Application of a Stool Antigen Test To Evaluate the Incidence of *Helicobacter pylori* Infection in Children and Adolescents from Tehran, Iran

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Helicobacter pylori* infection is acquired mainly in childhood, especially in developing countries, where a low-cost, rapid diagnostic technique which is reliable for all age groups may be useful for the management of *H. pylori* infection. For this purpose, we used an HpSA test (Equipar) to detect *H. pylori* infection in children and adolescents from Tehran, Iran. Thirty-five children who were positive or negative for *H. pylori* infection by endoscopy-based tests were used as positive and negative controls for the HpSA test. Stools were collected from 430 randomly selected children and adolescents (4 to 18 years old) from southwest, near the center, and northwest of Tehran. A questionnaire that included presence of recurrent abdominal pain (RAP), family history of infection and/or peptic ulcer disease (PUD), and income of parents was completed. A good agreement was found between the results of endoscopy-based tests and those of the HpSA test; the sensitivity and specificity of the Equipar-HpSA test were 100% and 83.4%, respectively. Among 430 children and adolescents, 47% were positive by the HpSA test, of whom 82% had RAP. No difference in incidence was observed between the two sexes; the various categories of age showed an increasing incidence, ranging from 24% (ages 4 to 6) to 58% (ages 16 to 18). The rate of infection in children and adolescents from the southwest was significantly higher (70%) than the rate in those from the northwest (32%), and a family history of *H. pylori* infection or PUD was observed in 59% of the HpSA positive subjects. The HpSA test is a useful test to detect *H. pylori* infection in children and adolescents from developing countries.

Helicobacter pylori infection is acquired mainly in childhood, especially in developing countries (40), where the influence of socioeconomic factors on the prevalence of *H. pylori* infection has been shown (11, 30, 37). Many investigators have studied the criteria for diagnosis and treatment of children infected by *H. pylori*, but association of symptoms with *H. pylori* infection in children presenting with nonulcer dyspepsia is controversial (3, 14, 22). One important controversy relates to the presence of recurrent abdominal pain (RAP) in children, where an important association was observed between RAP and *H. pylori* infection in some populations (4, 10, 19, 27, 28, 31, 34). Although endoscopy-based tests are the best methods to diagnose active *H. pylori* infection, their application in children is more difficult and unpleasant than in adults. Moreover, in developing regions, for socioeconomic reasons, most infected children are not diagnosed and/or treated for *H. pylori* infection. To circumvent these difficulties, a noninvasive test with reliability for all age groups of children and adolescents is required. Among the noninvasive methods, serological tests cannot be applied to young children because of low sensitivity. In addition, the ^{13}C urea breath test is cumbersome, expensive, and consequently unavailable in certain countries; furthermore, it is not reliable in very young children (13, 16, 19, 25). Therefore, a low-cost, rapid diagnostic technique may be useful for the management of *H. pylori* infection in children and adolescents from developing regions. The *H. pylori* stool anti-gen test has been introduced as a noninvasive, simple, relatively inexpensive, and reliable assay in the diagnosis of *H. pylori* infection of gastritis or peptic ulcer in adults and children (1, 2, 15, 17). Although it is not yet at the level to replace histology as a gold standard, it may be a promising tool in detection of *H. pylori* infection and could be used in the follow-up of adults and children undergoing antibiotic therapy (5, 8, 12, 18, 20, 23, 24, 29, 32, 33, 38, 39, 41).

The purpose of this work was to perform an evaluation of the *H. pylori* infection incidence in children and adolescents from Tehran, Iran, by using an HpSA test (Equipar, Italy).

MATERIALS AND METHODS

Positive and negative controls. Thirty-five patients (aged between 7 and 16 years) who were positive or negative for *H. pylori* infection were enrolled from of a series of children undergoing endoscopy at Tehran Medical Center for Children. A patient was considered an *H. pylori*-positive control if culture alone or histology plus rapid urease test (RUT) were positive. A patient was considered an *H. pylori*-negative control if culture, RUT, and histology were negative. Stool specimens from positive and negative controls were collected and stored at −20°C until use.

Assessment of histology, RUT, and culture. Histological examination of the biopsies was performed according to conventional histopathological methods after hematoxylin-eosin and Giemsa staining (35).

RUT was performed using a urea broth containing (per liter of water) peptone (1 g), dextrose (1 g), NaCl (5 g), K_2HPO_4 (0.4 g), urea (20 g), phenol red (0.01 g), and Tween 80 (0.1 ml). A positive RUT was read either within 2 h in the endoscopy room or after overnight incubation under a microaerophilic atmosphere at 37°C. To test the false positivity due to slow urease activity of microorganisms other than *H. pylori* (as either contaminating or transient microflora of the stomach), an aliquot from the overnight-incubated RUT tubes was cultured on a sheep blood agar plate (36). A negative control without biopsy was also incubated in the same conditions. For cultivation of the organisms, a biopsy

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sample from each patient was plated on Modified Campy-blood agar plates after enrichment in Modified Campy-thio medium as previously described (7).

Screening of subjects. Stool samples were collected from 430 children and adolescents aged from 4 to 18 years old (220 female and 210 male) living in three distinct districts of Tehran, i.e., district 19, district 6, and district 2, situated in the southwest, near to center, and the northwest of the city, respectively. The subjects or their mothers completed a questionnaire that included child's age, sex, presence of RAP, family history of *H. pylori* infection and/or PUD, and mean income level of the parents. To determine the relative socioeconomic status, the subjects were classified as poor, intermediate, or affluent according to the mean income level of the parents. The stool specimens were stored at −20°C until tested

Characteristics of the EIA kit used in this work. The HpSA test (Equipar Diagnostici, Italy) used in this work is based on a sandwich enzyme immunoassay (EIA) and uses microtiter plates coated with polyclonal rabbit anti-*H. pylori* antibodies as the capture antibody and a second antibody to *H. pylori*, labeled with peroxidase. The minimal concentration of *H. pylori* determined by this kit was <0.015 mg/g of stool. The optical densities at 450 nm for negative and positive controls were <0.200 and >1.000, respectively, and the cutoff value was defined as the negative control value + 0.200 (6).

Control assays before screening of stools. As we were the second group to use the Equipar HpSA test (6), we tested the HpSA kit alone with cultivated bacterial cells from the reference strain ATCC 26695. For this purpose, a suspension with turbidity equivalent to that of McFarland no. 5 standard was prepared in extraction buffer from the kit, 100 μl of the suspension was transferred to a well coated with anti-*H. pylori* immunoglobulin G (IgG) of the kit, and the EIA procedure was performed according to the kit manufacturer's instruction for the positive control. The results showed that optical density of the EIA reaction at 450 nm for this strain was in the range of the positive control of the kit (>1). We also tested the antigenic detection capability of the kit alone towards the *H. pylori* strains isolated in our area. For this purpose, 30 strains previously isolated in our area (7) were randomly selected. For all of them, a suspension with turbidity equivalent to that of a McFarland no. 5 standard was prepared and tested as described above.

To evaluate the minimal concentration of bacteria necessary to generate a positive reaction, 1-ml portions of suspensions of strain ATCC 26695 with turbidities equivalent to those of McFarland no. 0.5 to no. 5 standards were inoculated into the tubes containing 0.4 g stool obtained from a negative control (negative controls were considered healthy children). The stools seeded in these suspensions were tested by the HpSA assay, and the initial concentration of bacteria inoculated into the stool was determined by CFU analysis. To test for cross-reaction between the antiserum of the kit and *Campylobacter jejuni* antigens isolated in our area, a suspension with a turbidity equivalent to that of a McFarland no. 5 standard was prepared for two randomly selected *C. jejuni* isolates and tested as described above.

HpSA test. The HpSA test was performed using the same test series of the EIA for all of the samples. First, a small portion of stool (0.1 g) was transferred to a vial containing 500 μl of extraction buffer by using the applicator stick, vortexed for 15 s, and centrifuged for 10 min at 400 to 500 × *g*. One hundred microliters of supernatant was transferred into each well, and EIA was performed according to the manufacturer's recommendations. Positive and negative results were evaluated as recommended by the manufacturer.

RESULTS

Control assays. Our 30 previously isolated strains showed a positive reaction with anti-*H. pylori* IgG of the Equipar kit. *C. jejuni* strains showed no cross-reaction; their optical densities were in the range of the negative control of the kit (<0.200). The approximate minimal concentration of bacteria (for *H. pylori* strain ATCC 26695) required to produce a positive reaction was ~9 ×10^8 bacteria/ml of initial suspension inoculated into the stool (as determined by CFU).

Performance of the HpSA test with controls. Twenty-three out of 35 patients diagnosed at Children's Medical Center of Tehran for *H. pylori* infection were positive by biopsy-based tests, of whom 16 were positive by culture and 17 were positive by RUT and histology (Table 1). Of 35 positive and negative controls, 14 showed a positive RUT at endoscopy (within ~2

h), and 11 displayed a color change in the RUT tube after overnight incubation but showed no growth on a sheep blood agar plate. Also, the negative RUT control without biopsy remained unchanged after overnight incubation. As a result, the positive RUTs shown in Table 1 are the sum of the positives from 2 h (14 cases) and from overnight incubation (11 cases).

Twenty-three patients who were positive by endoscopy-based tests were also positive by the HpSA test; the agreement between the *H. pylori*-positive endoscopy-based tests and the positive HpSA was 92%. Ten patients diagnosed as negative by endoscopy-based tests were also negative by the HpSA test. There were no false negatives for the HpSA test; the agreement between *H. pylori*-negative endoscopy-based tests and negative HpSA was 100%. Two patients who were positive in both RUT and the HpSA but negative in both histology and culture were considered false-positive cases for statistical analysis (Table 1). The sensitivity and specificity of the Equipar HpSA test were 100% and 83.4%, respectively.

Screening results. Of 430 randomly recruited subjects, 47% were positive for *H. pylori* infection (Table 2). An increasing incidence of *H. pylori* infection was observed between different age categories (Table 2), but no significant difference was observed between the two sexes (Table 3). Comparison of the HpSA test results for subjects from the southwest, near the center, and the northwest of Tehran showed a considerable difference in the incidence between the southwest and northwest of Tehran (Table 4). Comparison of the mean income levels of the parents showed that the majority of children and adolescents living in the southwest were in the relatively poor group (lowest income) and that those living in the northwest were in the relatively rich group (highest income). Further-

TABLE 1. Performance of Equipar HpSA test with the control patients ($n = 35$)[a]

No. of patients	Result by:				%
	RUT	Histology	Culture	HpSA	
10[b]	Positive	Positive	Positive	Positive	28.6
6[b]	Positive	Negative	Positive	Positive	17.1
7[b]	Positive	Positive	Negative	Positive	20
2[c]	Positive	Negative	Negative	Positive	5.7
10[d]	Negative	Negative	Negative	Negative	28.6

[a] Sensitivity [number of true positives/(number of true positives + number of false negatives)] = 100%; specificity [number of true negatives/(number of true negatives + number of false positives)] = 83.4%.
[b] True-positive patients.
[c] False-positive patients.
[d] True-negative patients.

TABLE 2. Age distribution of HpSA-positive subjects

Age group (yr)	No. tested	No. (%) positive
4–6	90	22 (24.44)
7–9	125	62 (49.6)
10–12	105	55 (52.38)
13–15	60	34 (56.66)
16–18	50	29 (58)
Total	430	202 (47)

TABLE 3. Sex distribution of HpSA-positive subjects
(total positive = 202)

Gender	No./% positive	Total no. tested
Female	103/46.8	220
Male	99/47.14	210

TABLE 5. Relationship between results of HpSA test, recurrent abdominal pain, and family history among 430 investigated children and adolescents

Result of HpSA (n)	Relationship of HpSA test result (no./%) to:			
	RAP		Family history[a]	
	Positive	Negative	Positive	Negative
Positive (202)	165/82	37/18	120/59	82/41
Negative (228)		228	8	220

[a] Family history of *H. pylori* infection and/or peptic ulcer disease.

more, 82% of the HpSA-positive children and adolescents complained of RAP, and 59% of them had a history of *H. pylori* infection or peptic ulcer diseases in the family (Table 5).

DISCUSSION

Our 30 previously isolated strains showed a positive reaction with anti-*H. pylori* IgG from the Equipar HpSA kit. In addition, no false-positive reaction was observed between two *C. jejuni* isolates from our area and the anti-*H. pylori* IgG of the Equipar HpSA test, which confirmed its good specificity. Twenty-three out of 35 control patients were positive for *H. pylori*, of whom 16 were positive by culture and 17 were positive by both histology and RUT (Table 1). Our protocol for reading the RUT reduced the false-negative results (due to a low density of *H. pylori* inside the biopsy) without an increase in the number of the false-positive cases, since false positivity due to contaminating microorganisms or to overnight incubation of the biopsies was excluded. The concordance of *H. pylori*-positive endoscopy-based tests and positive HpSA, as well as the concordance of *H. pylori*-negative endoscopy-based tests and negative HpSA, was good (Table 1).

The analytical sensitivity of the test for *H. pylori* strain ATCC 26695 as evaluated by CFU analysis was ~9×10^8 bacteria/ml (theoretically equivalent to 0.9 mg). Comparison of this value to the analytical sensitivity of <0.015 mg/g of stool for *H. pylori* strain CCUGI17874, as evaluated by the Equipar research group (6), shows a wide difference. Although this permits us to think that this large difference is related to antigenic diversity between the two strains, it would be a premature conclusion, since the methods of evaluation were different; furthermore, more analysis with larger numbers of *H. pylori* strains is required to determine the analytical sensitivity for an HpSA test. A variety of experiments have been performed with HpSA tests, including Premier Platinum HpSA, Immuno Card STAT HpSA, and FemtoLab *H. pylori* CnX (1, 2, 12, 15, 16, 18, 20, 39–41). However, no uniformity was observed in different populations in regard to the cutoff level required to consider the test positive or negative. This lack of

uniformity may be related to antigenic difference between *H. pylori* strains present in the stools of different populations. Furthermore, the divergence in cutoff levels of various commercial HpSA kits may also be related to antigenic differences between the *H. pylori* strains used for antiserum preparation and those tested.

Screening of children and adolescents showed that 48% were positive for *H. pylori*; however, the rate of infection increased progressively with age and reached a maximum of 58% in adolescence. However, there was no significant difference between the two sexes with respect to *H. pylori* infection. These results were in accordance with the results obtained on *H. pylori* infection in Tehran in our previous work. In that work, among 250 children admitted to Children's Medical Center of Tehran, 50% demonstrated active *H. pylori* infection (as proven by endoscopy-based tests), and the rate of infection increased with age (26).

Analysis of the questionnaires showed an association between RAP and positive HpSA, since 82% of positive cases complained of abdominal pain. A similar association was observed in children from Russia, Greece, Pakistan, Tunisia, and Turkey (4, 10, 19, 27, 28, 31, 34), but no association was observed in other regions (3, 14, 22). These controversies suggest a possible role for either the geographical location of the population or the genetics of strains involved in the infection. To better understand the pathobiology of *H. pylori* infection, prospective studies with larger numbers of children from multiple geographic regions involving analysis of demographic characteristics and the genetics of the *H. pylori* strains are needed (9).

We observed that the poor socioeconomic status of the people living in southwest Tehran, which was characterized by their low income level, was related to higher incidence of infection. Therefore, as was previously reported (11, 22, 30), the socioeconomic level of children and adolescents is a risk factor for *H. pylori* infection. Interestingly, 59% of the HpSA-positive subjects had a history of either *H. pylori* infection or peptic ulcer disease in the family, which may indicate the role of interfamilial transmission in *H. pylori* infection (21).

In conclusion, the noninvasive, low-cost *H. pylori* stool antigen test is a useful method to detect *H. pylori* infection status in children and adolescents from developing regions, especially for the population in which *H. pylori* associated-pathology is prevalent.

TABLE 4. Incidence of *H. pylori* infection (by HpSA) in three different districts of Tehran

District of sampling[a]	No. tested	No. (%) positive
2	170	54 (32)
6	109	43 (39.5)
19	151	105 (70)
Total	430	202 (47)

[a] Districts 2, 6, and 19 are in the northwest, near the center, and in the southwest of Tehran, respectively.

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