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(57) Abstract

A colourimetric method which is advantageously applicable to a culture medium of the liquid type, and which is further suitable for the evaluation in vitro of sensitivity and resistance of Helicobacter pylori to antimicrobial pharmaceuticals, is based on the colourimetric detection of bacterial growth of Helicobacter pylori stemming from an increase in bacterial urease concentration, said colour variation being made possible by a pH colour indicator injected into the culture medium. A colourimetric kit for the assessment of Helicobacter pylori's sensitivity and/or resistance to antimicrobial pharmaceuticals, said assessment being carried out with the naked eye and/or by spectrophotometric means, comprises: a) a plurality of microwells and/or vessels made of transparent material and containing predetermined antimicrobial substances and at suitably predetermined concentrations, said vessels being advantageously assembled in printed modules and further being packed in a sterile manner; b) a culture medium for Helicobacter pylori containing urea and a colour pH indicator.
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COLORIMETRIC ASSESSMENT OF THE SENSITIVITY OF HELICOBACTER PYLORI TO ANTIMICROBIAL SUBSTANCES

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TECHNICAL FIELD

The present invention relates to a colourimetric method suitable for the evaluation of the sensitivity of Helicobacter pylori bacteria to single antimicrobial substances, said method being applicable to a liquid culture medium.

Moreover, a further object of the present invention is that of proposing a suitable kit for an easy and efficaceous use of the above method.

The present invention finds a particularly advantageous application in the field of diagnostics at the microbiology laboratory level, as it renders the Helicobacter pylori sensitivity test to antibiotics extremely simple, and according to a preferred form of embodiment of the present invention, it further makes it possible to entirely carry it out automatically.

STATE OF THE ART

Helicobacter pylori is a urease positive bacterium that infects human stomach and is accountable for as the main aetiologic agent of gastroduodenal diseases.

Regarding the above point, numerous studies conducted at a global level have demonstrated that recovery of most of the patients affected by peptic ulcer is made possible by eradicating the infection.

This means, in other words, that ulcer is nowadays to be considered an infective disease and treatment against it should be first carried out by the employment of antimicrobial pharmaceuticals.

Until now, there have been proposed several therapeutic strategies aimed at the eradication of the infection and the results obtained highlight that success is most likely thanks to the combination of more than one pharmaceutical, that is generally between two and five of them.

Those that were studied the most are antibiotics like amoxycillin, clarythromycin, tetracycline; chemotherapeutics like metronidazole; and antiacids like omeprazole, lansoprazole, ranitidine.

A first important aspect of the above operational method is the fact that the above mentioned pharmaceuticals must be administered over a period of time generally
ranging between few days and few weeks.

Hence it is of paramount importance to accurately choose among the above pharmaceuticals in order for them to be really effective in eliminating the infection without treating the patient with completely useless substances even for considerable periods of time.

The chances of success of this type of therapy depend on a number of concurring factors, among which are the combination of the pharmaceuticals employed, the length of the treatment and the methodicity which the patient carries it out with until the end.

In order for it to be possible to attain satisfactory results with the treatment of a patient affected by an infection from *Helicobacter pylori*, it is necessary to be aware of the resistance built by *Helicobacter pylori* to the specific antimicrobial pharmaceuticals that are going to be administered.

It is widely known that *Helicobacter pylori* is always and constantly sensitive to some types of antibiotics, among which for example amoxyceillin. It is further known on the other hand that other antibiotics like for example metronidazole and chlarythromycin exhibit a very variable biological activity towards it, although they are meant to be molecules of choice for the eradication of the infection deriving from it.

Furthermore, the effects they have differ depending on the patients that are subjected to the treatments and on their specific geographical areas of origin.

Several studies conducted in the field so far highlighted how the chance of success of the therapeutic protocols currently available and employed for the elimination of the infection determiningly depend on the sensitivity of the infecting strain to metronidazole and chlarythromycin.

Hence before starting whatever type of pharmaceutically based treatment of a patient affected by an infection caused by *Helicobacter pylori*, the infecting strain should be isolated by culture and tested *in vitro* in order to assess its sensitivity to antibiotics so as to rule out the employment of ineffective pharmaceuticals and therefore be able to immediately devise the most suitable therapy whose features derive from those of the specific case under treatment.

Such an operational procedure which is suitable for carrying out an evaluation of the sensitivity of the infecting strain to antibiotic pharmaceuticals, yields to an antibiogram as its final result; that is the reason why it is never resorted to as a starting
point in the choice of the specific therapy to prescribe, but it is often conducted at later stages, that is in patients for whom a first or second tentative therapy did not yield to any results.

The scanty use made of antibiograms should be accounted for by a plurality of factors, among which that Helicobacter pylori bacteria are cultured with a certain difficulty, that it has a low proliferation rate and that the operational methods nowadays available to technicians are cumbersome and relatively costly.

According to the techniques traditionally employed, antibiograms are carried out on solid substrates by means of plates whereon the bacteria to test have been implanted.

A further operational method is moreover represented by the E-test® that is basically a quantitative variation of the plate diffusion test outlined above.

It is further known and detailedly described in science journals in the field that Helicobacter pylori is capable of producing very high amounts of urease, and such a feature that it has can be exploited as a suitable parameter for quantitatively assessing the growth of said bacterium in a given culture substrate.

Methods known so far are based on the determination of urease activity and in these, said parameter is considered uniquely in order to diagnose the presence of Helicobacter pylori in a sample of biological material.

In such cases therefore, the aim is exclusively diagnostic and it is centred on verifying the presence or else of the bacterium.

Regarding that, US Pat. No A-4,748,113 discloses a method of the colourimetric type which is suitable for detecting urease, aiming at determining the presence of Helicobacter pylori in a gastric mucose sample.

It is known in fact that as the proliferation of Helicobacter pylori causes an increase in the production of the urease enzyme, it also yields to a remarkable increase of pH in the culture medium which is due to the fact that the bacterium hydrolyses urea to yield carbon dioxide and ammonium ions, which are well known for their alkalinity.

The increase in the alkalinity of the medium yields to a colour change and it is therefore an expression of bacterial growth.

According to what disclosed in US Pat. No 4,748,113 which was already mentioned above, a sample of gastric mucose is placed in an aqueous solution containing urea at a concentration ranging between 10 and 40 g/l, a coloured pH
indicator, preferably Phenol red, and a bactericide geared towards inhibiting the
growth of other urease positive bacteria.

The above document discloses therefore a method of the colourimetric type
which is exclusively suitable for diagnosing the presence of *Helicobacter pylori* in the
tested sample, said presence being shown by a change in colour of the colour indicator
employed following an increase in urease production as this is secreted by *Helicobacter pylori*.

Document US-A-5,498,528 discloses a colourimetry based method which stems from urease production for diagnosing the presence of *Helicobacter pylori* in a
biological sample, particularly saliva.

In this last document instead, the sample is remarkably enriched with
*Helicobacter pylori*, by incubation in a specific culture medium which has been
suitably chosen on account of its selectivity towards *Helicobacter pylori*.

The existence of a microdilution test is further known, said test being suitable
for use on a culture sample of the liquid type and for the determination of the
responsivity of *Helicobacter pylori* to antibiotics. Diagnostic parameter of this test is
the turbidity of the sample.

That method consists in fact in the dilution of the sample by placing it on
multiwell microplates and in the successive measurement of the turbidity of the
solution which is used as an index of bacterial growth as it took place within the
sample contained in said wells.

That test does not make it possible to give a reliable assessment of the
sensitivity of *Helicobacter pylori* to the several antibiotics that were tested, as the
turbidity differences that were obtained had necessarily to be measured and estimated
by dint of apparati which are suitable for reading and making quantitative evaluations
of the turbidity index that has been detected.

Furthermore it is known to the skilled in the art that an analysis as it is carried
out on a liquid culture medium whereon to execute a progressive dilution of the
antibiotic bound to be tested surely bears advantages as compared with the systems
which are applicable to solid culture substrates.

Indeed in a liquid medium it is possible to quantitatively ascertain the growing
potential by exactly knowing the concentration of the antimicrobial bound to be tested,
object which cannot be as easily accomplished in a solid substrate wherein a diffusion
in the agar gel is observed.

In such a case in fact, there are numerous variables to take in consideration such as for example the diffusion rate of the antibiotics, the entity of the bacterial inoculate and the bacterial growth rate.

It is an object of the present invention therefore to propose a method for the determination of the sensitivity of *Helicobacter pylori* to certain pharmaceuticals, said method having the same advantageous features of the methods which are known to be applicable to a liquid medium, such as the accuracy of the results and method standardisation, also bearing enhanced features as regards ease of handling, rapidity and cost effectiveness with respect to the analyses that are traditionally carried out on a substrate of the solid type.

**DETAILED DESCRIPTION OF THE INVENTION**

Given the remarkable spread of the infection from *Helicobacter pylori* in the world population and the consequent remarkable relevance acquired by the need to devise methods which are effective in contrasting it, a new method was optimised which is capable of evaluating the sensitivity of *Helicobacter pylori* to antibiotics in a simple and speedy fashion, so being easily applicable to routine laboratory practice.

The present invention therefore relates to a colourimetric type method which was devised in order to be applied to a culture medium of the liquid type, said method being suitable for the determination of *Helicobacter pylori*’s sensitivity to the antimicrobial substances that are meant to be tested.

According to the present invention in fact, colour change towards alkalinity, in other words towards bacterial growth, as described above, is an index of resistance to antibiotics, whereas a scarce colour change, or the absence of it, is a clear evidence that an inhibition of bacterial growth is taking place, therefore that there is a sensitivity to antibiotics.

Moreover the present invention relates to a kit by which said method can be advantageously and efficaciously applied.

According to the invention, one or more *Helicobacter pylori* colonies are suspended in a predetermined amount of a culture medium of the liquid type, after their identification has been made in a primary culture made up starting from a sample of human gastric mucose.

To said suspension a pH colour indicator such as phenol red and urea as a
substrate are added.

In these conditions, the culture substrate has acquired a yellow or yellow
orange colour, since its initial pH ranges between 4.2 and 6.

The suspension is then scattered among the plastic material microwells whose
size is the same as that generally employed for immunoenzyme tests.

A predetermined number of said microwells contains different types and
amounts of the antimicrobial substances to be tested, whereas other microwells are not
filled with any antimicrobial substance to be tested as they have to uniquely supply a
reference parameter.

Once the samples have been prepared, the wells are subjected to
microaerophilic incubation at a 35-37°C temperature and they are inspected after 16,
24 or 48 hours.

According to a first form of embodiment of the present invention, the
inspection is an examination with the naked eye of the colour acquired by the
microwells.

The growth of *Helicobacter pylori* in one or more wells is shown by a change
of their colour, that turns to red or red-blue from yellow or yellow-orange.

The colour change of the indicator occurs as a consequence of the pH increase
within the culture medium that coincides with a substantial and remarkable bacterial
growth.

On the grounds of the methodology described so far, if a well filled with a
given antibiotics at a given concentration has shown a colour change, this means that
the *Helicobacter pylori* isolate under test is to be considered resistant to that specific
antibiotics and to that specific concentration of the antibiotics used.

Conversely, if the well does not show any colour change and has a colour
which is the same or similar to that of the substrate where no bacterial growth has
taken place, the isolate is bound to be rated as sensitive to that antibiotics and that
concentration of the antibiotics which is being tested.

According to the present invention therefore, the microbial suspension under
exam is scattered among wells containing different amounts of more than one
antibiotics to be tested and it will be thus possible to determine the minimum
concentration of antibiotics that is necessary to inhibit the growth of *Helicobacter
pylori* (MIC).
According to a further form of embodiment of the present invention, the colour change of the culture medium is determined by a spectrophotometric reading of the wells.

More particularly, measurements are carried out by an immunoenzymatic dosage microplate detector, and they are advantageously elaborated by a suitable electronic calculator.

Such a solution obviously offers undoubted advantages as compared with naked eye reading; these are for example first of all a more precise and completely automated reporting of the results obtained, and the possibility to carry out a high number of determinations in a speedy and handy manner.

The employment of an electronic calculator further makes it possible to carry out the automatic interpolation of the optical densities measured in the single wells containing the antibiotics at different concentrations, and to work out the MIC with a high degree of precision.

By dint of a suitable software it is also possible to automatise reporting and printing the results on paper.

According to a further particular form of embodiment of the present invention, the pH colour indicator used is phenol red and measurements are made using a 545 nm wavelength.

It is clear that the present invention does not result to be limited to the utilisation of microwells as described above, but whatever other type of vessel is employable, as well as there results to be possible to carry out the measurements of which above, using other types of spectrophotometric equipment of the automatic type.

A further object of the present invention is that of proposing a suitably equipped kit so as to make it possible to carry out the method detailed above.

Said kit comprises:

a) a plurality of vessels made of transparent material containing different antimicrobial substances in suitably predetermined amounts, said vessels being advantageously assembled in printed modules and further being packed in a sterile environment.

b) A culture medium for *Helicobacter pylori* containing urea and a pH coloured indicator.

According to the present invention, the culture medium can also be supplied
ready for use or in the form of a lyophile preparation to be resuspended in sterile distilled water.

It is worth emphasising how the present invention provides a method with a kit relative thereto, based on the urease activity of *Helicobacter pylori*, said parameter being measured as a growth index of said bacterium in order to test its sensitivity to antibiotics in a culture medium of the liquid type, therefore not aiming at anything diagnostic, in order to evaluate the occurrence or lack thereof of *Helicobacter pylori* in a given culture medium, as it was mentioned in the state of the art outlined above.

**DESCRIPTION OF SOME FORMS OF EMBODIMENT**

The *Helicobacter pylori* isolate to test is generally obtained from a culture, for example a blood agar plate previously inoculated with gastric material excised from a patient during an endoscopic exam.

The identification of *Helicobacter pylori* in a culture is accomplished by the well known methods based on the determination of the morphological features (curved or spiralled shape), Gram negativity, biochemical features (catalase, urease and phosphatase positivity; hyppurate hydrolysis and nitrate reduction negativity).

The bacterium can further be identified by resorting to the immunologic method which is disclosed in Italian patent application N. VR96A000109 in the name of the Applicant, patent application wherein there is found to be claimed a hybridome (2H11), identified thanks to the ECACC deposit N. 96122033, which is capable of producing a monoclonal antibody (Helix-1) that immunoreacts with a *Helicobacter pylori* with an apparent molecular weight of 16 ± 2 kDa.

In said patent application the Applicant further claims an antigen with an apparent molecular weight of 16 ± 2 kDa of *Helicobacter pylori* as an indicator of the occurrence of *Helicobacter pylori* and an immunological type method which is suitable for the detection of said antigen.

According to that method, the following phases can be identified:

a) supplying a generic sample for analysis as a solid, liquid or in suspension;

b) supplying a monoclonal and/or polyclonal antibody in a biologically active form which immunoreacts with the antibody having an apparent molecular weight of 16 ± 2 kDa of *Helicobacter pylori*;
c) mixing the sample with the antibody of step b) in order to obtain an immunoreaction mixture;

d) keeping the mixture in the suitable conditions for favouring the reaction between antigen and antibody for a period of time which ranges between few minutes and some hours;

e) making sure that a reaction between antigen and antibody has already taken place, and determining whether within the sample under exam there is found to occur the antigen with an apparent molecular weight of 16 ± 2 kDa corresponding to *Helicobacter pylori*, and, possibly, determining the concentration thereof.

Once the occurrence of *Helicobacter pylori* has been detected according to one of the methods detailed above, according to the present invention, one or more colonies of *Helicobacter pylori* are resuspended in a liquid type culture medium test tube.

The culture media of the liquid type that can be employed for the growth of *Helicobacter pylori* are well known to the skilled in the art, and anyone of them can be efficaciously be resorted in order to carry out the test according to the present invention.

According to a particular form of embodiment of the present invention, a preferred culture medium is made of Brucella broth additioned with bovine foetal serum and Isovitalex®.

Urea, at a concentration ranging between 1 g/l and 4 g/l, preferably between 2 and 3 g/l, is added to the liquid culture medium, said urea representing the enzyme substrate for bacterial urease.

The amount of urea given above is sufficient to produce an appreciable increase in pH whenever there is the occurrence of growth of *Helicobacter pylori*, and it is not of any obstacle to the growth of the bacterium itself.

It can be highlighted that these urea concentration values are remarkably lower than those in the 20 g/l range, used in the tests described in the documents belonging to the state of the art described above.

In so far as the employment of the colour pH indicator is concerned, according to a preferred form of embodiment of the present invention, the culture medium is additioned with phenol red to obtain a concentration which is higher than 0.001%
(g/100 ml), more preferably between 0.005% and 0.02%.

In that case it is also possible to verify that the pH colour indicator concentration is remarkably different from that used in the documents mentioned above, said concentration reaching values which are even 20 times higher than those given in the above mentioned publications.

Such a high pH indicator concentration makes it possible to carry out a better measurement of the optical densities both in case the assessment is carried out at a glance, and in case spectrophotometry is resorted to.

It is clear that even other pH indicators can be used so long as in the culture medium used they change colour at a pH range between 5.5 and 9.0; among these are for example bromothymol blue, para-nitrophenol, neutral red, cyanin, cresol red, metacresol purple and thymol blue.

Generally the culture medium where the test is carried out according to the invention has a pH which is preferably in the range between 4.2 and 6.0, more preferably between 5.0 and 5.5.

It is clear that the medium has a pH that can suitably be adjusted by the addition of acids or bases.

For instance, 1 litre of Brucella broth culture medium can be taken from pH 7.0 (red coloured medium), to pH 5.5 (yellow coloured medium) by the addition of about 8 ml of 2N HCl.

According to a preferred form of embodiment of the present invention, the microwells used are made of polystyrene, and they are located in such a way to form strips each with 8 microwells (said strips being located in suitable support frames), and they are commonly used for traditional immunoenzyme laboratory assays.

16 well strips are also employable (2 X 8) or entire 96 well plates which are normally employed in cell culture immunoenzyme assays.

It is however evident that the test according to the present invention can be carried out in any other type of sterile plates, or in any other vessel such as a test tube or whatever vessel made of plastic material, both rigid and flexible.

At the beginning of the test, each of the dry and sterile microwells, which are transparent looking, has a specific antibiotics present in a predetermined amount, sticking to its inside surface, said antibiotics and said amounts being different between successive microwells.
According to the operational process of the present invention, one or more microwells do not contain any antimicrobial substance.

Said microwells have in fact the role of providing a test positive control, that is no growth inhibition whatsoever, as described below within the present specification.

Likewise, one or more microwells result to have been prepared so as to contain a mixture of antimicrobial substances which are consistently lethal against Helicobacter pylori, said microwells aiming at providing a test negative control, that is the greatest growth inhibition, as it will be made clearer below.

The addition of the suspension of Helicobacter pylori in a liquid medium to the microwell gets the antibiotics to dissolve at the desired concentration.

For example, if 0.5 µg metronidazole is present in a microwell, sticking onto the inner surface of the well itself, when this is filled with 100 µl of culture medium, metronidazole is dissolved yielding a concentration equalling or slightly below 5 µg/ml.

The amount of antimicrobial substances contained in the microwells can be worked out in such a way as to obtain final concentrations ranging between 0.0001 and 50 µg/ml.

According to a form of embodiment of the present invention, by the assay, the effect of the antimicrobial substance to be tested on Helicobacter pylori is analysed, at the concentration that the skilled in the art believe to be the end concentration that defines the degree of sensitivity and/or resistance of Helicobacter pylori to that given antimicrobial substance.

That end concentration defined above is called “breakpoint” concentration.

The antimicrobial substance of choice is then assayed even at higher or lower concentrations than the “breakpoint” concentration.

For instance, if the “breakpoint” concentration for metronidazole is found to be 8 µg/ml, this will be in the microwells in such amounts as to obtain a final concentration in the medium equalling 1, 2, 4, 8, 16, 32 µg/ml.

Once the wells have been inoculated with the suspension bound to be tested, they are made to undergo incubation in a microaerobic or anaerobic atmosphere.
This incubation can also be carried out in a specific incubator for cell cultures and with an atmosphere containing 5-10% CO₂ or it can be carried out in an ambient atmosphere if suitable liquid media are employed that make it possible for *Helicobacter pylori* to grow outside the incubator itself.

After a time period ranging between 8 and 48 hours, the wells are inspected in order to check the colour of the culture medium, and according to different operational embodiments of the present invention, the inspection can be carried out both at a glance and by an instrumental examination of the spectrophotometric type.

In both cases, the evaluation is anyhow carried out by comparison of the colour of the reference microwell, that is containing a predetermined concentration of a predetermined antibiotics, with the colour of the positive and negative control wells.

In the positive control wells, that is in those wells that do not contain any antimicrobial substance, *Helicobacter pylori* proliferates without finding any hindrance, in so doing causing the colour change of the medium yielded to by urease production.

Therefore, the positive control colour is the evidence of a 100% growth, according to the methodology so far described.

Conversely, in the negative control wells, that is in those wells where *Helicobacter pylori* has not had a chance to proliferate because of the presence of a suitable lethal mixture of antimicrobial substances, the urease present therein is that corresponding to that contained in the initial bacterial inoculate.

This means that in this case the urease enzyme concentration is much lower than that present in the microwell where the bacterial growth has taken place and, consequently, colour change within that well is bound to be minimal or even absent.

The negative control colour is therefore an index of a 0% growth, according to the methodology described so far.

On the basis of the values obtained in the extreme situations given above, whenever the colour obtained in a given well is the same as that of the positive control, it is possible to state that in that given well there has occurred a bacterial growth and the *Helicobacter pylori* strain assayed can be defined as being resistant to that antibiotics under test and to that given concentration employed of it.

On the contrary, whenever the colour obtained in a given well is the same as that of the negative control, this means that in that given well there has taken place no
bacterial growth, therefore the *Helicobacter pylori* strain under assay can be defined as being sensitive to that given antibiotics at that given concentration.

Intermediate concentrations are instead an index of a partial growth inhibition.

As specified above, the inhibiting concentration having been defined as MIC, that is the antimicrobial substance minimum concentration which causes inhibition of over 99% of the bacterial population, the operational method according to the present invention, comprises the assessment of the *Helicobacter pylori* strain under consideration at different concentrations of antimicrobial substance, therefore providing minimum inhibiting concentration values for the strain under assay.

It is clear that different strains of *Helicobacter pylori* have different MIC values, with respect to the same type of antimicrobial substance.

This means that, once a suitable antibiotics has been chosen, if a certain strain of *Helicobacter pylori* has an MIC that is below "breakpoint" concentration, then that strain is considered to be sensitive to that specific antibiotics; conversely, if its MIC is higher than "breakpoint" concentration, then that strain is bound to be considered to be resistant to that given antibiotics.

On analysing the lots of wells prepared at ever increasing concentrations of a given antimicrobial substance, the following conditions may arise:

a) all the wells have a colour that is the same as that of the positive control, therefore a strain under consideration is resistant and MIC results to be higher than the antibiotics maximum concentration under assay;

b) wells show a hue that is in the range between the two colours of the positive and negative controls: in fact as the antimicrobial concentration increases, the colour of the lots of wells moves towards the negative control colour, therefore it is necessary to find the first well where the colouring seen is the same as or very similar to that of the negative control. The antimicrobial concentration relative to that given well corresponds to MIC;

c) all the wells have a colour that is the same as that of the negative control, therefore the strain under consideration is sensitive and MIC results to be lower than the minimum concentration of the antibiotics which is being assayed.

As it was mentioned above, whenever phenol red is used as a pH indicator, measurements are preferably carried out using a 545 nm electromagnetic radiation as at this wavelength optical density differences on reading between the positive control
(red) and the negative control (yellow) are higher.

According to a preferred form of embodiment of the present invention, measurements are made by availing of a microplate detector that offers numerous advantages when compared with simple naked eye measurements.

For example, a first advantage is given by the greater sensitivity attained by spectrophotometric measurements, which is a particularly important aspect when it turns out to be necessary to quantify MIC.

For instance, 2 wells respectively containing 4 and 8 μg of antibiotics for each ml of medium may seem to have the same colour as that of the negative control well when looked at with the naked eye, and MIC would then accountably be given a value approximately equalling 4 μg/ml.

Actually, by dint of spectrophotometry it could be possible to highlight that the well containing 4 μg antibiotics has an optical density that is slightly higher than that of the negative control, which is an index of partial growth, whereas the well containing 8 μg/ml of antibiotics has an optical density that equals that of the negative control.

In such a case then, MIC would be quantified as equal to or greater than 8 μg/ml.

An optimum medium though minimises the possibility of errors, even if a simple assessment with the naked eye is carried out.

A further advantage offered by an instrumental reading, as it was emphasised above, is the possibility there is to automatise reporting and to easily carry out a great number of determinations.

**EXAMPLE 1**

Three patients were subjected to gastroscopy and a sample of gastric mucose
(or stomach lining), excised from each patient was inoculated onto a blood agar plate additioned with antibiotics suitable for ensuring a selective growth of *Helicobacter pylori*.

The three plates are then incubated in microaerophiles in a jar at 37° C for three days.

After that incubation period has elapsed, from an examination of the plates, there appear to be evident numerous little translucent colonies and one or more colonies are picked from each plate by means of a sterile loop.

These colonies are then scrutinised with Gram colouring and with urease,
catalase and oxidase biochemical tests.

On the basis of the above tests, the colonies are identified as *Helicobacter pylori* colonies and after that the test for sensitivity to antimicrobials is carried out according to the present invention.

In the present example the three *Helicobacter pylori* isolates were assayed in order to test their sensitivity to metronidazole and chlorthymycin.

The culture medium used was Brucella broth additioned with 2 mg/ml urea, 0.01% phenol red, 2% bovine foetal serum, 1% ISOvitalex®, and then taken to pH 5.5 by adding 2N HCl.

The medium was then sterilised by filtration after which it had a yellow colour. The process heretofore described was then carried out for each and everyone of the three samples obtained from the three patients of which above.

By dint of a sterile loop 2-10 colonies of *Helicobacter pylori* were picked from the plate and they were further added to 200 μl of medium.

The suspension was then further diluted by introducing 20 μl of said suspension in 5 ml of medium inside a sterile test tube.

Using a sterile Pasteur pipette the suspension that was obtained was swirled and 4 drops of it were placed in each of the 16 wells forming two strips of 8 sterilised wells each, used for immunoenzyme determinations, said microwells containing the antimicrobials to test detailed above.

The amounts of the two antimicrobials chosen were positioned in the microwells so as to yield for the two strips each with eight microwells, to the final situation shown in Table I, after the addition of a microbial suspension.

As it can be verified in Table I, wells A1 and A2 do not contain any antimicrobial substance and they can therefore be taken to be the positive control, that is the situation when an undisturbed growth of the bacterium takes place.

H1 and H2 wells instead both contain a mixture of amoxicillin and gentamycin, each with a 5 μg/ml final concentration, and they can be taken as the so called negative control, that is a total inhibition of bacterial growth.

The two antimicrobial substances to test have been supplied in serial concentrations according to a log 2 progression, as detailed in Table I.

After that, the six strips, two for each of the three samples under test, were placed in a suitable frame, and they were then covered with a sterile 96 well plate lid,
and incubated for a 16-48 hour period in an incubator set at 37°C and with a 5% CO₂ concentration.

The plate was then scrutinised for the first time after 16 hours.

Measurements on the plate containing the six strips was made with an ELISA microplate detector at a 545 nm wavelength and the optical density values obtained using the spectrophotometric reading of the negative control well are given in Table II.

On the basis of the measurements that were made the minimum antimicrobial concentration which gave the same or very similar values to that of the negative product was identified.

From this concentration it was then possible to make assessments on the MIC as it was exemplified in Table III where the results of the tests that were carried out are shown; it should be borne in mind that the highest MIC values necessary to consider the Helicobacter pylori isolate sensitive to metronidazole and clarythromycin are respectively 8 and 2 μg/ml.

**EXAMPLE II**

In Example II an operational strategy similar to what was done in Example I was followed, except for the difference that the assessment of the results was carried out with the naked eye.

On the basis of such an operational method, the colour obtained in each well was observed, and for each antimicrobial substance used, the lowest concentration that was capable of giving a colour that was the same as or very similar to that of the negative control was identified.

The results of the level of sensitivity and/or resistance of the three Helicobacter pylori isolates with respect to the two antimicrobials under scrutiny gave the same results as those obtained in Example I.

**EXAMPLE III**

Three patients were subjected to gastroscopy and a sample of gastric mucose (otherwise known as stomach lining) picked from each patient, was inoculated onto a blood agar plate additioned with antibiotics suitable for guaranteeing a selective growth of Helicobacter pylori.

The three plates were then inoculated in a microaerophile jar at 37°C for three days: from an assay of the plates, there resulted to be evident a number of small translucent colonies, and one or more colonies were picked from each plate by means
of a sterile loop.

The identification of the colonies was conducted using the immunological method which is the object of Italian patent application n° VR96A000109 in the name of the Applicant on the basis of which the colonies were identified as *Helicobacter pylori*.

The test for the assessment of sensitivity towards antimicrobials was carried out as described in examples I and II.
### Table I

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<td>POSITIVE CONTROL (growth)</td>
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<td>clarythromycin 0.25µg/ml</td>
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<td>C</td>
<td>2µg/ml metronidazole</td>
<td>clarythromycin 0.5µg/ml</td>
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<td>4µg/ml metronidazole</td>
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<tr>
<td>E</td>
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<td>Clarithromycin</td>
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CLAIMS

1. Colourimetric method advantageously applicable to a culture medium of the liquid type, suitable for the evaluation in vitro of sensitivity and resistance of Helicobacter pylori to antimicrobial pharmaceuticals, said method colourimetrically detecting bacterial growth of Helicobacter pylori on the basis of an increase in bacterial urease concentration, said colour variation being made possible by a pH colour indicator injected into the culture medium.

2. Colourimetric method according to claim 1, characterised by the fact that the colour change towards alkalinity, that is towards bacterial growth, is an index of resistance towards antibiotics, whereas a scarce and/or absent colour change is an index of inhibition of bacterial growth and of sensitivity.

3. Colourimetric method according to either claim 1 or 2, characterised in that it comprises the following steps:
   a) picking one or more colonies of Helicobacter pylori from a culture or a subculture advantageously isolated from an excision of gastric mucose;
   b) suspending the colonies picked in a liquid culture medium containing urea and a suitable pH coloured indicator;
   c) placing suitable amounts of the suspension obtained in microwells and/or vessels with the antimicrobial substances at the different concentrations to test;
   d) incubating for a predetermined period of time, for instance for a maximum 72 hours at a predetermined temperature, for instance between 35°C and 37°C;
   e) assessing the colour of the suspension contained in the microwells and/or vessels in order to define the resistance and/or sensitivity of Helicobacter pylori towards the antimicrobial substances used and define the minimum concentration of antimicrobial substance necessary to inhibit bacterial growth (MIC).

4. Colourimetric method according to anyone of the preceding claims, characterised in that the assessment of the colour change that takes place in the suspension contained in the microwells and/or vessels is made with the naked eye and/or by spectrophotometric means.
5. Colourimetric method according to anyone of the preceding claims, characterised in that the pH colour indicator is present in the culture medium at a concentration which is higher than 0.001% (g/100 ml).

6. Colourimetric method according to anyone of the preceding claims, characterised in that the pH indicator is phenol red.

7. Colourimetric method according to claim 3, characterised in that the urea present in the culture medium has a concentration ranging between 1 and 4 g/l.

8. Colourimetric kit for the assessment of *Helicobacter pylori*’s sensitivity and/or resistance to antimicrobial pharmaceuticals, said assessment being carried out with the naked eye and/or by spectrophotometric means, using the method according to anyone of the preceding claims, characterised in that said kit comprises:
   a) a plurality of microwells and/or vessels made of transparent material and containing predetermined antimicrobial substances and at suitably predetermined concentrations, said vessels being advantageously assembled in printed modules and further being packed in a sterile manner;
   b) a culture medium for *Helicobacter pylori* containing urea and a colour pH indicator.

9. Colourimetric kit according to claim 8, characterised in that the microwells are assembled and/or assemblable in plates having a capacity equalling that necessary for housing 96 of said wells.

10. Colourimetric method according anyone of the claims between 1 and 7, characterised in that *Helicobacter pylori* is detected by means of a hybridoma (ECACC deposit N. 96122033) that produces a monoclonal antibody (Helix-1), said monoclonal antibody immunoreacting with a *Helicobacter pylori* having an apparent molecular weight equalling 16±2 kDa.
A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/18 C12Q1/58 G01N33/62

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q G01N C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<th>Category</th>
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<tr>
<td>P.X</td>
<td><strong>MIRSHAHI F; FOWLER G; PATEL A; SHAW G:</strong> &quot;Omeprazole may exert both a bacteriostatic and a bacteriocidal effect on the growth of Helicobacter pylori (NCTC 11637) in vitro by inhibiting bacterial urease activity&quot; JOURNAL OF CLINICAL PATHOLOGY, vol. 51, no. 3, March 1998, pages 220-224, XP002103046 see the whole document *<strong>-/-</strong></td>
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Further documents are listed in the continuation of box C. Patent family members are listed in annex.

Date of the actual completion of the international search | Date of mailing of the international search report
--- | ---
18 May 1999 | 04/06/1999

Name and mailing address of the ISA
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax. 31 851 epo nl, Fax: (+31-70) 340-3016

Authorized officer
Hart-Davis, J
### INTERNATIONAL SEARCH REPORT

#### C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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<td>VASQUEZ, A.; VALDEZ, Y.; GILMAN, R. H.; MCDONALD, J. J.; WESTBLOM, &quot;Metronidazole and clarithromycin resistance in Helicobacter pylori determined by measuring MICs of antimicrobial agents in color indicator egg yolk agar in a miniwell format&quot; JOURNAL OF CLINICAL MICROBIOLOGY, vol. 34, no. 5, 1996, pages 1232-1234, XP002082359 see the whole document</td>
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<td>US 5 439 801 A (JACKSON FRANK W) 8 August 1995 see column 5, line 23 - line 33; claim 6</td>
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<td>Y</td>
<td>US 5 498 528 A (KING WING) 12 March 1996 cited in the application see claims 11,14</td>
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<td>US 4 748 113 A (MARSHALL BARRY J) 31 May 1988 cited in the application see examples 1,2</td>
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