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In vitro evaluation of the potential antibacterial effect of artemisinin on *Campylobacter jejuni*

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Abstract

Artemisinin, an extract of sesquiterpene lactone endoperoxide obtained from Artemisia annua, is routinely used in the treatment of malaria and various forms of human cancer. In order to extend / establish the therapeutic range on animals, in the context of restrictions imposed by organic agriculture and bacterial antibioresistance with a high impact in cases of food toxi-infections in humans, Artemisinin was evaluated in this study for a potential antibacterial effect against Campylobacter jejuni 81-176. The experiments were carried out by disc diffusion technique on Mueller Hinton agar supplemented with 5% sheep blood and microdilution assay in Mueller Hinton broth supplemented with 5% fetal bovine serum, on cultures obtained in microaerophilic conditions. The four concentrations of Artemisinin tested by disk diffusion, 10 μg, 20 μg, 40 μg and 80 μg showed an antibacterial effect on Campylobacter jejuni. The inhibition diameters were of 24-41 mm, with lower values for the solution made in DMSO. The minimum inhibitory concentrations ranged between 156.25 ng / ml and 312.5 ng / ml for Artemisinin solution in DMSO and between 39.0 ng / ml and 78,125 ng / ml for Artemisinin in DMF.

Keywords: Artemisinin, *Campylobacter jejuni*, disk diffusion assay, MIC

1. Introduction

Thermophilic strains of *Campylobacter* spp. (*C. jejuni, C. coli*) produce the most frequent and numerous cases of gastroenteritis, acute diarrheal disease or food toxi-infections in humans. For immuno-compromised patients, these strains are also involved in Guillain - Barré syndrome, a severe autoimmune disease, and quasi-incurable, characterized by progressive neuromuscular acute paralysis. Thermophilic strains of *Campylobacter* spp. are present in the intestinal tract of many animal species, but the main source is poultry (especially for *C. jejuni*), where clinical symptoms may include manifestations of depression, polydipsia, loss of appetite, diarrhea, faeces with abnormal consistency and ruffled feathers.

The control strategies for reducing the incidence of *Campylobacter* spp. on poultry include the infectious pressure drop through the establishment of biosecurity measures, the increase of chickens resistance obtained by competitive exclusion, vaccination or genetic selection and use of antimicrobial alternatives (treatment with bacteriophages, bacteriocins, etc.), the latter being imposed by restrictions due to organic agriculture and more serious phenomenon of antibioresistance, with impact on food toxi-infections in humans (1, 2, 3, 4, 5, 6).

In birds, intestinal colonization with *Campylobacter* strains is frequently concurrent or consecutive to the infections with various *Eimeria* species. The prevention and control measures against coccidiosis in poultry are also related to the phenomenon of resistance installed against the antiparasitic drugs used until now or to the restrictions due to the level of detectable medicinal residues in food of avian origin. All of them generated the need to find alternative treatments (7, 8).

Artemisinin, also known as Qinghaosu, used in standard therapy of malaria and some forms of human cancer, belongs to the endoperoxide sesquiterpene lactone group and was isolated in 1972 from *Artemisia annua* L, an herbaceous plant from the *Asteraceae* family, used in traditional Chinese medicine. Artemisinin (with IUPAC (3R, 5aS, 6R, 8aS, 9R, 12S, 12aR)-octahydro-3,6,9-trimethyl-3,12-epoxy-12H-pyrano[4,3-j]-1,2-benzodioxepin-10(3H)-one and empirical formula C15H22O5) is an odorless and colorless compound, forms crystals with a melting point of 152-157^{o}C, has a molecular weight of 282,332 g / mol, and contains an unstable peroxide group on which its intra-cellular action is based (9).

Currently, Artemisinin and extracts from *Artemisia annua* or other plants are intensively studied in order to bring alternative solutions to the problems concerning the resistance to antimicrobial / antiparasitic and organic agriculture (10, 11, 12).

2. Purpose of the study

In order to extend / establish the therapeutic range on animals, Artemisinin was evaluated in this study concerning the possible antibacterial effect on *Campylobacter jejuni*.

3. Materials. Methods

Artemisinin is soluble in organic solvents and unstable in aqueous solutions. The stock solutions of artemisinin (*Sigma*) tested in our experiments were prepared on the day of each experiment, at concentrations of 0.5 mg / ml, both in DMF and DMSO (*Merck*).

The potential anti-bacterial effect on *Campylobacter jejuni* was studied by disk diffusion technique and broth microdilution assay, which assessed the minimum inhibitory concentration (MIC).

3.1. For **Kirby - Bauer assay** we used filter paper disks impregnated with Artemisinin in concentration of 80, 40, 20 and 10 μg / disk (13), dissolved in organic solvents (DMSO and DMF). To check the test and strain there were also used commercial disks with 25 μg amoxicillin, 10 μg gentamicin and 5 μg enrofloxacin (*Biorad*). The culture medium was Mueller Hinton agar (*Biorad*) supplemented with 5% defibrinated fresh sheep blood.

3.2. Microdilution test was performed in Mueller Hinton broth (*Biorad*), supplemented with 5% fetal bovine serum (FBS), (*Gibco*). In Linbro 96 well flat bottom sterile tissue culture microplates with cover, 100 μl of Mueller Hinton broth were distributed in each well and binary serial dilutions of Artemisinin were made starting from the dilution of 1/50 of the stock solution made in Mueller Hinton broth also. The concentrations of Artemisinin obtained were in the range of 19.53 ng / ml - 10 μg / ml. The bacterial suspension was added in each well in volume of 100 μl.

3.3. For both techniques, the **inoculum** was prepared from a *Campylobacter jejuni* 81-176 culture of 48 hours obtained on TSA agar (*Himedia*), supplemented with 5% defibrinated fresh sheep blood, grown in microaerophilic conditions (CampyGen, *Oxoid*) at 42°C, for 48

hours. The bacterial suspension in a concentration of 0.5 McFarland was made in physiological saline (*I. Pasteur*) for Kirby-Bauer test and Mueller Hinton broth (*Biorad*) for microdilution test.

3.4. For both techniques, the **results** were recorded after 48 hours of cultivation at 42°C in microaerophilic conditions. The results of disk diffusion tests were recorded by measuring the diameter of inhibition zone (in mm). For the commercial antibiotics the interpretation criteria (resistant, R, susceptible, S) were applied, taking into account the recommendations of the Antibiogram Committee of the French Society of Microbiology regarding to the breakpoints (2010), as follows: amoxicillin (as for ampicillin), R≤14 mm, S≥21 mm, gentamicin R≤16 mm, S≥18 mm, and enrofloxacin (as for ciprofloxacin), R≤22 mm, S≥25 mm. The results of MIC test were spectrophotometrically measured at 492 nm (Multiskan EX, *Labsystems*). The determination of MIC range and MIC expression graphics were made by scoring the viability, meaning the growth / inhibition percentage of *Campylobacter jejuni* culture in wells treated with Artemisinin compared to controls (average absorbance values of *Campylobacter jejuni* culture in wells treated with Artemisinin compared with average absorbance values of the control - *Campylobacter jejuni* culture untreated with Artemisinin, considered as 100%).

3.5. All the experiments were performed twice, in quadruplicates for CMI tests. **Statistical significance** of the results was analyzed by the ANOVA: Single factor test (MS Excel 2003).

4. Results

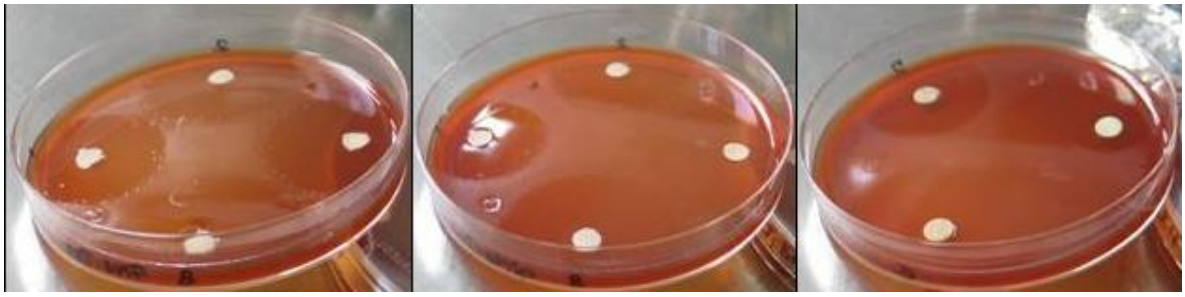
The results regarding to the antibacterial effect of Artemisinin on *Campylobacter jejuni* 81-176 are summarized in Tables 1 and 2 and Figures 1 and 2.

Table 1. Evaluation of the antibacterial effect of Artemisinin on *Campylobacter jejuni* 81-176, by Kirby - Bauer assay. Inhibition zone diameter (mm)

Bacterial strain	Amx		Gen		Enr	ART:DMSO				ART:DMF	
	25µg	10µg	5µg	80µg	40µg	20µg	10µg	80µg	40µg	20µg	10µg
Campylobacter jejuni 81-176	44	33	45	36-39	34-38	31-37	24-30	41-43	39-41	36-39	29-35

Table 2. Evaluation of the antibacterial effect of Artemisinin on *Campylobacter jejuni* 81- 176, by broth microdilution assay (MIC)

Culture	ART:DMSO		ART:DMF		Control					
					MH+FBS		MH+FBS+DMSO (DMSO 1%)		MH+FBS+DMF (DMF 1%)	
Artemisinin concentration	OD_{492}	SD	OD_{492}	SD	OD_{492}	SD	OD_{492}	SD	OD_{492}	SD
5 µg/ml	0.141	0.009	0.147	0.013	-	-	-	-	-	-
2.5 µg/ml	0.250	0.064	0.207	0.050	-	-	-	-	-	-
1.25 µg/ml	0.398	0.099	0.293	0.043	-	-	-	-	-	-
0.625 µg/ml	0.432	0.065	0.461	0.079	-	-	-	-	-	-
312.5 ng/ml	0.458	0.054	0.477	0.039	-	-	-	-	-	-
156.25 ng/ml	0.484	0.052	0.495	0.041	-	-	-	-	-	-
78.125 ng/ml	0.485	0.041	0.494	0.046	-	-	-	-	-	-
39 ng/ml	0.485	0.056	0.494	0.037	-	-	-	-	-	-
-	-	-	-	-	0.471	0.052	0.468	0.051	0.493	0.051



A B C

Figure 1. Evaluation of antibacterial effect of Artemisinin on *Campylobacter jejuni* 81-176 by disk-diffusion assay. A: disks with Artemisinin 10 µg, 20 µg, 80 µg and 160 µg in DMF; B: disks with Artemisinin 10 µg, 20 µg, 80 µg and 160 µg in DMSO; C: disk with amoxicillin 25 µg, gentamicin 10 µg and enrofloxacin 5 µg.

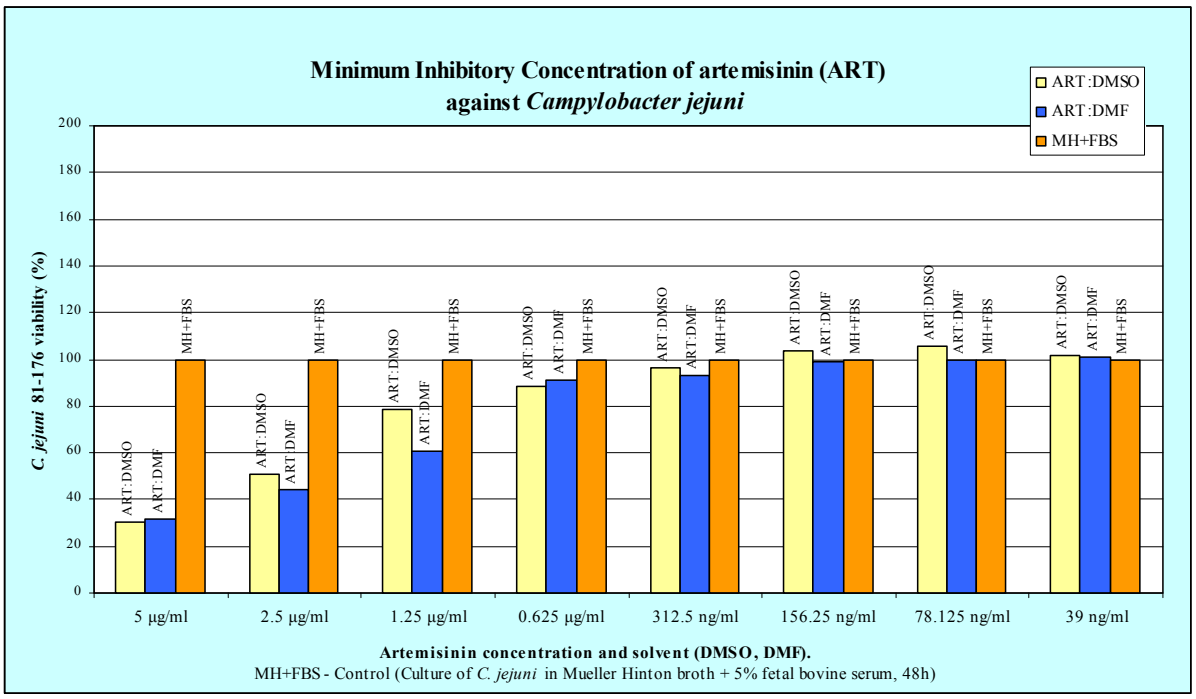


Figure 2. Evaluation of antibacterial effect of Artemisinin on *Campylobacter jejuni* 81-176 by broth microdilution assay. Minimum Inhibitory Concentration of Artemisinin based on the organic solvent used.

In Kirby – Bauer assay, *Campylobacter jejuni* 81-176 strain has proved to be sensitive to all 4 concentrations of Artemisinin, regardless of the solvent in which Artemisinin was dissolved. The inhibition diameters were of 24-41 mm, with lower values for the solution made in DMSO (differences statistically insignificant, P = 0.129927). For the three commercial antibiotics tested, the strain proved to be susceptible (all the recorded inhibition zone diameters were higher than the accepted breakpoints).

Minimum inhibitory concentrations ranged between 156.25 ng/ml and 312.5 ng/ml for Artemisinin solution in DMSO, and between 39.0 ng/ml and 78.125 ng/ml in the case of Artemisinin solution in DMF. As is shown in Table 2, the influences of the DMSO or DMF, tested as controls, were statistically insignificant (P = 0.377137), even if the DMSO values were lower than the DMF values.

5. Discussions

The *Campylobacter jejuni* 81-176 strain, used in our experiments, was isolated in 1981 from a 9 year old girl with an episode of food toxi-infection associated with the consumption of cow milk, but without a proved direct implication of the strain in this case (14). The strain, serotype 23/36 as Penner scheme, was fully sequenced (GenBank: CP000538.1), and is carrying two plasmids, pVir, which encode pathogenicity factors, and pTet, which encodes resistance to tetracycline (15, 16). This strain has proven to be also highly pathogenic for humans, monkeys and chickens, being able to invade the intestinal epithelial cells by endocytosis and to survive into cells by blocking lysosomal fusion (17, 18, 19, 20, 21, 22). *Campylobacter jejuni* strains resistant to tetracycline, as is *Campylobacter jejuni* 81-176 strain, were isolated at a rate of 55.3% in conventional turkey farms, and for farms of chicken grown in organic conditions it was found that strains tet-resistant become prevalent, in proportion of 66.7%, from the 5th week of life, and reach 100% in the 6th week of life (23, 24).

The antibacterial effect of Artemisinin and the organic extracts of *Artemisia annua* on strains of *Escherichia*, *Salmonella*, *Staphylococcus*, *Bacillus* and *Helicobacter* has been studied by other researchers (25, 26, 27). Artemisinin recorded MIC values of 0.25 – 1 μg/ml against *Helicobacter illory* associated with peptic ulcer in humans. Crude aqueous extracts of *Artemisia annua* proved to be effective against tested bacteria at the concentration of 50 mg/ml, while chloroform extracts were active against *E. coli* at the concentration of 26 mg/ml. *Salmonella* strains proved to be the least susceptible against various concentrations of *Artemisia annua* crude extracts. But all of these studies used non-standardized methods for the *in vitro* evaluation of the effects of artemisinin / *Artemisia annua* extracts.

6. Conclusions

Artemisinin has been shown as one of the strong and promising antimicrobial agents that might be used in the control of zoonotic infections associated with *Campylobacter jejuni* strains, including those resistant to tetracycline, as is the *C. jejuni* 81-176 strain. The effect of Artemisinin against *Campylobacter jejuni* was revealed for the first time, by our knowledge, in this study.

The MIC values ranged from extremely low levels, but further studies are needed in biotechnology and pharmacology to establish ways of extraction, formulation / stabilization and use of Artemisinin for the therapy of animals grown in organic and / or conventional farms.

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