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Helicobacter pylori FliD protein is a highly sensitive and specific marker for serologic diagnosis of *H. pylori* infection

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ABSTRACT

Screening for *H. pylori* in large populations continues to be a challenging task, since available tests have limited sensitivity and specificity, which, in population-based approaches, leads to significant numbers of false positive and false negative results. Various *H. pylori* proteins associated with virulence are highly immunogenic and therefore candidates to detect the infection. There are currently no defined markers that are recognized in all *H. pylori* infected patients and that do not show cross-reactivity with other bacterial proteins.

We identified the *H. pylori* "hook-associated protein 2 homologue", FliD (UniProtKB/Swiss-Prot: P96786.4) as a novel marker of infection for serological analysis. The *H. pylori* FliD protein is an essential element in the assembly of the functional flagella. However, this virulence factor has not yet been tested as a diagnostic marker in serology. For this purpose FliD was recombinantly expressed in *E. coli*, purified by affinity chromatography and gel filtration and used to coat ELISA plates or immobilized on nitrocellulose stripes. To evaluate its antigenicity we screened a defined panel of patient sera. The recombinant *H. pylori* FliD protein reacted with a high percentage of human sera. Among 318 samples reported positive by histology, 310 (97.4%) were tested positive by FliD Line assay, and 165 out of 170 samples were tested positive by ELISA (97%). We could also reconfirm 297 out of 300 (99%) negative sera by Line assay and 73 from 76 (96%) by ELISA. Taken together, application of FliD in serological diagnosis of *H. pylori* infection presents a high specificity of up to 99% and a sensitivity of up to 97%. This makes especially the FliD ELISA a simple, cost effective and highly efficient tool to detect *H. pylori* infection in developing countries where prevalence is high and other screening methods are either not available or are unaffordable.

Introduction

Helicobacter pylori (*H. pylori*), a microaerophilic, Gram-negative and spiral bacterium is colonizing approximately half of the world population and considered to be a human-specific gastric pathogen (Michetti et al., 1999). Most infected individuals show asymptomatic chronic gastritis. However, in some subjects the infection causes chronic active gastritis, peptic ulceration and atrophy, and plays an important role in the development of mucosa-associated lymphoid tissue (MALT) lymphoma, gastric adenocarcinoma and primary gastric non-Hodgkin's lymphoma (Suganuma et al., 2001).

The World Health Organization has categorized *H. pylori* as a class I carcinogen (Goto et al., 1999), and direct evidence of carcinogenesis has been demonstrated in animal models (Honda et al., 1998; Watanabe et al., 1998). Eradication of *H. pylori* can prevent gastric cancer in humans (Uemura et al., 2001). Test and treat strategies have been considered in populations with high gastric cancer risk (Yamaoka et al., 1998). However, such approach is hampered by the lack of efficient and affordable screening systems especially for countries of lower socioeconomic status. In these countries only

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serologic tests are applicable, most of which suffer from poor performance or are not well validated.

For *H. pylori* serology there are several specific single markers known and described. These factors have been applied in many diagnostic approaches, but almost all of them have significant limitations which make them unsuitable for *H. pylori* diagnosis. For instance, the cytotoxin-associated protein (CagA) is a very well characterized *H. pylori* protein. It is encoded on the cag-PAI (cytotoxin associated gene Pathogenicity Island) and is described as an oncogenic protein (Franco et al., 2005; Murata-Kamiya et al., 2007). This protein is also a highly immunogenic antigen, making it a frequently employed marker for serologic tests. CagA positivity can be used as an indicator of *H. pylori* virulence because individuals infected with CagA positive strains are at a higher risk for developing gastroduodenal diseases. However, it is not suitable as a single marker, since only a subgroup of *H. pylori* strains are CagA positive. Moreover, CagA positivity is not a hallmark of active infection as *H. pylori* eradicated patients maintain antibodies against CagA for many years (Fusconi et al., 1999). Therefore it should always be combined with other suitable antigens in serologic tests to confirm positivity.

Another well-characterized *H. pylori* protein is the vacuolating cytotoxin (VacA). It was reported to induce vacuolation in cells exposed to *H. pylori* supernatants or purified protein (Cover and Blaser, 1992). The vacA gene codes for a 140 kDa pro-toxin, where the amino-terminal signal sequence and the carboxy-terminal fragment are proteolytically cleaved during secretion, leading to an active protein with a molecular mass of 88 kDa that aggregates to hexamers and forms a pore (Montecucco and de Bernard, 2003). This protein consists of two different regions. A signal sequence (s1a, s1b, s2) and a mid-region (m1, m2), both with high allelic variations which appear to regulate cytotoxic activity (Atherton et al., 1995). The high diversity of VacA makes this protein unsuitable for serologic testing.

Another well characterized *H. pylori* protein, GroEL, belongs to the family of molecular chaperones, which are required for the proper folding of many proteins under stress conditions (Dunn et al., 1992). In different studies it was shown that this protein is highly conserved among different *H. pylori* strains and that its seropositivity was even higher than for the CagA in infected patients (Macchia et al., 1993; Suerbaum et al., 1994). Also, in our own studies we observed that a positive serostatus for GroEL was more often found in German gastric cancer patients compared to matched controls (unpublished data). Also, it is suggested that antibodies against GroEL might persist longer after disease-related loss of *H. pylori* infection. Thus, GroEL may be a suitable marker of either current or past infection, and may be helpful to overcome the underestimation of *H. pylori* – related gastric cancer risk due to clearance of infection (Gao et al., 2009; Haas et al., 2002; Krah et al., 2004).

In the present study, we employed the secreted *H. pylori* protein FliD as a novel marker of infection for serological analysis. The *H. pylori* FliD protein is an essential element in the assembly of the functional flagella and a *FliD* mutant strain is completely non-motile. Flagellin plays a central role in bacterial motility and is necessary for colonization and persistence of *H. pylori* infection (Eaton et al., 1996). Motility of *H. pylori* is a virulent factor in the pathogenesis of gastric mucosal injury (Watanabe et al., 1997). The *H. pylori FliD* gene encodes a 76-kDa protein (Kim et al., 1999).The *FliD* operon of *H. pylori* consists of *FlaG*, *FliD*, and *FliS* genes, in the order stated, under the control of a Sigma (28)-dependent promoter. In order to evaluate the applicability and suitability of this protein in diagnostic assays, we first analyzed its sequence by bioinformatics tools to assess conservation between Helicobacter species similarity to proteins of other bacteria. We then employed a recombinant FliD protein for the detection of specific antibodies in sera from *H. pylori* infected patients in a novel ELISA assay. Subsequently, the recombinant protein was applied for further development of a line immunoassay. The results of this study may contribute to the development of a simple, accurate and sensitive method for diagnosis of the *H. pylori* infection.

Materials and methods

Samples

A total of 618 patients (307 men, 309 women) were enrolled in the study. After receiving an explanation of the purpose of the study, informed consent was obtained from each patient and a blood sample was taken at the time of endoscopy, before any therapy was initiated. Sera were separated and stored at −20 °C. Diagnosis of infection was based on the histopathology as gold standard. Patients were considered *H. pylori* positive when the results of histopathology were positive. All patients were screened by FliD Line assay, and a subset of 246 sera was tested by FliD ELISA.

Cloning of the H. pylori FliD gene

All DNA manipulations were performed under standard conditions as described by Sambrook et al. (1989). Briefly, the *FliD* gene was amplified by PCR using genomic DNA from *H. pylori* strain J99 as the template. Following oligonucleotides were used as primers: 5′-CAT ATG GCA ATA GGT TCA TTA A-3′ and 5′-CTC GAG ATT CTT TTT AGC CGC TGC-3′. Using this approach a NdeI site was introduced at the 5′-end of forward primers and a XhoI site at 5′-end of the reverse primers. After PCR amplification, the product (2058 bp) was ligated into the pTZ57R/T cloning vector (InsTAclone™ PCR Cloning Kit, MBI Fermentas, Lithuania). Subsequently, the insert was confirmed via PCR and sequencing, and was cloned into a PET-28a(+) expression vector (Qiagen, USA) using NdeI and XhoI restriction enzymes.

Expression, purification and recognition of recombinant FliD

E. coli BL21 (Qiagen, USA) competent cells were transformed with pET-28a(+)-*fliD* and inoculated in LB broth with antibiotic (kanamycin, 50 μg/ml). Expression was induced by addition of 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) at an optical density (OD600) of 0.6. After 4 h cells were harvested and protein analysis of whole lysate was carried out by Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The soluble histidin-tagged proteins were purified using affinity chromatography (HisTrap crude, GE Healthcare). As a second polishing step and for buffer exchange, size exclusion chromatography (Superdex 75, GE Helthcare) was performed. The relevant fractions where collected and concentrated with a centrifugal filter device (Millipore) with a cut off of 10 kDa and stored at −80 °C. Purified recombinant protein was evaluated by western blot using an anti-His Tag-HRP antibody and also a mouse anti *H. pylori*-HRP antibody (Pierce, Rockford, USA) and detected by ECL system (GE Healthcare, Uppsala, Sweden).

Production and purification of rFliD specific antibody

A mature white New Zealand rabbit was immunized with purified protein according to the protocol of Hay et al. with light modifications (Hay et al., 2002). Briefly, immunization was carried out by i.m. injection of 250 μg purified recombinant protein (0.5 ml) with the same volume (0.5 ml) of Freund's complete adjuvant. For the recall immunizations, the rabbit was boosted with 125 μg purified protein prepared in the same volume (0.5 ml) of Freund's

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