Institute of Pharmacy and Molecular Biotechnology[1], Department of Biology, Institute of Hygiene[2], Department of Hygiene and Medical Microbiology, University of Heidelberg, Heidelberg, Germany, Department of Internal Medicine[3], Section of Complementary Medicine, Hospital of the University of Zurich, Switzerland

A novel colorimetric broth microdilution method to determine the minimum inhibitory concentration (MIC) of antibiotics and essential oils against *Helicobacter pylori*

A. Weseler[1], H. K. Geiss[2], R. Saller[3], J. Reichling[1]

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Prof. Dr. Jürgen Reichling, Insitute of Pharmacy and Molecular Biotechnology, Department of Biology, University of Heidelberg, Im Neuenheimer Feld 364, 69120 Heidelberg, Germany
juergen.reichling@urz.uni-heidelberg.de

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Helicobacter pylori infections have been associated with the pathogenesis of a number of stomach and gastroduodenal diseases. In order to find alternative drugs for their treatment the search is increasingly focused on new antimicrobial products. However, no standardized methods are available to test the anti-*Helicobacter pylori* activity in particular of natural substances. Therefore we developed a broth microdilution assay to investigate the susceptibility of this fastidious slow growing bacterium against 15 essential oils widely used to treat disorders of the gastrointestinal tract. The MIC values were determined colorimetrically using *p*-iodonitrophenyltetrazolium violet (INT) as an indicator for bacterial cell viability. The test sytem was evaluated with three common antibiotics: amoxicillin, ampicillin and levofloxacin. The antibiotic MICs were controlled by Etest®. The *Helicobacter* reference strain was remarkably susceptible to both the antibiotics (amoxicillin MIC: 0.02 μg/ml, ampicillin MIC: 0.064 μg/ml, levofloxacin MIC: 0.39 μg/ml) and the essential oils. Most of their MICs ranged from 0.015 to 0.064% (v/v) and about 140.0 to 280.0 μg/ml, respectively. Interestingly, chamomile oil, orange flower oil and ginger oil inhibited the bacterial growth in extraordinarily low concentrations of 0.0075% (v/v) and about 65 μg/ml, respectively. The bactericidal concentrations were generally one to two dilution steps higher. In conclusion, we could develop an innovative assay for the MIC determination of essential oils and antibiotics against *Helicobacter pylori*, which is simple to handle, accurate, reproducible and not as time- and material-consuming as traditional agar dilution techniques.

1. Introduction

Since the first description of *Helicobacter pylori* in 1983 (Warren and Marshall 1983) the association of this pathogene with a number of gastrointestinal diseases such as chronic gastritis (Warren and Marshall 1983), gastroduodenal ulceration (Blaser 1990), gastric non-Hodgkin's lymphoma (Eidt et al. 1994), mucosa-associated lymphoid tissue (MALT) lymphoma (Parsonett et al. 1994) and adenocarcinoma of the stomach (Dunn et al. 1997; Parsonett et al. 1991) has clearly been recognized. An increased density of *H. pylori* in the gastric mucosa is associated with more severe gastritis and an increased incidence of peptic ulcer. The observation that an eradication of this microorganism seems to cure both the infection and the accompanying disorder, therapeutic regimes were increasingly focused on antimicrobial agents. However, eradication is difficult and the use of antibiotics is more and more limited by the emerging resistance of *H. pylori* especially to metronidazole and clarithromycin (Midolo et al. 1996; Versalovic et al. 1999). There is a need for alternative or additional strategies for the treatment of *H. pylori* infections. Therefore, in the last decade a search for new antimicrobial agents which may successfully support the healing of a *H. pylori* infection has been started. For this purpose the *in vitro*-measurement of anti-*Helicobacter* activity is an indispensable guide for the selection of potential drugs.

During the last years a number of natural substances and plant extracts like sesquiterpene lactones, flavonoids, tea catechins, garlic extracts, wine and propolis have been proven to be active against *H. pylori in vitro* (Bea et al. 1999; Bayona et al. 2003; Cellini et al. 1996; Konstantinopoulou et al. 2003; Landvatter et al. 2002; Mabe et al. 1999; Marimon et al. 1998; McNulty et al. 2001; Meletiadis et al. 2001; O'Gara et al. 2000; Ohno et al. 2003). Moreover, recent studies reported the *in vitro* and *in vivo* (e.g. mice and rats) efficiency of different essential oils against antibiotic-susceptible and -resistant *H. pylori* strains (Bergonzelli et al. 2003; Imai et al. 2001; Kalpoutzaki et al. 2001; Ohno et al. 2003; Tzakou and Skaltsa 2003). Furthermore, it was of special interest that the bactericidal activities of the essential oils tested were enhanced at acidic pH values (Bergonzelli et al. 2003; Ohno

et al. 2003; Tzakou and Skaltsa 2003). Essential oils are of special interest if used as food additives to complement an anti-*Helicobacter* therapy (Bergonzelli et al. 2003). Unfortunately, no standardized methods for the susceptibility testing of *H. pylori* currently exist, neither for synthetic single substances nor natural plant products. Generally data generated by different antimicrobial test assays (e.g. agar diffusion, agar dilution, broth dilution) are difficult to compare and less indicative (King 2001; Piccolomini et al. 1997).

In our previous investigations a modified broth microdilution method was successfully used for the determination of the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of essential oils against a wide spectrum of microorganisms (Harkenthal et al. 1999; Reichling et al. 1999; Weseler et al. 2002a; Weseler et al. 2002b). But in contrast to those formerly tested bacteria, the fastidious bacterium *H. pylori* grows slowly and only with a slightly visible haze in liquid nutrient solution under microaerophilic conditions (5% O_2, 10% CO_2, 85% N_2). So the MIC determination by a visual assessment of bacterial growth as turbidity or pellet in the wells of the microtitre plate is very difficult or almost impossible. To overcome this problem, we have developed an alternative test method reading the MIC endpoint by colorimetry. Colorimetric methods are attractive, since they have the potential to generate clear-cut endpoints based on a visually detectable colour change. For our purpose we used the tetrazolium salt *p*-iodonitrophenyltetrazolium violet (INT) as an indicator of bacterial growth. INT is a compound that is reduced by bacterial dehydrogenases of metabollically active bacterial cells to a violet INT formazan product.

2. Investigations and results

2.1. Chemical composition of the essential oils

Essential oils are lipophilic multi-compound systems with a characteristic pattern of mainly monoterpenes, phenylpropanes and sesquiterpenes. The specific combination of these chemical elements effects their different biological activities (Blaschek et al. 2004). To confirm the pharmaceutical quality of the essential oils the chemical compositions were qualitatively and quantitatively analyzed by GC- and GC-MS methods as reported previously (Harkenthal et al. 1999). All essential oils tested met the standard demands of either current pharmacopoea monographies or literature data (Blaschek et al. 2004; Teuscher et al. 2002).

Table 1: Anti-*Helicobacter pylori* activity of three known antibiotic substances – comparison of MIC values obtained by a colorimetric broth microdilution method and Etest® strips

	Colorimetric broth microdilution method		Etest® strip MIC (μg/ml)
	MIC (μg/ml)	MBC (μg/ml)	
Amoxicillin-HCl	0.02	0.037–0.085	0.032
Ampicillin-HCl	0.064	0.127–0.273	0.047
Levofloxacin-Na	0.39	0.39	0.38

2.2. Anti-Helicobacter activity of antibiotics

The MICs obtained by the colorimetric determination of amoxicillin (MIC: 0.02 μg/ml), ampicillin (MIC: 0.064 μg/ml) and levofloxacin (MIC: 0.39 μg/ml) correlated very well with those obtained by the Etest® method (amoxicillin MIC: 0.02 μg/ml; ampicillin MIC: 0.05 μg/ml; levofloxacin MIC: 0.4 μg/ml) (see Table 1). The values were highly reproducible, the results of the duplicate tests were within ±1 dilution steps. Besides, our results were in accordance with published data (Bamba et al. 1997; Goodwin et al. 1986; Irie et al. 1997; Piccolomini et al. 1997; v. Recklinghausen et al. 1993; Takahashi et al. 1997).

2.3. Anti-Helicobacter activity of the essential oils

The *H. pylori* strain used for this study was highly susceptible to most of the 15 essential oils tested (see Table 2). The majority of the MIC values ranged between 0.015 to 0.064% (v/v) and 140 to 280 μg/ml, respectively. Chamomile oil, orange flower oil and ginger oil were the most active plant products with MIC values between 0.0038 to 0.0075% (v/v) and about 38 to 70 μg/ml, respectively. The MBC values of most of the oils were one to two dilution steps higher than the MICs.

Table 2: Antibacterial acitivity of fifteen essential oils against *Helicobacter pylori*

	MIC (% (v/v))	MBC (% (v/v))	MIC (μg/ml)	MBC (μg/ml)
Chamomile oil	0.0038–0.0075	0.015	35.7–70.4	140.8
Orange flower oil	0.0075	0.03–0.06	65.1	260.4–520.8
Ginger oil	0.0075–0.015	0.015	65.4–130.9	130.9
Lemon balm oil	0.015	0.06	135.7	542.9
Peppermint oil	0.015	0.06	135.6	542.2
Sage oil	0.015	0.06	137.6	550.3
Rosemary oil	0.015	0.06	137.0	547.9
Orange peel oil	0.015–0.03	0.03	127.4–254.8	254.8
Cardamom oil	0.015–0.03	0.06	139.0–278.0	556.0
Thyme oil	0.03	0.03	275.2	275.2
Coriander oil	0.03	0.06	259.3	518.6
Fennel oil	0.03	0.06–0.125	288.3	576.6–1153.2
Caraway oil	0.03	0.06–0.125	273.1	546.1–1092.2
Basil oil	0.03–0.06	0.06	286.7–573.4	573.4
Anise oil	0.03–0.06	0.06–0.125	294.7–589.4	589.4–1178.8

MIC = minimum inhibitory concentration
MBC = minimum bactericidal concentration

3. Discussion

Using the broth microdilution technique the major problem of MIC testing of *H. pylori* is to detect visible growth. Usually microbial growth is indicated by forming a white pellet, precipitation or at least a milky turbidity in the test wells. To overcome this problem the use of an indicator for the viable but not visible *H. pylori* cells is proposed.

A number of different tetrazolium salts is described to detect the viability of eukaryotic as well as prokaryotic cells, e.g. to study cytotoxic and inhibitory effects of agents on cell proliferation (Meletiadis et al. 2001; Roslev and King 1993; Roehm et al. 1991; Thom et al. 1993). The biochemical principle of such tetrazolium-based test assays is the redox activity of the colourless compound. Tetrazolium salt acts as an electron acceptor which is reduced to the coloured formazan by the oxidative metabolism in living organisms (see Fig. 1). In general the colour change can be assessed visually. Particularly, bis-methoxynitrosulfophenyl-phenylaminocarbonyl-tetrazolium hydroxide (XTT) (Meletiadis et al. 2001; Roehm et al. 1991; Roslev and King 1993), dimethylthiazolyldiphenyl-tetrazolium bromide (MTT) (Eloff 1998; Thom et al. 1993) and *p*-iodonitro-phenyltetrazolium violet (INT) (Eloff 1998; Gribbon and Barer 1995) have been proposed as indicators of viable bacteria and could successfully be established in bacterial and fungal susceptibility test systems. Based on the results of (Eloff 1998) we chosed INT to develop a novel method to determine MICs of antibiotics and essential oils against *Helicobacter pylori*.

The best performance of our test system was found with an 0.6 mg/ml INT solution. The pink colour of the formazan product obtained by lower INT concentrations (e.g. 0.2 to 0.4 mg/ml) was weak and breakpoint was difficult to assess. On the other hand higher concentrations of the indicator (up to 1 mg/ml) did not improve the optical analysis of the test trays. The colour intensity also depended on the duration of the incubaction period after the addition of the INT solution. Best results were seen after a 2 h incubation at 37 °C in a microaerophilic atmosphere.

These methodical settings were evaluated with amoxicillin, ampicillin and levofloxacin – three antibiotics with known MIC's for *H. pylori* (Bamba 1997; Goodwin et al. 1986; Irie et al. 1997; Piccolomini et al. 1997; v. Recklinghausen et al. 1993; Takahashi et al. 1997). Additionally, we determined the MIC values of the antibiotics with the Etest® strips, which are recommended as a standard method for routine susceptibility testing of *H. pylori* (King 2001; Piccolomini et al. 1997; Recklinghausen et al. 1993). The MIC results obtained by the colorimetric determination and by Etest® did not differ significantly. Even after repeated testing all results were within a range of ±1 dilution step. Thus the INT test system showed a high degree of reliability and reproducibility.

In addition the test system proved an excellent suitability when testing essential oils against *H. pylori*. To emulsify

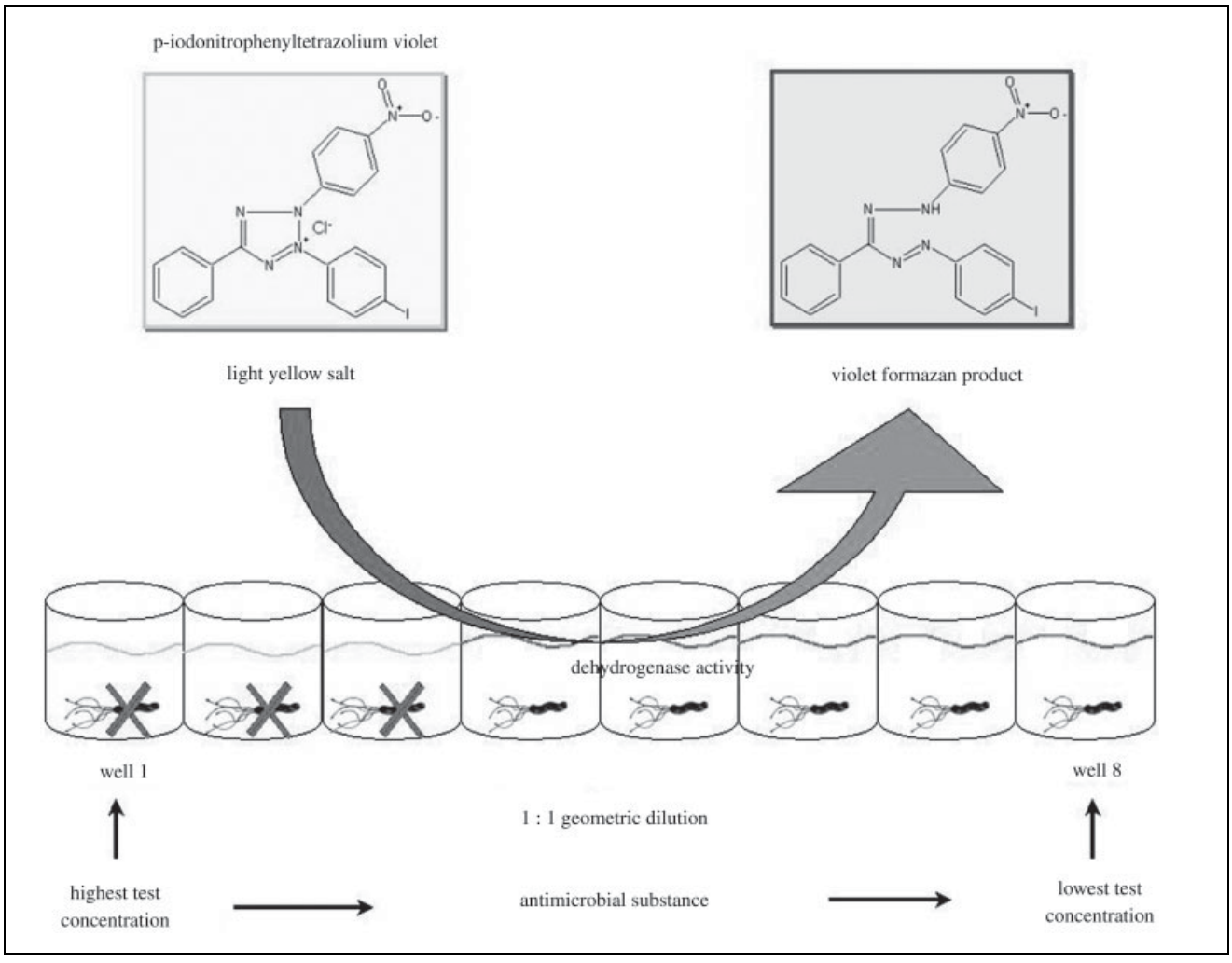

Fig. 1: Principle of the colorimetric MIC determination

the essential oils in the aqueous test medium we used ultrasonification which allowed a stable stock solution of 0.5% (v/v) essential oil. All essential oils tested showed a high anti-*Helicobacter* activity with MIC values of 0.015 to 0.064%. After conversion of the MIC values given in % (v/v) into μg/ml concentrations it was clear that results of 140 to 280 μg/ml are far beyond the activity of the three antibiotics tested (0.02 to 0.4 μg/ml). However, these results revealed a much higher activity than other natural products tested (Bae et al. 1999; Boyanova et al. 2003; Cellini et al. 1996; Imai et al. 2001; Konstantinopoulou et al. 2003; Landvatter 2002; Mabe et al. 1999; Marimon et al. 1998; O'Gara et al. 2000).

On search of novel antimicrobial compounds *in vitro* testings are indispensable to discover and preselect potential substances. Therefore we developed a test assay that may universally be used to determine MIC and MBC values of essential oils as well as novel single antibiotic agents against *H. pylori*. By the addition of the redox indicator INT we found a way to utilize the microdilution technique. Especially low required amounts of test substances, low contamination rates, exactness during performing and an usually easy reading of the test results make it to a simple to handle valuable alternative test system that is less time- and material-consuming as the widely recommended agar dilution and disc diffusion methods of anti-*Helicobacter* activity testing.

4. Experimental

4.1. Bacterial strain

The test strain *H. pylori* (DSM 4867) was obtained from the Deutsche Stammsammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). The bacterial cells were stored at −70 °C in a glycerol supplemented skim milk. Before testing bacteria were thawed and grown for 2 to 3 days on blood agar at 37 °C in a microaerophilic environment (5% O_2, 10% CO_2, 85% N_2) (AnaeroJar® and CampyGen Pak®, OXOID, Wesel, Germany). Colonies were identified and checked for their catalase, oxidase and urease production by routine methods.

4.2. Antibiotic substances

The antibiotic substances tested against *H. pylori* included amoxicillin (Sigma Aldrich Laborchemikalien GmbH, Seelze, Germany), ampicillin (Grünenthal GmbH, Aachen, Germany) and levofloxacin (Aventis Pharma Deutschland GmbH, Frankfurt a. M., Germany). Stock solutions of each antimicrobial agent (1500 mg × l^{-1}) were freshly prepared in 0.9% saline for each test performance. The Etest® strips were purchased of AB Biodisk (Solna, Sweden).

4.3. Essential oils

Fifteen different essential oils were investigated: anise oil (*Illicium verum*), basil oil (*Ocimum basilicum*), caraway oil (*Carum carvi*), cardamom oil (*Elettaria cardamomum*), chamomile oil (*Matricaria recutita*), coriander oil (*Coriandrum sativum*), fennel oil (*Foeniculum vulgare*), ginger oil (*Zingiber officinale*), lemon balm oil (*Melissa officinalis*), orange flower oil (*Citrus aurantium*), orange peel oil (*Citrus aurantium*), peppermint oil (*Mentha piperita*), rosemary oil (*Rosmarinus officinalis*), sage oil (*Salvia officinalis*), and thyme oil (*Thymus vulgaris*). All essential oils were provided by Caesar & Loretz GmbH, Hilden, Germany. The qualitative and quantitative chemical compositions of the essential oils were analysed by GC and GC-MS methods as described previously (Harkenthal et al. 1999).

To prepare stock solutions the lipophilic essential oils had to be solved in the aqueous nutrient medium. Since *H. pylori* does not tolerate the presence of emulsifiers such as Tween 80 even in concentrations of 0.5% (v/v) (Landvatter 2002), the essential oils were emulsified in broth by ultrasonication.

4.4. Antimicrobial susceptibility testing

For inoculum preparation colonies were suspended in a 100-ml Erlenmeyer flask containing 10 ml of brucella broth (BB) (OXOID, Wesel, Germany) supplemented with 5% fetal calf serum (FCS). The flask was covered with a loosely fitted cap and incubated for 18 ± 2 h at 37 °C under microaerophilic conditions. Before inoculation the bacterial cells were checked for their morphology by gram-staining and light microscopy at a final magnification of × 1000. Cultures showing a high proportion of coccoid cell shapes (see Fig. 2) were discarded. The inoculum was adjusted to a final cell count of approximately 5×10^5 cfu/ml, which was controlled by the spiral plating counting method (Spiral System Cincinnati, OH, USA).

Determination of the MIC and MBC based on a modified broth microdilution method according to DIN 58940 part 8 and appendix 1 (1997). 96-well-microtitre plates were prepared with geometric serial dilutions ranging from 0.0003 to 75 μg/ml of the antibiotics and 0.00024 to 0.5% (v/v) of each essential oil, respectively. In view of essential oils' density ($\delta < 1$ g × ml^{-1}) the results in % (v/v) may be converted into μg/ml, which offers the opportunity to directly compare the antibacterial potency with other natural compounds or antibiotics. At least one growth control (BB + NaCl 0.9%) and one sterility control (BB + stock solution of each test substance) were included on a test tray. Except for the sterility control 100 μl of the adjusted inoculum was added to each well of the microplate. Each test was performed in duplicate and repeated three times. Plates were incubated at 37 °C in a microaerophilic environment (AnaeroJar® and

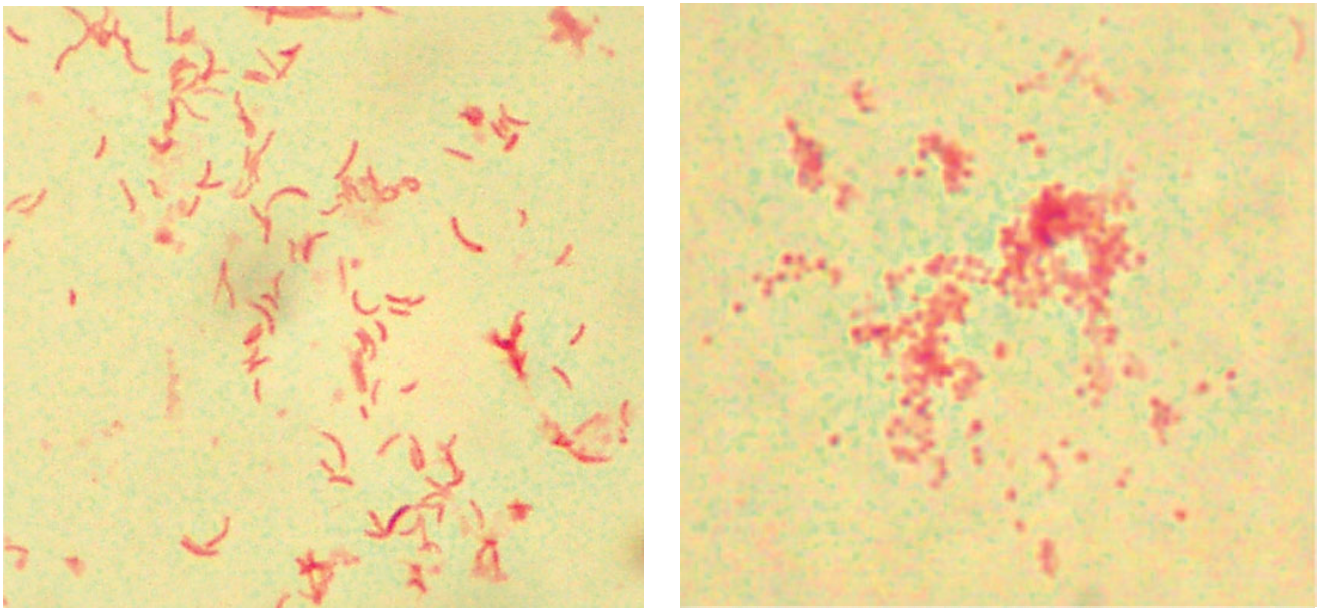

Fig. 2: Different morphological forms of *Helicobacter pylori* (gram-staining, light miscroscope, magnification × 1000)

a) mainly rod-like cells from a 18 h culture (Brucella broth, 37 °C, microaerobic environment);

b) mainly coccoid cells from a 60 h culture (Brucella broth, 37 °C, microaerobic environment)

CampyGen Pak®, OXOID, Wesel, Germany). After an incubation period of 20 ± 2 h a sample of 10 µl was removed from each well and transferred onto blood agar plates. These control agar plates were incubated for 3 to 5 days. The lowest concentration without any visible colony growth on the blood agar was defined as MBC. In order to ascertain the MIC values, 40 µl of an *p*-iodonitrophenyltetrazolium violet (INT) (Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany) solution (0.6 mg/ml) was added to each well. After 2 h of further incubation at 37 °C the MIC was determined visually as the lowest concentration of antimicrobial test substance at which no colour change occurred (see Fig. 1).

The MIC determination of the three antibiotics was additionally performed with the Etest® method. The antibiotic agents were tested across a range of 0.002 to 32 µg/ml. Blood agar plates were inoculated by confluent swabbing of the surface with the bacterial suspension. After the surface of the inoculated plates had dried at 37 °C inside a microaerobic jar two Etest® strips of each antibiotic were placed onto the surface of the agar plates and incubated. After an incubation period of 3 to 5 days in the microaerophilic atmosphere MICs could be read on the basis of the intersection of the elliptical zone of growth inhibition with the concentration scale on the Etest® strip.

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