

FIRST CASES OF CULTURE PROVEN LEGIONNAIRES' DISEASE IN BULGARIA

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ABSTRACT

Background. *Legionella pneumophila* is the most common cause of the potentially fatal Legionnaires' disease with worldwide increasing incidence reports. The bacterium is fastidious and slow growing and most laboratories do not perform cultivation.

Aim. To present results from the first culture proven cases of Legionnaires' disease in Bulgaria.

Materials and methods. Ten lower respiratory tract materials from 10 patients were cultured for legionellae with GVPC supplement. Identification was based on growth characteristics, latex agglutination and monoclonal typing.

Results. Seven *L.pneumophila* strains (serogroup1 and serogroup6) were isolated. Five belong to monoclonal sub-types Allentown/France, Knoxville and Philadelphia. One culture-positive sputum was received in the lab 5 days after sampling. The other materials were plated on the day of sampling, but three of them were obtained long after specific therapy was started and remained negative on culture. Five from the ten patients were with fatal outcome. Three were reported to ELDSNet in real-time as travel-associated.

Conclusions. Isolation of legionellae from patients remains the diagnostic 'gold standard'. The use of selective supplement designed for water samples had no obvious impact on our results in contrast with late sampling. Isolates were from the most frequently reported *L. pneumophila* serogroups

and five of them – from the virulence-associated Pontiac sub-groups. Legionnaires' disease is still underdiagnosed in Bulgaria. Clinicians must be encouraged to send appropriate and timely obtained respiratory materials. This should happen even in cases with other positive microbiological results, since co-infection with viral, fungal or other bacterial pulmonary pathogens might be of importance for patients' treatment and health.

Keywords: *Legionella pneumophila*, Legionnaires' disease; culture from clinical samples; typing.

INTRODUCTION

Legionnaires' disease is atypical bacterial pneumonia, occurring predominantly as a result of inhalation or sometimes microaspiration of legionella-contaminated water. For decades Legionnaires' disease has been considered as underdiagnosed, despite of its individual and public health importance. The overall incidence rate in many states is increasing, but at the same time there is also high heterogeneity in reporting across EU/EEA and other countries (1,2). Members of genus *Legionella* are ubiquitous in water/moist environments, especially man-made water systems. At the same time they are nutritionally fastidious and slow growing on artificial media. Isolation of *Legionella* spp. is the 'gold standard' for diagnosis. The yield from patient cultures depends on the severity of the illness but there are also other considerations. Cultivation requires: proper and timely obtained material from the lower respiratory tract; buffered charcoal yeast extract agar with addition of growth supplements (BCYEa) and antibacterial mixture to reduce background microbiota in sputum; 3-5-7-10 days stereo-microscopic plate observation; various tests for further identification and considerable technical laboratory expertise. Another issue is that patients with Legionnaires' disease are often non-productive of sputum and in such cases invasive procedures are required to obtain lower respiratory tract sample. All of the above explains why hospital-based laboratories in our country do not perform *Legionella* spp. cultivation. Nevertheless, isolates are critically important for epidemiological investigations, where comparison between clinical and environmental strains allows identification of the infection source and thus contributes to public health prevention strategies. *Legionella pneumophila* Sg 1 is considered

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as the most pathogenic species and serogroup (Sg). It accounts for 85% of culture-confirmed Legionnaires' disease cases in Europe (1). In Bulgaria *L. pneumophila* Sg1 was first isolated from a thermal mineral lake in 1981(3) and subsequently from numerous man-made water systems (4-6). Conversely, there were no isolates from patients with Legionnaires' disease. In this paper we present results from the first culture proven cases of Legionnaires' disease in Bulgaria.

MATERIALS AND METHODS

We investigated ten patients with severe pneumonia, admitted at intensive care units and having at least one positive result for legionella infection: Legionella antigen in urine (ICT or ELISA), direct immunofluorescence staining (MonoFluo *Legionella pneumophila* IFA, BIO-RAD), antibody index > 11 in single serum sample (ELISA *L. pneumophila* Sg 1-6 Ig M+IgG, Vircell). We obtain one sample per person, as follows: sputa (n=5), bronchoalveolar lavages (n=3), pleural punctates (n=2). Sputa were digested (Digest-EUR, Eurobio Sci.) for 15 minutes at 22°C and centrifuged in 10 ml dH₂O for 5 min at 2000 rpm. Liquid materials were concentrated for 15 min at 2000rpm. The obtained native deposits were used for direct seeding without further pre-treatments (e.g. acid-treatment with HCL/KCL solution at pH 2.2). The materials were plated on charcoal yeast extract agar with growth supplements - BYCE α (Oxoid). For inhibition of other bacteria we used GVPC (Oxoid), containing glycine, vancomycin, polymyxin B and cycloheximide – a selective supplement routinely used in our laboratory for investigation of water samples for legionellae. Incubation was performed at 35°C with 2.5%CO₂ and increased humidity. Plates were observed daily up to 10 days and suspected ground glass colonies were further sub-cultured and confirmed by: l-cystein dependence, +/- autofluorescence under long-wave (365-nm) UV light, latex agglutination (Oxoid; Prolab) and for *L. pneumophila* Sg1 when appropriate for further investigation purposes - monoclonal sub-typing with the Dresden Panel (7).

RESULTS

Samples for cultivation were actively searched by us in our attempt to confirm Legionnaires' disease by the "gold standard" method – isolation of *Legionella* spp. We were able to obtain lower respiratory tract

materials from 9 males and 1 female with median age 54 yrs (range 29-72 yrs). Because of severe pneumonia, all patients were admitted at intensive care units in seven hospitals (**Fig .1**). All cases had negative results in hospital laboratories when cultured for respiratory pathogens by routine methods, but had at least one positive for legionella infection test in our laboratory. Case-based characteristics are shown in **Table 1**. They were with the following primary categorization as: confirmed cases with positive Legionella antigen in urine (n=8) and probable cases (n=2) - one with positive direct immunofluorescence staining without other tests for *Legionella* and one with a single positive serum sample but with negative urine test for Legionella antigen. Sputum samples were five, followed by three bronchoalveolar lavages and two pleural punctates. Five cases were with fatal outcome as a result of acute respiratory or multiorgan failure. One culture positive sputum sample, stored at 6°C, was received in the lab 5 days after the expectoration. All the other materials were processed on the day of sampling, but three of them were taken late (\geq 7-10 days) after specific therapy with Levofloxacin or Azithromycin was started and remained negative for legionellae up to 10 days of incubation. In the remaining 7 samples suspicious ground glass colonies, which do not grow on sheep blood agar, appeared predominantly after 3-5 days of incubation and in one case – on day 7. *L. pneumophila* was isolated from seven samples, including those from the two initially probable cases. Six isolates were identified as *L. pneumophila* Sg1 and one - as *L. pneumophila* Sg6 (**Fig .2**). Five *L. pneumophila* Sg1 strains belonging to the Pontiac group were found to be Allentown/France, Knoxville and Philadelphia monoclonal sub-types.

Our supplementary investigation revealed that none of the patients was epidemiologically related to the others, but three cases were associated with travelling during the incubation period and were notified to the European Surveillance Scheme for Travel-associated Legionnaires' disease (ELDSNet). For one of them, who traveled in Bulgaria, an environmental isolate was also available, showing complete monoclonal and sequence type match with the clinical one (data not shown). The source of infection was established for 2 other cases and instructions for appropriate behavior at the corresponding locations were given.

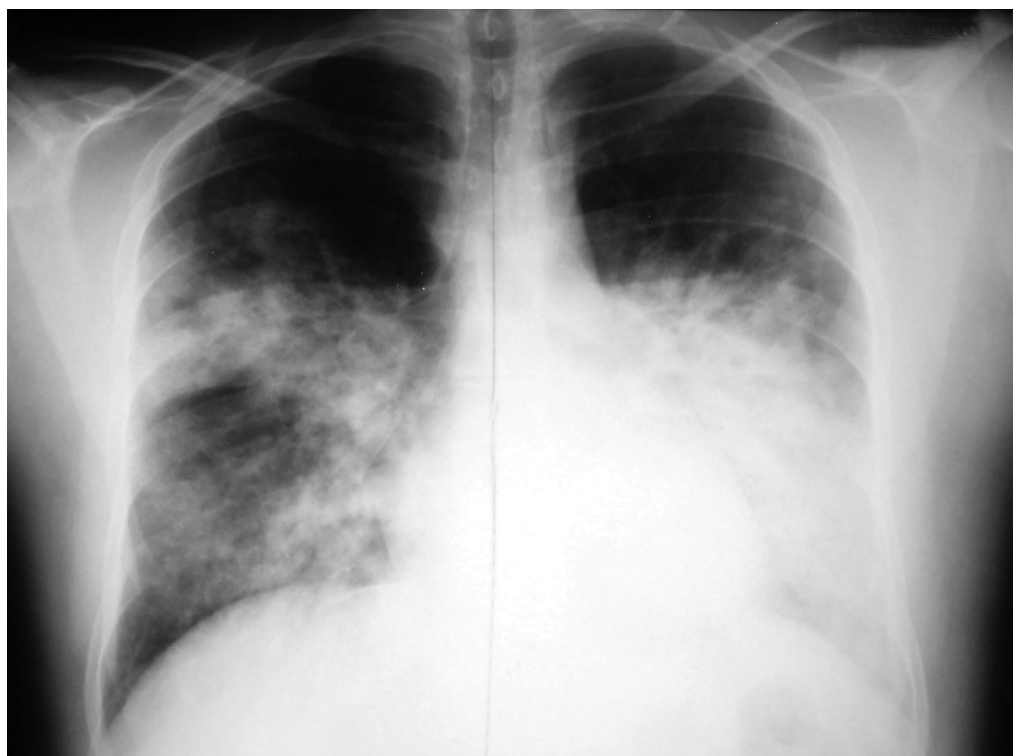


Figure 1. Radiologic manifestation of severe Legionnaires' disease in a 29 year-old patient with no co-morbidities and no risk activities during the incubation period.

Table 1. Main characteristics of the patients with lower respiratory tract samples used for *Legionella* spp. culture.

No	Gender ¹	Age	Initial diagnostic test ²	Initial category zation	Sample ³	Legionella ⁴	Final categorization	Outcome
1.	F	40	LUA	Confirmed	SP	<i>L.pn.</i> Sg 1 ⁶	Confirmed	Recovered
2.	M	60	LUA	Confirmed	BAL	<i>L.pn.</i> Sg 1	Confirmed	Dead
3.	M	58	LUA	Confirmed	BAL	<i>L.pn.</i> Sg 1	Confirmed	Dead
4.	M	72	LUA	Confirmed	SP	<i>L.pn.</i> Sg 1 ⁵	Confirmed	Dead
5.	M	50	LUA	Confirmed	BAL	<i>L.pn.</i> Sg 1 ⁵	Confirmed	Recovered
6.	M	70	DIF	Probable	SP	<i>L.pn.</i> Sg 1 ^{5,6}	Confirmed	Dead
7.	M	67	LUA	Confirmed	PP	None	Confirmed	Dead
8.	M	53	LUA	Confirmed	PP	None	Confirmed	Recovered
9.	M	41	SHT	Probable	SP	<i>L.pn.</i> Sg 6 ⁶	Confirmed	Recovered
10.	M	29	LUA	Confirmed	SP	None	Confirmed	Recovered

¹ F- female; M – male

² LUA – Legionella urinary antigen; DIF – direct immunfluorescence; SHT- single high titer

³ SP- sputum; BAL - bronchoalveolar lavage; PP - pleural punctuate

⁴ *L.pn.* Sg – *Legionella pneumophila* serogroup

⁵ clinical strains for comparison with corresponding environmental ones

⁶ strains from a travel associated cases

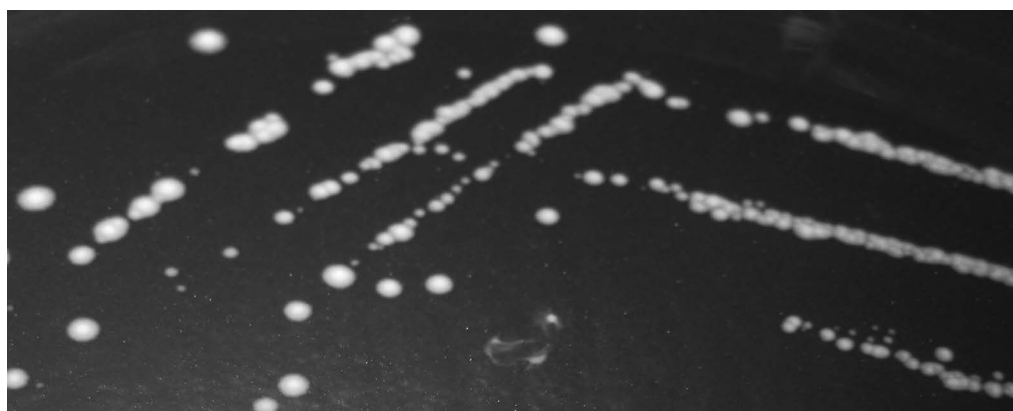


Figure 2. Pure culture of Legionella pneumophila Sg1 from BAL sample

DISCUSSION

Legionnaires' disease accounts for 2% to 15% of all community-acquired pneumonias requiring hospitalization in Europe and North America (8). *L. pneumophila* has been found to be a common cause of bacterial pneumonia, just after *S. pneumoniae* in patients who required intensive care unit admission (9). The case-fatality rate in Legionnaires' disease varies between 10–40% and may approach 50% in nosocomial outbreaks (10). Five of our patients were with fatal outcome, most probably as a result of late diagnosis and/or co-morbidities.

Because of the fastidious legionella nature (lack of laboratory capacity) and their slow growth (3–10 days), cultivation of patient materials is rarely requested and requires active lab approach. Clinicians prefer the easier and quicker test for detection of legionella antigen in urine where depending on the format used, results are obtained within minutes or several hours. However, these tests are mainly designed for detection of *L. pneumophila* Sg1 and thus Legionnaires' disease caused by other *Legionella* spp. or non- Sg 1 *L. pneumophila* could be overlooked. Our results show one such case with *L. pneumophila* Sg 6 infection.

Selective combinations recommended for patient specimens differ from the GVPC designed for water investigation and routinely used by us for environmental testing and in the present study. This had no obvious impact on our results. Delayed obtaining of the material, long after initiation of appropriate anti- *Legionella* therapy, appears to have a more significant negative impact as was the case with our three culture- negative samples. Based on positive isolation results, the two initially probable cases were re-categorized as confirmed ones. Timely obtained and/or appropriately stored and sent materials are crucial for legionella isolation. The disease is not only a personal health issue, but can cause epidemic outbreaks (11–16) with great economic and public impact (17,18). Comparison between clinical and environmental isolates is essential for infection source location, implementation of appropriate control measures, and thus - solving a multiauthority problem.

We have succeeded in culturing *L. pneumophila* from seven out of ten materials. These numbers may look insignificant but are important for such a neglected in our country infection, as well as for

further epidemiological investigations. Five from our *L. pneumophila* Sg1 isolates expressed the virulence-associated epitope recognized by the MAb 3/1 (Dresden Panel). Such strains present a problem in the Mediterranean countries and the UK for both community-acquired and nosocomial cases (7). We were able to locate the infection source in three of our cases, one of which was part of an international cluster so that the result contributed to its limitation.

Legionnaires' disease still remains an important cause of potentially preventable morbidity and mortality in EU/EEA with 23% notification increase in 2018 vs. 2017 and no indication of decreasing burden (1). In cases of atypical pneumonia, clinicians must be aware of the importance to send a set of materials for legionella testing. These must include at least urine, combined if possible with a lower respiratory tract sample, both obtained before/early after starting anti-*Legionella* therapy.

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