An outbreak of leptospirosis imported from Germany to Poland

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Advances in Clinical and Experimental Medicine, ISSN 1899-5276 (print), ISSN 2451-2680 (online)

Adv Clin Exp Med. 2017;26(3):415–419

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Funding sources

Financial support: NIPH-NIH funds (18/EM/2015).

Conflict of interest

None declared

Received on July 06, 2015

Revised on January 03, 2016

Accepted on March 02, 2016

Abstract

Background. Leptospirosis is a zoonotic disease caused by spirochetes of the *Leptospiraceae* family. In both humans and animals the main route of infection is indirect contact – through water or other products contaminated with urine containing spirochetes. Infection most commonly occurs through ingestion of water or food contaminated with *Leptospira* spp.

Objectives. The aim of the study was to characterize cases of leptospirosis imported to Poland from Germany in 2014 and to analyze methods that are helpful for making a diagnosis.

Material and methods. The 10 patients examined were reported as suspected leptospirosis cases on the basis of clinical symptoms and epidemiological investigations. They originated from different regions of Poland and had been working together at a strawberry plantation in the Cloppenburg district of Lower Saxony in Germany. Blood and urine samples were tested by PCR and serum samples by serology. All ELISA positive and negative cases were examined using a reference microscopic agglutination test (MAT).

Results. In the tested group, 6 individuals (60%) were seropositive according to the ELISA, and 2 of them were confirmed by the MAT. The PCR results for the blood and urine samples were negative.

Conclusions. Using the ELISA in the diagnosis of leptospirosis allowed the disease to be identified much faster, differentiating classes of antibodies and recognizing levels of them that are too low to be detectable by the MAT.

Key words: ELISA, outbreak, leptospirosis, diagnosis

DOI

10.17219/acem/62022

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In August 2014, a leptospirosis outbreak was recognized in Poland. All the infected people originated from different regions of Poland, and all had worked during the summer on a strawberry plantation in Germany.

Leptospirosis is a zoonotic disease caused by spirochetes of the *Leptospiraceae* family. In animals, the course of the disease is asymptomatic. Spirochetes colonize the renal tubules of the hosts and a large number of them are excreted into the environment with the urine.^{6,10} In both humans and animals the main route of infection is indirect contact –through water or other products contaminated with urine containing spirochetes. Infection most commonly occurs through ingestion of water or food contaminated with *Leptospira* spp.

In humans the disease is usually biphasic. The first stage – bacteremia (leptospiremia) – persists for one week, followed by the second stage, with immune response and leptospiruria. The second stage can lead to tissue damage directly by leptospira or by inflammatory processes developed as a result of the infection. In humans, leptospirosis has various clinical pictures, from fever of unknown origin or flu-like illness to the classic form of Weil's disease or meningitis. Many patients show spontaneous recovery within 10–14 days.^{9–11} *Leptospira* spp. can survive several months in the intraocular fluid, occasionally causing chronic, recurrent uveitis.¹¹ These bacteria may be also excreted with the urine for several months after infection.⁶

Certain professions are associated with increased risk of infection, including farmers, miners, veterinarians, sewage systems workers and garbage collectors. Leptospirosis outbreaks have been observed in Poland in various regions, mainly in Southern Poland.^{3,8,15} Most cases were identified by the clinical symptoms and physical examination. In the period from 2009 to 2012 sporadic cases requiring hospitalization were reported. According to national epidemiological data, from 2011 to 2013, only 6 cases were reported. However, the number of recognized cases probably underestimated the actual number, due to limited laboratory capacity. In Poland, only 3 medical laboratories are equipped to diagnose leptospirosis and this is probably the reason for such a low number of recognized cases.

The host's humoral response plays a major role in *Leptospira* spp. infection, so serological tests are useful in identifying the infection. IgM antibodies specific for *Leptospira* spp. are detectable as early as 5–7 days after the onset of the first symptoms of the disease, and they persist for at least 5 months. Detecting these antibodies is evidence of acute and/or active infection. IgG antibodies are detected 7 days after the onset of symptoms and persist for years.^{1,7}

Laboratory methods for identifying leptospirosis include urine culture, a reference microscopic agglutination test (MAT), an enzyme-linked immunosorbent assay (ELISA), and an immunofluorescence assay (IFA). Bacteria are isolated from human blood, urine and cere-

brospinal fluid, depending on the stage of the disease.^{9,12} Molecular diagnostic methods are also used, mainly the polymerase chain reaction (PCR). DNA gene fragments of *Leptospira* spp. have been detected in various clinical specimens including serum, urine, aqueous humor of the eye and tissue sections.¹³

Based on the results of amplification reactions and gene fragment sequencing it is possible to determine pathogenic and non-pathogenic human and animal leptospira strains.^{4,13} It is especially useful to apply molecular methods in the incubation stage of the disease, when the specific antibodies are produced. DNA is detected in the blood during the first 5–15 after the onset of symptoms. The number of bacteria in the serum at that time ranges from 10^5 to 10^9 leptospira/L. The results of an ELISA (class of IgM antibodies) and PCR in clinical materials taken at the same time are positive in about 60% of infected individuals.¹³

The aim of the present study was to characterize all the cases of leptospirosis imported to Poland from Germany in 2014, and to analyze laboratory methods helpful for identifying the infection.

Material and methods

Material was collected from 10 individuals returning from Germany (4 women and 6 men) with suspected leptospirosis. Two of the women were 45 years old, and the others 22 and 23 years old; the men were 18, 20, 21, 23, 26 and 56 years old. They presented with high fever (39–40°C), chills, myalgia, and flu-like symptoms. All but patient 5 were hospitalized 1 to 7 days after the onset of symptoms, and they were treated with antibiotics immediately. Patient 5 was treated at a community health center. Detailed characteristics of the patients are presented in Table 1. In differential diagnosis, sciatica was initially diagnosed in patient 5. Patient 9 was tested for HAV, HBV, HCV, *Mycobacterium tuberculosis*, *Chlamydomphila pneumoniae* and *Bordetella pertusis* infections. Urine and blood cultures were performed in all the patients; in patients 6 and 9 the cerebro-spinal fluid was cultured as well.

The blood and urine samples for leptospirosis tests were obtained from the patients between August 18 and September 3 of 2014.

Serological tests

ELISA

IgM and IgG antibodies to *Leptospira* spp. spirochetes were tested in sera with the ELISA method (Serion Classic, Institut Virion-Serion GmbH, Wurzburg, Germany). Seropositive sera were examined using a reference MAT.

MAT (microscopic agglutination test)

The 16 serovars used were *Leptospira interrogans* species: Icterohaemorrhagiae, Canicola, Zanoni, Autumnalis, Bataviae, Hebdomadis, Australis and Pomona; *Leptospira borgpeterseni* species: Poi, Ballum, Tarassovi, Sejroe and Mini; *Leptospira weilii* species: Celledoni; and *Leptospira kirschneri* species: Cynopteri and Grippotyphosa.

Titers of 1 : 400 and higher were considered a confirmation of leptospirosis.

DNA preparation

Bacterial DNA was extracted from serum and urine samples with the QIAamp Tissue kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. Concentrations of the extracted DNA, expressed as optical density, were measured with a Bio-Photometer (Eppendorf, Hamburg, Germany) at 260 nm. The DNA samples were stored at -20°C until tests were performed.

PCR

The extracted DNA samples were amplified to detect fragments of *Leptospira* spp. gene using primer pairs:

a) Amu1 (5'-CGC GCT GCA GTT ACT TAG TCG CGT CAG AAG-3') and Amu2 (5'-CGC GGT CGA CGC TTT CGG TGG TCT GCC AAGC-3'), which are complementary to the LipL32 gene fragment (occurring only in pathogenic species of spirochetes). These primers amplify a 756 bp fragment of the LipL32 gene.

b) G1(5'-CTG AAT CGC TGT ATA AAA GT-3') and G2 (5'-GGA AAA CAA ATG GTC GGA AG-3'), which amplify a 285 bp fragment (plasmid DNA).

The 50 µL reaction mixtures contained PCR buffer with 200 µM dNTPs, 50 pmol of each primer and 2.5 U Hot-Star Taq Plus DNA polymerase (QIAGEN GmbH, Hilden, Germany). An aliquot of 5 µL of DNA template was added to each reaction mixture. The cycling conditions were as follows: 3 min at 95°C, followed by 40 cycles of 30 s of denaturation at 95°C, 30 s of annealing at 60°C and 61°C, 1 min of elongation at 72°C and finally 7 min of elongation at 72°C. PCRs were performed in a Mastercycler gradient apparatus (Eppendorf, Hamburg, Germany). Each run of PCR testing included positive and negative controls (DNA from the strains collection and water, respectively). All the amplicons were analyzed in electrophoresis with 1.5% agarose gel stained with Midori Green Advance DNA Stain (Nippon Genetics Europe GmbH, Dueren, Germany).

Results

The patients examined had been reported as suspected leptospirosis cases on the basis of clinical symptoms and

epidemiological findings. Epidemiological investigations showed that the examined individuals originated from different regions of Poland and that they had been working together at a strawberry plantation in the Cloppenburg district of Lower Saxony in Germany. All 10 of the examined individuals worked on the strawberry plantation along with 4 other Polish individuals hospitalized in Germany due to leptospirosis presenting as Weil's disease. The precise dates when they worked in this region during the summer were not available.

All but 1 of the 10 patients were hospitalized in Poland; the exception (Patient 5) was treated at a community health center. IgM specific *Leptospira* spp. antibodies were detected in 6 of the patients and IgG in 2 of them. The main symptoms of the infected individuals were fever (approx. 40°C), chills, muscle and joint pain and headaches. Two patients presented meningitis, and 1 patient developed renal impairment (Table 1).

Four seronegative individuals had similar symptoms to those presented by seropositive ones. The diagnosis of leptospirosis in those patients was based on epidemiological data from anamnesis, negative blood and urine cultures and other negative test results. Those 4 patients did not agree to, or did not report for, the control tests.

All ELISA positive and negative cases were examined using a MAT. Positive results were confirmed by MAT for 2 individuals. Based on the results of the MAT, *Leptospira kirschneri* (Grippotyphosa titer 1 : 800 and Cynopteri titer 1 : 400) was the species that caused the infection.

All of the PCR results for the blood and urine samples were negative: Bacterial DNA was undetectable in the blood and urine.

Ceftriaxone was the medication most frequently used to treat the patients examined; other antibiotics used were ampicillin, amoxicillin and ciprofloxacin. Because of the serious condition of patients 8, 9 and 10, empirical treatment with ciprofloxacin and amoxicillin or ciprofloxacin and tarcefoksym was recommended. All the seropositive patients were cured and discharged in good general condition.

Discussion

In August 2014, a leptospirosis outbreak was recognized in Poland. The patients had been infected outside Poland, on a strawberry plantation in Germany, and the disease spread across the border. International travel as well as regional environmental and occupational exposure now constitute a major independent risk factor for disease acquisition.³

Saito et al. reported that in 2014 soil and environmental water were collected and cultured using "a novel combination of five antimicrobial agents for selective isolation of *Leptospira* from contaminated samples".¹⁶ They found that both saprophytic *Leptospira* spp. and pathogenic

Table 1. Characteristics of patients suspected of leptospirosis

No.	Age	Sex	Clinical symptoms on admission to the hospital	Onset of symptoms / date of hospitalization	Date of blood sampling (no. of days after the onset of symptoms)	ELISA		MAT	PCR	Treatment duration
						IgM U/mL	IgG U/mL			
1	45	female	fever up to 40°C, chills, myalgia, pain in the calves	5.08.2014 / 6.08.2014	18.08.2014 (13)	19	4	negative	negative	ceftriaxone i.v. 3 x 1 g (12 days)
2	23	female	fever up to 40°C, chills, myalgia, arthralgia, headache	2.08.2014 / 4.08.2014	18.08.2014 (14)	29	6	negative	negative	ceftriaxone i.v. 3 x 1.5 g (14 days)
3	20	male	fever up to 40°C, abdominal pain, myalgia	3.08.2014 / 8.08.2014	18.08.2014 (15)	78	30	negative	negative	ceftriaxone i.v. 3 x 1 g (10 days)
4	23	male	meningitis, fever, headache, arthralgia, stiff neck	24.08.2014 / 28.08.2014	29.08.2014 (5)	10.5	11	negative	negative	ampicillin i.v. 4 x 1.5 g (10 days)
5	22	female	fever up to 39°C, myalgia, arthralgia, headache, nausea, abdominal pain, renal dysfunction (pollakiuria)	30.07.2014 (not hospitalized, treated from 16.08.2014)	02.09.2014 (35)	31	1.8	negative	negative	amoxicillin p.o. 2 x 1 g (21 days)
6	18	male	meningitis, headache	29.08.2014 / 30.08.2014	01.09.2014 (4)	58	4.6	positive	n. d.	ceftriaxone i.v. 3 x 1 g (6 days)
7	21	male	fever up to 40°C, nausea, pain of muscles, bones, joints	10.08.2014 / 13.08.2014	18.08.2014 (8)	11	9	negative	negative	Ceftriaxone i.v. 3 x 1 g (8 days)
8	45	female	fever up to 39°C, abdominal pain, headache, vomiting, urinary tract infection of unknown etiology	7.08.2014 / 12.08.2014	19.08.2014 (5)	4.7	0	negative	negative	ciprofloxacinum 2 x 0.2 g (3 days) amoxicillin p.o. 2 x 1 g (14 days)
9	26	male	fever up to 40°C, chills, headache, sore throat, hepatitis (SIRS-systemic inflammatory response syndrome)	11.08.2014 / 18.08.2014	03.09.2014 (23)	3.8	1.5	negative	negative	ciprofloxacinum 2 x 0.2 g (17 days) tarcefoksym i.v. 3 x 2 g (17 days)
10	56	male	fever up to 39°C, hacking cough, RCC susp. (renal cell carcinoma)	20.08.2014 / 27.08.2014	03.09.2014 (14)	0	3	negative	negative	ciprofloxacinum 2 x 0.2 g (9 days) tarcefoksym i.v. 3 x 2 g (9 days)

The level of specific antibodies to *Leptospira* spp. by ELISA: IgM class: negative < 15 U/mL, borderline range from 15 U/mL to 20 U/mL, positive > 20 U/mL. IgG class: negative < 10 U/mL, borderline range from 10 U/mL to 15 U/mL, positive > 15 U/mL, n.d. – not done.

Leptospira spp. are widely distributed in the environment, and “hypothesized that soil serves as a reservoir for *Leptospira* spp. and infectious source for leptospirosis”.¹⁶

Similar outbreaks of leptospirosis among seasonal strawberry harvesters that have been reported recently pointed out the difficulties with early recognition of the disease.⁵ Leptospirosis has frequently been overlooked or misdiagnosed due to its varied clinical presentations, which range from relatively mild symptoms, such as headache or nausea, to more severe forms, associated with meningitis or renal impairment.

This highlights the need for rapid diagnostic tests for early screening of leptospirosis to replace the less sensitive MAT in outbreak investigations. In the group examined in the present study only 2 patients with seropositive ELISAs were confirmed by MAT (Table 1).

Cultures and the serological MAT method are both laborious and time-consuming. At the same time the results obtained are not clear-cut. Identification of *Leptospira* species using MAT requires constant maintenance of live cultures of multiple strains representing different serotypes of each spirochete species (recognized species of these bacteria have been classified into 25 serogroups and 300 serotypes, therefore it is very difficult to collect and maintain cultures of all of them). Interpretation of the MAT result of a single serum sample is also difficult. Therefore, examinations of paired sera or several repetitions for every patient are needed to monitor dynamic of antibody levels.¹⁴ ELISA kits, on the other hand, detect antibodies specific to all *Leptospira* serogroups. Moreover, these tests allow the class of antibodies to be determined.

For the purposes of the current study, ELISA IgM class antibodies were the best parameter, regardless of the stage of disease, because not all patients produce IgG antibodies. A diagnostic ELISA test was used to detect the presence of specific antibodies in both IgM and IgG classes to obtain information about the complete immune condition of the patient. In 1995 Silva et al. studied 2 groups of leptospirosis patients, 57 of them in the acute phase and 10 during convalescence. IgM class antibodies were detected on the 2nd day of symptomatic disease and were observed in 100% of patients up to the 5th month. Further, 66.7% of the patients had IgM class antibodies after 7 months, and 50% had them after the 12th month of the onset of symptoms. IgG class antibodies were detected on the 7th day of symptomatic disease in 9.1% of the patients, with maximum reactivity (87.5%) between the 2nd and 3rd months; they were not detected at all in 1 patient.¹⁷ Early detection of specific IgG antibodies and IgM antibodies may indicate reinfection.¹⁶

Moderately increased IgM titers accompanied by low IgG titers have also been found. As early as 1985, Terpstra et al. observed that the combination of moderately increased IgM titers and low IgG titers was most often observed in the first 10 day of the disease. This characteristic pattern was found in approximately 2/3 of the sera

obtained from leptospirosis patients during the acute phase of the disease. Terpstra et al. also confirmed observations by Adler et al. that it was not always possible to demonstrate specific IgG in the first few weeks after the onset of disease.^{2,18}

Conclusions

Using the ELISA in diagnosing leptospirosis allowed the disease to be identified much faster, differentiating the class of antibodies and recognizing them at levels too low to be detectable by the MAT. In the near future, however, it is necessary to develop an appropriate, sensitive PCR method and to establish appropriate materials and testing times using the ELISA and MAT, to complete the determination and recognition *Leptospira* species.

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