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First isolation of *Leptospira fainei* serovar Hurstbridge from two human patients with Weil's syndrome

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***Leptospira fainei* serovar Hurstbridge is a recently discovered *Leptospira* species and so far it has only been cultured from animal sources. Based on positive serology and positive PCR for *L. fainei* among patients suspected of having leptospirosis, a role in human disease seems likely. This study describes two patients with Weil's disease from whom *L. fainei* was cultured. A local source of the infections was suspected, as these two patients resided in the same area of Denmark, were hospitalised approximately at the same time and had not been travelling recently. The *Leptospira* species was determined by serology, PCR and sequencing of bacterial DNA. One patient developed autoimmune hepatitis in the course of the *L. fainei* infection and was treated with both antibiotics and immunosuppression with good effect. The other patient had a self-limiting disease and did not receive any treatment.**

Introduction

Leptospirosis is a world-wide zoonosis affecting wild and domestic mammals. It is transmitted to man most commonly by indirect contact with infected animals. Human leptospirosis may present with a wide range of signs and symptoms ranging from mild influenza-like illness to severe multi-organ failure. Classical leptospiraceae are species of *Leptospira biflexa*, *L. parva* and *L. interrogans*. *L. interrogans* includes the human pathogens consisting of 22 serogroups, covering more than 200 serovariants. Overall, the serogroups Icterohaemorrhagiae, Sejroe and Saxkoebing have most clinical relevance. Recently, *L. fainei* has been suspected as a new human pathogen [1, 2]. *L. fainei* serovar Hurstbridge was first isolated from culled sows in Australia in 1994 [3], and it is both a new species and a previously unknown serogroup (Hurstbridge). This report presents what are believed to be the first two cases in which *L. fainei* serovar Hurstbridge has been cultured from patients with suspected leptospirosis.

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Materials and methods

Culture

Each specimen (blood or urine) was inoculated into Bacto Leptospira Medium Base, supplemented with Bacto Leptospira Enrichment (EMJH; Difco Laboratories), incubated at 30°C for 4 weeks and examined twice weekly by dark field microscopy.

Electron microscopy

EMJH medium with *L. fainei* grown for 7 days was centrifuged for 20 min at 5000 rpm. The pellet was resuspended in 50 μl of medium and preparations were made for negative staining with ammonium molybdate 1% [4].

Serology

Patient sera were tested by the microscopic agglutination test (MAT) with serotypes known in Denmark (Patoc, Copenhageni, Sejroe, Icterohaemorrhagiae, Poi, Canicola, Ballum, Bratislava, Pomona, Grippotyphosa, Saxkoebing, Bataviae) and subsequently by Dr P. Perolat at the Pasteur Institute, Nouméa, New Caledonia, with antisera including the serotype Hurstbridge from *L. fainei* strain BUT6^T.

Sequencing of 16S RNA

Preparation of crude DNA extract, performance of PCR and sequencing of 16S rDNA were performed as described previously [5], with different 16S primers. The complete sequence of the described *L. fainei* isolate SSI 5402-98 was submitted to the EMBL Nucleotide Sequence Database under the accession number Y19243.

Results

Patient A

A 49-year-old Danish male was admitted to hospital with increasing jaundice, fatigue and weight loss (10 kg). Jaundice was first noted 1 month before admission and at the same time the patient had observed darkening of the urine and clay-coloured stools. For 6 months before admission, the patient noted a tendency to subconjunctival bleeding and pains behind the left eye, and during the same period, occasional temporal light reflexes were noted. The patient's employment involved cleaning aircraft (including toilets) at a Danish international airport. The patient had no prior history of blood transfusion, illicit drug use, hepatitis, chronic liver disease or alcohol abuse. He had not travelled outside Denmark since a visit to southern France 9 months earlier and a visit to Turkey 2 years earlier. No other occupational or recreational risks could be established. The initial diagnosis in this patient was obstructive liver disease in view of the increase in alkaline phosphatases. Ultrasound scanning showed no abnormalities in liver, gallbladder, kidney or pancreas. Results of investigations on admission were: haemoglobin concentration 7.9 mM; white cell count 5.1×10^9/L; platelet count 309×10^9/L; serum creatinine 87 mM; serum electrolyte values, potassium 4.7 mM, sodium 138 mM; total serum bilirubin 372 mM; aspartate aminotransferases (ASAT) 1112 U/L; alkaline phosphatase 320 U/L; PP 36%.

Because of an elevated IgG (28.4 g/L), a moderately positive value of smooth muscle-cell antibodies (detected by immunofluorescence, Statens Serum Institut, Copenhagen, Denmark) and negative hepatitis serology (including hepatitis A, B and C and cytomegalovirus), a negative *Leptospira* MAT (with the previously known common serotypes in Denmark, not including *L. fainei*) and negative microscopy for *Leptospira* in the urine, the patient started immunosuppressive treatment with prednisolone and azathioprine. This treatment resulted in a prompt two-fold fall in ASAT and a simultaneous fall in serum bilirubin concentration. Subsequently, a blood culture for *Leptospira* was found positive. Although the bacteria did not agglutinate with antibodies against the previously known *Leptospira* serovars, a riboprint pattern showed familiarity with patterns obtained from *L. interrogans* serogroups Icterohaemorrhagiae, Grippotyphosa, Javonica and Poi (data not shown).

In addition to the immunosuppressive treatment, the patient was treated with intravenous penicillin for 7 days. Further cultures indicated treatment failure, as the *Leptospira* could still be isolated (>1 month after initial culture). Treatment was changed to amoxycillin for 4 weeks and later culture was negative. Because of the prompt effect of the immunosuppressive treatment and biochemical parameters suggesting autoimmune hepatitis, this treatment was continued. Eye symptoms disappeared after treatment with both immunosuppression and antibiotics.

Subsequent analysis of the isolated strain. Sequencing of 16S rDNA has become a generally accepted tool in the species identification of micro-organisms. Ribosomal RNA genes are ubiquitous and highly conserved among bacteria, and long enough to contain a reasonable amount of phylogenetic information [6, 7]. 16S DNA sequencing placed the isolate in the *Leptospira* genus closest to *L. fainei* (99.4% identity). In addition, growth at 13°C, with 8-azoguanine and with copper sulphate, and the fact that 1 M NaOH produced no spherical forms, were consistent with *L. fainei*, but not with known pathogenic *L. interrogans* serovars.

The negatively stained cells showed a regular wavy outline and were 10–15 μm long and 0.1 μm wide (Fig. 1). Generally, the cells had hook-shaped ends. The wavelength of the coils was 0.5 μm and the amplitude was *c*. 0.2–0.3 μm. A single flagellum was inserted at each end of the cell (endoflagellum).

At the Pasteur Institute, Nouméa, New Caledonia, the strain was confirmed to be *L. fainei* serogroup Hurstbridge by agglutination with positive sera (titre 400, no cross-agglutination at a significant level with other reference sera). Serological examination showed an MAT titre of 100 in the patient's blood from the day of admission and a similar titre in a blood sample taken 2 weeks later (serogroup Hurstbridge, no cross-agglutination with other *Leptospira* species).

Patient B

A 46-year-old Danish male was admitted 1 week after patient A to the same department, with pain in the upper right abdomen and in the lower back. During the last 5 months the patient had experienced similar pains on three occasions, and *c*. 2 months before admission the patient had severe bilateral headaches and dizziness; these symptoms were present for 4 days. The patient worked in information technology, had no prior history of blood transfusion, illicit drug use, hepatitis, chronic liver disease or alcohol abuse. He had not travelled since a 14-day visit to Venezuela 18 months earlier. The patient lived in an old farmhouse but had had no apparent exposure to sources of leptospires. The

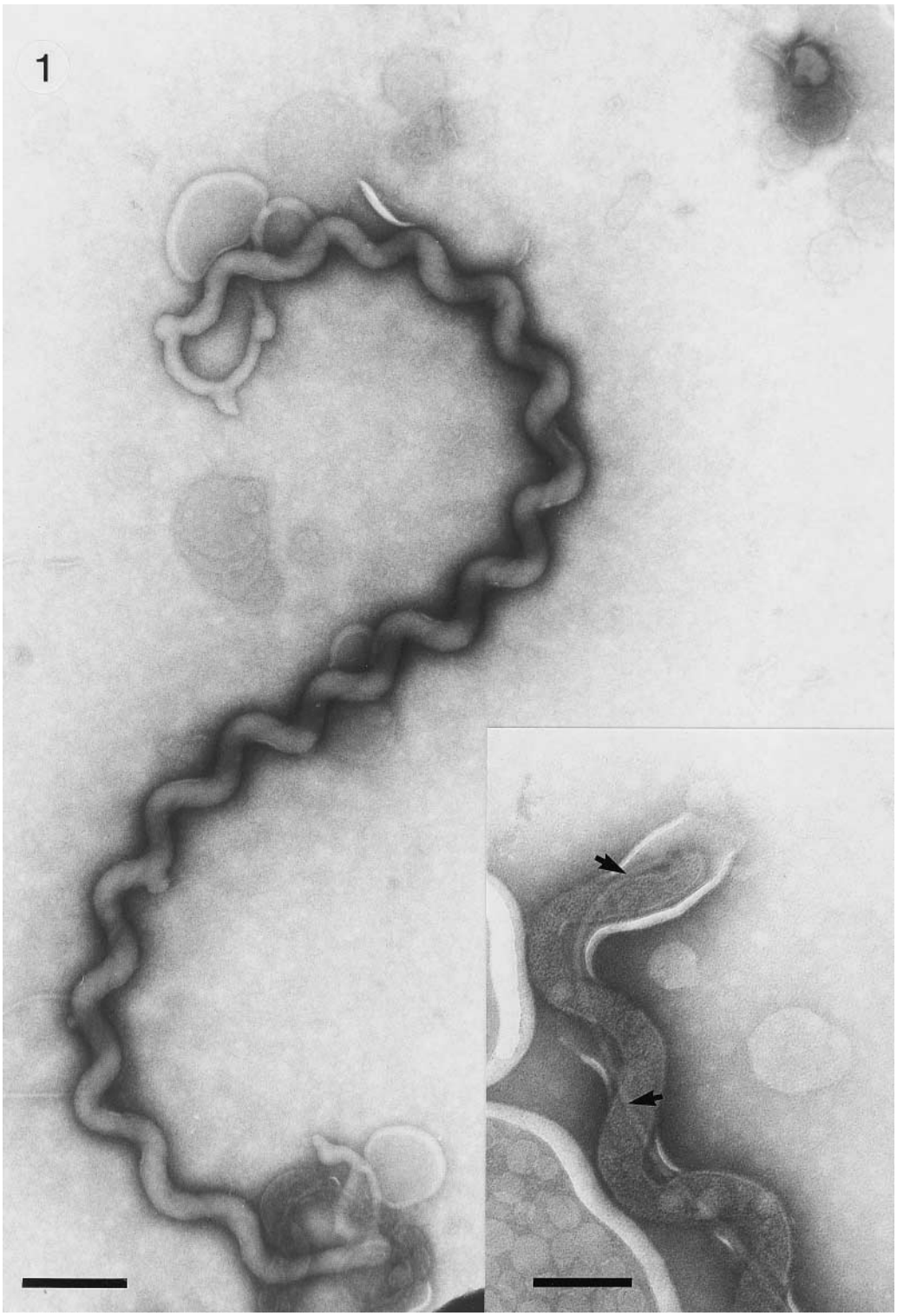

Fig. 1. *L. fainei* negatively stained with ammonium molybdate 1%, showing regular waved cell with hook-shaped ends (×32 000; bar 0.5 μm). Insert shows the hook-shaped cell with the endoflagellum (arrows) (×75 000; bar 0.2 μm).

patient was pyrexial (39°C) and icteric with tenderness in the upper right abdomen. Results of biochemical investigations on admission were: haemoglobin concentration 8.9 mM; white cell count 6.3×10^9/L; platelet count 181×10^9/L; serum creatinine concentration 108 mM; serum electrolyte values, potassium 3.8 mM and sodium 135 mM; total serum bilirubin 76 mM; ASAT 111 U/L; alkaline phosphatases were normal; there was microscopic haematuria. Ultrasound examination revealed possible parenchymatic liver disease. An echo-rich process 1.5 cm in diameter was noted in the right kidney. The lesion in the right kidney was noted again in a subsequent ultrasound examination and was believed to represent a benign haemangioma not related to the patient's symptoms.

During the following days, the patient's abdominal pain disappeared and the jaundice declined. Because of

continued microscopic haematuria, intravenous urographic examination and endoscopy of the bladder was performed, but no abnormalities were demonstrated. An MAT test for *Leptospira* antibodies with previously known *Leptospira* serotypes and urine microscopy for *Leptospira* were negative. Subsequently, a leptospira-like micro-organism was cultured from the urine. However, a control culture 1 week later was negative.

Subsequent analysis of the isolated strain. PCR examination of the blood sample (taken for serology) for the presence of *L. fainei* was positive and an MAT titre of 100 was found (serogroup Hurstbridge, no cross-agglutination with other *Leptospira*). Furthermore, the cultured strain was confirmed as serogroup Hurstbridge (titre 400, no cross-agglutination at a significant level with other reference sera; Pasteur Institute, Nouméa, New Caledonia).

Discussion

It has been suggested previously that the new *Leptospira* species, *L. fainei*, is a human pathogen. Of 723 diagnostic sera from Australian human patients suspected of having leptospiral infection and tested for antibodies against *Leptospira*, 13.4% had antibodies against *L. fainei* with titres >128. This was related to the fact that none of 62 control sera (from laboratory staff) gave similar titres and no cross-reactions to other leptospiral serovars could be demonstrated [1]. A leptospirosis surveillance programme in the Seychelles identified *L. fainei* by a PCR assay and by an MAT as the second most frequent serotype infecting human patients with suspected leptospiral infection, and a correlation to severe cases of human infection was confirmed [2].

Infection with *Leptospira* species usually involves contact with water, soil or vegetation contaminated with *Leptospira* from urine of infected animals. None of the patients described in this paper had any obvious risks of such transmission. Patient A's employment involved cleaning of toilets in aircraft, and renal carriage of *Leptospira* has led to rare examples of human-to-human transmission by urine or sexual contact [8, 9]. Patient A always wore gloves while cleaning toilets, and patient B had no obvious risks in this regard. Although no apparent environmental or personal connections between these two patients could be established, a common source seems likely, as both patients became ill at approximately the same time and lived in the same area of Denmark. Furthermore, neither of the two patients with confirmed *L. fainei* infection had travelled abroad recently. Thus the data suggest that this new species is present in the local environment.

Patient A was treated with steroids and azathioprine, which rapidly led to an improvement in his condition, including a normalisation of liver parameters. This occurred before the patient started antibiotic therapy. It has been reported previously that immunosuppressive treatment, although combined with antibiotics, can give symptomatic relief in severe leptospirosis [10–14]. It has been speculated that most of the multisystemic manifestations of leptospirosis result from indirect mechanisms and that the presence of bacterial antigens in some tissues for a long time after the elimination of live leptospiral organisms may perpetuate an inflammatory reaction responsible for much of the pathology [15]. In addition, severe chronic hepatitis in dogs has been linked to leptospiral infection [16]. Patient A had hypergammaglobulinaemia and it has been reported previously that autoimmune hepatitis with hypergammaglobulinaemia may be induced by infectious agents [17, 18].

In patient A, *L. fainei* was cultured twice with an interval of more than a month. This carrier state had probably lasted longer, as the patient developed jaundice at least 1 month before admission, and had subconjunctival haemorrhages for >6 months before admission. Chronic infection has, on rare occasions, been described for other *Leptospira* species [19]. Treatment with steroids and the unknown infection characteristics of this new *Leptospira* species may be contributory factors.

Treatment for >1 week may be necessary for *L. fainei*, because in patient A *L. fainei* could still be isolated after 1 week of intravenous penicillin, but not after 4 weeks of subsequent amoxycillin treatment. Data obtained by PCR in another study gave a similar indication of longer treatment being necessary for *L. fainei*, as PCR remained positive in 8 of 15 patients despite a 5-day course of intravenous penicillin [2]. Furthermore, subsequent susceptibility testing of the *L. fainei* strains isolated in this laboratory suggested reduced sensitivity (inhibition of growth rather than killing) when compared with an *L. icterohaemorrhagiae* reference strain (data not shown).

In conclusion, a new species of *L. fainei* seems to be involved in human disease in different parts of the world, and the inclusion of this species in micro-agglutination screening would seem to be justified. So far, treatment with amoxycillin for 4 weeks seems sufficient.

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