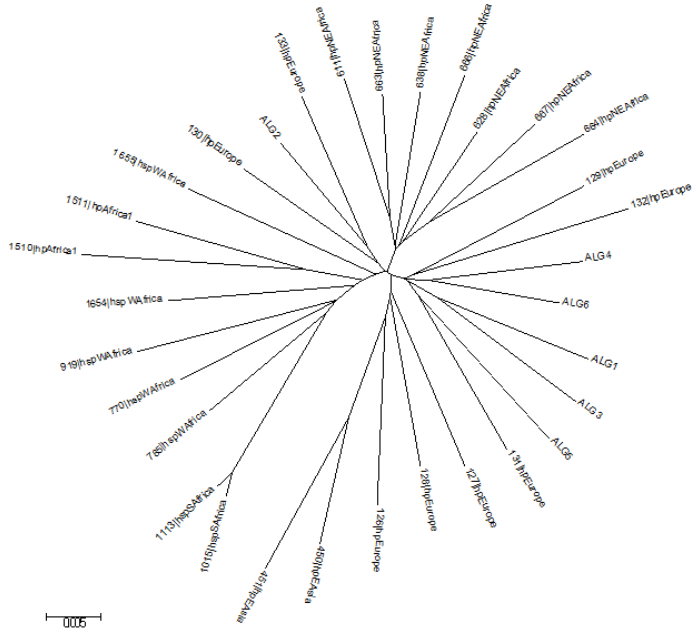

Figure 1: MLST analysis of 6 Algerian strains of *Helicobacter pylori* (ALG) with 25 reference strains.

Phylogenetic tree constructed by using neighbor-joining-tree with MEGA v6.

DISCUSSION

The prevalence of *H. pylori* infection in Algeria is high [13,14]. Infection is usually acquired during childhood [1] and highly dependent on socioeconomic conditions [3]. Pediatric infection rates vary considerably from one country to another. They are low in industrialized countries (15% in Spain [15], 10% in Sweden [16]) and high in developing countries (30% in Tunisia [17], 82% in Iran [18]). These rates also vary according to the diagnostic techniques used [19]. There are no published studies on the current prevalence of *H. pylori* infection in Algeria. In this study 38.7% of children had a positive *H. pylori* culture. This is the only *H. pylori* diagnostic technique available in our hospital. The results of the culture are very specific, the identification of the isolates was confirmed by PCR, but this is not the most sensitive technique due to the fragility of the bacterium. Moreover, although it makes it possible to obtain the results of sensitivity to antibiotics, the culture remains an invasive test requiring a digestive endoscopy poorly tolerated by children. Clinicians use these tests only when necessary, which explains the low number of patients.

Antibiotic resistance in this study is low. Few large-scale studies on antibiotic resistance in children are available. A European multicenter pediatric study reports primary and secondary resistance to clarithromycin of 20% and 42%, respectively [20]. In our case, no primary resistance and only one secondary resistance to clarithromycin was detected. The use of clarithromycin in the eradication treatment depends on the level of resistance in the region [2]. The rate of resistance to clarithromycin is to be monitored in pediatrics with studies including more patients because resistance in adults seems to be increasing in Algeria [14]. A single strain was resistant to metronidazole, but in contrast to clarithromycin, resistance to metronidazole *in vitro* has little impact on the efficacy of *in vivo* eradication therapy [20]. We found that 4 children were still infected with *H. pylori* after eradication treatment although the strains were susceptible to antibiotics. Studies show that in addition to antibiotic resistance, the non-adherence to eradication therapy, common in pediatrics, is an important factor in eradication failure [21].

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 in the bacterial genome of the *cag*PAI pathogenicity island expressing the *cag*A protein increases the risk of developing duodenal ulcers and gastric carcinomas [22]. The *s1m1* genotype of *vac*A is associated with the most severe pathologies in contrast to the non-cytotoxic *s2m2* genotype [23]. In our study, 4 strains out of 6 possess the island of pathogenicity *cag*PAI, which can potentially lead to serious lesions, and 5 out of 6 strains express non-cytotoxic *vac*A, one strain combined *cag*PAI and the *vac*A *s1b* allele.

Phylogenetic analysis by MLST of 6 strains revealed that they are all of hpEurope type which is in agreement with the North African location of Algeria. This distribution is the result of human migrations since the Palaeolithic period illustrated by Faluch and Moodley [24,25].

CONCLUSION

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

ACKNOWLEDGEMENTS

We gratefully acknowledge Professor Francis Mégraud, Lucie Bénéjat and the members of the French National Center for Campylobacter and Helicobacter for their assistance.

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