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Research Article

Rapid Identification of Viable *H. pylori* Cells in Feces by DVC-FISH

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Abstract

Helicobacter pylori isolation in fecal samples is a less invasive and more comfortable practice than those that require patient endoscopy, particularly in children. However, culture of this pathogen from stools is usually unsuccessful. Other techniques such as PCR or *H. pylori* Stool Antigen (HpSA) are used to detect the presence of *H. pylori* in feces; nevertheless, a positive result by using these techniques does not involve viability of the pathogen. Direct Viable Count combined with Fluorescent *in situ* Hybridization (DVC-FISH) technique has been successfully applied to detect viable *H. pylori* cells in highly contaminated environmental samples. To assess the suitability of DVC-FISH technique to identify viable *H. pylori* cells in stools, experimentally inoculated feces and fecal samples from infected patients were analyzed. qPCR and culture techniques were also used. Viable *H. pylori* cells were detected by DVC-FISH in all inoculated samples with a specific DNA/LNA probe. *H. pylori* colonies were also identified on agar. DVC-FISH gave positive results in all the patients' fecal samples, while qPCR only detected *H. pylori* in two patients.

DVC-FISH technique with LNA/DNA probes has the potential to be used as a specific and effective non-invasive method for the detection of viable *H. pylori* in stools samples. Moreover, our results evidence the presence of viable *H. pylori* cells in fecal samples from infected patients, supporting the evidence that *H. pylori* is transmitted via the fecal route.

INTRODUCTION

H. pylori is the cause one of the most common chronic infections in humans [1]. It is estimated that approximately 50% of the world population is infected; however, only about 10-15% of infected individuals will develop peptic ulcer, and in 1-3% gastric cancer will appear.

Currently a range of techniques are being applied for diagnosis, each with their own advantages and disadvantages. Invasive gastroscopic biopsy-based methods, such as the Rapid Urease Test (RUT), histological examination and culture have been widely used to diagnose *H. pylori* infection [2]. Endoscopy is an expensive procedure, not free of risks, that requires highly qualified personnel to be carried out. It is also an uncomfortable procedure, especially in non-collaborative patients and children. Other non-invasive diagnostic techniques, urea breath test (UBT), stool antigen detection or serology, are also widely used. However, these present some problems, such as being technically demanding, presenting non-optimun specificity or difficulty in assessing the existence of an active infection [3]. Moreover, they do not allow for isolating the bacteria. Thus, no antibiotic resistance tests or genetic characterization can be performed.

Bacteriological culture is a tedious, time-consuming procedure, and unnecessary for the routine diagnosis of *H. pylori* infection in the majority of patients. The sensitivity of culture has been reported to vary greatly among laboratories, because *H. pylori* is very fastidious, and even experienced laboratories recover it from only from 50% to 70% of infected biopsies [4]. However, culture is the most specific way to diagnose *H. pylori* infection. It allows for testing of the antibiotic sensitivity of isolated *strains*,

enables us to have a better understanding of the pathogen-host interaction and facilitates to determine the main epidemiologic features relative to *this pathogen [*4]. Especially, culture from clinical or environmental samples is crucial to demonstrate the potential of this sample to act as a transmission vehicle, as the bacterium must be present in a viable form to be infective.

There is much controversy regarding the transmission pathways of *H. pylori* [5]. Different routes have been proposed, such as oral-oral, fecal-oral and indirect transmission via water or even foods. *H. pylori* have been found in feces, saliva or vomit [4] and may even be acquired through other routes [6].

Among all the transmission mechanisms, fecal-oral transmission seems to be one of the most relevant, especially in developing countries, as suggested by different epidemiological studies [7]. *H. pylori*, present in gastric mucosa, is constantly eliminated to the gut via gastric juice. Thus, feces or any material contaminated by them, such as water, vegetables or milk, could be the vehicle of transmission for the infection [8].

The existence of a fecal-oral route means that the bacterium should be isolated from stool specimens. Extensive indirect evidence suggests that the organism is present in feces. Momtaz *et al.* [9] detected *H. pylori* in different areas of the digestive tract (10, 72% in saliva, 77, 66% in gastric biopsies and 71, 67% in feces). By PCR, *H. pylori* DNA has been detected it in fecal samples [4]. Equally *H. pylori* Stool Antigen (HpSA) test supports the view that *H. pylori* is excreted in the feces. Even so, there is still the doubt about how *H. pylori* can remain viable. The bacterium is sensitive to bile, so it should not be able to survive during its transit through the enteric tract [10].

A positive result obtained by PCR or antigen tests does not mean that the pathogen maintains its viability in the feces, as it may just be that they are detecting the antigen or DNA from dead bacteria. Therefore, except for some reports of culture from the stool, there is little evidence that *H. pylori* survives in the colon.

Attempts to culture *H. pylori* from feces have had limited success. Recovery from stool is very difficult because of the complex nature of the sample: stools are rich in bile salts, hydrolytic enzymes [11] and other commensal biota hampering the growth of *H. pylori [*2]. It may also be possible that the bacterium is viable but not culturable (VBNC) due to the stressful environmental conditions during its passage along the intestinal tract.

The use of sensible and specific methods for detecting viable cells of *H. pylori* in feces could be an alternative to invasive diagnostic methods, particularly for children, as feces can be comfortably obtained at home and without active collaboration [12]. Moreover, these techniques could facilitate epidemiological studies about its mode of transmission.

Direct Viable Count combined with Fluorescent *in situ* Hybridization (DVC-FISH) has been previously used to detect and identify viable *H. pylori* cells in water samples [13] and it can be easily applied to feces. Thus, the objective of this work was to assess the suitability of DVC-FISH technique to detect and identify viable *H. pylori* cells in fecal samples. HpSA, qPCR and culture techniques were also used for comparison.

MATERIAL AND METHODS

Helicobacter pylori Strains

H. pylori NCTC 11637 and B67 (isolated from a gastric human biopsy) strains were used for inoculation assays. Bacteria were grown on Columbia agar plates (Difco Laboratories, Detroit, MI, USA) supplemented with 10% horse blood, Dent selective supplement (Oxoid) and 0.0025% sodium pyruvate, at 37ºC under microaerobic conditions.

Inoculated faecal samples

Exponential (48 h) cultures of both *H. pylori* strains were used to spike *H. pylori*-free faecal samples. Briefly, cultures from both NCTC 11637 and B67 strains were resuspended in PBS buffer (130 mmol/L sodium chloride, 10 mmol/L sodium phosphate, pH 7.2), diluted to give 10^{7}-10^{8} CFU/mL, and used to inoculate 1 g of feces mixed previously with 9 mL of PBS. Samples were shaken for 5 min at 160 rpm to enable bacteria to attach to fecal particles. One mL aliquots of each inoculated sample were taken for later processing by q-PCR and DVC-FISH. One hundred µl samples were also spread onto Dent selective agar. In addition, 200 µl were inoculated on a 0.65 µm membrane (Whatman, Maidstone, England) and placed onto selective agar. Plates were incubated at 37 ° C under microaerobic conditions. After 24 h incubation, membranes of the plates were removed and incubated further for 48 -72h. Presumptive colonies were identified by FISH and qPCR as described below.

q-PCR

DNA from 1 mL sample was isolated by using the GeneJet™ Genomic DNA Purification kit (Fermentas, Germany) according to the manufacturer´s instructions.

Two µl of the eluted DNA was used for specific *H. pylori* SYBR Green-based qPCR with VacA primers, described previously, [14] to amplify a 372-bp fragment from the vacA gene of *H. pylori* [14]. Real time was performed in a final volume of 20 µl containing: 4 µl of LightCycler Fast-Star DNA Master SYBR Green I (Light Cycler Fast Start DNA Master SYBR Green I. Roche Applied Science.), 1.6 µl of MgCl2 (4mM), 0.5 µl of each primer and 2 µl of template. The amplification consisted of an initial DNA denaturation step at 95ºC for 10 minutes, followed by a 40-cycle reaction: 95ºC for 10 seconds, 62ºC for 5 seconds, 72ºC for 16 seconds and after that one cycle of extension at 72ºC for 15 seconds and finally one cycle at 40ºC for 30 seconds.

PCR products were visualized under 1.5 % agarose gel electrophoresis stained with 0.01% GelRed (Biotium, USA)and then purified with the GenElute PCR Clean-Up Kit (Sigma, USA) according to manufacturer's instructions and subsequently analyzed by sequencing (IBMCP, CSIC). The homology of the amplified sequences with the correspondent *H. pylori vac*A gene fragment was determined by a BLAST alignment (www.ncbi.nlm.nih.gov/BLAST).

DVC-FISH analysis

One mL of sample was inoculated in 9mL of DVC broth (Brucella broth supplemented with 5% Fetal Bovine Serum (FBS) and 0.5 mg/L novobiocin) supplemented with 0.0025% sodium

piruvate and incubated for 24 hours under optimal conditions for *H. pylori* [15].

After incubation, DVC tubes were centrifuged at 8500 rpm for 8 minutes. The supernatant was removed and the pellet was resuspended in 1 mL of PBS 1X and then fixed for FISH analysis as described for Gram-negative bacteria by Moreno *et al.* [16]

The fixed samples were deposited on the wells of hybridization slides, air dried and dehidrated by successive immersing in volumes of 50, 80 and 100 % ethanol for 3 minutes each. Fixed samples were subsequently hybridized with LNA- HPY and EUB 338 16S rRNA probes labelled with CY3 and fluoresce in respectively, according to conditions described by Moreno et al. Briefly, hybridization buffer (0.9 M/L NaCl , 0.01 % SDS, 20 mM/L Tris-HCl and 40 % formamide, pH 7.6) containing 50 ng of each probe were deposited on the wells and then, slides were incubated at 46ºC for 2 h in darkness. After washing, hybridized samples were examined with an Olympus microscope BX50 equipped with a 100W mercury high-pressure bulb and set filters U-MWB, U-MWIB and U-MWIG. Color micrographs were taken with a digital camera Olympus DP 12 (Olympus Optical Co., Hamburg, Germany). The fluorescent green intensity signal was measured with Olympus DP Soft program.

Faecal samples

Outpatients sent to the Endoscopy Unit of the Hospital de Sabadell for evaluation of dyspeptic symptoms were recruited for the study. Patients were contacted prior to the endoscopy, and asked to participate. Those who agreed signed informed consent. Patients were instructed to avoid antisecretory drugs during the two weeks before the test and they were asked to bring a faecal sample on the day the endoscopy was performed. Before the endoscopy, a 13[C]-urea breath test (UBT) (UBiTest 100 mg, Otsuka Pharmaceutical Europe Ltd, UK) was performed. During endoscopy, two antral biopsies for histology and one for rapid urease test (RUT, JATROX HP test CHR Heim Arzneimittel GmbH, Germany) were obtained. A total of 5 faecal samples from infected patients (positive for all tests above described) were also analysed to test the suitability of DVC-FISH technique in order to identify viable *H. pylori*.

Five grams of fresh faeces were homogenized with 45mL of PBS buffer and vortexed. One mL aliquots were processed for DVC-FISH and qPCR as previously described for inoculated samples. One hundred µL was also analysed directly by culture on Dent agar under the above describe conditions and 400µL were placed onto a 0.65 µm cellulose acetate membrane filters and deposited on selective agar media as previously described [13]. Samples were also analysed after an enrichment step. Briefly, 10 mL of the homogenized samples were suspended in 20 mL of Dent enrichment broth and incubated under microaerobic conditions at 37ºC for 24 h. After enrichment, aliquots were obtained for further analysis by DVC-FISH, qPCR and culture as for non- enriched samples.

RESULTS AND DISCUSSION

Histology is one of the most common tests used to detect the *H. pylori* infection and is considered by most authors the "gold standard" [17]. Nevertheless, it is also an invasive and expensive screening technique. *Helicobacter pylori* detection in faecal samples is an easier and more comfortable practice than invasive techniques, mainly in children. However, culture of this pathogen from stools is difficult and normally unsuccessful, and colonies confirmation tests are necessary. Other techniques such as PCR or *H. pylori* Stool Antigen (HpSA) are used to detect the presence of *H. pylori* in faeces. Nevertheless, a positive result by these techniques does not recognise the viability of the pathogen. This is an important point, as transmission of the bacteria via fecal-oral route, although generally admitted, is not fully assessed, due mainly to our lack of knowledge about the cellular state and infectiveness of *H. pylori* in feces.

In this work we have improved the specific method DVC-FISH to use as a non-invasive method to detect the pathogen in infected individuals.

Presumptive *H. pylori* colonies were observed from both NCTC 11637 and B67 inoculated samples, but in all cases with a high nonspecific microbiota growth on agar plates (Figure 1) Colonies from inoculated faeces were only appreciated on the plates in which the samples were passed through a membrane.

Viable *H. pylori* cells were detected by DVC-FISH in all samples of inoculated faeces with specific DNA/LNA probe (Figure 2). In spite of the presence of great amount of competitive microbiota, viable (elongated) and non- viable *H. pylori* cells were easily observed. After enrichment, samples showed an increase of unspecific microbiota, hindering *H. pylori* identification. Inoculated samples were also qPCR positive directly and yielded negative results after enrichment. These results were in accordance with previous reports about *H. pylori* detection [18], showing that a previous enrichment step does not favor the detection of the pathogen by qPCR.

As shown with the inoculated samples, some *H. pylori* presumptive colonies from patient fecal samples were only observed on the plates cultured by membrane technique in 2 out of 4 patients. Colonies could not be purified because of growth of unspecific microbiota on the selective plates. Thus, identification of *H. pylori* cells within the cultures was only possible by molecular methods such as qPCR and FISH. The low efficiency on *H. pylori* detection by culture could be due not only to the lack of specificity of culture media but also, the environmental stress on the pathogen through the gastro intestinal tract, which become *H. pylori* to VBNC state, diminish the pathogen recovery [11]. Other

Figure 1 Bacterial growth on Dent selective agar plates inoculated with feces.

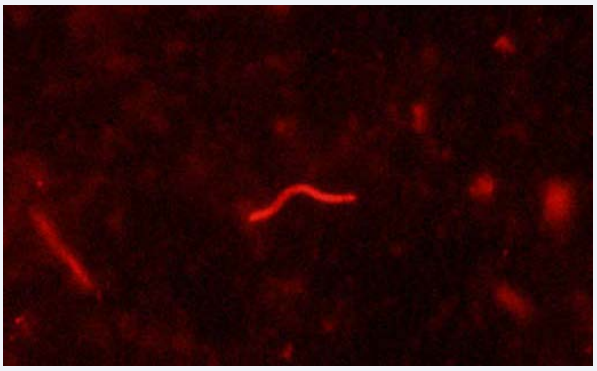

Figure 2 DVC-FISH detection of viable (elongated) *H. pylori* with *H. pylori* LNA probe labelled with CY3, from feces samples.

authors have reported the difficulty to culture *H. pylori* from feces and the need for a pretreatment of the patient prior to the analysis in order to reduce the bile salts content [12].

qPCR analysis detected *H. pylori* in feces from 2 out of the 4 patients, one of them after enrichment. Although qPCR is a highly sensitive technique, stools are complex mixtures that often contain PCR inhibitors and other commensal bacteria making false-negative and false-positive results a larger problem [19].

DVC-FISH showed the presence of viable *H. pylori* in all the patients samples according with the results obtained by invasive methods (histology and RUT). It seems that the factors affecting the cultivation of *H. pylori* in feces do not affect their metabolic activity, thus remaining viable and potentially infective. Although DVC-FISH is not as sensitive as other molecular techniques for less contaminated samples [18], it was more efficient than qPCR in this kind of samples. In addition, this technique incorporated an overview of the habitat and microbiota companion of the species studied, as well as morphology that adopt these bacteria in the feces, allowing for its count.

Thus, DVC-FISH technique would be an effective alternative to culture for the detection of viable cells of *H. pylori* in feces. It is a less invasive, more comfortable practice and more cost-effective than culture from gastric biopsy, and the results could be obtained in 48h, a short time with regard to culture.

Moreover, our results evidence the presence of viable *H. pylori* cells in fecal samples from infected patients, supporting the evidence of the fecal route of *H. pylori* transmission.

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