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(54) Title: PEPTIDE NUCLEIC ACID (PNA) PROBE, KIT AND PROCEDURE FOR SPECIFIC DETECTION OF HELICOBACTER PYLORI AND APPLICATIONS THEREOF

(57) Abstract: The current invention relates to the development of a peptide nucleic acid (PNA) probe for the specific detection of *Helicobacter pylori*. These probes are used in a process based on molecular biology techniques, namely the fluorescence in situ hybridization (FISH), and can be applied in a variety of samples such as biopsies, blood, air, food, water and other environmental samples. Due to the physical and chemical characteristics inherent to their structure, these probes are able to provide a more rapid and sensitive analysis than the DNA probes. Another aspect of the current invention is the development of a kit, based on the PNA probe and the FISH process, for the detection and/or quantification of the *H. pylori* in biological, clinic and/or environmental samples.

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DESCRIPTION

"PEPTIDE NUCLEIC ACID (PNA) PROBE, KIT AND PROCEDURE FOR SPECIFIC DETECTION OF HELICOBACTER PYLORI AND APPLICATIONS THEREOF"

Field of the Invention

This invention relates to a method for the detection of clinically relevant microorganisms, and as such a peptide nucleic acid (PNA) probe has been developed to detect *Helicobacter pylori*. Another aspect of the current invention is the application of the PNA probe and detection procedure to a kit for the detection, identification and/or quantification of *H. pylori* in biological samples which might be used by the pharmaceutical industry.

Background of the Invention

Helicobacter pylori is an important human pathogen that causes chronic gastritis and is associated with the development of peptic ulcer disease, atrophic gastritis and gastric cancer. Infection with *H. pylori* can be diagnosed either by invasive or by non-invasive techniques. The invasive techniques require upper endoscopy with collection of gastric biopsy specimens. Non-invasive tests are the most usual methods for routine *H. pylori* detection because they are clinically effective, less expensive and more convenient to the patient. The most usual non-invasive tests/kits used are those which that detect urease, an enzyme that is virtually specific for *H. pylori* (US2003068656 e EP0920531).

However, besides not being completely specific or sensitive, these tests fail to provide complementary information on *H.* pylori location in the stomach and on the histopathological lesions underlying the presence of the bacteria. Therefore,

there are situations where invasive techniques should be performed to provide a more complete diagnosis.

Gastric biopsy specimens obtained by upper endoscopy can be analyzed for the presence of the bacterium by culture or by other molecular methods.

In recent years, molecular methods, including RAPD (Random Amplified of Polymorphic DNA), PCR (Polymerase Chain Reaction)(JP9065899 and WO9109049), and fluorescence *in situ* hybridization (FISH), for the identification of several bacteria, have been imposing themselves over the more time-consuming culture methods.

FISH is arguably the most common method used for the detection and localization of a microorganism or particular groups of microorganisms within a sample. It detects nucleic acid sequences by a fluorescent labelled probe that hybridizes specifically to its complementary target sequence within the intact cell.

So far, FISH methods have been based traditionally on the use of conventional DNA oligonucleotide probes containing around 20 bases. More recently, peptide nucleic acid (PNA) probes have been developed and optimized for bacterial detection.

PNA molecules are DNA mimics, where the negatively charged sugar-phosphate backbone is replaced by an achiral, neutral polyamide backbone formed by repetitive units of *N* - (2-aminoethyl) glycine.

PNA can hybridize with complementary nucleic acid targets obeying the Watson-Crick-base pairing-rules. When compared to

the traditional DNA probes and due to the uncharged backbone, PNA probes have superior hybridization characteristics, exhibiting rapid and stronger binding to complementary targets and absence of electrostatic repulsion. As such, the optimum length for a PNA probe is generally of 15 base pairs (bp).

This technique has been shown to be a rapid, sensitive and very specific method for microbial detection and could be an important alternative or complement for the culture methods because using the probes produces faster results and additional information such as the location of the bacteria in gastric samples. Since the non-invasive techniques could not be adapted for the detection of the bacteria in different environments, this method has also more comprehensive application.

Several PNA probes have been designed and brought to public, through patent documents, to detect microorganisms of clinical interest such as Mycoplasm, Acholeplasm, or Ureaplasm (US2006252081; WO2006122049); species of Staphylococcus other than *Staphylococcus aureus* (WO2005054516; EP1709198); *Enterococcus faecalis* and/or other species of Enterococcus (WO2005018423; US2005153307); Pseudomonas (US2004259132) *Escherichia coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Streptococcus agalactiae*, *fungi*, *and Acinetobacter* (WO2006130872). For *H. pylori* only one PNA probe has been presented and although very specific the probe was not very sensitive (Table 1), possibly due to the high genetic diversity observed within *H. pylori*.

Table 1- Predicted specificity and sensitivity of the PNA probes for *H. pylori* detection.

Probe	Specificity[a]	Sensitivity[b]	References
Hprobe	100 %	25 %	Azevedo *et al*, 2003
Hpy769	85 %	89 %	This patent

[a]Specificity was calculated as NHp/(TN)x100, where NHp stands for the number of *H. pylori* strains and TN for the total number of bacterial strains detected by the probe.

[b]Sensitivity was calculated as NHp/(TNHp)x100, wherein NHp stands for the number of *H. pylori* strains detected by the probe and TNHp for the total number of *H. pylori* strains in the databases.

The procedure of the present invention, with the application of the PNA probe, does not involve the use of reagents or enzymes for the formation of pores in the cellular membranes before the hybridization step and consequently the PNA probe can be directly applied immediately after the preparation of the sample in the slide.

However, some of the usual components used in hybridization are still necessary, so, the probe is included in kits that facilitate the application of FISH procedure by the users. There are already kits that use PNA probes for the electrophoretic separation of DNA samples (US2005053944, WO9712995, EP1477572).

Summary of the Invention

The present invention is a peptide nucleic acid (PNA) probe for *Helicobacter pylori* specific detection. PNA probes have physical and chemical characteristics inherent to their structure that, when applied to a method based on the FISH technology, provide a more rapid and sensitive analysis than when DNA probes are used. Other aspect of the present invention is the development of a kit, based on the application of this probe to the methodology referred above, that permits a simplest and faster detection of *H. pylori*, in biological samples.

General Description of the Invention

The PNA probes have physical and chemical characteristics inherent to their structure that allow a more rapid and sensitive analysis than the DNA probes, namely when applied to FISH. Identical to ADN FISH, the sequence of the nucleotides is the functional part of the probe and is constructed in order to hybridize with a complementary rRNA sequence in the target cell. A successful hybridization allow us, for instance by fluorescence microscopy, to check the presence/absence and concentration of the target microorganism. To accomplish this goal, the probe has to meet the specific requirements of each trial, particularly the length and the sequence, to form a stable complex with the target in the ideal hybridization conditions. The addition of the fluorescence molecule that emits the signal, such as fluorescein or Alexa Fluor fluorochromes family, is accomplished using a chemical compound 8-Amino-3,6-dioxaoctyl that binds the two molecules.

The hybridization procedure consists in three steps: fixation, hybridization and washing step.

In this case, the utilization of paraformaldehyde and ethanol for ten minutes each to fixate the sample is advisable, but not necessary.

During hybridization, usually takes between 30 to 120 minutes, the probe of this invention with the sequence 5'-GAGACTAAGCCCTCC-3' is mixed in a solution named hybridization solution.

Those skilled in the art of DNA FISH will easily identify formamide concentration (or other denaturating agent), saline concentration (i. e. ionic force), hybridization temperature, detergent concentration, pH and the probe concentration as the usually referred factors that control the specificity of the hybridization.

The optimal procedure to obtain the probe/target sequence complex is usually achieved using the well known technique of maintaining several of the said factors constant and then determine the effect of the alteration of just one of the factors. The same factors can be modulated for the PNA and DNA hybridization, however this hybridization is relatively independent from the ionic force of the solution. For this probe, with a solution of 10% (wt/vol) dextran sulfate, 10 mM NaCl, 30% (vol/vol) formamide, 0.1% (wt/vol) sodium pyrophosphate, 0.2% (wt/vol) polyvinylpyrrolidone, 0.2% (wt/vol) Ficol, 5 mM disodium EDTA, 0.1% (vol/vol) Triton X-100, 50 mM Tris-HCl (pH 7.5) and 200 nM PNA probe a successful hybridization is obtain at 59°C.

Finally, during the washing procedure, the probe that has not completely hybridized is removed from the sample to ensure the specificity of the process. As a general rule, the more similar other sequences of the sample are from the target sequence, the more important this step is. In this case, the immersion of the sample in a solution of 5 mM Tris Base, 15 mM NaCl and 1% (V/V) Triton X (pH 10) at hybridization temperature for 30 minutes is the optimal condition to ensure the desired specificity.

In the end of this procedure, the sample can be observed using fluorescence microscopy with the right filters for the detection of the fluorochrome bonded to the probe. In samples without the target sequence, the microscope is unable to detect any signal emission.

The procedure can be applied in the diagnosis and quantification of *H. pylori* in different kinds of samples, including biopsies, blood, air, food, water and other environmental samples.

In addition, the PNA probes can also be used in real time PCR, a process wherein the product amount is continuously evaluated during the amplification process, allowing the quantification of the genetic material in the initial sample.

In a simple way, the PCR product is detected through the bonding of dyes to the DNA double chain. Since these dyes are not specific, they recognize any product formed during the procedure, which requires a purification of the DNA of interest before the amplification step. To solve this problem, DNA probes have been developed to recognize specific

sequences and such as with the FISH, the PNA molecules revealed additional vantages.

In detail description of the Invention

1. Sample preparation

The samples can be from biopsies, blood, air, food and water. In the case of biopsies, cuts of 3 to 5 μm are made and put in slides. If *H. pylori* is present in the suspension, such as in water samples, air or blood, the samples are filtered through a polycarbonate or equivalent black membrane. The membranes are then put in slides. In food samples, it is necessary to recover *H. pylori* from the food matrix, using either an ultrasonication step or a mechanical process of removal by batting the sample embedded in water or buffer solution with flat paddles. Once *H. pylori* is in suspension, the procedure used is the same as in the water, air and blood. As an alternative, the samples with *H. pylori* in suspension can be directly hybridized.

2. PNA probes design

To identify oligonucleotides suitable to be used as probes, the 16S rRNA sequences of several databases were aligned. Because several 15-base sequences were able to detect the majority of *H. pylori* strains, additional criteria was used for the selection of the final sequence. Some of the criteria used was the absence or near absence of complementary sequences in the probe sequence and also the detection of as few microorganisms as possible. According these criteria, the selected sequence was 5′-GAGACTAAGCCCTCC-3′.

3. **PNA hybridization**

3.1. Fixation - To prevent the loss of 16S rRNA during the hybridization step, the sample is exposed to a 4% (wt/vol) paraformaldehyde solution and a 50% (vol/vol) ethanol solution for 10 minutes each.

3.2. Hybridization - During this step, an hybridization solution containing the probe is in contact with the sample. During this time period, the probe goes through the cellular membrane and binds to complementary sequences of the 16S rRNA. The hybridization usually occurs between 30 and 120 minutes, at 59°C and can be performed to both suspension and adhered *H. pylori*. In the first case, a concentrated hybridization solution is added in a way that the final products concentration should be as follows. In the second case, the necessary volume of hybridization solution to completely cover the sample is added directly in the sample. During the hybridization period, it is essential that the hybridization solution does not evaporate. For such, a cover slip is added on top of the hybridization solution and the incubation environment is kept moist by the insertion of a humid paper around the slide.

Hybridization solution - A solution of 10% (wt/vol) dextran sulfate, 10 mM NaCl, 30% (vol/vol) formamide, 0.1% (wt/vol) sodium pyrophosphate, 0.2% (wt/vol) polyvinylpyrrolidone, 0.2% (wt/vol) Ficol, 5 mM disodium EDTA, 0.1% (vol/vol) Triton X-100, 50 mM Tris-HCl (pH 7.5) and 200 nM PNA probe was prepared.

3.3. Washing - During this step, the non completely hybridize probe is removed from the sample, by means of the immersion

of the sample in a solution of 5 mM Tris Base, 15 mM NaCl and 1% (V/V) Triton X (pH 10) at an hybridization temperature for 30 minutes.

3.4. Results - The results are achieved by observation in a fluorescence microscope with the correct filters for the detection of the fluorochrome bond to the probe, when the target sequence of the probe is not present there is no signal detection.

CLAIMS

1. Peptide nucleic acid probe for *Helicobacter pylori* detection, characterized in that it is 86% identical to the 5'-GAGACTAAGCCCTCC-3' sequence and has a length between 8 and 18 nucleotides.

2. Use of the PNA probe, according to the previous claim, characterized in that it is applied in a detection, identification and/or quantification method of *Helicobacter pylori* in clinical and/or environmental biological samples.

3. Method for the detection, identification and quantification of *Helicobacter pylori* in a sample, characterized in that a PNA probe is used, according to the previous claims, comprising the following steps:

- PNA probe contact with the sample
- PNA probe hybridization with the target sequence of the microorganisms within the sample
- Hybridization detection as an indication of the presence, identity and/or quantity of microorganisms in the sample.

4. Method, according to claim 3, characterized in that the said sample in the analysis is preferably, but not exclusively, from biopsies, blood, air, food and water.

5. Method, according to claim 3, characterized in that the hybridization step of the PNA probe with the target sequence is performed at 59°C during one and a half hours and the washing is carried out at the same temperature for half an hour.

6. Method, according to claim 5, characterized in that the hybridization is fluorescent and performed *in situ*.

7. Method, according to claim 3, characterized in that the presence, identity and/or quantity of *Helicobacter pylori* in the sample is detected by a conjugate, a radioisotope, an enzyme or a luminescent or fluorescent compound.

8. Use of the method, according to claims 3 to 7, characterized in that it is used for the detection, identification and/or quantification of *Helicobacter pylori* in clinical and/or environmental biological samples.

9. Kit for the detection, identification and/or quantification of *Helicobacter pylori* in biological samples, characterized in that it contains the probe described in claim 1 and is based on the method described in claims 3 to 7.

10. Use of the kit, according to claim 9, characterized in that it is applied in a real time PCR process.

11. Use of the Kit, according to claim 10, characterized in that it is applied to the detection, identification and quantification of *Helicobacter pylori* in clinical samples such as biopsies and/or environmental samples.