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Antibiotic Susceptibility Surveillance of Environmental *Legionella* Strains: Application of the E-Test to Bacteria Isolated From Hospitals in Greece.

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Introduction

Legionella bacteria are the causative agent of legionellosis, an infection mainly acquired through aspiration of contaminated water [1]. Macrolides and flouroquinolones are the treatment of choice for legionellosis. The selection of these categories is mainly based on clinical experience [2]. Especially erythromycin was the first line drug in the treatment of legionellosis [3], even though erythomycin resistant strains can easily be obtained in vitro [4]. Flouroquinolones has been shown to succeed high intracellular inhibition of *Legionella* compared with erythromycin [5].

According to literature, there are no clinical *Legionella* isolates resistant to antibiotics [6], even though some studies show in patient's failure of treatment [7, 8] and change of the antibiotic therapy. The in vitro activity of these antibiotics are not yet well established in clinical or environmental isolates and in addition, MIC wild-type (WT) distributions and epidemiological cut off values are not described yet [9]. As there is no specific regulation on susceptibility testing and *Legionella spp.* has particular nutritional requirements, various methods have been used to determine MIC values, with E-test, broth dilution and disc diffusion methods, being the most popular ones [9].

In order to assess the antibiotic susceptibility of environmental *Legionella* isolates in our region, we committed an in vitro antibiotic resistance monitoring, determining the minimal inhibitory concentration values (MIC) of erythromycin and ciprofloxacin against isolated *Legionella spp.*, using E-test on BCYEa agar.

Materials and Methods

Legionella spp.

A total of 48 previously isolated environmental *Legionella spp.*, were used in the present study. The strains were isolated during environmental surveillance, from four hospitals in Northern Greece during 2009-2011 (data not shown). From the 48 isolates, 17 were *Legionella pneumophila* serogroup 1, 26 *Legionella pneumophila* serogroup 2-15, 3 *Legionella anisa* and 2 *Legionella spp* other than *Legionella anisa*. All isolates were stored at -80 ^{0}C in glycerol stocks.

E-test analysis

Each isolate was subcultured on BCYE with L-cysteine agar and incubated at 37°C for 48 to 72 hours. E-test analysis was performed as described before [9]. Briefly, the inoculum was prepared by swabbing a portion of growth from the plate using a sterile cotton swab. The swab was transferred to a tube containing 5 ml of sterile water and the turbidity was adjusted to a 0.5 McFarland standard by visual examination. The inoculum was spread on BCYE agar plates and E-test strips were applied to the surface according to the manufacturer's directions (Biomerieux). The plates were incubated at 37°C for 48 h before reading the MIC values. The MIC value was determined as the lowest concentration of antibiotic that completely inhibited visible colonies. Isolates that had shown the highest MIC values, or revealed no growth during E-test analysis were retested [10]. As there are no official breakpoints for *Legionella spp.* yet, we used the National Committee for Clinical Laboratory Standards (NCCLS) guidelines, as it was described before [10]. According to these, bacteria are consider susceptible (S) to erythromycin (ERY) when the MIC values are =< 0.5 μg/ml and resistant (R) when there are >= 8 μg/ml. For ciprofloxacin (CIP) these values are as follow: S=< 1 μg/ml and R=> 4 μg/ml [10].

Results

MIC values

The susceptibility range determined was 0.02-2 mg/l for ciprofloxacin and 0.016-2 mg/l for erythromycin (table 1).Nine of the 48 isolates revealed no growth during E-test analysis (3*Legionella spp.* and 6*Legionella pneumophila* serogroup 2-15), thus were excluded from the analysis.

Discussion

Our results indicated that even though ciprofloxacin and erythromycin had common MIC ranges (0.02-2 mg/l and 0.016-2 mg/l), 18% (7/39) of isolates were considered as low-level resistant in erythromycin, while only 5% (2/39) were considered as low-level resistant in ciprofloxacin. This result indicated that ciprofloxacin was more active than erythromycin, against the majority of *Legionella* isolates, in accordance with previous studies [11, 12].

In previous studies, where in vitro susceptibility of *Legionella spp.* was determined in clinical and environmental isolates, the environmental *Legionella* isolates did not yield so high MIC values, especially for ciprofloxacin [11, 12]. An explanation of this may be was the use of E- test in this study, instead of other methods. It is known that this method yields higher MIC values compared with other methods [9].

The presence of antibiotic less susceptible isolates in the environment is not impossible. *Legionella* bacteria are ubiquitous in

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erythromycin MIC mg/l	**<0.016**	**0.016**	**0.064**	**0.094**	**0.16**	**0.125**	**0.23**	**0.25**	**0.38**	**0.47**	**0.5**	**0.75**	**1**	**2**	
number of isolates	6	10	1	1	3	1	3	2	1	1	3	2	4	1	
ciprofloxacin MIC mg/l	**<0.016**	**0.016**	**0.02**	**0.04**	**0.064**	**0.08**	**0.094**	**0.12**	**0.125**	**0.19**	**0.25**	**0.5**	**0.64**	**1**	**2**
number of isolates	3	3	7	1	3	1	2	1	1	4	2	5	1	3	2

Table 1: MIC values of environmental Legionella bacteria against erythromycin and ciprofloxacin.

aquatic and man-made environments where they can be exposed to antibiotics from medical or veterinary practice, or even from those physically secreted from other microbial [13].

It is known that intracellular life of *Legionella* bacteria protects them from various toxic agents, including antibiotics used in clinical treatment [14]. Nevertheless, the presence of antibiotic less susceptible environmental strains could increase the risk of a failed antibiotic treatment in patients with legionellosis. There is a need of antibiotic susceptibility surveillance of environmental *Legionella* strains, in order to assess any changes in resistance patterns.

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