Misleading Serological Identification of *Legionella anisa* as *Legionella bozemanii*

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Abstract

Identification of six *Legionella* species, which we previously identified by serological test as *Legionella bozemanii* (*L. bozemanii*), was performed by DNA-DNA hybridization using a commercial DNA-DNA hybridization kit (Kobayashi Pharm. Co., Japan) introduced by Ezaki et al. All strains were identified as *Legionella anisa* (*L. anisa*), this being the first identification of *L. anisa* in Japan. Conventional laboratory tests were performed following the DNA-DNA hybridization. In this study the results of biochemical examination obtained, corresponded closely with those described in previous reports, but the oxidase reaction was very weak and varied according to the age of the culture, indicating the unreliability of this test in our case. All strains examined under long wave ultraviolet (UV) light (366 nm) revealed a blue-white fluorescence, the intensity of which ranged from strong to weak. Serological identifications were performed by both the slide agglutination test (SAT) and indirect immunofluorescent assay (IFA). SAT using commercially available antiserum (Denka Seiken., Japan) supposedly specific for *L. bozemanii* showed cross-reaction between *L. bozemanii* and *L. anisa*. Hyperimmune rabbit antisera prepared in this study for both *L. bozemanii* and *L. anisa*, from which cross-reactive antibodies were removed by the absorption of each antigen, reacted only with homologous antigens. IFA using a commercially available antiserum and hyperimmune rabbit antiserum previously described, gave positive reactions with each strain.

Introduction

Since one of the causative agents of *Legionnaires’* disease, *Legionella pneumophila*, was isolated and identified over 14 years ago, a large number of new species and serogroups of *Legionella* have been added to the genus *Legionella* and now at least 51 serogroups are listed representing 32 species. As biochemical characteristics of each *Legionella* species are very similar, serological identification has been widely used in clinical laboratories. Generally in undertaking serological examination, cross-reaction should always be taken into consideration. There were several reports of the cross-reaction among *Legionella* species. Most cross-reactive antibodies can be removed by absorption with the appropriate heterologous strains. Another problem is antisera that appear to be specific when tested against currently known *Legionella* species, cross-reacting with antigens of species not yet discovered because of nonabsorption. We suppose that isolates identified previously as *L. bozemanii* by serological methods may have been misidentified, since antiserum to this species has numerous cross-reactions. We have reexamined stock strains previously tested with reagents, using a commercially available DNA-DNA hybridization kit, followed by conventional laboratory examination, Oxidase test, long wave UV light...
Table 1 Characterization of six strains of Legionella used in this study

<table>
<thead>
<tr>
<th>No. of strains tested</th>
<th>SAT for L. bozemanii&lt;sup&gt;a&lt;/sup&gt;</th>
<th>L. anisa&lt;sup&gt;b&lt;/sup&gt;</th>
<th>IFA for L. bozemanii&lt;sup&gt;a&lt;/sup&gt;</th>
<th>L. anisa&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Long Wave (366nm) U.V test</th>
<th>Oxidase test</th>
</tr>
</thead>
<tbody>
<tr>
<td>81-167</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+++</td>
<td>Blue-White</td>
<td>−</td>
</tr>
<tr>
<td>81-168</td>
<td>+</td>
<td>+++</td>
<td>−</td>
<td>+++</td>
<td>Blue-White</td>
<td>+</td>
</tr>
<tr>
<td>81-174</td>
<td>+</td>
<td>+++</td>
<td>−</td>
<td>+++</td>
<td>Blue-White</td>
<td>−</td>
</tr>
<tr>
<td>82-178</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+++</td>
<td>Blue-White</td>
<td>+</td>
</tr>
<tr>
<td>82-190</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+++</td>
<td>Blue-White</td>
<td>−</td>
</tr>
<tr>
<td>82-191</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+++</td>
<td>Blue-White</td>
<td>−</td>
</tr>
<tr>
<td>L. bozemanii (ATCC 33217)</td>
<td>+++</td>
<td>−</td>
<td>+++</td>
<td>−</td>
<td>Blue-White</td>
<td>−</td>
</tr>
<tr>
<td>L. anisa (ATCC 35292)</td>
<td>+</td>
<td>+++</td>
<td>−</td>
<td>+++</td>
<td>Blue-White</td>
<td>+</td>
</tr>
</tbody>
</table>

1) Commercially available antiserum for L. bozemanii (Denka Seiken, Co., Japan).
2) Antiserum prepared in our laboratory for L. anisa.

illumination SAT, IFA, to evaluate their comparative differentiating ability.

Materials and Methods

Bacteria: All Legionella strains used in this study are listed in Table 1, and had been previously reported<sup>14</sup>. They had been isolated from water in cooling towers in different geographical areas throughout Japan, and serologically identified as L. bozemanii. The reagents used in those studies were kindly donated by Centers for Disease Control (CDC) in the United States.

Antisera: Antisera were prepared with whole cell immunization by modified Thacker’s method<sup>13</sup>. Briefly described, the 2 day growth colonies from five buffered charcoal yeast extract agar plates supplemented with 1% α-ketoglutarate (BCYE-α) were suspended in 30 ml of phosphate buffered saline (pH 7.6) containing 1% formalin. The suspension was kept overnight at room temperature to kill the bacteria, then filtered through sterile gauze, and centrifuged at 2000 ×g for 15 min. The cell pellet was resuspended in phosphate buffered saline containing 0.5% formalin. A 2 ml portion of a suspension containing equal volumes of cells and incomplete Freund adjuvant (Sigma Chemical Co., USA) was injected intracutaneously in approximately 20 sites along the shaved backs of two young adult Japanese white rabbits. On day 10, the rabbits were injected with 2 ml of the above suspension divided into two deep muscles injections in the hindquarters. On days 20 and 30, the rabbits were injected intravenously with 2 ml of cell suspension without adjuvant. On day 37 and 44, a further 2 ml of cell suspension was injected intramuscularly. On day 51, the rabbits were exsanguinated. Antiserum titers were determined by preparing twofold dilutions in phosphate buffered saline (pH 7.6). The reactions were scored on a scale from 1+ (barely visible) to 3+ (strong agglutination). The serum titer was determined as the highest dilution which gave 3+ agglutination of homologous antigen within 30s.

Absorption of antisera: Cross reactive antibodies were removed by absorption with cross-reacting Legionella strain or strains. Cells for absorption were prepared as follows; the cells were mixed at a 1:5 ratio (vol/vol) with undiluted antiserum, incubated for 2 h at 37°C, and then kept at 4°C overnight. The cells were removed by centrifugation at 2,000 ×g for 15 min at room temperature, and the antiserum was tested for residual cross-reactivity. Absorption of the antiserum was continued until it contained only homologous agglutinating antibodies at its working dilution.

DNA probe: A commercial kit of the DNA-DNA hybridization test was used in this study. By using the following equation, the homology of each well with strains tested was calculated as follows:

\[
\text{Homology value} = \frac{\text{the measured value} - \text{the mean value of control}}{\text{the maximum value} - \text{the mean value of control}} \times 100
\]
Misleading serological identification of \textit{L. anisa}

Table 2 List of standard strains immobilized in Microdilution Plate

<table>
<thead>
<tr>
<th>Well No.</th>
<th>Labeled organism</th>
<th>ATCC No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>\textit{E. coli} (control)</td>
<td>25923</td>
</tr>
<tr>
<td>2</td>
<td>\textit{L. jordanis}</td>
<td>33623</td>
</tr>
<tr>
<td>3</td>
<td>\textit{L. pneumophila}</td>
<td>33152</td>
</tr>
<tr>
<td>4</td>
<td>\textit{L. bozemanii}</td>
<td>33217</td>
</tr>
<tr>
<td>5</td>
<td>\textit{L. micdadei}</td>
<td>33218</td>
</tr>
<tr>
<td>6</td>
<td>\textit{L. gormanii}</td>
<td>33297</td>
</tr>
<tr>
<td>7</td>
<td>\textit{L. longbeachae}</td>
<td>33462</td>
</tr>
<tr>
<td>8</td>
<td>\textit{L. dumoffii}</td>
<td>33279</td>
</tr>
<tr>
<td>9</td>
<td>\textit{L. oakridgensis}</td>
<td>33761</td>
</tr>
<tr>
<td>10</td>
<td>\textit{L. wadsworthii}</td>
<td>33877</td>
</tr>
<tr>
<td>11</td>
<td>\textit{L. feeleii}</td>
<td>35072</td>
</tr>
<tr>
<td>12</td>
<td>\textit{L. sainthelenis}</td>
<td>35248</td>
</tr>
<tr>
<td>13</td>
<td>\textit{L. hacheliae}</td>
<td>35230</td>
</tr>
<tr>
<td>14</td>
<td>\textit{L. jamestowniensis}</td>
<td>35298</td>
</tr>
<tr>
<td>15</td>
<td>\textit{L. cherrii}</td>
<td>35272</td>
</tr>
</tbody>
</table>

Standard strains immobilized on the microdilution plate are listed in Table 2.

Oxidase test: The oxidase test was performed by moistening a piece of filter paper of No 5A (Toyo Roshi Co., JAPAN) with a freshly prepared 1% solution of tetramethyl-phenylenediamine. A loopful of culture from a BCYE-α plate was rubbed onto the filter paper and any color development recorded. The development of a dark blue color within 30s was determined as a positive reaction.

IFA: Formalin-killed \textit{Legionella} antigens were added to wells on glass slides, air dried, and acetone fixed. These slides were overlaid with a dilution of antiserum previously described and incubated at 37°C for 40 min. After rinsing in phosphate-buffered saline to remove unbound globulin, the slides were then similarly incubated at 37°C for 40 min with anti-rabbit FITC conjugate (Organon Teknica N.V. Capple Product., USA), rinsed, and mounted in 10% glycerol. Specimens were examined at dilutions of 1:64 to 1:1024. The level of decreasing fluorescence was recorded for each serum dilution as 3+ through 1+, to ± (equivocal) and negative.

Results

DNA-DNA hybridization: Identification results for the six strains are shown in Figure 1. When the DNA of the six strains which had previously been serologically identified as \textit{L. bozemanii} were labeled, all strains hybridized most strongly to unlabeled DNA of \textit{L. anisa} within 90 min. However they also showed cross-hybridization strongly to unlabeled DNAs from \textit{L. parisiensis}, \textit{L. bozemanii} and \textit{L. cherrii}, but estimated homology values were less than 70% of that for \textit{L. anisa}. Thereby all strains were identified as \textit{L. anisa} by DNA-DNA hybridization method. Three of the six strains had showed the second most intense fluorescence with the \textit{L. parisiensis}, and of the remaining three strains, two with \textit{L. bozemanii}, and one with \textit{L. cherrii}.

SAT: Three of the six strains agglutinated with commercially available antiserum for \textit{L. bozemanii} (see Table 1). Hyperimmune rabbit antiserum for \textit{L. anisa} prepared in our laboratory reacted with both \textit{L. anisa} and \textit{L. bozemanii}, but after the absorption, this antiserum reacted only with the homologous antigen. All of the six strains agglutinated with antiserum for \textit{L. anisa}. The optimal dilution titer was 1:10.

IFA: Using the hyperimmune rabbit antiserum specific for \textit{L.anisa} described above, a strongly positive reaction with the \textit{L. anisa} antigen was obtained and also a very weak reaction with \textit{L. bozemanii} antigen was observed. Differentiation between \textit{L. bozemanii} and \textit{L. anisa} was possible down to 1024-fold.
Fig. 1  Homology value (%) of control wells (immobilized E. coli) and 25 reference wells (immobilized type strain DNAs). Dashed lines show value of 70%. For species in each well see Table 2.

Dilution of the antiserum. Using commercially available antiserum specific for L. bozemanii also enabled clear differentiation between L. bozemanii and L. anisa down to 512-fold dilution.

Oxidase test:  Two of the six strains showed oxidase positive, but the reactions were very weak and varied with the age of the culture.

Long wave UV light test:  All the experimental strains showed the same blue-white fluorescence as L. anisa (ATCC 35292), but the intensity varied from strong to weak. During the growth period three of the six strains showed a yellow-green fluorescence after 10 days.

Discussion

Accurate identification of Legionella can hardly be done by conventional laboratory tests; their phenotype characteristics are very similar and most species cannot be differentiated on this basis. Serological examination is thus widely used, with the advantages of speed and convenience, requiring no special equipments. But cross-reactions between serum and antigens of as yet undiscovered species cannot
Misleading serological identification of L. anisa

be ruled out. Newly isolated unidentified strains may then be mistakenly identified and stocked indefinitely as currently known strains, unless laboratories have the necessary techniques for DNA-level confirmation. Serum must be reexamined for cross-reactions with new species every time these are discovered, and the relatively successful technique of absorption for removing cross-reactive antibodies should be continued.

In this study we describe our own experience in testing the above hypothesis. Isolates identified serologically as L. bozemanii were subsequently found by DNA-DNA hybridization method to be L. anisa, a species assigned to the genus Legionella after L. bozemanii. Strains had been stocked under the mistaken identification for a long time, since antisera to these species were cross-reactive, which was unknown at that time. The situation is the same today, this being an unavoidable limitation to serological examination.

Species were initially established on the basis of their DNA-DNA homology value, so DNA-DNA hybridization is the rational method for their identification. Ezaki reported on the use of fluorometric hybridization in microdilution wells to identify Legionella species without using radioisotopes. We evaluated a commercially available DNA-DNA hybridization kit based on Ezaki's report, the specificity of which was found to be 100%. Members of the blue-white fluorescing Legionella species, such as L. parisiensis, L. bozemanii, L. cherii, and L. gormanii, were known to have numerous serologic cross-reactions within the group, and they were highly cross hybridized with L. anisa in this study. This indicated a correlation between serological relatedness and genetic relatedness except in the case of L. jordanis which is well known to have very low genetic relatedness to the other Legionella species but serological relatedness to L. bozemanii, and L. anisa. This correlation will produce significant savings of time and cost in finding cross-reactive serum for a new strain's antigen. In this kit the relative homology values of wells coated with DNAs of reference strains were calculated relative to the well which emitted maximum fluorescence, this being assigned the value 100%. This relative evaluation gives rise to a limitation of the process, this being that when a new strain is tested, it may well be misidentified in the following way: The strain in the test plate with the nearest homology value will be assigned the relative homology value 100%. The new strain will then be identified as this most similar strain unless a strain which emits a second fluorescence intensity is similar enough to give a tell-tale fluorescence intensity greater than 70% of the nearest strain. The absence of such a signal may be simply because a near enough strain does not exist, or because it is not coated on the test plate, i.e. it has not yet been isolated or is not yet available. To avoid this situation, we suggest that self-prepared unlabeled DNA of the strain to be tested might be included in the microplate, unfailingly giving an absolute intensity of 100%, a positive control with which one can compare the strongest homology value from the plate. If the strongest reaction gives a fluorescence intensity well below this control value, then identification is not possible from the isolated species, even though the remainder of the intensities fall below 70% of this maximum.

In this study we compared serological examinations using the IFA and SAT techniques. Commercially available antiserum specific for L. bozemanii gave a pseudopositive reaction with L. anisa by SAT but not by IFA test. There have been comparisons between the DFA test and SAT technique before but not to the author's knowledge between the far more specific yet practical IFA test technique and SAT. Our results suggested that in identifying Legionella the former was more effective than the latter. Vesey reported that the oxidase test was unreliable for Legionellas. In our study the same result was obtained; the oxidase reaction was very weak and varied with the age of the culture.

The proportion of species reported so far in Japan does not accurately reflect the distribution of Legionellae. This results from the selective manner in which isolates have been chosen for study. Our investigation using DNA-DNA hybridization and antisera resulted in the first identification of L. anisa,
which had not been reported in Japan until then. Koide had surveyed the distribution of *Legionella* species between 1987-1989 in Kinki in the central district of Japan. Five isolates were identified as *L. bozemanii* using commercially available antiserum and stocked at −70°C for two years. We reidentified the isolates by using serum prepared in this study, all strains being identified as *L. anisa*. It is a very important result that all the strains we investigated turned out to be *L. anisa*.

Wilkinson reported the proportion of species in Australia, where *L. anisa* was isolated as often as *L. pneumophilia* serogroup 1. It seems likely that when the antiserum for *L. anisa* becomes commercially available, the reported proportion of *L. anisa* might increase in Japan. Wide use of DNA-DNA hybridization kits may thus change the proportion of discovered species and further increase the number of species which have not yet been isolated.

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**Literature Cited**


Misleading serological identification of *L. anisa*


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**Legionella anisa** に対する血清学的検査は

*Legionella bozemanii* と誤同定される危険性の検討

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**要観**

*Legionella* 属の菌種の同定には血清学的検査が広く用いられている。1980年〜1982年にかけて血清学的に*Legionella bozemanii* と同定された6菌株について市販のDNA-DNA hybridization kit（小林製薬）をもって再同定を施行したところ全菌株ともに*Legionella anisa* の同定結果を得た。本菌株は本邦では初の同定である。引続き同菌株に対し、生物化学的検査をおこなった。 oxidase 検査は、反応が非常に弱く信頼度が低い為、本菌に対する検査としては不適当と思われた。長波長紫外線照射では全株とも blue-white fluorescence を示したが、蛻光の強さにはかなりのバリエーションが認められた。血清学的検査では、slide agglutination test（SAT）にて、市販のデンカ生研の* L. bozemanii* に対する血清は*L. anisa* に対し交叉反応を呈した。当教室で作製した両菌株に対する自家家児免疫血清も交叉反応を示した。 indirect immunofluorescent assay（IFA）では、デンカ血清及び自家家児免疫血清ともに明瞭に同定できた。以上より* L. bozemanii* と血清学的に同定されている菌株の中に*L. anisa* が含まれている可能性があるの再検討が必要と思われた。また*L. anisa* に対するDNA-hybridization による同定法は普及するにともない本邦での分離頻度が増加することが予想された。