® Postepy Hig Med Dosw (online), 2013; 67: 261-268
e-ISSN 1732-2693

www.phmd.pl
Reviev article

Received: 2012.06.01
Accepted: 2013.02.05
Published: 2013.04.09

Role of PCR in *Helicobacter* pylori diagnostics and research – new approaches for study of coccoid and spiral forms of the bacteria

Rola metody PCR w diagnostyce i badaniach naukowych *Helicobacter* pylori – nowe możliwości poznawcze formy kokoidalnej i spiralnej bakterii

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Summary

Helicobacter pylori since Marshall and Warren's discovery has been an object of interest of gastroenterologists and many researchers of other specialties. What needs to be highlighted is also the growing interest of dentists in the role of oral residue of *H. pylori* in oral pathologies such as burning mouth syndrome, periodontitis and gingivitis.
With the development of medical techniques more studies using highly specific diagnostic methods are performed in order to determine the transmission pattern of bacterial infection. Suggested faecal-oral and oral-oral routes of bacterial transmission raised interest in molecular biology methods as tools for the study of these environments. Additionally, co-existence of helical and coccoidal forms of *H. pylori* in the mentioned niches raised the question whether the latter is potentially pathogenic. This is why molecular biology is now giving a great opportunity to explore parts of the human body that could not have been diagnosed before using only gold standard diagnostic methods. Molecular techniques have shown their usefulness in examining the potential virulence of coccoid forms of bacterium. This review was created also to summarize the knowledge about molecular methods, especially different PCR techniques, as diagnostic tools that can help medical teams during regular diagnosis of gastritis.

Keywords: ***Helicobacter pylori*** **review •** ***Helicobacter pylori*** **diagnostics • PCR diagnosis • PCR detection**

Full-text PDF: http://www.phmd.pl/fulltxt.php?ICID=1044005

Word count: 5346
Tables: 1
Figures: 1
References: 40

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The use of molecular biology techniques in *H. pylori* epidemiological research

H. pylori is a spiral, Gram-negative, microaerophilic bacterium which colonizes human gastric mucosa and causes severe gastritis and related complications leading to stomach and duodenum ulceration as well as – in the long term – to gastric cancer and gastric MALT lymphoma. Since 2005 when two Australian researchers [23] received the Nobel Prize in medicine for discovery of *H. pylori* and its role as the causative agent in ulcer disease, the number of scientific studies conducted in order to understand the characteristics of *H. pylori* have increased significantly. More extensive epidemiological studies using a high number of patients were performed in order to determine the transmission pattern of bacterial infection. Ways of transmission of *H. pylori* infection have not been well specified and any attempt to systematize this knowledge has divided the medical community [9]. Epidemiological studies carried out so far enable establishment of reliable types of transmission which undergo modifications with the development of medical knowledge. The suggested classification of the types of *H. pylori* transmission is shown in the table below (Table 1).

Despite the unsure and hypothetical transmission of *H. pylori* infection, two routes which have been confirmed by epidemiological studies on large populations of patients seem to be most likely: the transmission of infection through the faecal-oral and oral-oral routes [5].

The suggestion that the faecal-oral route of transmission may be of great significance and the easy access to biological material through which the transmission of infection may occur results in frequent use of faeces as biological material in the epidemiological studies [16]. Many techniques are used in order to detect *H. pylori* in the faeces, e.g. bacterial cultures, enzyme assays and molecular medicine techniques such as polymerase chain reaction (PCR) [5,9].

Due to the abundance and diversity of bacterial flora, faeces as a diagnostic material is problematic as far as obtaining specific results for *H. pylori* microbial cultures is concerned. From the point of view of detecting *H. pylori* in the stool, there are two obstacles to the specific determination of *H. pylori* with the use of microbiological methods. The first methodological problem is related to the high probability of *H. pylori* occurrence in the coccoid form [29], which makes culture on agar media difficult, and therefore makes the microbiological method demanding and challenging to use in epidemiological studies. The second problem is possible overgrowth of *H. pylori* by other bacterial strains which in turn may lead to false negative results. Both arguments make the establishment of *H. pylori* microbiological cultures from faeces technically difficult and, subsequently, diagnostically difficult [18, 24]. Equally problematic is detection of *H. pylori* by the PCR method, which, despite the great potential of generic material detection, involves the removal of faecal PCR inhibitors prior to the DNA extraction process [24]. Steps that are recommended in order to remove PCR inhibitors are filtration processes on the polypropylene membranes or chromatographic separation on columns [18].

For the process of bacterial DNA amplification to be consistent with the current presence of bacterium, and in order to reduce false positive results, which in the case of faeces is particularly important, it is vital to use those fragments of selected genes from bacterial DNA which give the possibility of obtaining a specific result. There are several preferred reactions during DNA amplifications of *H. pylori*. Among them is the nested PCR method, which proved to be effective during an investigation conducted by Vinette et al. among paediatric patients [39].

Infection through contamination of drinking water by human faeces has been suggested as one of the routes of in-

Table 1. Types and transmission routes of *H. pylori* infection

Type of transmission	**Routes of transmission**		
Direct transmission	Oral-oral route	Fecal-oral route	Gastro-oral route
Intermediate (indirect) transmission	Environmental factors	Faecal contaminated water source and H. pylori biofilm that forms on the surfaces of water pipes.	1. Small children in clusters or families with many children; transmission through contaminated with gastric contents toys or items. 2. Professional exposure of nurses and gastroenterologists performing endoscopic procedures; transmission via contaminated endoscope surfaces.

fection transmission. The morphological type that exists in this environment that is harsh for *H. pylori* has proved to be a coccoidal form of *H. pylori* [24]. Queralt et al. in their research aimed to confirm the presence of *H. pylori* in water, in faeces, and in water reservoirs contaminated with human faeces, amplifying successfully the fragments corresponding to the A subunit of the urease gene using nested PCR techniques [29].

The biological fluids used in epidemiological screening are typically excretions and residues of the initial part of the digestive system – saliva, dental plaque and residue from interdental spaces. Considering the suggestions of the importance of faecal-oral transmission in the populations of developing countries and oral-oral transmission as the main route of transmission in developed countries [9], it seems appropriate to assume that the reservoir of *H. pylori* in the oral cavity may be important during the entire infection process and also during the process of possible reinfection [5,9,32].

Coccoid form of *H. pylori* – potentially pathogenic?

Observations with the electron microscope conducted by the co-authors of this article, Giovanni Loi and Corrado Serra, showed that the *H. pylori* helical form converts into a degenerative coccoid form. The process is illustrated by the images taken by the co-authors with the electron microscope which accompany this article. With the use of electron microscopy it was possible to show the process of conversion from the bacillary form of *H. pylori* to the fully coccoid form (Figure 1). The morphological transformation process begins as a bleb at the bacterial surface (number 1 in the image); the bleb continues to grow and the rod bends, resulting in a U-shaped bacterium (numbers 2-3 in the image); finally, a "ball-like" coccoid form is achieved (numbers 4-6 in the image).

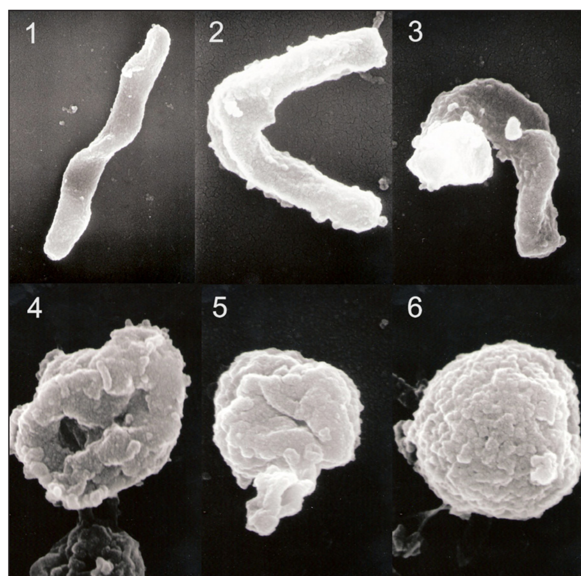

Figure 1. Conversion from the bacillary form of *H. pylori* to the fully coccoid form. (See description in the text)

This observation is also confirmed by many other scientists who devote their research to exploring the role of *H. pylori* coccoid form in gastric mucosa infection [11,13,17]. Coccoid *H. pylori* forms have been divided into three types: degenerative coccoid form representing dying bacteria of pyknotic structure; a living coccoidal bacterium that can be cultured in agar medium; and viable but non-culturable (VBNC) *H. pylori* [2,4]. Because of the complexity of the structure and different physico-chemical conditions affecting colonies which form a bacterial biofilm, it has been reported that it is precisely in bacterial biofilms that all forms of coccoid bacterium are present along with the coexisting helical forms. Their presence *in vivo* may be a result of one of the following conditions. It may be a part of the survival strategy of bacteria, an effect of differing antibiotic concentrations due to different biofilm structures, or due to unequal access of bacterial colonies to nutrients which leads to the differentiation of colonies in this environment [4]. Bacterial ability to transform in the form of grains and a possible return to its helical shape opens new horizons for our understanding of the infection and reinfection transmission with *H. pylori* [13]. The induction of coccoid form in drinking water reservoirs confirmed by several studies [17], and the presence of *H. pylori* in marine plankton provide insight into the gravity of the problem of possible infection transmission [11]. In the case of potential reversion from the coccoid to the helical form, there exists a possibility of inducing an active infection on the surface of gastric mucosa [11]; however, *in vitro* research which attempted to transform coccoid forms of *H. pylori* into a helical form was unsuccessful in humans. It has therefore been suggested that the transformation of bacteria into the coccoid form reduces its metabolism and induces minor modifications in the physiological processes of the bacterium. These changes lead to the inability to recover those bacterial traits which facilitate colonization and infection of gastric mucosa. Observations of *H. pylori* cultures have shown that the transformation process of the helical form into the coccoid form occurs in the presence of either of two conditions. The first is the occurrence of extremely unfavourable environmental conditions, such as temperature other than 30-37°C, no nutrient medium, gas content level other than the one in the preferred microaerophilic atmosphere, or low concentrations of antibiotics affecting the bacterial culture. In this case the helical form is reconstituted into a coccoid form. The coexistence of both forms (coccoidal and helical) in various environmental conditions, would explain in this case the survival strategy of bacteria [4,11,17]. Secondly, this transformation may occur during cell degradation due to the end of its life cycle and degeneration of its virulence factors [40].

As a result of transformation into the coccoid form there occurs bacterial DNA and RNA condensation, but without fundamental changes in the expression of genes responsible for virulence of *H. pylori*, i.e. genes encoding urease, vacuolating cytotoxin and the pathogenic island [40]. For this reason a coccoid form in drinking water and in seawater tanks accessible by humans would suggest a possible influence on the transmission of infection [4,11,17]. The

suspicion that the discussed coccoid form is a survival form in adverse conditions but may give the possibility of transmission of infection prompted scientists to test its potential virulence. Wang et al. with the aim of describing the virulence of coccoidal forms applied to their research molecular biology methods such as DNA amplification, creation of recombinant plasmids with the use of previously isolated DNA, and subsequent sequencing of obtained genes which were most important for bacterial virulence [40]. In that study, the researchers induced coccoid form formation by exposing the *H. pylori* NCTC11637 strain to low doses of antibiotics. The conclusion reached by the authors was that sequences of coccoidal and helical forms of *H. pylori* are 99.7% compatible. It was also observed that the *cagA* gene which provides pathogenicity of *H. pylori* was complete in the coccoid form of the bacterium [40].

Speculations about the possible pathogenicity of the coccoid form led to the search for solutions to this puzzle with the use of an animal model – laboratory mice. In this study bacteria isolated during a biopsy of gastric mucosa of a patient who suffered from gastric ulcers were used to infect the mice. Induction of coccoid forms creation was obtained after the standard microbiological diagnosis of bacterial infection through the stimulation of unfavourable environmental conditions by dissolving the bacteria in sterile water. The aim of the study was firstly to confirm the presence of cell adhesion factor in *H. pylori* coccoid form in the mouse gastric mucosa after administrating a suspension of the coccoid form of bacteria; and secondly, to determine whether it is possible to transform the coccoid form into a helical form *in vivo*. After the infection procedure of mouse gastric mucosa with coccoidal form of *H. pylori* was initiated, there was confirmed presence of flagella, an essential element in the process of infection with bacterium of helical morphology. She et al. indicate that the cause of infection may be the bacterial transformation from coccoid form into the spiral form, due to the presence of specific environmental factors, characteristic only for *in vivo* conditions. It was justified by the presence of spiral *H. pylori* forms in gastric mucosa biopsy specimens from mice that had been subjected to a solution containing initially only the inducible coccoid form of bacteria. Due to the unconfirmed information about the possibility of inducing the infection through the coccoid form among humans, it was said that the transformation from the form of grains (coccoid form) into the spiral form (helical form) of *H. pylori* in the human body has to be confirmed and requires further research in order to describe possible processes which regulate it [31].

Possible applications of molecular methods in detection of coccoidal and spiral *H. pylori* forms

Studies show a great diversity between different strains of *H. pylori* infecting one human. That is in agreement with results of an experiment presented by Lukeš et al. [20]. The researchers describe in their paper differences between gastric and oropharyngeal genotypes in viral forms of bacterium infecting one human. The described experiment was performed using the real-time PCR method based on DNA extracted from saliva and stomach tissue specimens of each patient. The study confirmed that heterogeneity between *H. pylori* strains can be present also in the level of genes that are most important for bacterium virulence such as *cagA* and *vacA* genes in strains involved in infection of one patient. This was also confirmed by Momtaz et al. [25], who claimed to have found different genotypes of *H. pylori* in saliva, gastric biopsies and stool of the same patient. The described experiment included testing for presence of *ureC* and *cagA* genes and the genotypes of *vacA* alleles (*s1a*, *s1b*, *s1c*, *m1a*, *m1b* and *m2*) using PCR technique. The conclusions made by Momtaz et al. were that because of high diversity between *H. pylori* genotypes in saliva, stool and stomach, the latter environment may be most supportive for growth of *H. pylori* strains of different genotypes. Genotype diversity in different strains may be caused not only by environmental characteristics, but also by genetic changes or co-infection with another strain.

In 2004 there were more than 23 *Helicobacter* species. Some of these non-*H. pylori* species are found in clinical samples, and others colonize animals. What has great importance is that these species have similar genomes. That makes differentiation with molecular techniques problematic. As described by Suzuki et al., the most closely related species to *H. pylori* is *H. acinonychis*, which colonizes the stomach of large felines. Many important virulence factors were also found in *H. felis*, which even if it lacks *cag* PAI and *vacA*, still has the whole ComB system [37]. Additionally, as mentioned before, non-culturable coccoid form of *H. pylori* is said to be present in both tap water and environmental water. These two facts create a problem with specific species differentiation. The problem seems not only to be in the detection of coccoid and spiral forms of the bacterium, but also distinguishing *Helicobacter* from the genotypically similar *Campylobacter* spp. There are researchers who have set themselves the aim to search for the combination of PCR-based reactions that would provide an effective assay for determining *H. pylori* either in coccoid or helical form. Shahamat et al. [30] reported in their article that specific species distinction depended on good preparation of the PCR process, based on genetics of *H. pylori* and the most common species showing similarity, *Campylobacter* spp. The targets of the Shahamat et al. PCR reaction were the unique region of *H. pylori* flanking the 16S rRNA gene sequence, urease (*ureA*), and phosphoglucosamine mutase (*glmM*) genes. PCR proposed by Shahamat *e*t al. using the 16Sr RNA hypervariable region complete the results obtained by PCR amplification with *glmM*-specific primers, the target of which was the phosphoglucosamine mutase gene. Specific *glmM* primers allowed detection of *Helicobacter* species (*pylori* and other members of the genus), and did not amplify the following non-*Helicobacter* species: 4 strains of *C. jejuni*, and 18 strains of other microbial species. Amplifications based on the primers specific for the region flanking the 16S rRNA gene of *H. pylori* discriminate the non-*H. pylori* species of this genus. Additionally, sequence comparison using the proposed hypervariable region upstream of the 16S rRNA gene of *H. pylori* can

be an easy way to distinguish *H. pylori* strains from other members of the genus, since this region has sequences that are highly specific for each *H. pylori* strain.

The molecular method prepared by Nayak et al. [26] was meant to be a valuable source of information about the contamination of ground water by viable but non-culturable (VBNC) *H. pylori*, with a basis that it may be a way of transmission of its infection. The reason why scientists prepared a helpful tool to detect a VBNC was to evaluate whether it is possible to use a rapid and effective test of water purity, to avoid waterborne disease transmission. Using an argument that there is a large difference between colonies and species genomes, it should be difficult to evaluate a specific test for detection of *H. pylori* VBNC form. According to Sisto et al., differences between genomes of coccoid and helical forms of bacterium are not relevant in some conditions. Researchers have shown that DNA and RNA of the coccoid form are not degraded and that they have expression of the most important virulence and colonization factors such as urease, pathogenic island and vacuolating toxin genes even after a period of 31 days. The only problem was induction of gene *vacA*, which was not present after 15 days of incubation in most strains apart from strain 190. This, the authors claim, may be due to decrease in mRNA production to below the lower detection limit of the PCR method used in the Sisto el al. research [33]. In other research Can et al. claimed that urease activity was detected in coccoid forms of *H. pylori* both phenotypically using the rapid urease test and genotypically by the PCR method [10]. The presence of urease activity in all coccoid cells induced by different factors showed that transformation from helical to coccoid form influenced urease activity independently from the transforming factor. Determination of an unimpaired *ureA* region in coccoid cells can confirm that this region of the *H. pylori* genome is highly protected.

The conclusion can be made based on the presented research that coccoid and spiral forms of *H. pylori* can be specifically detected by molecular biology methods with a mixed procedure of combination of two primers – one based on *glmM*-specific primers and the second using hypervariable region 16Sr RNA, or with primers directed to *vacA* regions of the bacterium genome [26,30] on the condition that in some cases primers flanking the *vacA* gene can give false negative results. This was previously described by Nayak et al. as due to prolonged incubation time longer than 16 days.

Differentiation between spiral and coccoidal forms of *H. pylori* with the PCR test that would specifically answer the question which form is present in the specimen is still problematic. As described in this section there is however a possibility to specifically distinguish both coccoidal and helical forms of *H. pylori* from other *Helicobacter* species and closely related bacteria such as *Campylobacter*. Still the greatest potential for differentiation is the possibility of microbiological culture of the spiral form, and distinguishing morphological characteristics of the bacterium using specific types of microscopes.

Application of PCR in *H. pylori* infection diagnostics

Difficulties in biological materials analysis during *H. pylori* infection diagnostics are due to a long (lasting 6 days) and fastidious growth process of bacterial cultures on agar media. With the development of medical techniques, there appeared suggestions to speed up the diagnostics by performing parallel diagnostics using molecular biology techniques. Microbiological diagnosis cannot be abandoned; what is more, in the regular diagnostics it is considered a gold standard on a par with histological diagnostics of gastric mucosa and urease breath test [39]; however, a parallel PCR test can improve the work of medical teams by reducing the results turnaround time, which in turn could enable medical teams to make quicker decisions about patients' treatment. This is important especially for diagnosis of gastritis in children, in whom heterogeneity of distribution of the pathogen was observed. In the case of paediatric diagnosis of *H. pylori* infection, preparation of biological material and bacterial culture gives the possibility of obtaining a false-negative result [39].

The results of comparative studies testing the techniques used to diagnose *H. pylori* presence in gastric mucosa biopsy specimens from children, such as standard histological techniques, CLO test (Campylobacter-like organism test) and immunological techniques used as tests for antibodies to *H. pylori* performed using ELISA and Western blot methods and techniques of molecular medicine, confirm the usefulness and effectiveness of PCR in analysing the specific presence of infection. Sensitivity of well-constructed *H. pylori* detection reaction can reach even 100%, and its specificity might be greater than any serological tests. Its additional advantage is the fact that it does not require any waiting time for the formation of antibodies [39]. According to the recommendations [6, 21], the diagnosis of active *H. pylori* infection should be designed primarily to treat the patient if the result is positive. It is important therefore to select the correct diagnostic methods in order to confirm the presence of bacteria in patients who require antibiotic treatment (it is recommended to first of all diagnose patients with active peptic ulcer disease, patients with peptic ulceration confirmed in their medical history, and patients with gastric MALT lymphoma). What should also be underlined is the position of Sugimoto et al., who pointed out the disadvantages of using techniques of molecular medicine when decisions to use the PCR technique as the unique diagnostic method confirming *H. pylori* infection are made. That is mostly linked with unspecific oligonucleotides used for bacterium detection in PCR reaction [36].

In patients whose first line eradication treatment has failed, the test for drug resistance of the bacterium should be carried out [6,21]. An important diagnostic aid with respect to this problem is molecular medicine methods (PCR) which specifically detect resistance to clarithromycin in biopsy specimens [36]. Because of bacterial resistance to antibiotics which according to Malfertheiner et al. in various European countries ranges between 5% and 28% for clarithromycin and between 20% and 40% for metronidazole, the possibility of recognizing the initial bacterial resistance to

the planned use of antibiotics is usually of key importance for the effective eradication of *H. pylori* before selection of drugs to be used in the therapy. Resistance to clarithromycin is also a major risk factor when using the treatment recommended in Europe, which, besides clarithromycin, consists of amoxicillin and proton pump inhibitors. According to current recommendations presented in the Maastricht III Consensus Report [21], *H. pylori* resistance to clarithromycin test should be performed prior to the treatment if the prevalence of primary resistance is 15-20% [38]. Tankovic et al. in their study show that resistance to clarithromycin is generally caused by two mutations in the 23S ribosomal RNA (rRNA): adenine to guanine transition at position 2142 or 2143, or adenine to cytosine transversion at position 2142. These two positions participate in the formation of the peptidyl transferase loop of the ribosome. There is a possibility of these mutations' detection using molecular methods, especially real-time PCR. The technique of assessing clarithromycin resistance in biopsy material of gastric mucosa by real-time PCR was introduced as a routine in Henri-Mondor Hospital, described by Tankovic et al. in January 2003. The routine histological examination has not been abandoned; however, the microbiological culture was excluded. Microbiological assessment of resistance to drugs was preserved as a basis for the diagnosis in assessing the sensitivity of other antibiotics used in the third-line therapy and in case of differences between the results in PCR and histological diagnostics, and not for evaluation of resistance to clarithromycin [38]. Moreover, the results of the Chrisholm et al. study showed that *H. pylori* DNA could be detected by PCR directly in human gastric biopsy samples with high specificity and sensitivity. Chrisholm et al. also developed a PCR assay targeted to the *vacA* gene – a species-specific and highly conserved locus in *H. pylori*. This gives a possibility to obtain a valuable result of PCR diagnosis [14].

For most specific primer pairs, the polymerase chain reaction method may be used only to confirm the gold standards: culture microbiological tests, histological examination of slices taken during biopsy, or urease tests. Diagnosing an active *H. pylori* infection only on the basis of a positive PCR result, without the use of the achievements of gastroenterology, microbiology and histology, is not recommended [36].

PCR DETECTION OF *H. PYLORI* IN MATERIAL FROM THE ORAL CAVITY

Beginning with the discovery of Warren and Marshall [23], *H. pylori* infection has been in the field of interest of scientists from many areas of life science, not only gastroenterologists. With the techniques of molecular medicine, it has become possible to examine niches that so far have rendered research problematic due to the lack of diagnostic methods which would be considered as gold standards in diagnostics. The reasons for the rising interest in the oral cavity as a potential nesting place of *H. pylori* which affects the transmission process have already been mentioned [15]. It should be noted that the presence of bacteria in the mouth and its relation to infection of the gastric mucosa shows no agreement in the scientific community. This may result either from suggestions of co-existence in the mouth of both helical and coccoidal forms, which is recognized by most researchers as a degenerative artefact unable to induce an active infection [40] and whose transformation into a helical form in the human organism has not been confirmed [31], or because of lack of confidence in molecular biology techniques in the diagnostic assessment of the presence of bacteria in the mouth [36]. Lack of confidence in molecular biology methods has already been pointed out, but the overall assessment of the literature on the subject of the presence of *H. pylori* in the oral cavity seems to be necessary for a full description of the issue of *H. pylori* infection.

According to Parrish et al. [27], 325 bacterial strains have been grown so far from dental plaque. However, the development of techniques for distinguishing all of the actual microbes colonizing the oral cavity is difficult, especially because of the lack of possibility of developing a bacterial culture of some microbes during routine diagnostics [27]. In the explanation of the issue why the gold standard of microbiological culture cannot be used in the oral cavity, the arguments are similar to those presented above, in the section about isolating the bacterial DNA from the stool. *H. pylori* during microbiological diagnosis of gastritis is problematic because of the long incubation time of the bacterial culture, and the requirement of highly specific substrate and culture conditions. In the case of dental plaque and saliva diagnosis performance, there is a high possibility of outgrowth by other bacterial colonies whose natural reservoir is located in the mouth, and this process can subsequently affect the growth of *H. pylori*. Additionally, the presence of *H. pylori* in the coccoid form inhibits bacterial cultures, which in effect can cause false negative results [15].

It is known that among bacterial flora of the mouth *H. pylori* is not the only bacteria responsible for urease production. This enzyme, which belongs to the hydrolase class, owes its presence in the mouth to *Streptococcus* spp., *Haemophilus* spp., and *Actinomyces* spp.; hence a positive urease test result in this environment cannot specifically confirm the presence of *H. pylori*. Taking into account the possibility of receiving a false positive result, this method also does not seem to show any importance in scientific research [15]. The solution to both problems – false-positive urease test results as well as false-negative results of microbiological culture – is to conduct research using molecular techniques, so that the nucleotide sequence comprising a specific gene responsible for encoding the virulence factors of *H. pylori* will give a specific result [27]. It is particularly important for this reason to use sensitive methods of detection. A highly sensitive test which gives a specific view of the presence of bacteria in the mouth is a well-matched PCR test [15,34].

The use of a one-step PCR test may not be sufficient to carry out the detection of *H. pylori* in the saliva. The one-step reaction has been evaluated by Mapstone et al. as inadequate for assessment of the presence of *H. pylori* in material from the oral cavity. This is due to a lower degree of *H. pylori* colonization in the mouth in comparison with the biopsy of the active site of infection in the gastric mucosa.

As far as saliva is concerned, it is required therefore to apply a more specific and sensitive test. Furthermore, Mapstone et al. [22] in their work confirmed this assumption while underlining that the use of a single-step technique is associated with a risk of obtaining false-negative results, despite the use of specific primers [19,34].

Particular attention is paid to the association of the *H. pylori* reservoir in the oral cavity with gingivitis and chronic periodontitis [12,35]. It was indeed noted that patients with poor oral hygiene represent a group in which the presence of *H. pylori* in dental plaque is more likely to be confirmed [1]. It is also suspected that the presence of the bacteria in these two pathologies may lead to oral inflammation and the formation of gingival/periodontal pockets, which develop favourable conditions for bacterial growth [1,35]. Development of favourable conditions would allow the reinfection of gastric mucosa, particularly in the above-mentioned patients – with poor oral hygiene and periodontal infection – which could foster pathological state development of gastric mucosa infection [1]. This statement may be revised by comparing bacteria presence in the oral cavity in patients with good oral hygiene, in which the results of detection of bacteria, although statistically significant, included only 30% of healthy patients [1]. This information suggests the presence of a bacteria reservoir in the dental plaque which plays a role in transmission of *H. pylori* infection. In addition, researchers show a lot of interest in the ability of *H. pylori* to adhere to bacteria which cause periodontitis, i.e. *Fusobacterium* spp. [3], and the effect of this co-aggregation on the ability to colonize the mucous membrane, which could initiate the re-infection process [1,38]. One of the studies, conducted by Andersen et al. in order to assess the effectiveness of the adhesion of 79 bacterial strains to *H. pylori*, showed that *H. pylori* specifically adheres to *Fusobacterium nucleatum* and *Fusobacterium periodonticum* of human origin. This process shows the importance of the presence of adhesins on the surface of these two representatives of *Fusobacterium* and their respective receptors on the surface of *Helicobacter*, which may play a role in the development of periodontal disease as well as the process of reinfection of the gastric mucosa membrane by ingestion of *H. pylori* [3].

During periodontal disease progression an important factor in the inhibition of disease development is the composition of saliva, as well as its physical conditions (viscosity, proper saliva production of salivary glands) and, which proved to be equally important, a balance between the components of saliva which play an inhibitory role in periodontal disease [28]. It should be noted that the role of proteases in the destruction of periodontal tissues is of major importance. The presence of protease inhibitors such as salivary leukocyte protease inhibitor (SLPI) has a direct effect on maintaining integrity of the tissue. The protease inhibitors prevent the spread of the disease by neutralizing the action of proteases. Linking stress with increased probability of the occurrence of oral disease, especially in periodontal inflammation in animal and human models, is a subject of interest for researchers [7]. Stress and psychological care of patients with oral mucosa problems are important not only because of their association with diseases such as periodontitis and gingivitis. Psychiatric disturbances may also be connected with burning mouth syndrome (BMS). A high percentage of patients suffering from BMS complain of depression of unknown origin (which results either from chronic mouth pain or otherwise is not connected with any burning symptoms).

In the case of patients with symptoms of burning in the oral cavity and clinically healthy oral mucosa detailed medical history should reveal systemic factors which might result in burning symptoms. There exists a strong link between those patients and patients who complained of oral burning before treatment for *H. pylori*. As Brailo et al. confirmed in their research, "true" burning mouth syndrome diagnosis should be established when all other systemic and local reasons for burning are excluded. Unfortunately, as Brailo continues, the "true" burning mouth syndrome still shows limited therapeutic options [8]. Particular interest should be paid to infection with *H. pylori* due to the high percentage (79%) of patients whose burning symptoms were resolved after *H. pylori* eradication treatment. Additionally, in patients with burning symptoms clinical examination of the oral cavity – candida swab, oral galvanism measurement, salivary flow rate and haematological screening (with specific attention to serum ferritin, blood glucose levels and *H. pylori* antibodies) – should be performed in order to identify possible underlying disturbances. Brailo et al. also suggested that due to the significantly higher prevalence of gastritis in patients with BMS together with the reported incidence of 3.2 times more gastrointestinal symptoms in BMS patients, each patient with burning mouth syndrome should consult a gastroenterologist [8].

Acknowledgments

The linguistic and editorial assistance of Irena Wypych is gratefully acknowledged.

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The authors have no potential conflicts of interest to declare.