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Chapter 3

Pre–Endoscopy Screening of *Helicobacter pylori* Infection: Implication and Advantages

Maria Teresa Mascellino, Alessandra Oliva and Barbara Porowska

Additional information is available at the end of the chapter

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1. Introduction

Over the last 30 years, upper gastrointestinal endoscopy has become the investigation of choice for patients with symptoms referable to upper gastrointestinal tract. Owing to the increasing number of patients who should be undergone endoscopy with a consequent high cost and a marked workload and medical expenses for the hospitals, it has been recommended that pre-endoscopy screening strategies might identify patients at low risk of having major pathology. These patients could avoid prompt endoscopy and might safely undergo different management.

Considering that *Helicobacter pylori* (*Hp)* is the most frequent aetiologic agent in these pathologies, several invasive and non-invasive diagnostic tests have been taken into account for the diagnosis of *Hp* in the individual patient. The non-invasive tests obviate the need for endoscopy and can be surely more accepted by the subjects.

It has been proposed [1,2,3] that younger patients with symptoms of dyspepsia with non-alarming symptoms could be screened non-invasively for the infection in order to reduce endoscopy procedure. In addition, non-invasive tests are suitable, other than for pre-endoscopy screening of younger dyspeptics, also for use in research and for epidemiological surveys as well as for confirming successful eradication after treatment and for screening asymptomatic population.

The pre-endoscopy screening is based on different methodologies (such as serological markers, molecular markers, etc.) that will be discussed in the present chapter.

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2. Serological markers

Serological testing has been recommended for initial pre-endoscopy or pre-treatment screening in dyspeptic patients. Serology is cheap and convenient and thus should be preferred in situations where the additional information yielded by an endoscopy is not needed.

Patients are prone to undergo this analysis because it only requires a simple peripheral blood collection for the investigation of anti-Hp IgG, IgM and IgA antibodies. The presence of even high levels of immunoglobulines does not appear to influence eradication of the bacteria from the stomach: the microorganism in fact is rarely eliminated and when it is not treated adequately, the infection generally persists in the rest of an individual's life [4].

For these reasons, the use of serological tests are very commonly used for clinically diagnosis of Hp-related infections. In general, the serum levels of anti-*H. pylori* IgG antibodies increase in the presence of infection and can be used as a marker. On the other hand, even if anti-Hp IgA antibodies are less appropriate for this purpose [5], serological findings of anti-Hp IgA in symptomatic patients might have significant clinical value for the diagnosis of infection, especially if the patient is seronegative for IgG. The disadvantage for serology is that past or current infections are not distinguished owing to the fact that past infections may lead to false positive, so that this test cannot be used for determining therapy success after treatment even if successful eradication can follow a substantial drop in antibody title, using repeat serology after a delay post-treatment. [6]

2.1. Serology as diagnostic tool

Serological testing is recommended for initial pre-endoscopy or pre-treatment screening in dyspeptic patients. The systemic response typically comprises a transient rise in IgM followed by a rise in specific IgA and IgG maintained throughout infection.

The consideration that patients with IgG antibodies to Hp have a greater risk of peptic ulcer disease as a cause of their dyspepsia, has led to screen dyspeptic patients under the age of 45 years using Hp serology. Three strategies are proposed after serology screening:

1. endoscopy of Hp seropositive patients and treatment of seronegative patients symptomatically;
2. treatment of seropositive patients for Hp and endoscopy of seronegative patients
3. eradication of infection from Hp seropositive patients, treatment of seronegative patients symptomatically and endoscopy for those with recurrent dyspepsia.

The attitude in both gastroenterologists and general practitioners with interest in gastroenterology towards the current pattern of use of pre-endoscopic Hp serology screening of young dyspeptics has been evaluated [7].

The most popular strategy among general practitioners is that of eradicating infection from seropositives and treating seronegatives symptomatically. In contrast, the most popular

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strategy among gastroenterologists is that of endoscoping seropositives and treating seronegatives symptomatically.

There is then wide variation in attitudes and practice between these two groups: general practitioners like more serological tests and strongly prefer eradicating infection in seropositives before addressing to endoscopy (even for cost consideration). On the contrary, the majority of gastroenterologists would endoscope seropositives before treating the infection.

In any case, it is recommended that non-invasive Hp testing should be used in place of endoscopy with all those testing positive being given anti-Hp therapy and those testing negative being treated symptomatically. The above strategy of "test and treat" used in clinical practice may include some inconveniences: expense morbidity from drug side effects and introduction of antibiotic resistance both in Hp and in other pathogens [8].

An important serological tool for the pre-endoscopy screening in patients at risk of carcinoma includes the quantitative determination of the different subclasses of IgG. In fact, a selective reduction of anti-Hp IgG subclass antibody is proven to occur in gastric carcinoma [9]. Cell-mediated immunity influences the outcome of infection including the development of gastric carcinoma (CG). The T-cell response comprises a secreted cytokine profile which influences the B-cell response including the production of the different IgG subclass antibody. In the adenocarcinoma, a fall in IgG level is demonstrated resulting to be particularly predictive of cancer [10]. This is thought to reflect premalignant gastric atrophy with loss of colonization and antigens stimulus [11]. A diminuished IgG antibodies response due to low immunogenicity of Hp-LPS or to the loss of Hp in some subjects evolving to GC, could reflect the premalignant phase of gastric atrophy. Significantly lower IgG2 levels are found in subjects with gastric carcinoma compared with those with reflux oesophagitis, chronic gastritis, gastric ulcer and peptic ulcer whereas IgG1 antibody remains at similar levels (Figure1). The levels of IgG 3 and IgG 4 are not affected and in most subjects are undetectable. The decreasing of IgG 2 subclass level, noticed in patients with adenocarcinoma and not in other Hp-related pathologies, depends on both the switching of mucosal cytokine secretion and the different kinetics of IgG response to gastric colonization by B-lymphocyte that can be influenced by cytokine profiles in secreting different antibody patterns.

Consequently, the patients showing low levels of IgG especially of subclass IgG 2 (below an established cut-off value) can be considered subjects at high risk of developing pre-malignant disease, gastric atrophy and adenocarcinoma [9]. These data show that above certain levels of antibody, irrespective of age, the risk of cancer is low and that primary endoscopy could be restricted to those with antibodies values below this level. In this way, the endoscopy could be avoided, as initial investigation, in 42% of dyspeptic subjects [9].

The value of this test as a predictive diagnostic tool in the pre-endoscopy screening strategy is crucial.

In conclusion, the screening strategy based on Hp serological status, determined with the enzyme-linked immunoadsorbent assay (ELISA) and Western blotting (WB), in patients with uncomplicated, simple dyspepsia up to 55 years of age, is able to identify 95%-100% of patients with significant gastroduodenal lesions while potentially saving 47% of endoscopies [12].

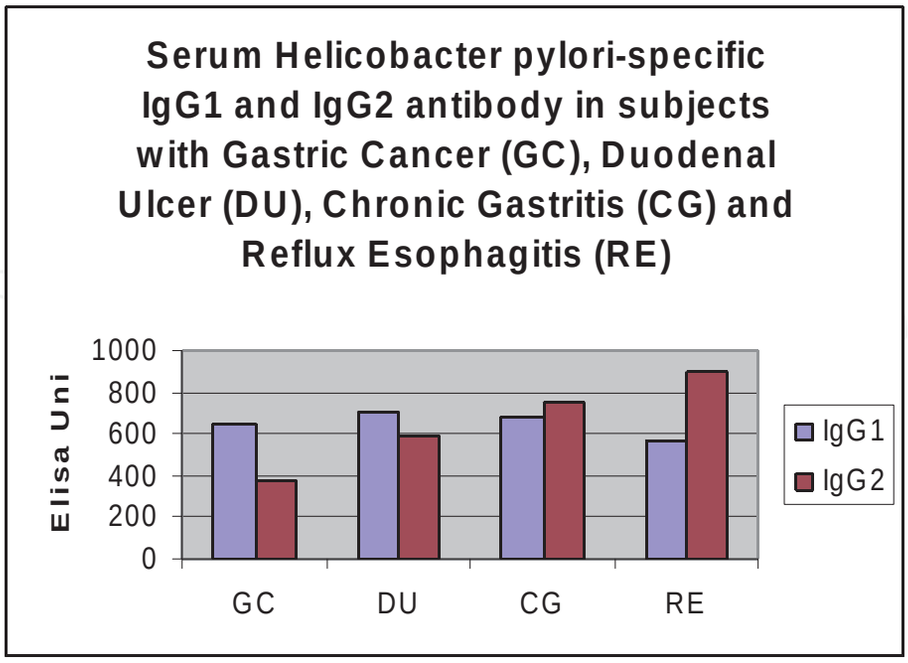


Figure 1. Serum Helicobacter pylori-specific IgG1 and IgG2 antibody in subjects with Gastric Cancer (GC), Duodenal Ulcer (DU), Chronic Gastritis (CG) and Reflux Esophagitis (RE)

2.2. Sensitivity and specificity of serological test

The concentration of serum IgG is reported to have sensitivity of 64%, specificity of 83.7 %, PPV (Positive Predictive Value) of 82%, NPV (Negative Predictive Value) of 66% and accuracy of 73.1% for the diagnosis of Hp infection [4]. For the same purpose, serum IgA has the following values: 72.0%, 65.9%, 72.0%, 64.4% and 69.8% respectively [4]. If the serological tests are considered together (when both test are positive or negative), some of these values could increase: the accuracy could be 80%, sensitivity 86.6%, specificity 74.2%, PPV 74.2 % and NPV 86.6%. In synthesis, the serological tests are efficient in the diagnosis of the presence or absence of Hp infection and when used simultaneously, they are more efficient in accuracy, sensitivity and negative predictive value than when used alone. (Table 1)

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
IgG alone	64	83.7	82	66	73.1
IgA alone	72	65.9	72	67.4	69.8
IgG + IgA (both positive or negative)	86.6	74.2	74.2	86.6	80

Modified from A. Locatelli et al. (2004)

Table 1. Sensitivity, Specificity, Positive Predictive Value (PPV), Negative Predictive Value(NPV), Accuracy of IgG and IgA detection in serum.

The detection of Hp IgA and IgG antibodies in serum is useful in distinguishing between infected and uninfected patients whereas the concentration of antibodies in duodenal fluid is not suitable at this purpose [13]

2.3. Advantages and disadvantages

Screening strategies, based on the serology used as marker of virulence, surely results to be very useful as reported above. The main advantage of serology is that it is a non-invasive and simple method for diagnosing *Hp* infections and for screening individuals at high risk to develop malignant disease. Furthermore, it reduces endoscopies taking also into account the patient's compliance. A drawback of using serology as predictive diagnostic marker of disease is that it could miss a proportion (even if irrelevant) of severe pathologies and underlying malignancy. However, in western countries, this is rare in patients less than 55 years of age presenting with dyspepsia in the absence of sinister symptoms [14].

3. Molecular markers

Knowing in advance if a *Hp* strain in a specific patient is virulent or not is vital for the approach that the clinician should have towards the infected individuals. In other words, the presence of virulence determinants (such as CagA, VacA, Hsp60 proteins) can address the gastroenterologists to a correct and suitable therapy. For this aim, strain typing could be generally useful in pre-endoscopy screening; for example endoscopy might be unnecessary in young dyspeptic patients without severe symptoms who are infected with non- virulent strains. It would be better not only to treat young dyspeptic patients infected with virulent strains without performing an endoscopy but also to treat patients likely to develop ulcers or gastric malignancy before those conditions arise.

In consequence of this, it would seem preferable to screen for and treat only strains which are known to cause disease. For this purpose, the serology towards the virulence determinants can be used instead of invasive endoscopy.

3.1. Vac–A and Cag–A

VacA serology is uncommon because there are some uncertainties about its interpretation owing to the mosaicism of antigens and to the variety of existing subtypes which are correlated to the different diseases (for example vacA s1 strains are more commonly associated with ulcer than vacA s1b strains or vacA s2). In this situation, the vacA genotype should be determined but that requires a gastric biopsy so vacA genotyping cannot be used in non-invasive screening strategies.CagA serology is more reliable than VacA serology due to the strong immunogenicity and the less variability of CagA protein respect to VacA.

CagA seropositivity reflects the presence of cagA gene together with the cag PAI (pathogenicity island). Some problems linked to CagA serology could occur. First of all, the infection with CagA+ strains is common so that treating CagA seropositive subjects might result in

unnecessary treatment even if it has been demonstrated [15] that people with CagA seropositive infection are at higher risk of ulcers or more severe pathologies than CagA-negative subjects.

A second problem concerns the fact that avoiding treatment for CagA-negative patients would lead to miss some infected individual patients who later develop malignancy.

Third the presence of CagA-negative strains may be rare in some populations depending on geographical area. Further, it would be advisable to know, in CagA-negative subjects, if their risk of developing more severe disease such as carcinoma is higher than in uninfected people. If any significant risk is confirmed between CagA-negative infected and uninfected individuals, the treatment of CagA-negative patients would be strongly recommended.

In synthesis, if there is evidence that treatment of CagA-positive patients reduces the possibility of subsequent *Hp*-related malignancy, CagA serology can be considered a viable test for selecting strains to treat [16, 17].The *Hp* infectious status is determined serologically using a commercially available enzyme-linked immunosorbent assay ELISA with a sensitivity and specificity of 96% and confirmed by Western blotting (WB).

3.2. Hsp60 (Heath schock protein 60)

Antibodies to Hsp60 have been suggested as markers of chronic inflammation so the detection of anti-Hsp60 covers a crucial role as serological marker of strain-virulence and may therefore be good predictors for the risk of vascular diseases as well as it has been reported for Chlamydia species [18]. High levels of anti-Hsp60 antibodies may constitute a marker and/or a concomitant pathogenic factor of these pathologies.

Lenzi C et al, 2006 [19] found an increased prevalence of CagA-positive Hp infection as well as increased levels of antibodies to Hsp60 in patients with CHD (Coronary Heart Disease) compared with controls. The accurate definition of this new risk factor may lead to novel strategies for the prevention of ischemic heart disease since simple procedures such as the detection of anti-Hsp60 may be a good predictor of ischemic illness.

Wick et al [20] demonstrated that the association between high levels of anti-Hsps60 antibodies and atherosclerotic vascular disease is due to an autoimmune reaction to endothelial cells that express high levels of Hsps in response to different stimuli such as free radicals, local infections, cytokines etc.

Antibodies to Hsp60 are determined by ELISA test using a commercially available human hsp60 (Sigma Che. Co., Milan, Italy) (19).

4. Multiplex PCR assay (Molecular screening)

The molecular markers of virulence, listed above, can be easily detected, other than by the evidence of antibodies towards them through the serology, also by multiplex assays based on PCR. Multiplex PCR assay is an advancement, compared to uniplex or single locus PCR, because it is suited to diagnose and specifically identify virulence *Hp* strains and their main virulence genes

cagA, cagE, cagT, vacA, hrgA. This method is able to genotype *Hp* isolates based on the main virulence genes analysis of cagA alleles as well as vacA is performed by polymerase chain reaction (PCR). The methodology for performing Multiplex PCR is reported by Tiwari *et al.* 2007 [17]. Briefly, samples in sterile phosphate bufferd saline after being vortexed, are boiled, cooled in ice and centrifuged. The supernatant is transferred to another tube where 1 μl of the template for amplification is added. Multiplex PCR is carried out in 25-μl volumes using DNA, *Taq* polymerase, oligonucleotide primers of all the selected genes, deoxynucleotide triphosphate and $MgCl_2$ in standard PCR buffer for 35 cycles.

PCR products are electrophoresed in agarose gel with ethidium bromide in a Tris-borate-EDTA buffer. Gel is visualized under UV transilluminator. Polymerase chain reaction products of each target genes are sequenced directly after purification.

The PCR products were inspected by eletrophoresis on 2% agarose gels. Reference strain *H. pylori* ATCC 49503 is used as a positive control whereas water for cell culture grade was used as negative control. [21].

This method results very useful in distinguishing five potential virulence genes also including the two subtypes of vacA signal region (s1 and s2). This new strategy, which not only predicts mere presence or absence of *Hp* infection but also gives information about its genetic heterogeneity, is highly recommended especially because it is a fast and reliable alternative to others methods and also can be employed even in highly contaminated samples. Different genotypes are reported to be correlated to various infection kind by Tiwari *et al.* 2007 [17].

In this study, they report the distribution of the above genes in the different pathologies (Table 2).

	Gastric carcinoma	Duodenal ulcer	Pre-pyloric ulcer	Peptic ulcer	GERD*	NUD**
	%	%	%	%	%	%
vacA s1	85	64	100	100	50	50
vacA s2	14	35	/	/	50	50
cagA	100	78	100	50	100	66
cagE	100	85	100	100	100	83
cagT	100	92	100	100	100	83
hrgA	100	100	100	100	100	100

Modified from S.K. Tiwari et al. (2007)

GERD* : Gastric oesophageal reflux disease; NUD**: Non-ulcer disease

Table 2. Distribution of major virulence genes of *Helicobacter pylori* in various diseases.

An important finding of this study is that hrgA gene results to have 100% prevalence among all disease groups irrespective of clinical category. This result differs from that obtained by Ando 2002 [22] who reported a more marked presence of hrgA in patients with cancer than in those with other pathologies. These discordant data can depend on different geographical areas considered in the two researches and on the need of examining a more large number of subjects. Higher prevalence of the genotype cagT +, hrgA +, cagA +, cagE + and vacAs1 + is found among patients with pre-pyloric ulcer (100%) and gastric carcinoma (85.7%) followed by duodenal ulcer subjects (60.7%). Overall, this genotype is present in 67% of the total subjects analysed with higher occurrence among those with ulceration and gastric carcinoma than among those with GERD (gastric oesophageal reflux disease) and NUD (non-ulcer disease). The genotype cagT +, hrgA +, cagA-, cagE + and vacAs2 subtype is least prevalent. The vacAs1 subtype is more correlated with the presence of cagA than the vacAs2 subtype and only 2.44% CagA-negative strains possess the vacAs1 allele. Then with reference to the clinical status, vacAs1 is prominent in patients with pre-pyloric ulcer (100%), gastric carcinoma (85%) and duodenal ulcer (64%).However, this study has been performed using gastric tissues (biopsies). Consequently it is an invasive method and cannot be used as a pre-endoscopy screening. The same authors in a previous attempt, had reported saliva as one of the effective non-invasive specimen not only for the detection of *Hp* infection but also for genotyping the strain infecting [23]. The 16S rRNA gene of *Hp* is a highly specific target for amplification, able to confirm *Hp* infection. Positive amplification of *Hp* specific DNA may be considered as a direct evidence of the presence of the pathogen. Non-invasive methods for the rapid diagnosis of *Hp* in salivary secretion of patients with various gastric diseases using 16S rRNA PCR analysis result to be very useful in pre-endoscopy screening thus showing comparable results with those obtained when biopsies are used (Table 3).Consequently saliva of infected persons serves as a reliable non-invasive alternative to detect the presence of *Hp* infection compared to currently diagnostic invasive tests. Tiwari *et al* [24] in another research also report salivary secretion as a sample suitable for detecting cag PAI (pathogenicity island) of infecting *Hp* correlating this with the disease status of the patients. Hence, analysis of complete cag PAI of *H. pylori* isolated from saliva would be of immense importance in standardizing saliva as a reliable non-invasive diagnostic specimen and also to evaluate the type of *Hp* infection. cagE and cagT are found in a larger proportion of the ulcer group than in the non-ulcer group [24,25].

	Symptomatic subjects		**Asymptomatic subjects**	
	(80)		(20)	
	N°	%	N°	%
Stomach biopsy	72	(90)	10	(50)
Saliva	70	(87.5)	12	(60)

Modified from S.K. Tiwari et al. (2005)

Table 3. Detection of *H. pylori* in biopsies and in salivary secretions by multiplex PCR.

5. Multiplex bead array assay and pre–endoscopy screening

A number of new methodologies and assays have been defined during the last years in order to have reliable, rapid, precise and cost-effective results for the management of many diseases. Furthermore, these methods include the use of non-invasive specimens such as serum and plasma being then a useful tool for pre-endoscopy screening. Multiplex bead array assays (MBAA) and Luminex X-map constitute an advancement in detecting contemporaneously biomarkers in plasma and serum. They result comparable to ELISA method and in addition have the advantage of revealing, independently and quantitatively, a large number of analytes using an automated 96-well plate format. These methods also permit the molecular study of genetic variables involved in virulence mechanisms of important bacterial strains.

The clinical applications of MBAA are reported in Table 4.

Application	Available kits*
Autoimmune	ASCA (h), β-2 Microglobulin (h,m) Centomere B (h)
Cancer markers	α-Fetoprotein (h), Cancer antigen 125 (h), Carcinoe
Cytokine	Aβ40 (h), Aβ42 (h), BDNF (h) DR-5 (h), EGF (h,m)
Gene expression	1L6R (h), ACTB (h), BAD (h), BAK1 (BAK) (h), BCL
Genotyping	FlexMAP (G), Mitochondrial DNA Screening (h)

* (h)= human, (m)= mouse

Modified from F.M. Elshai et al. (2006)

Table 4. Principal clinical applications of MBBA.

The most important application of this test is the quantitative detection of cytokines. The measurement of soluble cytokines and other analytes plays a pivotal role in Hp-related infections. In fact, in Hp diseases, a number of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF- α), interleukin-1β (IL-1β), IL-6, IL-8, IL-2, IL-24 etc, is on the basis of the host immune response and of the immunopathology of this microorganism. Practically multiplex assays rely upon the determination of soluble analytes in serum or plasma through the utilization of specific beads for each ligand with subsequent detection of the captured ligand by a second "reporter" antibody. Positive reaction is detected by the fluorescences where ELISA method uses enzyme amplification of a colorimetric substrate.

Protein microarray kits that use capture antibodies in a multiplex fashion similar to MBAA, are relatively new but they are not accepted as a "gold standard" for clinical use and may be of limited sensitivity [26].

Problems for the MBAA technique can arise for the multiplex nature of the test that can lead to cross-reactions and to anomalies in quantifying some analytes. Interferences can also occur in anti-cytokine antibodies which may cross-react whit other cytokines and other interfering

substances. Kits have been optimized to eliminate or minimize any artefact from multiplexing. Nevertheless the problem of interferences can exist.

Test ELISA has been considered as a "gold-standard" for the determination of the analytes in plasma and serum but MBAA test is comparable to it [27]. Even if these two tests have been correlated in many studies [26, 27], it can be difficult to evaluate the results because various investigators use different methods of comparison between MBAA and ELISA. Most of published studies [28,29] have shown good correlation and reproducibility between these two methodologies for the majority of cytokines tested even if the degree of correlation has varied widely. MBAA test has proven to be easy to perform, reliable, time saving and cost-effective so that its use in the clinical practice and in the research area is suggested. (27)

6. Luminex X–MAP technology

Among various MBAA tests that generally incorporate an automatic software able to evaluate the cytokine levels in the samples (plasma and serum), significantly reducing the complexity of the assay and requiring less user interaction, Luminex X-MAP technology plays an important role. It uses digital signal processing capable of classifying polystyrene beads (microspheres) dyed with distinct proportion of red and near-infrared fluorophores.

A spectral address for each bead population can be defined by these proportions. In this case, different detection reaction can be carried out simultaneously on various bead populations. Some recent applications with Luminex-based fluorescent microspheres include cytokine quantitation [30] and polymorphism genotyping [31]. In conclusion we can say that it is possible to measure, with these new methodologies, the level of important cytokines involved in *Hp* immunopathology. These results can make us know, through non-invasive methods, the pattern of cytokines involved in the infection which accounts for the disease status and the strain virulence.

7. Conclusions

The non-invasive tests as diagnostic tool in *Hp* infections of patients with various gastrointestinal disorders, are strongly important because they make the endoscopy unnecessary in different situations. The pre-endoscopy screening may be performed principally through serological markers (detection of different kinds of immunoglobulines) or through molecular markers (presence of CagA or Hsp60).

For CagA detection, serology has proved to be useful, being CagA protein a factor with good antigenic properties, easy and realiable to perform and prone to reveal the presence of Cag pathogenicity island [12]. Hsp60 is also a good antigen so that its detection can be performed through the appearance of specific antibodies against it.[32]

Strain typing could also be useful in pre-endoscopy screening: in fact the invasive gastroscopy could be avoided in young populations with non-ulcer dyspepsia and with non-alarming

symptoms. It might be even better to treat patients infected with virulent strains without performing an endoscopy. For these problems, the fact to know in advance if a *Hp* strain is virulent or not, could allow us to treat only isolates with proved aptitude to cause disease.

What we would suggest concerns the rapid and easy detection of virulent strains avoiding both invasive techniques and the consequences of a long-lasting untreated infection. The best approach for this is the new development of multiplex PCR assay considered an advancement over other PCR-based methods which could contribute to gain insights at the genotypic variability exhibited by this pathogen. Multiplex PCR assay by which the presence of various markers can be detected in a single reaction constitutes an important tool [17].

Other new methods such as new multiplex assays (Multiplex Bead Array Assays-MBAA) and Luminex-X map technology, constitute a considerable advancement for genotyping *Hp* thus using non-invasive samples as serum, plasma and salivary secretions [26, 27].

Further problems that should be more deeply examined concern the possible link that may exist between strains with more combinations of virulence determinants and antibiotic resistance that is known to be a crucial drawback in the disease treatment.

Author details

Maria Teresa Mascellino[1*], Alessandra Oliva[1] and Barbara Porowska[2]

*Address all correspondence to: mariateresa.mascellino@uniroma1.it

1 Department of Public Health and Infectious Diseases, Sapienza University, Rome, Italy

2 Department of General and Specialistic Surgery, "Paride Stefanini", Sapienza University, Rome, Italy

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