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Antibiotic Minimum Inhibitory Concentrations and Time–killing against *Helicobacter pylori* Clinical Isolates in Douala, Cameroon: Therapeutic Potential of Routinely Antibiotics

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ABSTRACT

The choice of regimens treatment against *H. pylori* infection in the present time should be based on knowledge of local resistance patterns and antibiotic use. In the present investigation, we tested the susceptibility of *H. pylori* isolated from dyspeptic patients residing in Douala- Cameroon, to routinely used drugs; amoxicillin, metronidazole, clarithromycin, erythromycin, doxycyclin and ciprofloxacin alone and in combination with two drugs each, using broth micro dilution assay and their bactericidal effectiveness in time-killing studies. Our findings showed that doxycycline, clarithromycin and ciprofloxacin, when tested alone were the most active among the antibiotics used with MIC value of 0.125 µg/ml. All the 11 different combinations showed synergism, and no case of antagonism was observed. The best combinations with the highest minimum fold inhibition (128) were Amoxicillin-Doxycyclin, Metronidazole-Clarithromycin and Metronidazole-Doxycyclin. The bactericidal studies of doxycyclin, clarithromycin and ciprofloxacin showed that ciprofloxacin at 8 MIC, produced a viability decrease of 3 log against clinical isolates tested.

Our findings clearly support the efficacy of combination therapies against *H. pylori* and suggest that susceptibility testing alone may not be sufficient to provide evidence of the clinical potential of anti *H. Pylori* agents and that time-killing study may be a useful method for evaluating the efficiency of antibiotics.

Keywords: Antimicrobial susceptibility, Time-killing studies, *Helicobacter pylori*, Antibiotic, Cameroon.

INTRODUTION

The importance of *Helicobacter pylori* in the field of gastroenterology has increased because of its potential etiologic role in disorders of the upper gastrointestinal tract [1]. *H. pylori* with concurrent gastritis is common in patients with peptic ulcers, indicating a causal relationship to ulcer disease, and possible links may also exist between *H. pylori* and gastritis in the pathogenesis of gastric cancer [1]. As a result, much emphasis has been placed on the treatment of gastritis and ulcer disease with regimens which include antimicrobial agents [2-4]. *In vitro*, *H. pylori* is extremely susceptible to the

majority of antibiotics, however, most of them are unable to successfully eradicate this bacterium from infected subjects [5].

For example, *in vitro* susceptibility testing indicates that amoxicillin is one of the more active agents against *H. pylori*. However, amoxicillin produces long-term *H. pylori* eradication in only 20% of patients [5].

In vitro, *H. pylori* is moderately sensitive to metronidazole and to tinidazole (MIC range 0.5- 32 mg/l). However, both compounds have proved unsuccessful in clearing *H. pylori* from the gastric mucosa when used as a single agent (clearance rate, 3%-20%) [6].

Such observations clearly show that traditional MIC determinations are inadequate for determining an antibiotic's clinical effectiveness against *H. pylori* and indicate that an effective antimicrobial drug against this bacterial strain must be bactericidal rather than bacteriostatic. Hence, time-kill curves of *H. pylori* exposed to several drug concentrations, measuring bactericidal activity overtime are most appropriate for estimating the clinical efficiency of antibiotics against this mucosa associated bacterium.

On the other hand, none of the most commonly used antibiotics in the treatment of *H. pylori* infection (tetracycline, amoxicillin, metronidazole and clarithromycin) is active enough to be used as a monotherapy [7,8] and the successful eradication of *H. pylori* infections requires a combination of two or three of them and an antacid drug [5].

The first-line eradication therapy for *H. pylori* infection worldwide is the standard triple therapy, including a proton pump inhibitor and the antibiotics clarithromycin and amoxicillin/metronidazole [9-12]. However, the efficiency of this standard triple regime has decreased over the past decades, with the overall success rate of 74.6% in an Intention-to-treat analysis and 82% in a per-protocol analysis [13].

Knowledge of the entire genomic sequence of *H. pylori* may allow the rapid development of novel drugs that specifically target vital functions of *H. pylori* but until we have them, we must try stopping the rapid spread and induction of resistance [14]. For this purpose, monotherapy should never be used, and choice of regimens for patients in the present time should be based on knowledge of local resistance patterns and antibiotic use [15,16].

Cameroon does not have regional surveillance programmes that monitor the evolution of *H. pylori* resistance in order to allow timely adaptation of the treatment regimens. Taking into account this information, studies have been initiated by us to elucidate this question in order to bridge the gap in knowledge about an optimal therapeutic regimen for this infection adapted to Cameroon. Consequently, 20 Cameroonian *Helicobacter pylori* clinical isolates were evaluated for *in vitro* susceptibility on the basis of their MICs and their time–killing effects to six antimicrobial agents recently given for eradication therapy, alone and in combination.

MATERIALS AND METHODS

Chemicals and culture media

Culture media (Columbia Agar, Brain Heart Infusion (BHI), Lacked horse blood, Horse Serum, Vitox Supplement) and CampyGen gas pack were obtained from Oxoid, Basingstoke, England. Doxycyclin (Doxycycline 200 mg, Combitic Global Caplet, India), erythromycin (Erythromycin stearate 500 mg, cipla, India), amoxicillin (Amoxicillin trihydrate 500 mg, maxheal pharmaceutical, India), ciprofloxacin (ZOFLOX, Ciprofloxacine 750 mg, Odypharm), clarithromycin (Clarithromycin 500 mg, Aurechem Laboratories, India) and metronidazole (Metronidazole 500 mg, strides Arcolab, India) used as reference antibiotics routinely given for eradication therapy in Cameroon were purchased from a local pharmacy. Antimicrobial agents were also selected on the basis of published information on efficiency or lack of efficiency for the eradication of *H. pylori* in human clinical trials. P-Iodonitrotetrazolium chloride (INT, Sigma-Aldrich) was used to indicate microbial growth [17].

Bacterial strains

The 20 *Helicobacter pylori* isolates were obtained from gastric mucosal biopsy specimens from the Gastroenterology Department of Laquintinie Hospital, Douala, Cameroon. The collection of biopsy specimen from dyspeptic patients was approved by local ethical committee of Laquintinie Hospital (Approval No. 425/ AR/ MINSANTE/ HLD/ SCM/ CR). All isolates were removed from storage at -80 °C and subcultured on

supplemented Columbia Agar (Columbia Agar + 5% (v/v) lacked horse blood and 1% (v/v) Vitox). Subcultures were incubated at 37°C under microaerophilic conditions (CampyGen gas pack) for 96 hours and for two passages to ensure reliable growth. Gram staining and catalase, oxidase a nd urea hydrolysis were performed to confirm the identification.

Antimicrobial susceptibility tests

MICs were determined by the INT broth microdilution method [18] using 96-well plates. Two fold dilutions of each selected antibiotics were prepared in the test wells in BHI broth supplemented with 5% horse serum (BHI-serum). The final antibiotic concentrations ranged from 0.125 to 512 µg/ml**.** One hundred microliters of inoculums prepared from 48 h colonies of each isolate on supplemented Columbia Agar (Columbia Agar + 5% (v/v) lacked horse blood and 1% (v/v) Vitox) at McFarland turbidity standard 3 was added to 100 μl of the antibiotic-containing culture medium. Control wells were prepared with culture medium and bacterial suspension, and broth only.

The plates were covered with a sterile plate sealer; the contents of the wells were mixed with a shaker and incubated for 3 days at 37◦C under microaerophilic conditions. After incubation, 40 μl of 0.2 mg/ml INT was added per well and incubated at 37◦C for 30 min. Living bacteria reduced the yellow dye to pink. The antibiotic concentration that prevented the color change of the medium, exhibited complete inhibition of microbial growth known as the MIC was determined. Each MIC was determined in triplicate and the mean values were recorded. MIC value for each antibiotic was compared to the break-point MIC value recommended by the European Committee on Antimicrobial Susceptibility Testing 2015 on *H. pylori* and the isolate were classified as susceptible or resistant.

Synergic effect of antibiotics in combination

According to the CMI value obtained, antibiotics were considered as active or non-active. The anti-*H. pylori* activity of antibiotic in combination was carried out in order to check for any synergetic effect. Different combinations were then made between active and non-active antibiotics and between non-active antibiotics together. Those combinations were doxycyclin, clarithromycin and ciprofloxacin, taken as active antibiotics with each of the non-active antibiotics; metronidazole, amoxicillin and erythromycin. Within non-active antibiotics, the combinations were amoxicillin/metronidazole, amoxicillin/erythromycin, and metronidazole/erythromycin. Clinical isolates resistant to amoxicillin, metronidazole and erythromycin (triple resistant) were used.

The broth microdilution method as described above was used with BHI-serum as culture media. A two-fold serial dilution of each non-active antibiotic ranging from MIC and MIC fractions were mixed together with a fixed concentration of each active antibiotics corresponding to its MIC value. So, for the non-active antibiotics, the concentrations tested were ranging from 512 to 2 µg/ml, from 256 to 2 µg/ml and from 128 to 2 µg/ml respectively for amoxicillin, metronidazole and erythromycin; and 0.125 µg/ml for each active antibiotic.

Each non-active antibiotic was serially diluted in BHI-serum; into 96-well round bottom sterile plates and the active antibiotic solution separately prepared in test tubes were added. Then, 100 µl of triple resistant *H. pylori* suspension prepared from 48 h colonies on supplemented Columbia agar at McFarland turbidity standard 3 were distributed into wells containing various concentrations of the different compounds. The inoculated 96-well round bottom was incubated for 3 days at 37◦C under microaerophilic conditions. After incubation, 40 μl of 0.2 mg/ml INT was added per well and incubated at 37◦C for 30 min. the minimum inhibitory concentrations of drugs in combination were determined as mentioned above. Each MIC was determined in triplicate and the mean values were recorded. Interactions between combined drugs were considered synergistic if the FIC index was <0.5 and antagonistic if the FIC index exceeded 4.

Time-kill bactericidal activity

Bactericidal activity of the drugs was studied using a modified time-kill assay by evaluating the decrease in viable cells during exposure to the drug [18,19]. The assay was performed in BHI-serum as medium, incubated in microaerophilic environment. Active antibiotics at concentrations of 1, 2, 4, and 8 MIC of susceptible strain were prepared in BHI-serum medium in a 100-ml baffled flask. A 48-h culture of the selected isolate was diluted with fresh broth and then inoculated to each drug solution at a final concentration of 10^6 CFU/ml. The bacterial suspension was incubated with circular shaking, and samples

(0.1 ml) taken at 0, 4, 8, 24, 48 and 72 hours after drug exposure were tenfold serially diluted with saline and inoculated in duplicate onto drug-free supplemented Columbia agar plates for colony counts. The CFU/ml was calculated from the number of colonies that appeared after incubation at 37◦C for 72 hour under microaerophilic atmosphere.

RESULTS

Antimicrobial susceptibility tests

The results of *H. pylori* clinical isolates susceptibility to the tested antibiotics are shown in Table 1.

Table 1: MICs of various antibiotics against *H. pylori* clinical isolate (20 isolates).

Antibiotics	MIC of *H. pylori* susceptible strains	CMI range (µg/ml)	% resistance
Metronidazole	≤ 8 mg/l	256-512	100
Amoxicillin	≤ 0.5 mg/l	> 512	100
Erythromycin	< 4 mg/l	128-256	100
Clarithromycin	≤ 0.5 mg/l	0.0625-0.125	0
Doxycyclin	< 1 mg/l	0.125-1	10
Ciprofloxacin	≤ 1 mg/l	0.125-8	15

From these results, we noticed that doxycyclin, clarithromycin and ciprofloxacin with MIC value of 0.125 were the most active antibiotics tested. However, ten and fifteen percent of the tested isolates were resistant respectively to doxycyclin and ciprofloxacin, whereas none of them were resistant to clarithromycin. A 100% resistance was obtained with erythromycin, metronidazole and amoxicillin, indicating the multidrug resistant phenotype of these clinical isolates.

Synergetic effect

Eleven combinations of two drugs each were tested against resistant clinical isolates selected according to the MIC value obtained when antibiotics were tested alone. The tested drug combinations were Amoxicillin-Doxycyclin, Amoxicillin-Clarithromycin, Amoxicillin-Ciprofloxacin, Amoxicillin-Metronidazole, Amoxicillin-Erythromycin, Metronidazole-Doxycyclin, Metronidazole-Clarithromycin, Metronidazole-Ciprofloxacin, Metronidazole-Erythromycin, Erythromycin-Doxycyclin and Erythromycin- Ciprofloxacin. Results are given in Tables 2 and 3. All the 11 different combinations showed synergism and no case of antagonism were observed. However, the highest minimum fold inhibitions for the tested triple resistant bacterial isolates were obtained only in combination with active antibiotics. The best combinations with the higher minimum fold inhibition (128) at low antibiotic concentration were Amoxicillin-Doxycyclin, Metronidazole-Clarithromycin and Metronidazole-Doxycyclin.

We also found that the synergistic effect of the drugs combined was concentration dependent in the case of clarithromycin-amoxicillin, ciprofloxacin-amoxicillin and ciprofloxacin-metronidazole as shown in Table 2.

Table 2: MIC (µg/ml), FIC index and interaction's type of combination between active and non-active antibiotics against multidrug resistant phenotype *H. pylori* clinical isolates.

Antibiotics	Antibiotic concentration (µg/ml)	MIC (µg/ml) value of antibiotics in combination, FIC index value, Interaction's type		
		AMO	**MET**	**ERY**
CLAR	0	> 512	256	128
	CMI/2	16 (0.03125)^{S}	< 2 (0.0078)^{S}	/
	CMI	8 (0.015625)^{S}	< 2 (0.0078)^{S}	/

	2XCMI	< 2 (0.00390) [S]	< 2 (0.0078) [S]	/
DOX	0	> 512	256	128
	CMI/2	< 2 (0.00390) [S]	< 2 (0.0078) [S]	< 2 (0.0156) [S]
	CMI	< 2 (0.00390) [S]	< 2 (0.0078) [S]	< 2 (0.0156) [S]
	2XCMI	< 2 (0.00390) [S]	< 2 (0.0078) [S]	< 2 (0.0156) [S]
CIP	0	> 512	256	128
	CMI/2	256 (0.5)[S]	32 (0.125)	< 2 (0.0156) [S]
	CMI	64 (0.125)[S]	16 (0.0625)	< 2 (0.0156) [S]
	2XCMI	< 2 (0.00390) [S]	< 2 (0.0078) [S]	< 2 (0.0156) [S]

Non active antibiotics: Amoxicillin (AMOX), Metronidazole (MET), Erythromycin (ERY).

Active antibiotics: Doxycycline (DOX), Clarithromycin (CLA), Ciprofloxacin (CIP).

MIC: Minimum inhibitory concentration, FIC: fractional inhibitory concentration, [S]: synergy.

Synergism concentration dependent was also observed within non-active antibiotic combinations with high minimum fold inhibition at high drugs concentration (Table 3).

Table 3: MIC (µg/ml), FIC index and interaction's type of combination between non-active antibiotics against multidrug resistant phenotype *H. pylori* clinical isolates.

AMOX-ERY		**AMOX-MET**		**ERY-MET**	
Antibiotic concentration	**MIC, (FIC index), Interaction's type**	**Antibiotic concentration**	**MIC, (FIC index), Interaction's type**	**Antibiotic concentration**	**MIC (FIC index), Interaction's type**
ERY: 0	> 512	**MET: 0**	> 512	**MET: 0**	128
ERY: CMI/8	128 (0.25)[S]	**MET: CMI/32**	128 (0.25)[S]	**MET: CMI/32**	32 (0.25)[S]
ERY: CMI/4	64 (0.125) [S]	**MET: CMI/16**	64 (0.125) [S]	**MET: CMI/16**	32 (0.25)[S]
ERY: CMI/2	32 (0.0625) [S]	**MET: CMI/8**	32 (0.0625) [S]	**MET: CMI/8**	32 (0.25)[S]
ERY: CMI	16 (0.03125) [S]	**MET: CMI/4**	8 (0.015625) [S]	**MET: CMI/4**	32 (0.25)[S]
/	/	**MET: CMI/2**	< 4 (0.0078) [S]	**MET: CMI/2**	16 (0.125) [S]
/	/	**MET: CMI**	< 4 (0.0078) [S]	**MET: CMI**	16 (0.125) [S]

AMOX: Amoxicillin, MET: Metronidazole, ERY: Erythromycin. MIC: Minimum inhibitory concentration,

FIC: Fractional inhibitory concentration, [S]: synergy.

Time-kill bactericidal activity

Bactericidal effect overtime at different concentrations of each selected active antibiotics; clarithromycin, doxycyclin and ciprofloxacin were studied. The results are presented in Figures 1 - 3.

The bactericidal studies of doxycyclin (Figure 1) show that, at concentrations of 1, 2 and 4 MIC, doxycylin had very little effect on the growth of *H. pylori* isolate tested throughout the experimental period. However, at a doxycyclin concentration equal to 8MIC, bacterial numbers were approximately 1 log lower than those for the control at 72 h. thus; bactericidal effect of doxycyclin against the tested *H. pylori* was concentration and time dependent.

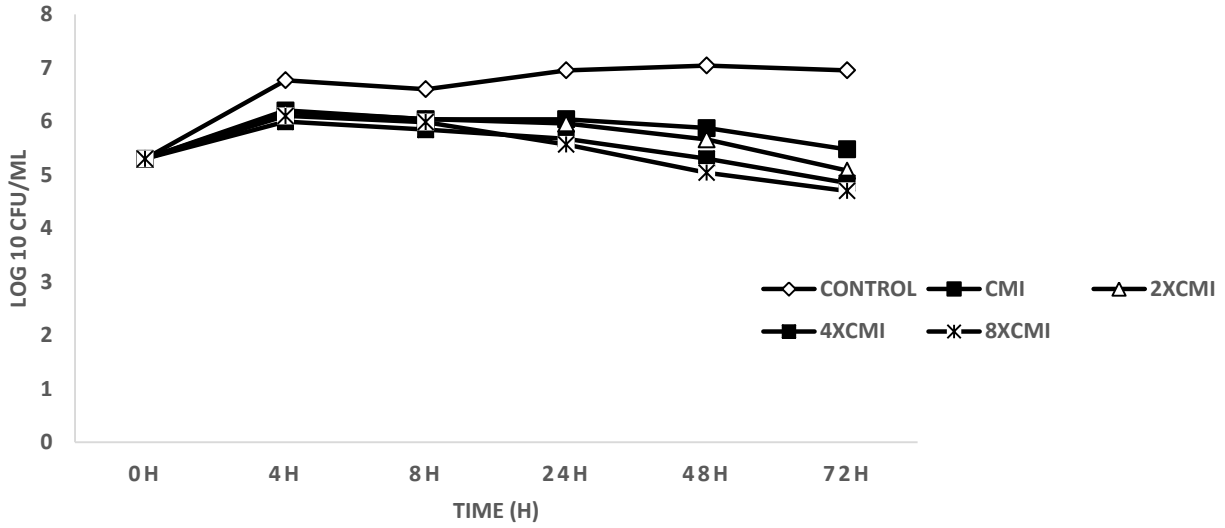

Figure 1: Bactericidal effect of doxycyclin against Cameroonian *Helicobacter pylori* clinical isolates at MIC, 2 MIC, 4 MIC and 8 MIC.

Bactericidal effect of clarithromycin against the tested *H. pylori* was also concentration and time dependent (Figure 2). At concentrations of 1, 2 and 4 MIC, clarithromycin had very little effect on the growth of *H. pylori* isolate tested throughout the experimental period. At 4 MIC concentrations, we noticed that bacterial numbers were approximately 1 log lower than those for the control at 72 h of incubation. A similar effect was also observed at a clarithromycin concentration equal to 8MIC, from 48 to 72 h.

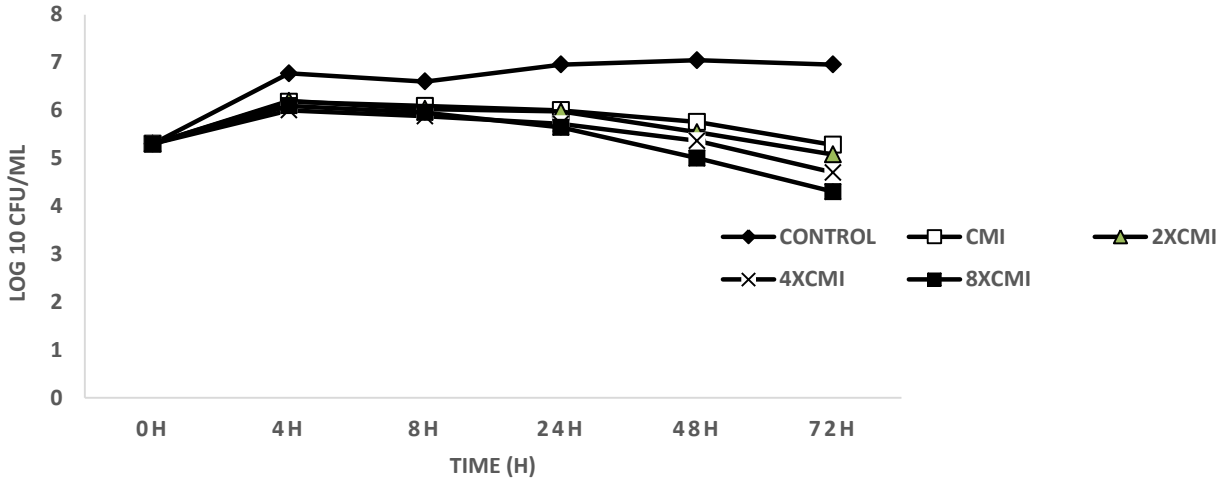

Figure 2: Bactericidal effect of clarithromycin against Cameroonian *Helicobacter pylori* clinical isolates at MIC, 2 MIC, 4 MIC and 8 MIC.

The bactericidal studies of ciprofloxacin (Figure 3) show that, at a concentration less than 4 MIC, ciprofloxacin had little effect on the growth of *H. pylori* isolate tested until 48 h. However, at 72 h, a bactericidal effect was observed at 2MIC and 4MIC concentrations. Ciprofloxacin at 2MIC and 4MIC produced a relatively similar effect, suggesting a possible break-point concentration which is reach at 8MIC. In fact, following exposure of the bacteria to 8MIC concentration of ciprofloxacin, bacterial numbers were approximately 1 log lower than those for the control from 4 to 8 h, and a 3log reduction was seen after 24 h.

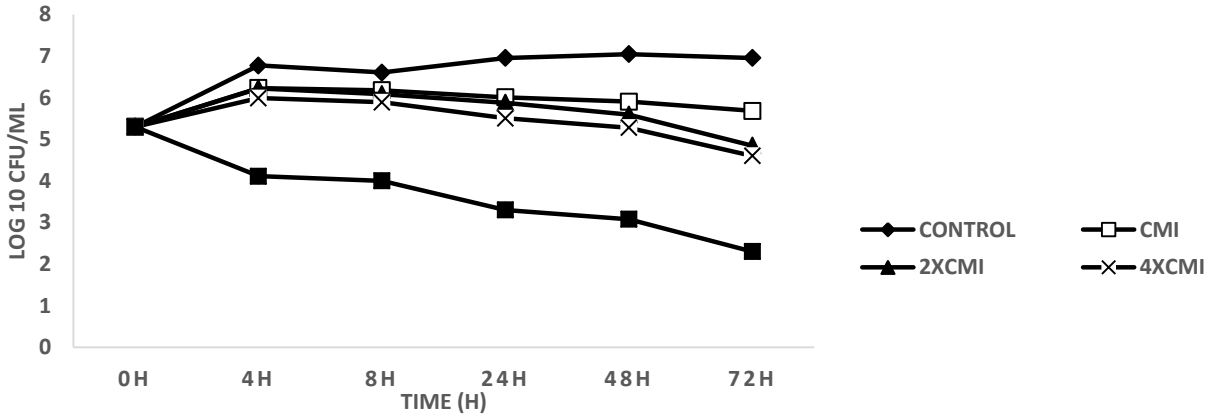

Figure 3: Bactericidal effect of ciprofloxacin against Cameroonian *Helicobacter pylori* clinical isolates at MIC, 2 MIC, 4 MIC and 8 MIC.

DISCUSSION

We have studied Cameroonian *H. pylori* clinical isolates susceptibility and found that doxycyclin, clarithromycin and ciprofloxacin were the most active among the six tested antibiotics routinely prescribed for *H. pylori* eradication in Cameroon. Clarithromycin and erythromycin are the two macrolides used in this study. Although these antibiotics belonging to the same family, they have shown opposite activity against the isolates tested. While all the strains tested were resistant to erythromycin, they were all susceptible to clarithromycin. Other authors have also studied the *in vitro* activities of macrolides. Hardy et al. found that clarithromycin is 4 to 32 times more active than other macrolides [20]. The MIC for erythromycin, roxithromycin, and azithromycin was 0.25, while for clarithromycin it was 0.03 mg/liter [20]. Clarithromycin (MIC, 0.03 mg/liter) was also found to be significantly more active than either erythromycin (MIC, 0.125 mg/liter) or azithromycin (MIC, 0.25 mg/liter) in the study of Malanoski et al. [21]. Macrolides are a group of antibiotics that bind to the bacterial ribosome and thus block the synthesis of proteins [22]. The antibacterial spectrum of clarithromycin is similar to that of erythromycin, but clarithromycin is better absorbed, more acid-stable, and hence more effective against *H. pylori* than erythromycin [23].

In this study, the lowest rate of resistance of the tested isolates (10%) was obtained with doxycyclin. It is reported that, almost all *H. pylori* strains (> 99%) are sensitive to tetracycline or doxycyclin (MIC < 4 g /l), both *in vivo* and *in vitro* [24]. The difference in the breakpoint concentration of tetracycline between our study and those previous ones may be the reason of such observations. Tetracycline acts by inhibiting bacterial protein synthesis, and its activity is barely affected by low pH, and hence more effective against *H. pylori* in its acidic niche.

Fifteen percent of the tested isolates were resistant to ciprofloxacin. Ciprofloxacin acts by inhibiting the bacterial DNA gyrase, which results in inhibition of bacterial replication [25]. Ciprofloxacin and most related fluoroquinolones display a significant decrease of their activity at low pH, rendering them less effective for the treatment of *H. pylori* infections. Resistance of *H. pylori* to ciprofloxacin depends on the development of a mutation in the gyrA gene that encodes the DNA gyrase A subunit [26,27]. The majority of the observed mutations occurs at a single position (Asp-91) within this subunit and is associated with resistance to (8 g /l) ciprofloxacin [26,27]. Ciprofloxacin and other fluoroquinolones are widely used for a variety of bacterial infections and ciprofloxacin should not be considered for treatment of *H. pylori* in patients with a history of chronic infections treated with this class of antibiotics [28].

Amoxicillin acts by interfering with the synthesis of the bacterial cell wall, resulting in the lysis of replicating bacteria (as do related penicillin derivatives). Its antimicrobial activity rapidly decreases under increasingly acidic circumstances [29], but it is actively secreted from the blood into the gastric juice [30], hence, intravenous amoxicillin can eradicate *H. pylori* infections [31]. All the tested clinical isolates here were resistant to

amoxicillin. Similarly, Van Zwet et al. had identified and characterized an amoxicillin-resistant strain in their study [32]. In contrast, many studies have shown that *H. pylorus is* sensitive to amoxicillin (MIC < 1 g/ l) both *in vitro* and *in vivo.* Some studies in Italy and in the US, have reported isolation of several amoxicillin-tolerant *H. pylori* strains from patients [33]. The frequency and inappropriate use of this antibiotic in our population may explain such difference. Amoxicillin resistance, mediated by a variety of different mechanisms including mutations in penicillin binding proteins, decreased permeability for the antibiotic, or development of efflux pumps [28,34].

In vitro, H. pylori is moderately sensitive to metronidazole (MIC range 0.5-32 mg/l), and, according to different studies between 15 % and 50 % of strains tested have been found to be primarily resistant to metronidazole (MIC > 8 mg/l). Upon entering the bacterium, metronidazole is reduced to an active anion radical [35]. This forms the active compound, which acts by causing lethal damage to vital molecules such as DNA, RNA, proteins and fatty acids. An extremely low redox potential is required to allow conversion of the drug into the active form, and the cells of the host and most aerobic bacteria lack such a low redox potential. There has been much speculation on the exact mechanism for resistance to metronidazole in *H. pylori*. Metronidazole resistance, mediated by mutations leading to inactivation of the bacterial enzymes needed to activate the antibiotic, is also fairly prevalent worldwide [14,36]. In fact, many studies postulated that high-level resistance to metronidazole (> 32 g/l) is associated with mutations in the rdxA gene in the majority of cases [14].

In order to understand the interactions of drugs for combination therapy of *H. pylori* infection, we examined the *in vitro* effect of different combinations of the 6 antibiotics tested using the accurate determination of the minimal inhibitory concentration (MIC). All the tested combinations showed various synergistic effects on each other. No case of antagonism was observed. The best combinations were Amoxicillin-Doxycyclin, Metronidazole-Clarithromycin and Metronidazole-Doxycyclin. This synergistic effects indicate that active compounds with different modes of action within the combination, contribute to neutralize and to kill this bacterium. In fact, each drug used in combination has its own mode of action. Clarithromycin is bacteriostatic and inhibits protein synthesis by binding to the 50S ribosomal subunit. Metronidazole is bactericidal and works via activation within the bacteria, leading to production of toxic metabolites. Amoxicillin is bactericidal and inhibits synthesis of bacterial cell walls. Tetracycline is bactericidal and works by inhibiting protein synthesis. Hence, association of antibiotics constitutes an alternative in the fight against *H. pylori* infections and to prevent the emergency of multidrug resistant strains. However, susceptibility testing should be carried out before treatment, and the selection of drugs to combine should be determined based on these results.

The synergistic effect of the drugs combined was concentration dependent in the case of clarithromycin-amoxicillin, ciprofloxacin-amoxicillin and ciprofloxacin-metronidazole. Consequently, for a synergistic effect with combination therapy, a sufficient dose of the agents is needed.

Synergism concentration dependent was also observed within non-active antibiotic combinations with high minimum fold inhibition at high drugs concentration. However, the administration of a higher dosage of drugs may also cause severe side effects to occur.

Our findings clearly support the good efficiency of combination therapies against *H. pylori*.

Such effects are due to the action of the active compounds of the combined antibiotics. Moreover, drug combination reduces the recruitment of mutant which could be with potential resistant factors. In fact, standard first line treatment for *H. pylori* infection has classically been triple therapy with proton pump inhibitor, clarithromycin, and either metronidazole or amoxicillin. Efficacy is equivalent when using either amoxicillin or metronidazole [37]. Use of high dose proton pump inhibitor will increase cure rates with standard therapy regimens by 6-10% [38]. Tetracycline resistance, mediated either by efflux proteins or ribosomal protection proteins [39], is less prevalent worldwide than resistance to clarithromycin, metronidazole. In patients in Taiwan receiving a 2nd course of treatment for a prior failure, addition of tetracycline to PPI, bismuth, and amoxicillin was more effective than addition of metronidazole [40].

Nevertheless, in some cases, the eradication of *H. pylori* was unsuccessful. This may, in part, be due to the lack of susceptibility to the drugs administered. In addition, the

relatively low efficiency of drug *in vivo* may be also due to insufficient local drug concentrations at the locus of infection after oral administration.

Judging from the obtained MIC value (0.125 µg/ml), doxycyclin, clarithromycin and ciprofloxacin were the most active among the 6 antimicrobial agents tested. In order to estimate the clinical efficiency of these active antibiotics (clarithromycin, doxycyclin and ciprofloxacin) against this mucosa associated bacterium, we studied the bactericidal effect of each of them overtime.

The bactericidal studies show that in spite of the fact that these drugs have the same MIC value, only ciprofloxacin at 8 MIC produced a viability decrease of 3 log against clinical isolate tested. It appears likely that 8MIC (2 mg) concentration of ciprofloxacin may reach to the site of *H. pylori* infection because 500 mg/kg body weight dose of ciprofloxacin can be administered orally. McNulty et al., [41] in their study found that ciprofloxacin attained very high concentrations (range 35-1762 mg/kg) in gastric mucosal and that the inhibitory concentrations (35 mg/kg) were still present at 6 h after the dose. Our data on bactericidal studies emphasizes once more the fact that susceptibility testing alone may not be sufficient to provide evidence of the clinical potential of anti-*H. pylori* agents, and those time-killing studies may be a useful method for evaluating the efficiency of antibiotics.

CONCLUSION

Our findings clearly support the good efficacy of combination therapies against *H. pylori* and found Amoxicillin-Doxycyclin, Metronidazole-Clarithromycin and Metronidazole-Doxycyclin as the best combination adapted to Cameroonian's clinical isolates tested. Our results also showed differences in activity between agents with similar MICs and suggest that time–killing studies may be a useful method for evaluating the efficiency of antibiotics against *H pylori*. Our data also clearly indicate the potential of ciprofloxacin as a therapeutic strategy against *H. pylori* infection.

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REFERENCES

1. W. G. Kwack, Y. J. Lim, C. Y. Lim, D. Graham., *Gastroenterology. Research. Practice.* **2016**, Article ID 1648047, 7 pages.
2. K. Wolle, A. Leodolter, P. Malfertheiner, W. Konig., *J. Med. Microbio.* **2002,** 51, 705-709.
3. T. Falsafi, F. Mobasheri, F. Nariman, M. Najafi., *J. Clinic. Microbio*. **2004,** 42, 387-389.
4. D. Y. Graham, W. A. Qureshi., *Cur. Pharma. Design.* **2000,** 6, 1537-1544.
5. A. E. Marcus, G. Sachs, R. D. Scott., *Cur. Gastroenterology. Reports.* **2016,** 18(7), 33.
6. Y. Glupczynsk., ©Spnnger-Verlag, Berlin Heidelberg, **1990.**
7. A. F. Mentis, E. Roma, A. Pangalis, E. Katsiyiannakis., *Antimicrob. Agents. Chemotherapy*. **1999**, 44, 720-722.
8. S. Mendonca, C. Ecclissato, M. S. Sartori, A. P. Godoy, R. A. Guerzoni, M. Degger., *Helicobacter*. **2000,** 5, 79-83.
9. J. W. Lee, N. Kim, J. M. Kim., *Helicobacter.* **2013,** 18(3), 206-214.
10. B. An, B. S. Moon, H. Kim., *Annals. Lab. Med.* **2013,** 33(6), 415-419.
11. K. H. Yoon, S. W. O. Park, S. W. O. Lee, B. J. I. Kim, J. G. Y. Kim., *J. Korean. Med. Sci.* **2014,** 29(9), 1240-1246.
12. P. Malfertheiner, F. Megraud, C. A. O'Morain., *Gut.* **2012,** 61(5), 646-664.
13. E. J. E. Gong, S. C. Yun, H. Y. Jungetal., *J. Korean. Med. Sci.* **2014**, 29(5), 704-713.
14. A. Goodwin, D. Kersulyte, G. Sisson, S. J. Veldhuyzen van Zanten, D. E. Berg, P. S. Hoffman., *Mol. Microbio.* **1998,** 28(2), 383-393.
15. S. P. Thyagarajan, P. Ray, B. K. Das, A. Ayyagari, P. Rajasambandam, B. Ramathilagam., *J. Gastroenterology. Hepatology*. **2003**, 18, 1373-1378.
16. L. Perez Aldana, M. Kato, S. Nakagawa, M. Kawarasaki, T. Nagasako, T. Mizushima., *Helicobacter.* **2002,** 7, 306-9.

17. L. B. Kouitcheu Mabeku, B. Nanfack Nana, B. Eyoum Bille., *Pharma. Bio.* **2017,** 55(1), 929-936.

18. S. P. N. Mativandlela, N. Lall, J. J. M. Meyer., *South. African. J. Botany.* **2006,** 72, 232-237.

19. J. Hindler., Antimicrobial susceptibility testing, 5.16.1-33. *Amer. Society. Microbio.* DC, **1995.**

20. D. J. Hardy, C. W. Hanson, D. M. Hensey, J. M. Beyer, P. B. Fernandes., *J. Antimicro. Chemotherapy.* **1988,** 22, 631-636.

21. G. J. Malanoski, G. M. Eliopoulos, M. J. Ferraro, R. C. Moellering., *European. J. Clin. Microbio. Infection. Disease.* **1993,** 12, 131-133.

22. R. C. Goldman, D. Zakula, R. Flamm, J. Beyer, J. Capobianco., *Antimicro. Agents. Chemotherapy.* **1994**, 38, 1496-1500.

23. D. H. Peters, S. P. Clissold., *Drugs.* **1992,** 44, 117-164.

24. J. C. Delchier, P. Malfertheiner, R. Thieroff-Ekerdt., *Alimentary. Pharma. Therapeutics.* **2014,** 40(2), 171-177.

25. P. M. Just., *Pharmacotherapy.* **1993,** 13(2), 4S–17S.

26. V. Cattoir, J. Nectoux, C. Lascols, L. Deforges, J. C. Delchier, F. Megraud., *Internat. J. Antimicro. Agents.* **2007,** 29(4), 389-396.

27. R. A. Moore, B. Beckthold, S. Wong, A. Kureishi, L. E. Bryan., *Antimicro. Agents. Chemotherapy.* **1995,** 39(1), 107-111.

28. P. Malfertheiner, F. Megraud, C. A. O'Morain, J. Atherton, A. T. Axon, F. Bazzoli., *Gut.* **2012,** 61(5), 646-664.

29. M. L. Grayson, G. M. Eliopoulos, M. J. Ferraro, R. C. J. Moellering., *Euro. Journal. Clinic. Microbio. Infection. Disease.* **1989,** 8, 888-889.

30. S. J. van Zanten, J. Goldie, J. Hollingsworth, C. Silletti, H. Richardson, R. H. Hunt., *J. Clinic. Pathology.* **1992,** 45, 225-227.

31. R. J. Adamek, M. Wegener, W. Opferkuch, G. H. Ru¨hl., *Americ. J. Gastroenterology.* **1993,** 88, 792-793.

32. A. A. van Zwet, J. C. Thijs, B. de Graaf., *Antimicro. Agents. Chemotherapy.* **1995,** 39, 250-252.

33. M. P. Dore, D. Y. Graham, A. R. Sepulveda, G. Realdi, M. S. Osato., *Antimicro. Agents. Chemotherapy.* **1999,** 43, 1803-1804.

34. N. N. Qureshi, B. Gallaher, N. L. Schiller., *Microbial. Drug. Resistance.* **2014,** 20(6), 509-516.

35. D. I. Edwards., *J. Antimicrobial. Chemotherapy.* **1993,** 31, 9-20.

36. P. J. Jenks, D. I. Edwards., *Internat. J. Antimicrob. Agents.* **2002,** 19(1), 1-7.

37. J. P. Gisbert, L. Gonzalez, X. Calvet, N. Garcia, T. Lopez, M. Roque., *Alimentary. Pharmacology. Therapeutics.* **2000,** 14(10), 319-328.

38. A. Villoria., *Gastroenterology. Hepatology.* **2008,** 31(8), 546-547.

39. M. M. Gerrits, M. R. de Zoete, N. L. Arents, E. J. Kuipers, J. G. Kusters., *Antimicrobial. Agents. Chemotherapy.* **2002,** 46(9), 2996-3000.

40. C. H. Chi, C. Y. Lin, B. S. Sheu, H. B. Yang, A. H. Huang, J. J. Wu., *Alimentary. Pharmacology. Therapeutics.* **2003,** 18(3), 347-353.

41. C. A. McNulty, J. C. Dent, G. A. Ford, S. P. Wilkinson., *J. Antimicrob. Chemotherapy.* **1988,** 22(5), 729-738.