Epidemiological and Environmental Investigations of *Legionella pneumophila* Infection in Cattle and Case Report of Fatal Pneumonia in a Calf

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A fatal pneumonia due to *Legionella pneumophila* was diagnosed in a young calf reared in a dairy herd located in northern Italy. Clinical symptoms consisted of watery diarrhea, hyperthermia, anorexia, and severe dyspnea. The pathological and histological findings were very similar to those observed in human legionellosis. *Legionella pneumophila* serogroup 1 (SG1) and SG10 were isolated from the calf's lung, and *L. pneumophila* SG1 was isolated from the calf's liver. *L. pneumophila* SG1 was also demonstrated in the lung tissue by immunofluorescence and immunohistochemical examinations. Nine of 10 *L. pneumophila* SG1 isolates belonged to the Olda subtype, and 1 belonged to the Camperdown subtype. A very low prevalence of antibodies to *Legionella* was detected in cows and calves reared in the same herd. Cultures of aqueous sediment of an old electric water heater which supplied hot water for the feeding of the calves yielded *L. pneumophila* SG1. Four of the colonies tested belonged to the Olda subtype. Ten clinical and four environmental isolates were examined for the presence of plasmids. Nine of them were also examined by pulsed-field gel electrophoresis assay, and the same patterns were found for *L. pneumophila* SG1 Olda strains isolated from the calf and from the electric heater. This is the first report of a documented case of a naturally occurring *Legionella* pneumonia in an animal. Cattle probably act as accidental hosts for legionellae, much the same as humans.

*Legionella pneumophila* is a well-known cause of infection in humans. In humans legionellosis occurs in two main forms: Legionnaires’ disease (19, 33) and Pontiac fever (21). Legionnaires’ disease is a severe pneumonia, often progressing to multisystemic disease and sometimes death, whereas Pontiac fever is a much less serious nonpneumonic illness. Legionellae are bacteria that are ubiquitous in natural aquatic ecosystems (18, 30). In Legionnaires’ disease legionellae grow intracellularly in macrophages and monocytes (24), whereas in aquatic habitats a variety of amoebae and ciliates act as hosts (17, 41, 42). Hot water systems are frequently colonized by legionellae (1, 45). Infection is acquired when water containing legionellae is inhaled (36) or aspirated (50) into the lungs.

However, the widespread distribution of legionellae is in contrast to a somewhat surprising lack of clinical reports of *L. pneumophila* infection in animals, which has prompted several investigators to assess the susceptibilities of different animal species to *Legionella* infection. Investigations have been carried out with both domestic animals (cattle, horses, swine, sheep, goats, dogs, and rabbits) and wild animals (antelopes, water buffaloes, camels, and pigeons) in order to detect a serological evidence of infection (2, 4, 8, 10, 12, 14, 38, 47), yet so far the results have not been conclusive. Among all animals investigated, horses yielded the highest prevalence of antibodies to *Legionella* (4, 12), even though the experimental infection of this species only prompted a marked serological response without clear signs of clinical illness (9).

In 1987, Boldur and colleagues (4) reported the isolation of *L. pneumophila* serogroup 1 (SG1) from the lung tissue of two calves which had died of a disease of unknown etiology (4). No macroscopic lesions were observed, however, and an association between the isolated organism and the disease was not documented. It was therefore suggested that the bacterium might have been aspirated with contaminated material during coma.

Due to the reasons mentioned above and because legionella organisms are difficult to identify and isolate, requiring specialized laboratories, routine cultures for this bacterium are not usually performed with veterinary specimens. In order to verify a possible role of *Legionella* in animal respiratory syndromes, specific media have started to be used for the routine diagnosis of animal pneumonias at the Istituto Zooprofilattico Sperimentale in Pavia. In 1993 we reported a severe case of pneumonia due to *L. pneumophila* SG1 in a calf (16).

In the present paper we extensively describe the investigations carried out to assess the relevance of the infection in the herd where the disease had occurred.

**MATERIALS AND METHODS**

A young calf was submitted for examination to the diagnostic laboratory of the Istituto Zooprofilattico Sperimentale in Pavia, which is in northern Italy. The calf came from a herd of 112 Italian-Friesian dairy cows in which a high calf mortality rate had occurred since the previous winter. About 40% of the calves were born weak and subsequently died from enteric and pulmonary diseases. The survivors were mostly in poor condition.

The herd was located in the Po Valley, a few kilometers from Pavia, and was reared in dilapidated buildings under poor hygienic conditions. The parturient cows were debilitated due to a low-protein diet lacking in vitamins and micro-
elements. As a measure to prevent digestive troubles, calves were fed for their first few days on colostrum diluted 3:1 with hot water and then with powdered milk reconstituted with the same hot water.

The calf submitted to our laboratory was about 20 days old, and it had had watery diarrhea for 2 days, when it suddenly became dyspneic, febrile, and anorectic; weakness and severe prostration followed. Penicillin and streptomycin were given intramuscularly, but the calf died a few hours later.

**Pathology.** Following postmortem examination, specimens of the calf’s lung, spleen, and liver were fixed in 10% neutral phosphate-buffered formalin, embedded in paraffin, and sectioned at a thickness of 5 μm. Hematoxylin-eosin, Gram, and Giemsa stainings were performed on sections from each specimen. Deparaffinized sections were studied by the avidin-biotin-peroxidase complex procedure (25) for the presence of *L. pneumophila* SG1 and *L. pneumophila* SG10. The rabbit antisera were prepared with the calf tissues in our laboratory in Pavia and at the National Legionella Reference Laboratory, Vyscow, Czech Republic, by V. Drašar, respectively.

Dewaxed lung tissue sections were also examined for the presence of *L. pneumophila* SG1 by indirect immunofluorescence assay (IFA) with homemade antiserum prepared with the reference strain *L. pneumophila* SG1 Philadelphia 1 (ATCC 33152).

**Bacteriology.** Samples of lung, liver, spleen, and kidney tissue from the calf were collected for culture. The surfaces of the organs were seared and material was collected with sterilized instruments from the core of the tissues to ensure aseptic sampling. Specimens were directly streaked onto the surfaces of the culture media for aerobic and anaerobic bacteria and mycoplasmas. The plates were incubated at 37°C in humidified air plus 2.5% CO₂. Suspect colonies of legionellae were checked for failure to grow in the absence of cysteine and for hippurate hydrolysis. Identities of species and serogroup were performed by direct immunofluorescence assay with an *L. pneumophila*-specific monoclonal antibody (Diagnostik Pasteur, Marnes-La-Coquette, France) and polyclonal monovalent *L. pneumophila* SG1 to SG6 antisera (Seimedx, Dasit, Cornaredo, Italy).

The *L. pneumophila* strains which did not react with the SG1 to SG6 reagents were tested by IFA with homemade rabbit antisera to SG7 to SG10 in Rome and by slide agglutination for SG10 to SG15 by V. Drašar in Vyscow, Czech Republic. The *L. pneumophila* SG1 isolates were further subgrouped by IFA according to the Oxford subtyping scheme (48) with a panel of six monoclonal antibodies (purchased from the Redcliffe Hospital, Oxford, United Kingdom).

**Virology.** Lung tissue fragments were ground in a mortar with sterile quartz sand and were resuspended in Eagle minimum essential medium supplemented with antibiotics (penicillin, streptomycin, amphotericin B). Bovine lung and bovine kidney primary cell cultures were used to check for the presence of viruses, and McCoy cells were used to check for the presence of *Chlamydia*. For virological examination, cell monolayers were inoculated with the lung suspension, incubated at 37°C in a 5% CO₂ atmosphere, and examined daily for cytopathic effects for a week. The cells were then serially passaged three times, and the third monolayer passage was allowed to grow on glass coverslips. To exclude the presence of noncytopathic bovine viral diarrhea virus, the cells were examined by IFA. The bovine viral diarrhea virus monovalent monoclonal antibody and the antimouse fluorescein isothiocyanate conjugate were supplied by the Department of Biotechnology of the Istituto Zooprofilattico Sperimentale, Brescia, Italy.

Cell cultures for *Chlamydia* were inoculated and propagated by routine methods (44). Cell monolayers were examined by direct immunofluorescence with an anti-*Chlamydia* monoclonal antibody (Merilfluor; Meridian Diagnostics, Cincinnati, Ohio).

**Analysis of plasmid and chromosomal DNAs from *L. pneumophila* isolates.** The DNA analysis was performed by the method described by Kado and Liu (27) with vertical 0.8% agarose gel electrophoresis. Estimation of the molecular size of the plasmid was done by reference to the size of plasmid pZM62, which is known to be 9 MDa (31).

Pulsed-field gel electrophoresis (PFGE) of the *L. pneumophila* isolates was carried out substantially as described by Lück et al. (29). DNAs were cleaved with the rarely cutting enzyme NorI (Boehringer Mannheim, Mannheim, Germany) by following the manufacturer’s instructions.

**Environmental studies.** One-liter specimens of water were collected from the water trough provided for the herd, the hot and cold water taps, and the aqueous sediment at the bottom of an old electric water heater (capacity, about 100 liters; Fig. 1) which had been used as the source of hot water for dilution of the colostrum used to feed the calves. All the specimens were examined for the presence of legionellae and amoebae. Samples were plated directly, and after concentration by filtration (Millipore membrane; pore size, 0.22 μm) on modified Wadowsky-Yee medium (Unipath, Oxoid, Garbagnate, Milanese, Italy), with and without heat and acid treatments (5, 7). Portions of the same samples were also kept at 35°C for 6 weeks and were replated for Legionella weekly after serial dilution as described by Sanden et al. (43).

Free-living amoebae were cultured on nonnutrient agar plates that had been seeded with a thick portion of *Escherichia coli* taken from a seeded nutrient agar plate (15). Briefly, two plates were seeded with 0.1 ml of unconcentrated water, and two plates were seeded with a sample resulting from the concentration by filtration (Millipore membrane; pore size, 0.45 μm) of 250 ml of water. The plates were incubated for 7 days at 30°C with 2.5% CO₂ and were checked daily for amoebic cysts and trophozoites. Amoebae were maintained on the same culture medium and were identified on the basis of their morphological characteristics and by the flagellation test (15).

**Follow-up investigations.** One hundred forty-two serum samples from 112 cows and 30 calves (1 to 4 months old) were collected from the herd and were tested for antibodies to *L. pneumophila* SG1 by IFA. A highly seropositive (titer, 2,048) but apparently healthy 3-year-old cow reared in the same herd was slaughtered and examined in the same way as the dead calf.

**RESULTS**

**Necroscopy.** Postmortem examination of the calf revealed a bilateral confluent lobular pneumonia mainly involving the lung cranial lobes. Fibrinous pleurisy was also evident in pneumonic areas. The cut surface of the lung was red and airless with multiple necrotic foci ranging from about 0.5 to 1.5 cm in diameter. No pathological changes in other organs were observed.

Areas of lung consolidation, mainly located in the cranial lobes and associated with mild chronic pleurisy, were detect-
Hematoxylin-eosin stain was used. Bar, 125 μm.

Histopathology and immunohistochemistry. An acute fibrinous and necrotic pneumonia was observed in the affected areas of the calf lung. The alveoli were filled with fibrinous material and inflammatory cells, mainly macrophages and neutrophils. The lumens of the bronchioles were filled with neutrophils and necrotic material, while the bronchi were unaffected (Fig. 2). Some well-demarcated foci of coagulative necrosis were scattered in the pulmonary areas. A few clusters of gram-positive bacilli were seen in the center of some necrotic foci. No histological lesions were observed in the liver or in the spleen.

Immunohistochemical examination for *L. pneumophila* SG1 antigen revealed the presence of immunoreactive material in the cytoplasms of macrophages in pneumonic areas. Groups of immunostained bacteria were also detected in some necrotic foci, particularly at the edges (Fig. 3). *L. pneumophila* SG1 antigen was not detected in unaffected areas of the lung tissue. Immunohistochemistry was negative when *L. pneumophila* SG10 antiserum was used, and no immunohistochemical reactivity was observed in the liver or spleen. Immunofluorescence demonstrated the presence of legionellae within areas of pulmonary consolidation (Fig. 4): many bacteria were seen in alveolar spaces and within macrophages. Immunofluorescence and immunohistochemical analysis for *L. pneumophila* SG1 and SG10 were negative for all tested samples collected from the cow.

Bacteriology and virology. Cultures of calf lung yielded several colonies of *L. pneumophila*. Legionellae were also isolated from the liver. Nine isolates from the lung and one from the liver were selected for typing. Eight were SG1 and two were SG10 (Table 1).

The *L. pneumophila* SG1 strains were further examined by monoclonal subtyping. Six isolates from the lung and one isolated from the liver belonged to the Olda subtype, whereas one isolate from the lung belonged to the Camperdown subtype. Also, a few colonies of *Actinomyces pyogenes* were isolated from the lung. Cultures for anaerobic bacteria, mycoplasmas, chlamydias, and viruses from the calf lung were negative.

All plates inoculated with lung, liver, spleen, and kidney from the slaughtered cow were negative for *Legionella*.

Environmental studies. Legionellae were not isolated by direct plating from the water samples. However, several colonies of *L. pneumophila* SG1 were isolated from the sediment in the electric heater (Table 1) after incubation of the serially diluted sample for 15 days at 35°C. *Pseudomonas aeruginosa* inhibited the growth of *L. pneumophila* on plates inoculated with the first three decimal dilutions of the incubated sample. Four single colonies of *L. pneumophila* SG1 were selected and identified as subtype Olda.

Free-living amoebae of the genus *Naegleria* grew from concentrated and nonconcentrated water samples. *Acanthamoeba* spp. grew only on the plates seeded with the concentrated sample. The electric water heater was supplied by water taken from a well 12 m deep. The temperature of the water in the electric water heater was 43°C.

Follow-up investigations. Of 142 serum samples tested for legionella antibodies, samples from three calves had a titer of 32 and a sample from one calf had a titer of 64. Two cows were positive; their titers were 128 and 2,048, respectively. The latter cow was slaughtered and was examined as reported above.

Plasmid and chromosomal DNA analysis. Table 1 summarizes the results of some genomic characteristics of the strains examined. Plasmid analysis was performed on 14 colonies, 10 from calf specimens and 4 from the heater deposit. A plasmid of approximately 9 MDa was detected in five isolates from the calf; no plasmid was found in the other five calf isolates or in any of the four strains recovered from the water.

The chromosomal DNA analysis by PFGE was performed with five clinical and four environmental isolates. The same patterns were found for *L. pneumophila* SG1 Olda strains isolated from the calf and from the electric water heater, whereas *L. pneumophila* SG10 strains showed a completely different pattern. The results of macrorestriction analysis by PFGE of NotI-cleaved DNAs are shown in Fig. 5. The *L. pneumophila* SG1 pattern is independent on the plasmid content.

**DISCUSSION**

There is a strong evidence that the calf that was the object of our studies died of a severe pneumonia due to a *Legionella* infection, since the other most common causes of pneumonia...
in calves (viral, bacterial, chlamydial, and mycoplasmal infections) have been explicitly ruled out. Moreover, other causes of respiratory diseases in calves such as parasites, fungi, and non-infectious agents were not recognized histologically; on the other hand, histological findings were indeed indicative of a bacterial etiology.

The immunochemical and the immunofluorescence analyses provided evidence of the bacterium in the affected tissues, sometimes in an intracellular location and in association with a severe necrotic inflammatory process (Fig. 2). The clinical signs observed in the calf were not specific, since they resembled those usually observed in enteric and respiratory diseases of calves; nevertheless, the pathological and histological pictures were indeed similar to those described for human legionellosis (49).

The immunohistochemical investigation performed very well for the detection of *L. pneumophila* SG1 in the lung tissue. However, the absence of reactivity for *L. pneumophila* SG1 in the liver and for *L. pneumophila* SG10 in the lung suggests that culture is more sensitive. Different legionellae were isolated from the calf. Simultaneous infection with different legionellae is rarely reported in humans (20, 23), but it is well known that

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**TABLE 1. Phenotypic and genomic characteristics of *L. pneumophila* strains isolated from a calf suffering from pneumonia and from electric water heater sediment**

<table>
<thead>
<tr>
<th>Source and strain no.</th>
<th>Serogroup and serotype&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Plasmid&lt;sup&gt;b&lt;/sup&gt;</th>
<th>PFGE pattern&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calf lung</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1, 3, 5, 6, and 7</td>
<td>Lp SG1 Olda</td>
<td>No plasmid</td>
<td>A&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>8</td>
<td>Lp SG1 Olda</td>
<td>9 MDa</td>
<td>A</td>
</tr>
<tr>
<td>9</td>
<td>Lp SG1 Camper-down</td>
<td>9 MDa</td>
<td>ND&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>2 and 4</td>
<td>Lp SG10</td>
<td>9 MDa</td>
<td>B</td>
</tr>
<tr>
<td>Calf liver, 10</td>
<td>Lp SG1 Olda</td>
<td>9 MDa</td>
<td>A</td>
</tr>
<tr>
<td>Electric heater sediment, 11, 12, 13, and 14</td>
<td>Lp SG1 Olda</td>
<td>No plasmid</td>
<td>A</td>
</tr>
</tbody>
</table>

<sup>a</sup> The *L. pneumophila* (Lp) serogroup and monoclonal subtype were determined by the Oxford scheme.

<sup>b</sup> PFGE was carried out after digestion of bacterial DNA with NotI endonuclease.

<sup>c</sup> Only strain 1 was tested.

<sup>d</sup> ND, not determined.
several *Legionella* species and serogroups coexist in aquatic habitats (3, 34, 46).

*L. pneumophila* SG1 was also isolated from the calf liver, a finding that may suggest a generalized infection. As to the route of infection, the calf could have inhaled, aspirated, and/or ingested the bacteria which were present in the contaminated milk. Two hypotheses should be considered: (i) *Legionella* first colonized the lung tissue and then the liver or (ii) first the liver and then the lung. The first hypothesis implies that inhalation or aspiration is the way of transmission, whereas the second one suggests a preliminary ingestion of the bacterium. In humans there is little evidence to support ingestion as a mode of transmission, whereas in animals (guinea pigs) the gastrointestinal tract has already been shown to be a portal of entry of the bacteria (28, 40). Our observations do not allow us to draw conclusions, because both hypotheses could be accepted; however, the severity and the localization of the lesions in the lung cranial lobes would, rather, suggest a respiratory portal of entry, since cranial lobes are the first ones to be thoroughly ventilated and exposed to the etiological agent.

Cultures of the sediment from an old electric water heater yielded *L. pneumophila* SG1, and there is a strong circumstantial evidence that the calf became infected by exposure to the milk which had been diluted with contaminated water from that heater. In fact, the water supplied was at 43°C, a temperature suitable for both the survival and the multiplication of *Legionella*.

Most of the clinical and environmental *L. pneumophila* SG1 isolates belonged to the Olda monoclonal subtype, and their chromosomal DNAs showed identical PFGE patterns after digestion with the NorI endonuclease (Table 1; Fig. 4). Although the Olda subtype does not contain the epitope monoclonal antibody 2 which is thought to be associated with virulence (48), its isolation from human patients with Legionnaires’ disease has been reported (13).

The strains isolated from the calf were either plasmidless or contained a plasmid of 9 MDa; plasmids were not detected in the four isolates from the electric water heater sediment. However, the presence or absence of plasmids in legionellae adds little information since this character has never been definitely associated with virulence (39).

The successful isolation of legionellae from the electric water heater sediment only after a prolonged incubation of the samples at 35°C confirms the usefulness of this method for increasing the sensitivity of culture for legionellae.

*A. pyogenes* has been reported to be able to inhibit the growth of *Legionella* on buffered charcoal yeast extract α-ketoglutarate agar medium (22, 26, 42), possibly due to the action of the pyocyanin, a metabolite produced by *Pseudomonas* known for its bactericidal properties (32). This can lead to a failure to detect legionellae either in clinical or in environmental samples. Whether such inhibition also occurs in nature is unknown. In our laboratory, we observed the phenomenon only on the plates inoculated with the most concentrated water samples, whereas legionellae grew well and were easily detected on the plates inoculated with the most diluted samples.

The concurrent isolation of amoebae from the water samples collected from the electric water heater leads to two considerations. First, legionellae could have survived and multiplied within the protozoa *Naegleria fowleri* and *Acanthamoeba* species, protected from the influences of the metabolic products of *Pseudomonas*, and then released in the electric water heater. Second, inhalation or aspiration of legionella-contaminated material containing amoebae could have enhanced the risk of infection for the calf; it has, in fact, been reported that legionellae replicate well within *Naegleria* and *Acanthamoeba* (35, 37) and that legionellae grown in protozoan hosts, such as *Acanthamoeba castellanii*, may be more efficient at entering mammalian cells (11). Briel and colleagues (6) also demonstrated that intrapulmonary amoebae potentiate replicative *L. pneumophila* lung infection in mice. Although the role of inhaled amoebae in the natural infection of our calf remains unknown, the occurrence of one or both of these events could explain the transmission of the infection to the animal and the severity of the disease.

The recovery of few colonies of *A. pyogenes* from the calf lung should be considered incidental; in fact, this bacterium is one of the commonest pathogenic opportunistic organisms isolated from the respiratory tracts of cattle. Moreover, the isolation of *A. pyogenes* probably corresponds to the histological finding of gram-positive bacteria in some necrotic foci of the lung. In humans, *Legionella* has often been isolated in association with other bacteria (23).

We found a very low seroprevalence of *L. pneumophila* SG1 in the herd; in fact, of 112 cows, only 2 were positive. One cow had a titer of 2,048, and subsequent investigations showed a chronic interstitial pneumonia and an interstitial nephritis. No legionellae were detected by cultural or immunohistochemical examination. These findings would suggest a previous infection.

Among 30 calves, we found *Legionella* antibodies in only 4 animals. The low titers detected do not necessarily demonstrate infection.

To the best of our knowledge, this is the first report of a naturally occurring *Legionella* pneumonia in an animal. In fact, the isolation of legionellae from two calves reported by Boldur et al. (4) was not associated with clinically manifest disease, and the lungs did not show any macroscopic lesions. The investigators themselves suggested that the transmission of the bacterium might have occurred during coma.

Our data suggest that *Legionella* disease in cattle should be considered an uncommon event. Predisposing factors such as poor hygiene, bad management, and insufficient and/or unbalanced feeding seem to be important in the occurrence of the disease. When diagnosing severe pneumonias in cattle, it would therefore be advisable to perform investigations aimed at the detection of *Legionella*.

Legionellosis in cattle should be regarded as a minor hazard to human health, since cattle probably act as accidental hosts for legionellae, much the same as humans.

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**REFERENCES**


