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The use of stool specimens reveals *Helicobacter pylori* strain diversity in a cohort of adolescents and their family members in a developed country

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ABSTRACT

Helicobacter pylori infection occurs within families but the transmission route is unknown. The use of stool specimens to genotype strains facilitates inclusion of complete families in transmission studies. Therefore, we aimed to use DNA from stools to analyze strain diversity in *H. pylori* infected families. We genotyped *H. pylori* strains using specific biprobe qPCR analysis of *glmM, recA* and *hspA*. Concentration of *H. pylori* organisms before DNA isolation enhanced subsequent DNA amplification. We isolated *H. pylori* DNA from 50 individuals in 13 families. T_m data for at least 2 of the 3 genes and sequencing of the *glmM* amplicon were analyzed. Similar strains were commonly found in both mothers and children and in siblings. However, 20/50 (40%) individuals had multiple strains and several individuals harbored strains not found in other family members, suggesting that even in developed countries sources of infection outside of the immediate family may exist. Whether infection occurs multiple times or one transmission event with several strains occurs is not known but future studies should aim to analyze strains from children much closer to infection onset. The presence of multiple stains in infected persons has implications for antibiotic sensitivity testing and treatment strategies.

1. Introduction

Helicobacter pylori colonizes the gastric mucosa of humans and induces a complex inflammatory response with the development of chronic antral gastritis in both children and adults (Blanchard and Czinn, 2017; McColl, 2010; Suerbaum and Michetti, 2002). A small proportion of those infected will develop peptic ulcer disease and gastric cancer (Leow et al., 2016; Sonnenberg, 2013). While *H. pylori* was considered one of the commonest bacterial infections worldwide, there has been a rapid decline in the prevalence of *H. pylori* over the last 100 years in developed countries, with an accompanying decline in the incidence of *H. pylori* associated peptic ulcer disease and gastric cancer (Lanas and Chan, 2017; Leow et al., 2016; McColl, 2010; Suerbaum and Michetti, 2002).

H. pylori infection almost always occurs in childhood, and persists for life unless specifically treated with antimicrobials (Leow et al., 2016; McColl, 2010; Rowland et al., 2006; Suerbaum and Michetti, 2002). There is no known reservoir of *H. pylori* outside the human stomach. While infection is clustered in families (Drumm et al., 1990), there is little information on the intra-familial factors which facilitate transmission. Some studies suggest that transmission between siblings is more likely than parent to child transmission (Cervantes et al., 2010), however, parents are an obvious source of infection for initial colonization of children in a family. Despite the serious consequences of *H. pylori* infection, particularly in countries with a high prevalence of gastric cancer, we still do not know how transmission occurs and what factors might disrupt transmission.

Potential transmission routes include oral-oral, gastric-oral or fecal-oral. Isolation of *H. pylori* from stool has rarely been reported (Kelly et al., 1994; Parsonnet et al., 1999). Reports of culture from the oral cavity are rare and a number of studies have suggested that the presence of *H. pylori* in the oral cavity is transient (Ferguson et al., 1993; Hirsch et al., 2012; Mapstone et al., 1993; Olivier et al., 2006). Furthermore epidemiological data do not support oral–oral transmission as co-habiting couples are infected with different strains; and treated adult patients are not infected by their untreated infected partner (Cutler and

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Schubert, 1993). A number of studies support the potential role of gastroenteritis in the transmission of *H. pylori* (Janzon et al., 2009; Perry et al., 2006). Parsonnet et al. demonstrated that *H. pylori* can be cultured easily from induced emesis in volunteers (Parsonnet et al., 1999) and on occasion from stool specimens folowing the use of cathartics (Parsonnet et al., 1999).

Studies of transmission that rely on upper gastrointestinal endoscopy to obtain gastric biopsies are limited by the number of asymptomatic family members or complete households that can be included (Krebes et al., 2014). The use of stool specimens for isolation of DNA from *H. pylori* to genotype strains present in different family members and conduct transmission studies is an attractive option as it facilitates the inclusion of large numbers and complete families. DNA detection circumvents the potential strain selection bias of culture based techniques. However, a particular challenge associated with using stool specimens is the low concentration of *H. pylori* DNA compared to that of other gastrointestinal organisms.

Puz et al. (Puz et al., 2008) described a novel genotyping protocol for *H. pylori* in stool specimens using biprobe qPCR assays and fragments of *glmM* and *recA* genes as target sequences. When strains had identical melting points they sequenced the *glmM* amplicon to confirm strain identity. The discriminatory capacity of the method was 100%. They reported clonal identities in 9/10 European families and 7/8 African households.

Between 1997 and 2001 we prospectively evaluated the incidence of *H. pylori* in a cohort of Irish families (Rowland et al., 2006). Young age (< 5 yrs) was the single biggest risk factor for acquisition of infection and older children did not get infected despite living in households with infected parents and siblings (Rowland et al., 2006). From this cohort we identified a number of families who currently are infected with *H. pylori* (using the carbon-13 labeled urea breath test (^{13}C UBT) and collected stool specimens from each of them with the aim of isolating *H. pylori* DNA from their feces and using this DNA to investigate the relatedness of strains within the families.

2. Materials and methods

2.1. Participants and sample collection

The families who participated in this study have been described previously (Rowland et al., 2006). Briefly, between 1997 and 2002 317 index children and their families were enrolled in the first prospective study of the acquisition of *H. pylori* infection in childhood (Rowland et al., 2006). The index children were between 24 and 48 months of age at enrolment and had ^{13}C UBTs carried out at baseline and annually thereafter for 4 years. 290 index children and their families completed the full follow-up. 28 index children (8.56%) were infected at baseline in 2007 and over the remaining 4 years of follow-up a further 20 children became infected with *H. pylori*. The incidence of *H. pylori* infection was highest among the 2–3 year olds (5.05 per 100 person years of follow-up, 95% CI 1.6-11.8) and declined progressively as children got older (Rowland et al., 2006).

In 2013 we traced 250/290 (86.2%) index children and their families and invited them to participate in a second follow-up study. The *H. pylori* status of families was again determined using the ^{13}C UBT as described elsewhere (Rowland et al., 1997). Families in which the index child and at least 1 other member were infected were invited to provide a fecal/stool sample. Participants were provided with stool collection kits and instructions on how to collect a sample of stool. Fresh stool samples were collected and transported to the laboratory within 3 h and either processed immediately or frozen at −20 °C until use.

2.2. ^{13}C urea breath test

The ^{13}C UBT was performed as described previously (Rowland et al., 1997). After a 2 h fast breath samples were collected at baseline and 30 min after ingestion of 75 mg ^{13}C urea. A cut-off of 5.0 $^{13}CO_2$ 0/00 was used to classify participants as infected with *H. pylori*. To ensure that the *H. pylori* status of all participants was accurately determined in 2013, participants with (i) a borderline ^{13}C UBT value (2.5–6.0 over baseline); (ii) a result different from 2002; or (iii) a new positive result (younger sibling not previously tested) had their breath tests repeated after an overnight fast. The result of the test performed after an overnight fast was defined as the correct result.

2.3. Ethics

Approval for the study was provided by the Irish College of General Practitioners. All participants over 18 years of age provided signed informed consent for the study while those under 18 yrs of age provided assent and their parents/guardians provided signed consent for their participation in the study.

2.4. Bacterial strains and culture

H. pylori strains J99, 26695, P12, N6, PU4, PU44, G27 and SS1 were routinely cultured at 37 °C on Columbia Blood Agar base (Oxoid) supplemented with 7% (vol/vol) defibrinated horse blood under microaerophillic conditions generated using CampyGen gas packs (Oxoid). *Listeria monocytogenes* EGDe was cultured at 37 °C on Brucella broth (Oxoid) supplemented with 1% (wt/vol) Agar (Oxoid). *Salmonella typhimurium* SL1344 and *Escherichia coli* HB101 were cultured at 37 °C on Luria-Bertani Agar (Oxoid).

2.5. DNA isolation from cultured bacteria

Following culture of bacteria biomass from one plate was collected into sterile PBS (Oxoid). The OD_{600} was adjusted to ~1 and 1 ml of bacteria was collected by centrifugation. The pellet was resuspended in 567 µl TE buffer (Sigma-Aldrich). Following the addition of 30 µl 10% (vol/vol) SDS and 3 µl proteinase K (20 mg/ml; Qiagen), the bacterial suspension was incubated for 1 h at 3 °C. Following incubation, 600 µl of phenol/chloroform/isoamyl alcohol (25:24:1; Invitrogen) was added, the solution was mixed well and centrifuged at 14,000 x *g* for 5 min at 4 °C. The aqueous layer was transferred to a fresh tube, an equal volume of chloroform (Sigma-Aldrich) was added, mixed well and centrifuged as before. The aqueous layer was transferred to a fresh tube and 2 volumes of ethanol (Sigma–Aldrich) and 1/10 volume 3 M sodium acetate (Sigma–Aldrich) were added, mixed well and incubated on ice for 30 min. Eluted DNA was pelleted by centrifugation for 15 min at 4 °C. The pellet was washed in 70% ethanol, air dried for 15 min and resuspended in 50 µl TE buffer at 4 °C.

2.6. Isolation of DNA from fecal material

A number of methods were used to isolate DNA from fecal material including the CTAB (cetyl trimethylammonium bromide) method of DNA isolation and the QIAamp DNA Stool Mini Kit (Qiagen). In addition, we developed an antibody capture method to enrich for *H. pylori* organisms present in stool followed by use of the PowerFecal DNA isolation kit (Mo Bio Laboratories). For the CTAB method 30 µl of 10% (wt/vol) SDS and 3 µl proteinase K (20 mg/ml) was added to 200 mg of fecal material resuspended in 567 µl of Tris EDTA (TE) buffer (10 mM Tris; 1 mM EDTA, pH 8.0) and the suspension was incubated for 1 h at 37 °C. Following incubation 100 µl 5 M NaCl and 80 µl CTAB/NaCl (100 mM Tris-HCl (pH 7.5), 25 mM EDTA, 1.5 M NaCl, 2% (wt/vol) CTAB, and 0.3% (vol/vol) β-mercaptoethanol) was added and, the sample was incubated for 10 min at 65 °C. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added, and after centrifugation the aqueous layer was transferred to a fresh tube and an equal volume of chloroform/isoamyl alcohol (24:1; Sigma-Aldrich) was added. After centrifugation the aqueous layer was transferred to a fresh