

Responses of Endoscopy Patients in Ladakh, India, to *Helicobacter pylori* Whole-Cell and CagA Antigens

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Although *Helicobacter pylori* is a cosmopolitan colonizer of the human stomach, the responses among persons in remote populations from whom *H. pylori* was cultured have not been studied. We report on studies of 189 persons in the Ladakh region of India in whom serum immunoglobulin G responses to *H. pylori* whole-cell and CagA antigens were measured. *H. pylori* was isolated from 68 of these patients. An *H. pylori* whole-cell antigen derived from Ladakhi strains outperformed a similar antigen from U.S. strains, as determined by antigen-specific enzyme-linked immunosorbent assays. In total, 95% of the population was seropositive, including individuals responding only to the CagA antigen. Correlation with culture results showed that these were true positives and, therefore, that the *H. pylori* whole-cell serology was falsely negative in some cases. In addition to establishing a collection of *H. pylori* isolates from a remote area in the world, we show that use of *H. pylori* whole-cell and CagA serology together increases the sensitivity for the detection of colonization.

Although humans in all parts of the world may be colonized with *Helicobacter pylori* (2, 11), most detailed studies correlating serology with bacterial culture have been conducted in industrialized countries or in urban centers in developing countries (5, 23). In part, this pattern stems from the relative difficulty of acquiring proper specimens from indigenous peoples in remote areas, where much of the infrastructure that permits clinical investigation and subsequent biomedical research is lacking (4).

Ladakh, India, is a remote trans-Himalayan region that is sparsely populated, owing to its high elevation ($\geq 3,500$ m) and lack of rainfall. Its population of about 220,000 consists of individuals of Tibetan-Buddhist ethnicity (about 70%) and of Muslims originating in the Baltistan area of Pakistan and other areas of India (about 30%). The majority of the Muslim subjects (Argons) are the descendants of Kashmiri Muslim fathers and Ladakhi Buddhist mothers. Anecdotally, residents of Leh, the capitol of Ladakh, have a high incidence of upper gastrointestinal (GI) tract complaints, and before the advent of antacids and histamine receptor-blocking agents, they were believed to have had a high incidence of peptic ulcers and upper GI tract bleeding. Given that most individuals in such an environment would be expected to carry *H. pylori* (11), we conducted field studies of *H. pylori* carriage and serology in Ladakh.

In an initial serological survey, performed during the summer of 1995, approximately 380 randomly selected individuals were screened for *H. pylori* antibodies by using a serological field test kit (Quidel, Inc., San Diego, Calif.). Included were

residents of small rural communities and of Leh, including Tibetan orphans. The results of this survey were that about 95% of Ladakhis were *H. pylori* seropositive whether or not they had any potentially relevant symptoms referable to the upper GI tract (S. Wall, N. Shah, and R. P. Novick, unpublished data).

On the basis of the results presented above, we now report on a second study of *H. pylori* in residents of Ladakh. This study of Sonam Norbu Memorial Hospital clinic patients with symptoms referable to the upper GI tract included endoscopy with biopsy to ascertain histopathological status and to culture the organism. The study was based on the assumption that any distinctive epidemiological findings with respect to *H. pylori* carriage were likely to be generalizable to the population as a whole, given that the Ladakhi lifestyle is conducive to the widespread dissemination of enteric organisms.

An important focus in this study is the *cag* island, which is associated with the virulence of *H. pylori* (6, 8, 33). Although serological recognition of the *cag*-encoded CagA antigen is a generally reliable indicator of the presence of *cag*-positive organisms, some individuals who respond to this antigen do not respond to preparations of whole-cell *H. pylori* antigens (3, 21, 32). It is thus unclear whether such individuals are actually carrying *H. pylori*. A meaningful interpretation of such serological data therefore requires that the correlation between anti-CagA antibodies, other serology, and the presence of viable organisms be established. We sought to ascertain the frequency of *H. pylori* carriage in Ladakh by isolating strains from biopsy specimens obtained during endoscopy and to determine whether (noninvasive) serological testing could be as reliable as the (invasive) endoscopic method for determination of *H. pylori* status.

In the course of this study, we have obtained direct evidence that persons with serological responses to CagA but not to

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whole-cell *H. pylori* antigens are indeed carrying *H. pylori* in their stomachs and that *H. pylori* antigens prepared from local strains are more sensitive for indicating seropositivity than those prepared from remote (U.S.) isolates.

MATERIALS AND METHODS

Patients studied. As part of an overall evaluation of their health status, 189 patients in Leh underwent upper GI tract endoscopy because of gastrointestinal symptoms. There were 117 women and 72 men, and the mean age was 38 ± 12 years (age range, 12 to 75 years). Endoscopes were decontaminated between patients by soaking in 70% ethanol for at least 10 min and then rinsing in sterile water. Sera were obtained from all 189 patients, and endoscopy with gastric biopsy was performed on 102 patients. Among these 102 patients, 1 patient had a duodenal ulcer and 1 patient had a gastric ulcer, whereas among the other 100 patients there were no specific endoscopic findings. The individual clinic patients were undergoing routine endoscopy for diagnosis of dyspepsia. Therefore, they were not participants in any formal clinical study, nor were they invited to serve as subjects. Under the circumstances, we considered it unnecessary and inappropriate to ask for informed consent. Moreover, there will be no means of identifying any of the individual patients in this or future publications. For comparative purposes, sera from 50 U.S. patients from Nashville, Tenn., were selected; each of these patients was a participant in a long-standing study to evaluate the relationship between *H. pylori* strain type and clinical and histological findings, as described previously (1, 26, 32). The *H. pylori* status of the Nashville patients was determined by attempts to culture the organism from gastric biopsy specimens, by histological examination, and by rapid urease testing (1, 26, 32). For those who carried *H. pylori*, the strain was examined by a *cagA*-specific PCR as described previously (25). We selected sera from 20 persons not carrying *H. pylori* and from 30 *H. pylori*-positive persons, with 15 persons in the latter group carrying *cagA*-positive strains and 15 carrying *cagA*-negative strains.

***H. pylori* culture.** Gastric biopsy specimens from the 102 patients from Leh undergoing upper gastrointestinal endoscopy were collected in sterile microcentrifuge tubes. They were maintained on dry ice during the remainder of the study period (≤ 2 weeks) and were then transported to the United States on dry ice, with more dry ice added during shipment by air to Nashville. Upon receipt in the reference laboratory in Nashville, the biopsy specimens remained frozen and were then stored at -70°C until thawed for culture. Culture was performed with selective media, as described elsewhere (1), and the isolates were characterized by colony morphology and catalase and urease status and were confirmed to be *H. pylori* by specific PCR, as described previously (25).

Western blotting. The antigens present in the *H. pylori* strains from the Ladakhi patients were assessed by Western blotting. After the bacterial cells of four isolates were sonicated and pooled, the lysates were solubilized in sodium dodecyl sulfate-sample buffer and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (26). The proteins were electrophoretically transferred to nitrocellulose and then incubated with individual Ladakhi patient serum samples diluted 1:100, as described previously (27).

Serological methods. The serologies of individual patients were determined by antigen-specific enzyme-linked immunosorbent assays (ELISAs). To assess the serum immunoglobulin G responses to *H. pylori* whole-cell antigens, an ELISA based on an extensively characterized pool of sonicates from five U.S. strains was used (8, 24, 26, 30). By using patient serum diluted 1:800, an optical density ratio ≥ 1.0 was defined as indicating seropositivity, as described elsewhere (24). A parallel antigen was prepared by using sonicates from four *H. pylori* strains (strains 98-65, 98-518, 97-690, and 97-693) isolated from Ladakhi patients, and the ELISA was performed by methods identical to those described previously (12, 16). To assess whether a patient was carrying a *cagA*-positive strain, an ELISA for detection of specific serum immunoglobulin G antibodies was performed by using as the antigen a 66-kDa CagA fragment that had been cloned in *Escherichia coli* as pORV220 (3). For the CagA ELISA, sera diluted 1:100 were considered positive when the optical density was ≥ 0.35 (3). Our previous studies showed that persons all over the world carrying *cagA*-positive strains recognize the conserved CagA antigen (16, 29).

Statistical methods. Chi-square analysis was used for comparisons of proportions between groups. For comparisons of distributions of values, Student's *t* test was used (two tailed). In each case, a *P* value < 0.05 was defined as significant.

RESULTS

Characterization of *H. pylori* antigen from Ladakhi strains.

TABLE 1. Evaluation of *H. pylori* antigen from Ladakhi or U.S. strains in 50 U.S. patients of known *H. pylori* status

<i>H. pylori</i> status of patient ^a	No. of patients studied	No. of patients positive		
		U.S. antigen		Ladakh <i>H. pylori</i> antigen ^c
		CagA ^b	<i>H. pylori</i> ^c	
<i>H. pylori</i> negative	20	0	0	1
<i>H. pylori</i> positive, Cag positive	15	15	15	15
<i>H. pylori</i> positive, Cag negative	15	0	15	14

^a As determined by culture of gastric biopsy specimens and *cagA* PCR (9,11,13).

^b The CagA antigen is pORV220, a recombinant 66-kDa CagA fragment expressed by *E. coli* cells (3).

^c The U.S. antigen is from a pool of five isolates from U.S. patients (14,19), and the Ladakh antigen is from a pool of four isolates from Ladakhi patients, as described in Materials and Methods.

The antigens present in the Ladakh *H. pylori* antigen were assessed by immunoblotting with sera from both Ladakhi ($n = 11$) and U.S. ($n = 2$) patients. For both preparations, several major antigens migrated at approximately 50 to 60 kDa, and for the Ladakh antigen the 120-kDa band that corresponds to the CagA antigen was especially prominent (data not shown). The sera from the Ladakhi and U.S. patients recognized the same bands in both preparations. Since these findings confirmed our general expectations (16, 27, 28), we next examined the utility of the Ladakh antigen in an ELISA format for the screening of human serological responses to *H. pylori*.

Characterization of *H. pylori* antigen from Ladakhi strains with sera from U.S. patients of known *H. pylori* status. To assess whether the *H. pylori* antigen from Ladakhi strains can be used to detect *H. pylori* seropositivity, we first examined its utility among 50 U.S. patients of known *H. pylori* status. Twenty of these patients were known to be *H. pylori* negative and 30 were *H. pylori* positive, with half of the *H. pylori*-positive individuals carrying *cagA*-positive strains (Table 1). By using the recombinant CagA antigen, only the 15 patients carrying a CagA-positive strain were positive, as expected. Similarly, all 20 *H. pylori*-negative patients were negative by the *H. pylori* assay with the U.S. antigen, while all 30 *H. pylori*-positive patients were positive. The antigen prepared from Ladakhi strains performed nearly as well, with one false-positive result (1 of 20 patients; specificity, 95%) and one false-negative result (1 of 30 patients; sensitivity, 97%) (Table 1). With these results for patients of defined *H. pylori* status, we could then examine serological responses in Ladakhi patients of unknown serological status.

Analysis of *H. pylori* serological status of Ladakhi patients. Of the 102 Ladakhi patients who underwent endoscopy, structural upper GI lesions such as ulceration were found in only 2 (2%); no cancers or lesions were seen. Among the total of 189 patients from whom serum was obtained, 127 (67.2%) were seropositive by assays with both the U.S. and the Ladakh antigens, whereas 22 (11.6%) were negative by both assays. Among the 40 (21.2%) persons for whom the results obtained with the U.S. and Ladakh antigens were discordant, the distribution was highly skewed; 37 (92.5%) were positive with the Ladakh antigen and negative with the U.S. antigen, and only 3

TABLE 2. *H. pylori* serological status of 189 persons from Ladakh in relation to antigens used for serological testing

Serological status		U.S. <i>H. pylori</i> antigen ^b		Ladakh <i>H. pylori</i> antigen ^b	
<i>H. pylori</i> antigen	CagA ^a	No. of patients	% of patients	No. of patients	% of patients
+	+	119	63	150	79
+	-	11	6	14	7
-	+	48	26	17	9
-	-	11	5	8	4

^a The CagA antigen is a recombinant 66-kDa fragment expressed in *E. coli* (3).
^b The U.S. antigen is from a pool of five isolates from U.S. patients (25,26), and the Ladakh antigen is from a pool of four isolates from Ladakhi patients, as described in Materials and Methods.

showed the opposite results ($P < 0.001$). Thus, in this population, the antigen derived from local strains increased the assay sensitivity by 29.1% over that obtained in the assay with the U.S. antigen alone, results suggesting the presence of a specific local antigen.

Combined use of the *H. pylori* and the Cag antigens indicated that nearly all (95 to 96%) persons in the Ladakhi population tested were *H. pylori* seropositive (Table 2). When the U.S. *H. pylori* antigen was used, 48 persons were *H. pylori* negative but CagA positive; however, use of the Ladakh *H. pylori* antigen reduced this group to 17 persons, with a concomitant increase in the number of persons who were both *H. pylori* positive and CagA positive. These results suggested that the apparent false-positive CagA result in relation to the result obtained with the U.S. *H. pylori* antigen was actually a false-negative *H. pylori* result for these 31 patients. In total, 167 (88.4%) of the 189 persons were CagA seropositive. Of 180 persons whose ethnicities were determined, 129 were Buddhist and 51 were Muslim; positivity rates were nearly identical for the two groups.

Comparison of serological and culture results. *H. pylori* cultures were attempted with the 102 Ladakhi patient biopsy specimens that had been transported from Ladakh to the United States. Considering the difficulties with transport from Leh, *H. pylori* was isolated from a surprising number of patients ($n = 68$; 66.7%). Each of these isolates was *cagA* positive by PCR. In a subanalysis of individual *H. pylori* colonies picked from the primary culture plate from 11 patients, all 206 isolates were *cagA* positive by PCR. Among the 68 culture-positive persons, 63 (92.6%) were CagA seropositive, 61 (89.7%) were seropositive by the assay with the Ladakh *H. pylori* whole-cell antigen, and 47 (69.1%) were seropositive by the assay with the U.S. whole-cell antigen. By use of the combination of the serological assay results obtained with the CagA antigen and the Ladakh *H. pylori* antigen, 66 (97.1%) of the 68 culture-positive persons were found to seropositive; use of the U.S. *H. pylori* antigen did not add any sensitivity (Table 3). Interestingly, five culture-positive persons did not respond to the CagA antigen, and two others did not respond to any antigen. The five patients with negative CagA serology did not differ substantially in demographic characteristics from the other patients, but the values of the *H. pylori* serological assay were slightly lower for these patients. Of the 34 persons from whom *H. pylori* could not be isolated, contamination of samples was

TABLE 3. Serology of 102 Ladakhi patients of known *H. pylori* culture status

Serological status			No. of patients by <i>H. pylori</i> culture status	
<i>H. pylori</i> antigen	CagA antigen		Culture positive ($n = 68$)	Culture negative ^a ($n = 34$)
Ladakh	U.S.			
+	+	+	45	17
+	+	-	2	3
+	-	+	13	5
+	-	-	1	2
-	+	+	0	1
-	+	-	0	0
-	-	+	5	3
-	-	-	2	3

^a May be falsely negative due to specimen contamination.

responsible or contributed to the result in nearly each instance. In total, 31 (91.1%) of these 34 persons also were seropositive, suggesting that their cultures were probably unsatisfactory. The distributions of positive serological assay results in the culture-positive and culture-negative groups were similar (Table 3).

Comparison of *H. pylori* and CagA serological assay results for persons of defined *H. pylori* status. The 189 persons studied can be divided into the 68 individuals who are known to be culture positive (Table 3) and the 121 individuals from whom tissue samples for culture were not obtained or whose tissue samples were unsatisfactory. On the basis of the results obtained with the U.S. *H. pylori* antigen, 27% of the culture-positive patients were CagA seropositive alone, and the proportion for the group whose culture status was unknown was similar (Table 3). Thus, the false-positive results for CagA serology (Table 2) among persons with negative *H. pylori* serology in fact represents true-positive results, as shown for the culture-positive persons. This same phenomenon was observed, although to a lesser extent, when the Ladakh *H. pylori* antigen was used.

***H. pylori* serological responses by age for Ladakhi patients.** Among the 189 patients, 88.4% were CagA seropositive, with similar prevalences in all age groups (data not shown). Although substantially fewer numbers of individuals were *H. pylori* seropositive based on use of the U.S. antigen, there also was no age-related trend (Table 4). Similarly, by use of the Ladakh

TABLE 4. Antibody levels in Ladakhi patients known to be seropositive, by age

Age group (yr)	Total no. of patients ($n = 189$)	Mean \pm SD optical density		
		CagA ($n = 167$) ^{a,b}	<i>H. pylori</i> antigen	
			U.S. ($n = 130$)	Ladakh ($n = 164$)
<30	43	0.76 \pm 0.26	1.61 \pm 0.41	1.92 \pm 0.48
30-39	55	0.68 \pm 0.19	1.59 \pm 0.34	1.85 \pm 0.47
40-49	56	0.67 \pm 0.17	1.67 \pm 0.50	1.90 \pm 0.51
≥ 50	35	0.60 \pm 0.15	1.55 \pm 0.42	1.79 \pm 0.56

^a n , number of seropositive patients who constitute the group examined for each assay.
^b A significant ($P = 0.003$) age-related decrease in optical density was observed.

antigen in conjunction with the CagA antigen, nearly (95.8%) everyone was seropositive. Among persons meeting the seropositivity criteria with either the U.S. or the Ladakh *H. pylori* antigen, no age-related trend in the level of antibody was observed (Table 4). However, among the CagA-seropositive individuals, a significant ($P = 0.003$) age-related decrease in the level of anti-CagA-specific antibodies detected at the screening dilution was observed (Table 4).

DISCUSSION

One important aspect of this study is that we were able to obtain *H. pylori* isolates from a population in a remote part of the world. Despite the possible loss of culture positivity due to prolonged transport, we were able to isolate *H. pylori* from the majority of specimens studied. Thus, rapid freezing of specimens on dry ice, with frequent replenishment of dry ice from a portable dry ice generator, may be useful to other investigators conducting field studies of the epidemiology and pathophysiology of *H. pylori* in remote areas. Contamination of plates may reflect the presence of atrophic gastritis in the population, with achlorhydria and overgrowth of oral and intestinal bacteria. Although we have no direct evidence for this hypothesis, atrophic gastritis may be common in adult populations in developing countries (12, 35). As a result of these studies, we now have a unique frozen collection of *H. pylori* isolates from Oriental (Buddhist) and Indo-European (Muslim) populations of Tibetan origin who are living in close proximity to one another. Although the primary purposes of this study were to ascertain the presence of *H. pylori* in the populations and the utility of serological screening, the preserved isolates are an excellent resource for future bacteriological studies.

The isolation of *H. pylori* strains enabled us to produce a local *H. pylori* antigen for serological studies. Although with sera from a U.S. population the U.S. (local) and Ladakh (foreign) antigens performed essentially equally, with sera from the Ladakhi population, the local antigen was clearly superior (Tables 2 and 3). In previous studies evaluating 132 Chinese subjects in whom *H. pylori* was visible in gastric biopsy specimens, the local (Chinese) and the U.S. antigens performed essentially equally (13, 16).

By PCR, we found that all *H. pylori* isolates from each patient studied was *cagA* positive. High rates of *cagA* positivity have been reported in other studies of Asian patients (19, 23). That our assessment of 206 individual isolates from 11 patients showed that each was *cagA* positive indicates that mixed *H. pylori* populations with respect to *cagA* status, as reported elsewhere (14, 17, 34), are uncommon at best in this locale. However, although most (92.6%) persons known to have *cagA*-positive strains developed a significant antibody response to the recombinant CagA antigen, a small percentage did not. That this percentage (7.4%) was nearly identical to that in our earlier study (7.5%) of U.S. patients known to be carrying *cagA*-positive strains (3) illustrates the difficulty in establishing a threshold that is both sufficiently sensitive and specific. Nevertheless, the finding that CagA serology showed nearly identical sensitivity (3) to the "gold standards" of culture and PCR for the two distinct populations studied confirms the cosmopolitan value of the antigen used. As with other single (or pooled) *H. pylori* antigens (10, 31), in every population of

culture-positive persons there are individuals who do not mount a sufficient serological response to be considered positive.

However, as shown in this study, the value of serology with the *H. pylori* whole-cell antigen and CagA antigen is clearly additive (Tables 2 and 3). When the results of assays with the whole-cell and CagA antigens are combined, use of the assay with the CagA antigen complemented use of the assay with the U.S. antigen as well as use of the assay with the Ladakh antigen. Because the U.S. antigen was less sensitive than the local antigen for the Ladakhi population, addition of the CagA antigen brought the total level of seropositivity to about the same level as that obtained by the assay with the Ladakh whole-cell antigen. In addition, there were clearly persons who were *H. pylori* culture positive who responded only to the *cagA* antigen and not to either the U.S. (26%) or the Ladakh (7%) whole-cell antigen. Thus, these data indicate that use of both the whole-cell and CagA antigens is a more accurate way to diagnose *H. pylori* infection than use of either antigen alone. Because of the difficulties with culture of the specimens that had been transported from Leh, we were unable to determine the specificity of the serological methods used. However, in other populations, the specificities of both the *H. pylori* (28) and the CagA (3) antigens exceeded 90%.

That nearly all (95.8%) patients were *H. pylori* positive (and carried *cagA*-positive strains) is typical for a preindustrial society (5, 15, 23). Our collection of strains, with multiple isolates from the same individuals, should be particularly useful in evaluating host adaptations since the isolates originated in this nonacculturated population.

The age-related decrease in the level of CagA antibodies is of interest and should be confirmed, especially in studies in which serum is titrated, since the assays that we conducted were done with a single dilution. If confirmed, one explanation for this phenomenon is that the antibody level reflects the intensity of the interaction between the population of *H. pylori* cells and the host mucosa. Since *cagA*-positive *H. pylori* cells inject the CagA protein into host epithelial cells (7, 22, 30), a diminished interaction might reflect a diminishing density of *H. pylori* cells with age, as has been postulated secondary to the development of atrophic gastritis (18), or selection for *H. pylori* cells without a complete functioning type IV secretion system (22).

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