Article

Legionella spp. Colonization in Water Systems of Hotels linked with Travel-Associated Legionnaires' Disease

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Abstract: Hotel water systems colonized with *Legionella* spp. have been the source of travel-associated Legionnaires' disease and cases, clusters or outbreaks continue to be reported worldwide each year. A total of 132 hotels linked with travel-associated Legionnaires' disease, as reported through the European Legionnaires' Disease Surveillance Network, were inspected and tested for *Legionella spp*. during 2000–2019 by the public health authorities of the island of Crete (Greece). A total of 3,311 samples were collected: 1,885 (56.93%) from cold water supply systems, 1,387 (41.89%) from hot water supply systems, 37 (1.12%) were swab samples and two (0.06%) were soil. Of those, 685 (20.69%), were collected from 83 (62.89%) hotels, testing positive (≥ 50CFU/L) for *Legionella pneumophila)* serogroups 1-10, 12-14 and non-pneumophila species (*L. anisa, L. erythra, L. tusconensis, L. taurinensis, L. birminghamensis, L. rubrilucens, L. londiniesis, L. oakridgensis, L. santicrusis, L. brunensis, L. maceacherii*). The most frequently isolated *L. pneumophila* serogroups were 1 (27.92%) and 3 (17.08%). Significantly higher isolation rates were obtained from hot water supply systems (25.96%) versus cold water systems (16.98%) and swab samples (13.51%). A Relative Risk (R.R.) > 1 ($p < 0.0001$) was calculated for hot water temperature <55 °C (R.R.: 4.43), chlorine concentrations <0.2 mg/L (R.R.: 2.69), star rating <4 (R.R.: 1.73) and absence of Water Safety Plan implementation (R.R.: 1.57).

Keywords: *Legionella*; water systems; risk; water safety plan; hotel

1. Introduction

Hotel water systems can be colonized with *Legionella* spp. and can provide the source for travel-associated Legionnaires' disease (TALD)[1]. Travelers infected in the country that they visit are usually diagnosed in their home country after returning from holidays. The European Legionnaires' Disease Surveillance Network (ELDSNet) at the European Center for Disease Prevention and Control (ECDC) conducts surveillance of Legionnaires' disease at the European Union level. Public health authorities in Crete conduct an inspection, including risk assessment and water sampling, each time a case, cluster or outbreak is reported through ELDSNet among tourists who have stayed in a hotel in Crete. Furthermore, the public health authorities inform through ELDSNet about the actions taken in accordance with the operating procedures [1].

A total of 1,657 cases of TALD with date of onset in 2019 were reported to ECDC by 28 countries, including Greece [2]. In Greece, studies conducted in previous years have demonstrated *Legionella* spp. colonization of hotels' water systems [3-7]. The national

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legislative framework requires regular monitoring, as well as preventive and control measures in accordance with the European and World Health Organization (WHO) guidelines [8, 9]. WHO makes suggestions regarding water system construction, design, routine operational monitoring and management incorporated in water safety plans developed by building owners or managers [8]. We report results of risk assessment and testing for *Legionella* spp. at hotels in Crete where tourists who developed Legionnaires' disease had stayed and had been reported through ELDSNet, together with an analysis of inspection results and water safety practices implemented in these hotels.

The objectives of the present study were to: (a) determine the level of colonization of *Legionella* spp. in hotel water supply systems that have been associated with TALD, and (b) identify the risk factors associated with *Legionella* colonization of hotel water systems that have been associated with TALD.

2. Materials and Methods

2.1.Sample Collection

From 2000 to 2019, the public health authorities in Crete, Greece inspected a total of 132 hotels that were associated with TALD as reported through ELDSNet. In accordance with national guidelines and procedures, samples were taken from hotel water systems including water tanks, hotel room outlets, showers located in swimming pools and spas, garden sprinklers and soil, where applicable.

The sample collection and site selection processes were in accordance to: (a) the European Technical Guidelines for the Prevention, Control and Investigation of Infections Caused by Legionella species and (b) the international standard methods: ISO 5667-2:1982–Part 2: guidance on sampling techniques, while since 2006 samples were collected following the ISO 19458:2006 Water quality ◎ Sampling for microbiological analysis methodology [9-11]. The samples were labeled and temporarily stored in a cool box at a temperature of up to 5 (±3) °C, protected from direct light, before being delivered to the laboratory immediately after the sampling (no more than 24 h).

2.2.Risk Assessment, Data Collection and Corrective Action

Inspections were conducted following the European technical guidelines**[9]**. Water temperature was measured by placing a calibrated thermometer sensor in the middle of the water stream, two minutes after flushing. A portable calibrated microprocessor-based meter was used to measure pH and free chlorine. One L sterile containers containing sodium thiosulphate (20 mg) were used for sample collection. Risk assessment and corrective actions were implemented in accordance with the European guidelines for water systems linked with TALD **[9]**. For each hotel water system the following information was recorded: chlorine concentration, water temperature, pH, type of water disinfection applied, hotel star rating, seasonal hotel operation, hotel capacity in rooms/beds, water safety plan (WSP) implementation, and type of water supply, type of hot water production system, water system maintenance and cleaning frequency.

2.3. Plate Culture Method

Legionella was isolated by culture in accordance with the international standard methods ISO 11731 (1998), and after 2004 with ISO 11731-2 (2004). Water samples were concentrated by filtration and were re-suspended in distilled deionized water. A volume of the suspension (200 μL) was spread on BCYE (Buffered Charcoal Yeast Extract), BCYE minus cysteine and GVPC (Glycine Vancomycin Polymyxin Cycloheximide) (Biomérieux, Craponne, France) Petri dishes: a) directly after filtration; b) after incubation at 50 °C for 30 min and c) after the addition of an acid buffer (0.2 mol/L solution of HCl, pH 2.2 for at least 15 minutes). The detection limit of the procedure was 50 CFU/L. The inoculated plates were incubated for 10 days at 36 ± 1 °C in 2.5% CO_2 with increased humidity. Suspected colonies were randomly chosen for subculture on BCYE minus cysteine, BCYE and GVPC agar.

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2.4. Typing of Legionella Isolates

The agglutination test (SLIDEX *Legionella*-Kit, Biomérieux, Craponne, France), was used to identify the isolated colonies, including distinction between *L. pneumophila* serogroup 1 and serogroups 2–14 and of *L. anisa*. Individual latex polyclonal reagents were used (Pro-lab, Richmond Hill, Canada) for the exact detection of each *L. pneumophila* serogroup.

2.5. Identification—MALDI-TOF Mass Spectrometry

Since 2010, identification of individual *Legionella* colonies against its microbial database (v 3.1.2.0) took place with the MALDI Biotyper (Microflex LT MALDI-TOF mass spectrometer) (Bruker Daltonics, Leipzig, Germany) equipped with a microSCOUT ion source. Spectra were recorded using the flexControl software with the default parameters for optimization set by the manufacturer (Bruker Daltonics, Leipzig, Germany). For each spectrum, 240 laser shots were collected and analyzed (6 * 40 laser shots from 120 different positions of the target spot). All identifications were evaluated according to the manufacturers' scoring scheme.

2.6. Statistical Analysis

The IBM SPSS Statistics Version 24 statistical package and the Epi-Info 2000 version 7.2.0.1 (Centers for Disease Control and Prevention, Atlanta, GA) were used for statistical analysis. Categorical risk variables from water distribution systems and hotel characteristics were assessed for association with *Legionellae*-positive test results. Proportional z-test was calculated to test for significant differences between sampling site and *Legionella pneumophila* serogroup 1 versus serogroups 2-15, serogroup 1 versus *Legionella* non *pneumophila,* and serogroups 2-15 versus *Legionella* non *pneumophila*. When the *p* value was <0.05 the results were considered statistically significant and highly significant when the p value was <0.0001.

3. Results

3.1. Descriptive Data

Of the 3,311 samples collected, 685 (20.69%) originating from 83 (62.89%) hotels, tested positive (≥ 50CFU/L) for *Legionella (Legionella pneumophila)* serogroups 1-10, 12-14 and non-pneumophila species:*L. anisa, L. erythra, L. tusconensis, L. taurinensis, L. birminghamensis, L. rubrilucens, L. londiniesis, L. oakridgensis, L. santicrusis, L. brunensis, L. maceacherii*). Table S1 presents the summary laboratory examination results for *Legionella* spp. by culture per sample type. The most frequently isolated *L. pneumophila* serogroups were 1 (27.92%) and 3 (17.08%). In 70 (55.12%) hotel cold water distribution systems, 297 (16.66%) samples tested positive. In 66 (53.23%) hotel hot water distribution systems, 345 (26.29%) samples tested positive. In 5 (35.71%) hotels, six (15.38%) swab samples tested positive. Table 1 presents the results of *Legionella* spp. colonization of hotel water systems linked with travel-associated Legionnaires' disease according to the sampling site, as well as the results of associations between the different serogroups and *L. pneumophila* in comparison to non *pneumophila*. Table 2 presents the level of *Legionella* spp. colonization of hotel water systems.

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Table 1. *Legionella* spp. colonization of hotel water systems linked with travel-associated Legionnaires' disease (TALD) on the island of Crete, Greece.

	Sample description	No of positive samples / total (%)				*p*-values (proportional z-test)		
		Legionella spp.	*L. pneumophila* serogroup 1	*L. pneumophila* serogroup 2-15	*L. non pneumophila*	Serogroup 1 vs serogroup 2-15	Serogroup 1 vs *non pneumophila*	Serogroup 2-15 vs *non pneumophila*
Cold water system	Cold water first catch sample (rooms closest to boilers)	31/127 (24.41)	6/127 (4.72)	16/127 (12.60)	17/127 (13.39)	0.026*	0.016*	0.852
	Cold water first catch sample (rooms distal to boilers)	102/212 (48.11)	27/212 (12.74)	61/212 (28.77)	32/212 (15.09)	<0.001**	0.468	<0.001**
	Cold water sample after two min flush (rooms closest to boilers)	21/87 (24.14)	6/87 (6.90)	9/87 (10.34)	11/87 (12.64)	0.419	0.202	0.634
	Cold water sample after two min flush (rooms distal to boilers)	25/106 (23.58)	6/106 (5.66)	16/106 (15.09)	8/106 (7.55)	0.024*	0.580	0.083
	Hotel room. cold water first catch sample	4/8 (50.00)	1/8 (12.50)	2/8 (25.00)	1/8 (12.50)	0.522	1.000	0.552
	Hotel room. cold water sample after two minutes flush	1/7 (14.29)	1/7 (14.29)	-	-	-	-	-
Hot water system	Hotel room. hot water first catch sample	5/8 (62.50)	1/8 (12.50)	4/8 (50.00)	1/8 (12.50)	0.106	1.000	0.106
	Hotel room. hot water sample after two minutes flush.	3/7 (42.86)	1/7 (14.29)	3/7 (42.86)	-	0.237	-	-
	Returning hot water sample	26/76 (34.21)	7/76 (9.21)	18/76 (23.68)	6/76 (7.89)	0.016*	0.771	0.008**
	Hot water first catch sample (rooms distal to boilers)	45/142 (31.69)	14/142 (9.86)	27/142 (19.01)	16/142 (11.27)	0.028*	0.966	0.069
	Hot water sample after two min flush (rooms closest to boilers)	47/110 (42.73)	22/110 (20.00)	28/110 (25.45)	12/110 (10.91)	0.335	0.062	0.005
	Hot water sample after two min flush (rooms distal to boilers)	81/158 (51.27)	32/158 (20.25)	60/158 (37.97)	19/158 (12.03)	<0.001**	0.047*	<0.001*
	Hot water sample heated from solar panels	2/7 (28.57)	1/7 (14.29)	1/7 (14.29)	-	1.000	-	-
	Water sample directly from boiler	38/115 (33.04)	9/115 (7.83)	27/115 (23.48)	8/115 (6.96)	<0.001**	0.801	<0.001**
	Water sample from alternative heating source	1/3 (33.33)	1/3 (33.33)	1/3 (33.33)	-	1.000	-	-
	Water sample from spa establishment showers	3/11 (27.27)	1/11 (9.09)	2/11 (18.18)	-	0.534	-	-
	Water sample from swimming pool showers	32/182 (17.58)	7/182 (3.85)	20/182 (10.99)	10/182 (5.49)	0.009**	0.548	0.056
	Hot water first catch sample (rooms closest to boilers)	194 (51.55)	38/194 (19.59)	59/194 (30.41)	27/194 (13.92)	0.014*	0.139	<0.001**

*Significant at 0.05 level, ** Significant at 0.01 level

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Table 2. Level of *Legionella* spp. colonization of hotel water systems (CFU/L).

Sample type	Number of samples			
	Low (⩽ 1.000)	Medium (>1.000 & <10.000)	High (⩾ 10.000)	Total
Cold water distribution system	177 (57.84)	79 (35.11)	64 (41.56)	320
Hot water distribution system	129 (42.16)	144 (64.00)	87 (56.49)	360
Sediment from room shower water sample and from filtering systems	0 (0.00)	2 (0.89)	3 (1.95)	5
Soil	0 (0.00)	0 (0.00)	0 (0.00)	0
Total (n)	306 (44.67)	225 (32.80)	154 (22.48)	685

3.2. Isolation and Identification of Legionella species

Legionella was isolated from both hot and cold water systems (Tables 2 and 3). The following serogroups of *Legionella pneumophila* were detected: 1, 2, 3, 6, 7, 8, 13, 14 and 2-15. Moreover, *L. anisa, L. erythra, L. taurinensis, L. birminghamensis* and *L. rubrilucens* were detected. Table S2 presents the concentrations of *Legionella* non *pneumophila* spp. in water samples (CFU/L).

Table 3. *Legionella* spp. concentration (CFU/L) per serogroup and level of colonization.

***Legionella* species and serogroup (sg)**	***Legionella* spp. concentration (CFU/L)**			
Species	**$\leq 10^3$ (%)**	**$>10^3$ and $<10^4$ (%)**	$\geq 10^4$ (%)	Total (%)
L. sg1	51 (68.00)	19 (25.33)	5 (6.67)	75 (22.20)
L. sgs 2- 15	99 (54.10)	45 (24.59)	39 (21.31)	183 (49.39)
L. non *pneumophila*	63 (55.75)	28 (24.78)	22 (19.47)	113 (30.46)
Total number of samples	222 (59.84)	84 (22.64)	72 (19.41)	320 (16.98)

Table 4 reports the water temperature and chlorine concentration of samples per *Legionella* spp. and serogroup.

Table 4. Water temperature and chlorine concentration of samples per *Legionella* spp. and serogroup.

Parameter		Number of samples	Positive samples (≥50 CFU/L) *Legionella* species and serogroup (sg)			
			Legionella spp.*	sg 1	sg 2-15	*L.* non *pneumophila*
Hot water temperature (Celsius)	**20-40**	197	62	19	45	16
	41-50	285	90	40	51	25
	51-55	170	38	11	26	10
	>55	304	17	5	13	3
	Total	956	207	75	135	54
Cold water temperature (Celsius)	10-20	140	20	6	12	2
	21-25	497	64	16	36	21
	26-30	315	65	12	33	34
	>30	71	15	2	8	8
	Total	1023	164	36	89	65
Residual chlorine (mg/L)	0-0.20	424	110	29	48	50
	0.21-0.50	226	31	5	16	12
	>0.51	345	20	2	12	8
	Total	995	161	36	76	70

*total number of samples that were tested positive to any *L. pneumophila* serogroup or any species

3.3. Univariate Examination of Factors

Table 5 presents the risk factors for *Legionella* colonization per hotel characteristics, water sampling site and physicochemical parameters.

Table 5. Risk factors for *Legionella* colonization per hotel characteristics, water sampling site and physicochemical parameters.

Risk factors	Odds Ratio (95% Confidence Interval)
Boiler outflowing water temperature <60 °C	**27.5455 (1.6349-464.1095)**
Boiler returning hot water temperature <55 °C	**9.1698 (1.1613-72.4041)**
No use of alternative disinfection procedures	**7.2528 (2.2864-23.0074)**
Hot water temperature <55 °C	**5.9124 (3.8358-9.4106)**
Boiler returning hot water temperature <50 °C	**4.6667 (1.5273-14.2593)**
Incorrect application of WSP measures	**3.4593 (2.0965-5.7078)**
Residual Chlorine <0.2 mg/L	**3.3242 (2.3876-4.6595)**
Start of season	**2.2562 (1.3326-3.8200)**
Star classification <4	**1.982 (1.6442-2.3894)**
Exclusive use of solar panels and hot water temperature <55 °C	**1.9438 (1.0398-3.6335)**
Absence of a Water Safety Plan	**1.7459 (1.4109-2.1604)**
Population using municipality water distribution system < 10.000 residents	**1.4624 (1.2243-1.7469)**
Cold water temperature >25 °C	**1.4238 (1.0979-1.8464)**
Residual Chlorine <0.2 mg/L & pH out of range	1.4075 (0.6882-2.8786)
No guidance by the public health authority (1st inspection)	**1.249 (1.0444-1.4965)**
End of season	1.0643 (0.7742-1.4630)
High season period	1.0578 (0.8626-1.2971)
Non-automated disinfection system	0.9769 (0.6039-1.5802)
Exclusive use of solar panels	0.9396 (0.5886-1.5000)
Seasonal operation	0.8499 (0.6968-1.0367)

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Hot water distribution system-distal room to boiler	0.8217 (0.6022-1.1212)
Unsatisfactory operations according to the checklist	0.6672 (0.3539-1.2576)
pH out of range	**0.5481 (0.3432-0.8753)**
Number of rooms >80	**0.4427 (0.3652-0.5366)**
Number of beds >200	**0.4351 (0.3529-0.5366)**
Groundwater as a source of water supply	**0.3717 (0.2564-0.5388)**

3.4. Inspection Results and Implementation of WSPs

The results of 101 hotel inspections were analysed. Water storage tank protection, cleaning of showers, residual chlorine concentration, and water temperatures were among the main findings. Table 5 presents test results for risk factors for *Legionella* colonization per hotel characteristics, water sampling site and physicochemical parameters. Inspection findings are summarized in Table S3.

4. Discussion

Our study demonstrated that approximately 63% of the hotels which were inspected following a Legionnaires' disease case notification were found to be colonized with *Legionella* spp. A retrospective cohort study of 357 touristic accommodations associated with two or more TALD cases conducted in 2011-2016, reported detection of *Legionella* spp. in 67.4% of the 340 accommodation sites for which results of environmental investigation were available [12]. The same study found that the detection of *Legionella* spp. in the water system was not shown to be associated with the risk of a further case [12]. A water system that has been tested negative for *Legionella* does not exclude the possibility that this site was the source of infection. Moreover, a positive Legionella test does not prove that the water system is the source of infection. Increasing competencies of public health authorities for risk assessment and water sampling could contribute to conducting thorough and comprehensive follow-ups of cases, clusters or outbreaks associated with hotels.

During 2011-2016, Greece was among the countries with the highest proportions of accommodations associated with a TALD cluster (Italy=42.6%, Spain=17.1%, France=14.6%, Greece=7.6%) [12]. Unfortunately, it was not possible for the authors to associate epidemiological data for Legionnaires' disease with environmental investigation results.

Previous studies in Greek hotels that were not considered associated with Legionnaires' disease cases have demonstrated *Legionella* colonization in hotels in Thessaly and Corfu in 2018, where 38 (75%) hotels were colonized by *Legionella* spp. [4]. Other studies revealed colonization rates of 86% in a 1989 study, 21% in 2007 in hotels across Greece, and 33% in hotels in southwest Greece [4, 6]. This percentage is also comparable to three similar studies in Turkey, where colonization rates ranged between approximately 60%, to 92% [13, 14]. Moreover, equivalent surveys in hotels in Italy showed colonization rates varying between 60% to 75% [15-17].

Apart from *L. pneumophila* (serogroups 1, 2, 3, 6, 7, 8, 13, 14, 15, and 2–15), other potentially pathogenic environmental species were also isolated, such as *L. anisa, L. erythra, L. tusconensis, L. birminghamensis, L. londiniesis, L. oakridgensis* and *L. maceacherii* [18]. Infection risks from non-*pneumophila* species should not be underestimated, especially in regard to *L. anisa*, which is the second most common species that has been reported worldwide, including on the island of Crete [7].

The most frequently isolated *L. pneumophila* serotype was 1 (30.96%) and 3 (18.27%). ECDC's annual report indicates that "*only 10% of cases were culture-confirmed (10%) probably meaning that disease caused by Legionella species other than Legionella pneumophila is under-estimated*" [2]. Significantly higher incidence rates were related to the hot water network (36.81%), compared to the cold water network (12.04%) and the sediment samples

(21.43%). Worldwide, cases of Legionnaires' disease are due to *L. pneumophila* and more than 80% of cases are due to *L. pneumophila* serotype 1 [19].

Cold water systems were found colonized in high proportion of approximately 57%. Cold water first catch samples, hot water samples after two min flush (rooms distal to boilers), water samples directly from boiler and hot water first catch samples (rooms closest to boilers) were significantly more frequently colonized with *L. pneumophila* serogroup 2-15, compared to *L. pneumophila* seroproup 1 and *L.* non *pneumophila*. Colonization of distal rooms and boiler water could be attributed to a possible low temperature of the boiler water, as well as the length of piping and reduction in water temperature as it reaches distal rooms. There were no significant differences in the sampling site for *L. pneumophila* serogroup 1 comparing to *L.* non *pneumophila*. Our findings demonstrated that hotel water systems with poor temperature control, no use of alternative disinfection procedures, residual chlorine <0.2 mg/L, non-automated disinfection system seasonal operation, star classification <4, population using municipality water distribution system < 10.000 residents, and no guidance by the public health authority had higher odds of *Legionella*-positive results. A systematic literature review identified as contributing factors for potable water systems colonization of hotels where cases of Legionnaires' disease had stayed the following: no water recirculation features, blind ends or closed loops in the main building where patients /guests stayed, stagnation of hot water in the feedback circuit, poor temperature control and lack of disinfectant residual [20]. The acceptable levels of chlorine concentration in hotel water systems are not effective to eradicate *Legionella* spp. in biofilms and/or when amoeba is present in the water system [21]. WSPs provide a multibarrier approach to ensure water safety, and do not rely only on routine chlorine disinfection to reduce Legionella risks [8, 19, 22]. Training of hotel operators and system maintenance staff can increase awareness and competencies in implementing prevention and control measures.

Larger capacity hotels (>80 rooms) had lower risk of testing *Legionella*-positive. Our findings contradict the results of a retrospective cohort study of 357 touristic accommodations associated with two or more TALD cases conducted in 2011-2016. This study reported that the risk of a further Legionnaires' disease case was higher in accommodation sites with 36 to 67 rooms,+ compared to those with less than 36 rooms, while accommodations with more than 67 rooms had the same risk as accommodations with 36 to 67 rooms. The same study found that "*neither the detection of Legionella in the water system nor the type of disinfection were found to be associated with the risk of a further case*" [12].

Hotel water systems that were linked with TALD and with the absence of a WSP had a higher risk of testing positive for *Legionella* spp. compared to hotels that were also linked with TALD but implemented a WSP. During the study period, water safety plan implementation was not mandatory for touristic accommodation facilities. However, it is expected with the recent legislation in Greece regarding water intended for human consumption, operators of hotels and other touristic accommodation sites will implement risk-based approaches [23]. The WSP methodology as described by WHO allows a site-specific process for description of the water system, identification of different hazards and the appropriate control measures [8, 19]. It is expected to improve water systems conditions and provision of safer water to consumers.

5. Conclusion

The present study found that approximately 63% of the hotels which were inspected following a Legionnaires' disease case notification were found to be colonized with *Legionella* spp.. The study also evaluated the significant factors that contribute to the maintenance, management and disinfection of water distribution systems, including the successful implementation of WSPs, to improve hotel water supply and sanitation systems.

Chemical treatment and monitoring of drinking water quality including chlorine disinfection, pH adjustment, and water temperature control of hot water systems are

recommended as control measures in water safety plans, in conjunction with other procedures. It has also been found that antiquated hotel buildings are at increased risk in terms of safety and quality of the water in their distribution system.

To conclude, risk assessment, environmental monitoring and disinfection of water systems, as well as the implementation of preventive control measures (WSPs) are the key elements to prevent contamination by pathogenic microorganisms in large public and private water distribution systems.

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