Minimum inhibitory concentration distribution in environmental *Legionella* spp. isolates

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ABSTRACT

In Greece standard tests are performed in the watering and cooling systems of hotels' units either as part of the surveillance scheme or following human infection. The purpose of this study was to establish the minimum inhibitory concentration (MIC) distributions of environmental *Legionella* isolates for six antimicrobials commonly used for the treatment of *Legionella* infections, by MIC-test methodology. Water samples were collected from 2004 to 2011 from 124 hotels from the four prefectures of Crete (Greece). Sixty-eight (68) *Legionella* isolates, comprising *L. pneumophila* serogroups 1, 2, 3, 5, 6, 8, 12, 13, 15, *L. anisa*, *L. rubrilucens*, *L. maceachernii*, *L. quinlivanii*, *L. oakridgensis*, and *L. taurinensis*, were included in the study. MIC-tests were performed on buffered charcoal yeast extract with α-ketoglutarate, L-cysteine, and ferric pyrophosphate. The MICs were read after 2 days of incubation at 36 ± 1 °C at 2.5% CO_2. A large distribution in MICs was recorded for each species and each antibiotic tested. Rifampicin proved to be the most potent antibiotic regardless of the *Legionella* spp.; tetracycline appeared to have the least activity on our environmental isolates. The MIC-test approach is an easy, although not so cost-effective, way to determine MICs in *Legionella* spp. These data should be kept in mind especially since these *Legionella* species may cause human disease.

Key words | antibiotics, *Legionella pneumophila*, *Legionella* spp., MIC, MIC-test

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INTRODUCTION

Legionnaires' disease is caused by bacteria of the genus *Legionella*; these are Gram-negative facultative intracellular bacteria that favor moist environments where the occurrence of free living amoeba is high (Diederen 2008). Aquatic, or moist, environments are the main gateway for human infection since this is usually a result of contaminated aerosol inhalation. Such conditions can be found in spa pools, water systems, and wet cooling systems (WHO 2007), while large water pipe-line networks serve as the prevalent environment for developing biofilms of *Legionella* (Murga *et al.* 2001).

The majority of incidents of Legionnaires' disease are attributed to *Legionella pneumophila* serogroup (sg) 1 strains; however, other *L. pneumophila* serogroups or *Legionella* spp. have also been implicated in human disease (Guyard & Low 2011).

In the current research, we tested the susceptibility of several *Legionella* spp. isolated from environmental samples. Susceptibility testing produced results that could be considered as indicative of the susceptibility of *Legionella* spp. in six antibiotics.

Azithromycin, erythromycin, moxifloxacin, rifampicin, tetracycline, and trimethoprim–sulfamethoxazole were tested against 68 *Legionella* spp. and/or serogroups. All antimicrobial agents used have a proven clinical application based on the national formulary of the National Organization of Medicines. The activity of these antibiotics against such a collection of different strains has not been performed before. The data revealed could be of great usefulness in the instance of human cases that do not respond well to standard therapeutic schemes.

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MATERIALS AND METHODS

Sampling

A total of 1,494 water samples were collected during 2004–2011 from 124 hotels from the four prefectures (Lassithi, Heraklion, Rethymno, and Chania) in Crete (Greece), both on the basis of routine surveillance and following a human case of *Legionella* infection (Chochlakis *et al.* 2013). Six of the samples analyzed herein were isolated during routine surveillance while the remaining 62 were from establishments where human cases of Legionnaires' disease have been reported.

The number of samples collected from each hotel was dependent on the establishment's size and architecture. Sample collection points and conditions were determined based on guidelines for Legionellosis surveillance. To neutralize the residual-free chlorine, sodium thiosulfate was added to the bottles. All water samples were stored at 4 °C and processed within 24 h of collection.

Plate culture method identification

Isolation of *Legionella* from water samples was performed by culture according to the International Standard method ISO 11731 (1998) and ISO 11731-2 (2004). Briefly, water samples were concentrated by filtration and were re-suspended in distilled deionized water. A volume of the suspension was spread on BCYE-α (buffered charcoal yeast extract with α-ketoglutarate, L-cysteine, and ferric pyrophosphate), BCY (buffered charcoal yeast extract without L-cysteine) and agar supplemented with vancomycin, polymyxin B, cycloheximide, and glycine (BioMerieux, Craponne, France) Petri dishes, directly after filtration, after incubation at 50 °C for 30 min and after addition of acid buffer. The inoculated plates were incubated for 10 days at 36 ± 1 °C in 2.5% CO_2 with increased humidity.

Legionella spp. were identified with MALDI Biotyper using the microbial database (v3.1.2.0) (Microflex LT MALDI-TOF mass spectrometer, Bruker Daltonics, Germany). The results were evaluated based on the MALDI Biotyper manufacturer scoring scheme, direct fluorescent antibody/agglutination test (MONOFLUO™ *L. pneumophila* IFA test kit, Bio-Rad), agglutination test (SLIDEX® Legionella-Kit, Biomérieux, Craponne, France), agglutination test of serotypes 2–15 independently (Prolex-Lab Diagnostics, Waltham, USA), polymerase chain reaction amplification and sequencing of 16S rRNA (Lane 1991) in order to discriminate the rest of the *Legionella* spp., for which the results of the Biotyper were under dispute.

In total, seven *L. pneumophila* sg 1, two *L. pneumophila* sg 2, seven *L. pneumophila* sg 3, one *L. pneumophila* sg 5, 15 *L. pneumophila* sg 6, three *L. pneumophila* sg 8, two *L. pneumophila* sg 12, one *L. pneumophila* sg 13, one *L. pneumophila* sg 15, 11 *L. anisa*, one *L. rubrilucens*, one *L. maceachernii*, one *L. quinlivanii*, one *L. oakridgensis*, and 14 *L. taurinensis* were tested.

Antimicrobial susceptibility testing

BCYE-α was used for susceptibility testing since it is not supplemented with antibiotics. Antimicrobial susceptibility testing (AST) was performed by minimum inhibitory concentration (MIC)-test methodology, and by disk diffusion according to the EUCAST (European Committee on Antimicrobial Susceptibility Testing) guidelines (EUCAST 2009).

All strains were re-cultured for 2–3 days at 36 ± 1 °C in 2.5% CO_2 with increased humidity. For the *in vitro* testing of the antimicrobials, colonies were suspended in sterile water and adjusted to a turbidity of McFarland 0.5 using a photometer. Two milliliters were drawn out of the suspension and spread to the entire surface of the 90-mm plate using a plate rotator.

Diffusion disks (Oxoid, Hampshire, UK) were used for each drug: azithromycin (15 μg), erythromycin (15 μg), moxifloxacin (5 μg), tetracycline (30 μg), trimethoprim–sulfamethoxazole (25 μg), and rifampicin (5 μg). Disks were applied within 15 min of inoculation. Zone edges were read at the point of complete inhibition.

Antimicrobial-gradient strips (Liofilchem, Italy) with an MIC scale in a continuous-range concentration of each drug were used: azithromycin (0.016–256 μg/mL), erythromycin (0.016–256 μg/mL), moxifloxacin (0.002–32 μg/mL), tetracycline (0.016–256 μg/mL), trimethoprim–sulfamethoxazole (0.002–32 μg/mL), and rifampicin (0.016–256 μg/mL); one MIC-test strip with antimicrobial gradient was applied to the plates according to the manufacturer's instructions; plates were incubated at 36 ± 1 °C in 2.5% CO_2 with an

increased humidity. The MICs were read after 2 days of incubation from the scale on the strip at the point where the ellipse of growth inhibition intercepted the strip, according to the manufacturer's recommendations.

Inocula of *Staphylococcus aureus* ATCC 6538 and *Escherichia coli* ATCC 25922 were used as control strains in order to record the influence of charcoal (present on BCYE-α) upon the diffusion of the antibiotic in the MIC-test strip. Inocula were prepared from a 24 h culture on Müeller-Hinton agar (BioMerieux) to a 0.5 McFarland standard. The plates were inoculated and MIC-tests were applied as above. The MICs were read after 24 and 48 h of incubation at 37 °C without CO_2, as described above.

A reference strain of *L. pneumophila* sg 1 (NCTC 12821) was also treated as described above, and tested against the above-described six antibiotics.

Statistical analysis

Statistical analysis was performed using the software IBM SPSS statistics v. 19, applying frequencies and analysis of variance (ANOVA) at a 95% confidence interval ($p > 0.05$).

RESULTS

MICs

A total number of 68 isolates were tested, isolated from variable collection points (Table 1). As regards the MIC-test methodology, a large variation in the MICs' distribution and range was recorded among the *Legionella* spp. and among the antibiotics used (Table 2).

MICs were statistically interpreted using ANOVA at a 95% confidence interval ($p < 0.05$). As regards azithromycin, *Legionella* spp. showed a large difference when compared with *L. anisa* ($p = 0.004$), *L. pneumophila* sgs 2–15 ($p = 0.005$), and *L. pneumophila* sg 1 ($p = 0.045$). Similar differences were observed when using erythromycin while comparing *Legionella* spp. with the rest of the species (*L. anisa* $p = 0.007$), *L. pneumophila* sgs 2–15 ($p = 0.001$), and *L. pneumophila* sg 1 ($p = 0.001$). In terms of moxifloxacin, *L. anisa* showed a difference compared to *L. pneumophila* sgs 2–15 ($p = 0.004$) and *L. pneumophila* sg 1 ($p = 0.009$), whereas *L. pneumophila* sgs 2–15 showed a difference compared with *Legionella* spp. ($p = 0.007$) and *Legionella* spp. showed a difference compared with *L. pneumophila* sg 1 ($p = 0.005$). Rifampicin and trimethoprim–sulfamethoxazole proved to be the most stable antibiotics; *Legionella* spp. showing a difference compared with *L. pneumophila* sg 1 ($p = 0.019$) and *L. anisa* showing a difference compared with *L. pneumophila* sgs 2–15 ($p = 0.002$), respectively. On the other hand, the isolates showed the greatest variation in terms of tetracycline MIC; in particular, *L. anisa* showed a difference compared with *L. pneumophila* sgs 2–15 ($p = 0.00$), *Legionella* spp. ($p = 0.008$), and *L. pneumophila* sg 1 ($p = 0.028$), whereas *L. pneumophila* sgs 2–15 showed a difference compared with *Legionella* spp. ($p = 0.00$) and *Legionella* spp. showed a difference compared with *L. pneumophila* sg 1 ($p = 0.012$). All results are given in Table 3.

The *L. pneumophila* sg 1 NCTC strain used as reference generally showed lower sensitivities (azithromycin: 0.38 mg; tetracycline: 48 mg; rifampicin: 0.047 mg; trimethoprim–sulphamethoxazole: 1 mg; and moxifloxacin: 0.38 mg) compared with our studied strains, with the exception of erythromycin (0.38 mg) where the sensitivity was equal to that of the environmental strains.

An attempt was made to correlate the results of the MIC-tests with those of the disk diffusion. However, the

Table 1 | Origin of samples from which isolates tested in the current study were isolated

Origin of sample	Type of sample	No. of isolates tested
Public douche		6
Swimming pool		2
Water storage tank		2
Collector		1
Water-cooled conditioning		2
Public water network		4
Hose		5
Water leaving boiler		5
Water returning to boiler		4
Douche at room	Cold (before flushing)	6
Douche at room	Cold (after flushing)	10
Douche at room	Hot (before flushing)	10
Douche at room	Hot (after flushing)	11
Total		68

Table 2 | The MIC range values for the antibiotics used on all *Legionella* isolates

	MIC range (mg/L)					
	Azithromycin	**Erythromycin**	**Moxifloxacin**	**Rifampicin**	**Tetracycline**	**Trimethoprim–sulfamethoxazole[a]**
L. pneumophila sg 1	0.19–6	0.125–0.38	0.25–0.75	0.047–0.125	64–256	2–32
L. pneumophila sg 2–15	0.094–6	0.094–6	0.25–0.75	0.032–0.5	24–256	2–32
L. anisa	0.19–1	0.19–1	0.5–1	0.023–0.5	12–32	0.094–0.5
L. maceachernii	1.5	1	0.38	0.032	8	1
L. oakridgensis	6	0.38	0.38	0.094	4	0.38
L. quinlivanii	1	0.5	0.38	0.032	16	0.5
L. rubrilucens	4	2	1	0.125	4	1.5
L. taurinensis	2–8	0.5–4	1	0.094–0.38	2–8	0.75–32

[a]Concentrations indicate trimethoprim.

effort was unsuccessful (data not shown). Although high MIC values recorded at the MIC-tests generally agreed with decreased zone edges in the disk diffusion, we could not devise an algorithm that would define a certain decrease in zone radius in the disk diffusion, with a certain increase in MIC in the MIC-test.

Influence of charcoal

Comparing the results in MICs obtained from *E. coli* and *S. aureus* grown on two different media, the antibiotic most influenced by charcoal was tetracycline (one-fold to five-fold increase). The influence of charcoal was pathogen- and time-dependent. All MICs increased over time, with the exception of azithromycin which showed a slight decrease in the 48 h incubation on BCYE-α, although it proved to be relatively stable regardless of the presence of charcoal when tested against *E. coli* but not against *S. aureus*. Rifampicin was relatively stable regardless of the pathogen. On the other hand, erythromycin showed a 1.3- to three-fold increase, moxifloxacin a 1.7- to five-fold increase and trimethoprim–sulfamethoxazole a two-fold increase, always depending on the pathogen (Table 4).

DISCUSSION

In Greece, there have been no data published before concerning the antimicrobial susceptibility of *Legionella* spp. In the current research, we tested environmental isolates, which gave results that could be considered as indicative of the susceptibility of *Legionella* spp. in six antibiotics.

All antimicrobial agents used have a proven clinical application based on the national formulary of the National Organization of Medicines.

The most potent drug was rifampicin (MIC range 0.125–0.5 mg/L). Azithromycin and erythromycin appeared to be less active against *Legionella* spp., rifampicin and trimethoprim–sulfamethoxazole were more active against *L. pneumophila* sg 1 and *L. anisa*, respectively, while little variation was observed with respect to moxifloxacin. Surprisingly, tetracycline was clearly the least potent of all (MIC range 16–256 mg/L), especially with regards to *L. anisa*, *L. pneumophila* sgs 2–15, and *L. pneumophila* sg 1. Tetracycline appeared to be more active on the rest of the *Legionella* spp. tested.

The MIC-test results appeared to be pathogen, medium- and incubation time-dependent. With the exception of the effect of azithromycin on *E. coli*, the MIC of which appeared to decrease after 48 h incubation on BCYE-α, the rest of the MICs either remained the same or increased. However, the increase was different both for *E. coli* and *S. aureus*. Therefore, it is rather unsafe to draw clear conclusions about the effect of charcoal on the MIC of different *Legionella* spp.

Few studies have been conducted using the E-test methodology to determine the MICs of *Legionella* spp. (Bruin *et al*. 2012). Apart from the E-test methodology, agar dilution

Table 3 | Cumulative distribution of azithromycin, erythromycin, moxifloxacin, rifampicin, tetracycline, trimethoprim–sulfamethoxazole MIC for the environmental isolates

			Cumulative % of strains inhibited at indicated concentrations (mg/L)																										
Agent tested	**Species**	**No. of isolates**	**0.023**	**0.032**	**0.047**	**0.064**	**0.094**	**0.125**	**0.19**	**0.25**	**0.38**	**0.5**	**0.75**	**1**	**1.5**	**2**	**3**	**4**	**6**	**8**	**12**	**16**	**24**	**32**	**64**	**96**	**128**	**192**	**256**
Azithromycin	*ani*	11							18.2		54.5	81.8		100															
	L. p.[a]	32					9.4	15.6	25	62.5	87.5	90.6		96.9					100										
	macea	1													100														
	oakri	1																	100										
	quinli	1												100															
	rubri	1																100											
	tauri	14														7.1		28.6		100									
	sg 1	7							14.3	42.9			57.1	71.4					100										
Erythromycin	*ani*	11							9.1	27.3	54.5	81.8	90.9	100															
	L. p.[a]	32						3.1	28.1	71.9	93.8	100																	
	macea	1												100															
	oakri	1									100																		
	quinli	1										100																	
	rubri	1														100													
	tauri	14										7.1		78.6		92.9		100											
	sg 1	7						14.3		42.9	100																		
Moxifloxacin	*ani*	11										18.2	90.9	100															
	L. p.[a]	32								37.5	87.5	93.8	100																
	macea	1									100																		
	oakri	1									100																		
	quinli	1									100																		
	rubri	1												100															
	tauri	14												100															
	sg 1	7								14.3	71.4	85.7	100																
Rifampicin	*ani*	11	9.1	81.8			90.9					100																	
	L. p.[a]	32		52.9	63.2	66.2	83.8	95.6	97.1	98.5		100																	
	macea	1		100																									
	oakri	1				100																							
	quinli	1		100																									
	rubri	1						100																					
	tauri	14					7.1	78.6		92.9	100																		
	sg 1	7			42.9	85.7		100																					
Tetracycline	*ani*	11																			9.1	36.4		100					
	L. p.[a]	32																					3.1		9.4	68.8	93.8	96.9	100
	macea	1																		100									
	oakri	1																100											
	quinli	1																				100							
	rubri	1																100											
	tauri	14														7.1	21.4	85.7	92.9	100									
	sg 1	7																							14.3	57.1	71.4	85.7	100
Trimethroprim/ Sulphamethoxazole	*ani*	11					9.1	18.2	81.8	90.9		100																	
	L. p.[a]	32														28.1	34.4	87.5	93.8					100					
	macea	1												100															
	oakri	1									100																		
	quinli	1										100																	
	rubri	1													100														
	tauri	14											7.1				21.4	85.7	92.9					100					
	sg 1	7														28.6	42.9		71.4					100					

Sg 1.: *L. pneumophila* sg 1. [a]: *L. pneumophila* sgs 2, 3, 5, 6, 8, 12, 15. *ani: anisa. macea: maceachernii. oakri: oakridgensis. quinli: quinlivani. rubri: rubrilucens. tauri: taurinensis.*

Table 4 | MICs of other bacteria grown on different media showing the potential influence of charcoal on E-test

	E. coli				*S. aureus*			
Antibiotic	**M.H. (mg/L)**		**BCYE (mg/L)**		**M.H. (mg/L)**		**BCYE (mg/L)**	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
Azithromycin	12	12	12	8	1.5	1.5	8	8
Erythromycin	24	24	32	32	0.125	0.19	0.5	0.75
Moxifloxacin	0.023	0.023	0.38	0.38	0.023	0.023	1	1
Rifampicin	>32	>32	>32	>32	0.008	0.016	0.125	0.38
Tetracycline	3	6	12	48	0.38	1	1.5	16
Trimethoprim– sulfametoxazole[a]	0.25	0.19	0.75	0.75	0.75	1	3	3

[a]Concentrations indicate trimethoprim.
M.H.: Muller Hinton.

and disk diffusion have also been used in an attempt to record the MICs of *Legionella* spp. (Gomez-Lus *et al.* 2001; Dunbar & Farrell 2007; Erdogan *et al.* 2010; Pandya *et al.* 2010; Sato *et al.* 2011; Sader *et al.* 2012).

Perhaps the difference in the number of surveys that have used either E-test or microdilution/agar dilution/disk diffusion may be due to the cost of the E-test. Nevertheless, it seems that the E-test/MIC-test is rather reliable and may prove of extreme usefulness to laboratories that perform isolation of *Legionella* spp.

To our knowledge, this is the first study reporting MIC values based on the MIC-test approach on *L. pneumophila* sgs 2, 5, 8, 12, 15, *L. anisa*, *L. rubrilucens*, *L. quinlivanii*, *L. maceachernii*, *L. oakridgensis*, and *L. taurinensis*. Regarding *L. anisa*, *L. rubrilucens*, *L. oakridgensis*, and *L. taurinensis*, no MIC data exist in the literature, while a single survey has been conducted that has included *L. maceachernii* (Edelstein *et al.* 1989).

Despite the fact that *L. pneumophila* sg 1 does indeed cause the majority of human cases (Guyard & Low 2011), and reports of deaths have also been recorded, other serogroups as well as other species have been implicated in human disease. With the exception of *L. pneumophila* sg 15 and *L. taurinensis*, which have still not been implicated in human disease, all our other isolates, *L. pneumophila* sg 2, *L. pneumophila* sg 5 (Mineshita *et al.* 2005; Kura *et al.* 2006; Brulet *et al.* 2008; Kusnetsov *et al.* 2010), *L. pneumophila* sg 6 (Chen *et al.* 2006; Fendukly *et al.* 2007), *L. pneumophila* sg 8 (Guillouzouic *et al.* 2008; Schuetz *et al.* 2009; Kawanami *et al.* 2011), *L. pneumophila* sg 12 (Meigh *et al.* 1989), *L. anisa* (Tanabe *et al.* 2009), *L. maceachernii* (van Dam *et al.* 2006; Chee & Baddour 2007), *L. oakridgensis* (Lo Presti *et al.* 2000), *L. quinlivanii* (Berger *et al.* 2006), and *L. rubrilucens* (Matsui *et al.* 2010) have been recorded in human cases of either pneumonia or pontiac fever.

Our results on the MIC ranges of *L. pneumophila* sg 1 and sg 2–15 are in agreement with all equivalent previous studies except for a slight elevation in the MIC of rifampicin and a greater increase in the MICs of tetracycline (Schulin *et al.* 1998; Tsakris *et al.* 1999; Dubois & St-Pierre 2000; Higa *et al.* 2005; Roch & Maurin 2005; Bruin *et al.* 2012; Alexandropoulou *et al.* 2013). For most of the remaining species, to our knowledge, this is the first report of antimicrobial susceptibility testing on environmental isolates.

It is obvious that not every *Legionella* strain isolated from either a clinical patient or the environment requires antimicrobial susceptibility testing. However, such tests should be performed in cases where a patient fails to respond on appropriate empiric regimens or in epidemiological surveys. In the latter cases, the recording of not only the presence of *Legionella* spp. but also of potential high MIC values should signal an alert towards strict compliance with the regulations on water safety (temperature, chlorine concentration, etc.).

CONCLUSION

Overall, despite the potential inhibitory effect of charcoal on most antibiotics, the MIC-test method on BCYE-α with 48 h incubation may be a good option to choose for the *in vitro*

testing of the susceptibility of *Legionella* spp. to antimicrobials. This is especially so since lately a number of human cases caused by species and serogroups other than *L. pneumophila* sg 1 have been described.

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