APPLICATION OF PNA-FISH AS A NEW DIAGNOSTIC METHOD FOR THE DETECTION OF *HELICOBACTER PYLORI* CLARITHROMYCIN RESISTANCE IN GASTRIC BIOPSY SAMPLES

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INTRODUCTION

Helicobacter pylori is an important bacteria that colonizes the human stomach. Its association with the development of gastric diseases (peptic ulcer disease, MALT lymphoma and gastric carcinoma), is already been proved. In addition, a major expenses outflow in hospitalization resulting by ulcer related diseases is pointed out.
Triple therapy comprising a proton pump inhibitor in association with two antibiotics (clarithromycin, amoxicillin or metronidazole) is the gold standard treatment for eradication of *H. pylori* from the human stomach. Nevertheless, increased resistance to clarithromycin became the main factor of treatment failure. Until now, culturing methods such as agar dilution or E-test has been used to assess resistance status. However, these methods are fastidious and time consuming (in some cases they take over one week to show results). In this study, a new and more prompt genotypic method to detect *H. pylori* clarithromycin resistance in clinical samples is proposed. Clarithromycin resistance in *H. pylori* is associated mainly with three point mutations in the positions A2142G, A2142C and A2143G in 23S rRNA of the bacteria. The method which is based on fluorescent *in situ* hybridization (FISH) using a set of peptide nucleic acid probes (PNA) that are complementary to that regions, was applied to paraffin-embedded gastric biopsy samples for validation proposes, after optimization of hybridization conditions. This new genotypic method will permit a more prompt response to *H. pylori* clarithromycin resistance, being less demanding to the patient and leading to a more cost effective patient treatment. The intellectual property of the PNA probes described here has been patented (PT PAT 40801-09) and a paper concerning this study is submitted to Journal of Clinical Microbiology.

METHODS

Three PNA probes were designed to detect strains with resistance to clarithromycin, corresponding to the three most frequent mutations in the 23S rRNA gene. The selected probes were Hp1 (A2143G) 5'-GGG TCT CTC CGT CTT -3', Hp2 (A2142G) 5'-GGG TCT TCC CGT CTT -3' and Hp3 (A2142C) 5'- GGG TCT TGC CGT CTT -3'. An additional probe to detect wild type (susceptible) strains (Hpwt 5'- GGG TCT TTC CGT CTT -3') was also included. Afterwards, the selected sequences were synthesized (Panagene, Daejeon, South Korea), coupling fluorochromes within probes.
Thirty-three isolates were used for optimization of hybridization conditions and to confirm the practical specificity and sensitivity of the probes. The temperature, formamide concentration and pH have to be all well defined to achieve reaction stringency. The standard method E-test and sequencing were used as control. A multiplex assay was carried out to check the performance of the probes working together. To validate the method in the stomach tissue, thirty paraffin-embedded gastric biopsy specimens from 24 patients (20 patients with a single biopsy sample, two with 2 biopsies samples and two patients with 3 samples) with known resistance antibiotic profile by antibiogram were used. The study was in accordance with the institutional ethical standards and consisted to adapt the hybridization conditions used in smears to gastric biopsies material. In order to confirm the susceptibility profile and prove the effectiveness of PNA-FISH method, antibiogram and PCR-sequencing were done to all the biopsies. Smears or histological slides were observed using an epifluorescence microscope equipped with filters adapted to signaling molecules within probes.

RESULTS AND DISCUSSION

Probes Hp1, Hp2, and Hp3, hybridized only with the resistant strains that have the corresponding point

mutations conferring clarithromycin resistance and as such presented 100% sensitivity (95% CI, 79.9-100) and 100% specificity (95% CI, 71.6-100). The set of probes can discriminate the resistant and susceptible strains, even though they only have one mismatch. Then, it was studied the applicability of the method, using simultaneously a mixture of the four probes for multiplex detection. In this case, the method for the detection of point mutations was even more robust, which is possibly due to the fact that all probes target the same locus, and as such there is a competition effect between them. However, with the mixture it is only possible to discriminate between clarithromycin resistant and clarithromycin sensitive strains, as opposed to the discrimination between point mutations that was conferred by using the probes separately. In practical terms and considering the application of the PNA-FISH to the clinical setting, the mixture of probes introduces an important simplification to the method.

Considering the application of the PNA-FISH method in clinical settings, results clearly show that it is possible to discriminate susceptible from resistant *H. pylori* strains and, in the latter group, to detect the three different mutations, using fluorescence microscopy.

Of the 30 histological samples, 26 presented total agreement in all the three methods (86.7% concordance), ten presenting susceptible strains and 16 presenting resistant ones. Overall, the PNA-FISH method was in full agreement with PCR followed by sequencing. Considering the antibiogram as the gold standard, the method presented a specificity and sensibility of 90.9% (95% CI, 57.1-99.5) and 84.2% (95% CI, 59.5-95.8).

CONCLUSION

Resistance to antibiotics, namely to clarithromycin, is one of the causes of treatment failure in *H. pylori* eradication. For this reason, it is the most beneficial to detect resistance to clarithromycin prior to antibiotic therapy. Herein, it is described the applicability of PNA-FISH methodology to clinical material, namely gastric biopsy samples, thus overcoming the need of culturing steps and/or PCR/sequencing procedures and enabling rapid initiation of appropriate antibiotic therapy until culture confirmation can be obtained several days later. Furthermore, the required equipment, a fluorescent microscope equipped with adequate filters for fluorochromes, is easy to handle for routine diagnostic purposes. For centers using routine cultures of *H. pylori*, the complementary use of PNA-FISH methodology to smears of bacteria will increase the sensitivity of the detection of resistant strains in clinical samples.

AUTHOR BIOGRAPHIE

LAURA CERQUEIRA was born in Braga, Portugal and went to the **School of Sciences of the University of Minho**, Braga, where she studied Applied Biology and obtained her degree in 2001 and her Master degree in Environmental Sciences in 2005. In 2006 she had a research grant in the European SAFER project – Surveillance and control of microbiological stability in drinking water distribution networks, *Helicobacter pylori*, under the responsibility of Prof. Maria João Vieira in Centre of Biological Engineering, University of Minho - School of Engineering. At the present, she is a PhD student (PhD in Chemical and Biotechnology Engineering in University of Minho) and the theme of her work is "Development and evaluation of peptide nucleic acid (PNA) probes for the rapid detection of antibiotic-resistant microorganisms". Her e-mail address is: lauracerqueira@deb.uminho.pt

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