Prolonged Occurrence of *Moraxella bovis* Infection on a Restricted Heifer Farm

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(Received 19 July 1993/Accepted 10 December 1993)

**ABSTRACT.** An epizootiologic study was conducted for infectious bovine keratoconjunctivitis (IBK) on a large grassland farm rearing a total of 1,300-1,400 heifers during May to October in both 1991 and 1992. Heifers were examined for infection with *Moraxella bovis*, and isolation of *M. bovis* from the affected eyes and from Asian face flies (*Musca bezzi* Patton et Cragg) swarming on the heifer face was carried out. During the observation period, 10.7% of the heifers in 1991 and 5.3% of the heifers in 1992 were affected with IBK. Eight *M. bovis* isolates were isolated from the ocular swabs of affected heifers with IBK for 2 years and identified by biochemical examinations and Southern DNA-DNA hybridization, but no *M. bovis* was obtained from 331 Asian face flies tested. Plasmid profile analysis showed that two plasmids (35-kb and 4.0-kb) were commonly found in all 8 *M. bovis* isolates. This indicates that *M. bovis* isolates with the same plasmid profile were widely disseminated on this farm for 2 years.—**KEY WORDS:** heifer, IBK, *M. bovis*, Musca bezzi, plasmid.

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*M. bovis* is considered to be the primary etiologic agent of infectious bovine keratoconjunctivitis (IBK), which is the most important ocular disease of cattle [1, 7]. This disease is characterized by inflammation of the cornea, lacrimation and conjunctivitis, and influenced by environmental factors such as ectoparasitic flies, dust and solar ultraviolet radiation [1, 10, 16]. Among them, face flies (*Musca autumnalis* De Geer) have been associated with symptoms of IBK in cattle and have been shown to play a role as a mechanical carrier in the dissemination of IBK in the U.S.A. [2, 6, 21]. The disease commonly occurs sporadically within herds from year to year.

Young animals have generally been thought to be more susceptible to IBK than adults, and the rate of infection and the incidence of keratitis in calves exceeds that in cows [1, 7, 9]. On Farm Y where young heifers were maintained in many pastures, epizootics of IBK have been a serious and contagious disorder for grazing cattle, occurring every year. The Asian face fly (*Musca bezzi* Patton et Cragg, 19) is another species that closely resembles the face fly and is thought to be a mechanical vector of IBK in the U.S.A. In Japan, this species is known as a particular nuisance, congregating especially around the eyes on cattle. It is unknown whether Asian face flies are able to transmit *M. bovis* to susceptible cattle in pastures. In Japan, a few reports have dealt with short-term observations of IBK infection and isolation of *M. bovis* from infected animals [14, 15], but there is no report concerning longitudinal observation of IBK under field conditions.

The purpose of the present study was to investigate lasting infection of IBK on a restricted heifer farm for two years, to isolate *M. bovis* from infected heifers and to characterize the plasmid profile in the *M. bovis* isolates. We also tried to isolate *M. bovis* isolates from Asian face flies during the epizootics of IBK to estimate the distribution and mode of spread of *M. bovis*.

**MATERIALS AND METHODS**

**Experimental farm:** Public grassland Farm Y (520 ha) is located in Hokkaido, Japan. On this farm, a total of 1,300-1,400 heifers (10 to 24 months old) were collected from various dairy farms and maintained in several pastures from May to October. Before heifers were brought to the farm, each one was checked for IBK infection. About three hundred heifers were assigned to the group observed in the IBK studies.

**Clinical observation:** The heifers were examined for IBK each week. The clinical examination was conducted by looking for photophobia, lacrimation, iridospasms, foreign bodies and conjunctivitis [1, 7]. During each weekly observation, samples of eye secretions were collected from the affected eyes for bacterial examinations.

*M. bovis* isolation from heifers: Each eye of heifers with IBK was swabbed with sterile cotton swabs (Mentin, Nippon Menbo, Tokyo). The swabs were kept in an ice box until cultured. They were inoculated onto brain heart infusion agar (Eiken Kagaku Co., Tokyo) with 5% sheep blood. The media were incubated at 37°C for 24 hr. Colonies with β-hemolysis were picked up and examined for identity with *M. bovis*.

*M. bovis* isolation from Asian face flies: Asian face flies (*Musca bezzi*) on the heifers’ eyes and faces were collected with a butterfly net. A pool of 2 to 5 flies was collected from each heifer with this net. The flies in each pool were immediately anesthetized with CO₂ and their labella were placed on blood agar plates. The flies were also allowed to walk on the plates for 5 min, after which the plates were incubated at 37°C for 24 hr and examined for *M. bovis*.

Identification of *M. bovis* isolate: The β-hemolytic
colonies on the blood agar were examined morphologically by Gram-staining, and for oxidase and catalase activities, motility, indole production, and acid-production from lactose, glucose and sucrose [14, 15]. The moraxella-like isolates selected in the biochemical tests were finally identified by the Southern DNA-DNA hybridization test. Two M. bovis strains, Matsu [15] and EPP-63 [17] were used as reference cultures.

Southern DNA-DNA hybridization: Chromosomal DNA was prepared from the moraxella-like isolates [8], and approximately 10 µg of chromosomal DNA was digested with restriction enzyme EcoRI (Takara Shuzo Co., Kyoto). The EcoRI-digested chromosomal DNA was separated by 0.7% agarose gel electrophoresis [8]. The separated DNAs were transferred to a nitrocellulose filter (Schleicher & Schuell, Dassel, Germany) according to the Southern method [20], and then were hybridized with the 32P-labelled EcoRI-digested chromosomal DNA of M. bovis Matsu at 65°C for 18 hr. The probe was labelled by random hexanucleotide priming with [α-32P]dCTP [5]. Filters were washed at 65°C with 2 x SSC and 0.1% SDS three times for 30 min [12].

Plasmid isolation and agarose gel electrophoresis: Plasmid DNA was extracted from each isolate by the procedures described by Maniatis and co-workers [12]. The plasmid DNA preparations were electrophoresed through a 0.7% agarose gel as previously described [8]. Plasmid bands were visualized with a UV transilluminator and were photographed through an orange filter. Plasmid DNA molecular size was estimated in kilobases (kb) by comparing the electrophoretic mobilities of M. bovis plasmids with those of known molecular sizes of four reference plasmids, R100 (100-kb) [11], Rs-a (34-kb) [11], pBR322 (4.3-kb) [11] and pBluescript (3.0-kb) [12].

RESULTS

Occurrence of IBK: The occurrence of clinical IBK on Farm Y during May to October of 1991 and 1992 is summarized in Table 1. Epizootics of IBK appeared on the farm in early June 1991, and peak incidence of IBK was observed in July, 1991. The same prevalence pattern was also observed in 1992, although the incidences of IBK in 1992 were lower than those in 1991. The initial signs consisted of an increased tear pool, lacrimation and photophobia, and subsequently progressed to conjunctivitis and keratitis. Clinical signs of disease were similar in the older heifers (15–24 months old; group I) and the younger heifers (10–14 months old; group II). However, the younger heifers (group II, 18.6% and 12.0%) were more highly affected than the older ones (group I, 4.3% and 1.3%) in 1991 and 1992, respectively, indicating that younger animals were more susceptible to IBK than older ones. Table 1 shows that a total of 147 (10.7%) and 83 (5.8%) heifers suffered from IBK on this farm in 1991 and 1992, respectively. During this study, heifers that are repeatedly affected with IBK were often observed.

Isolation and identification of M. bovis: Moraxella-like isolates that were Gram-negative, oxidase-positive and indole-negative rods were isolated from 13 ocular samples of heifers and nine Asian face flies, respectively, in the 2 years. Among the nine isolates from Asian face flies, 6 isolates (designated as the FL series) were isolated from the legs of flies, the remaining 3 isolates (designated as the FM series) were isolated from the labela of flies. These moraxella-like isolates were subjected to a Southern DNA-DNA hybridization test with the chromosomal DNA of M. bovis reference strain Matsu to finally determine if they were M. bovis isolates. Figure 1 shows that 8 isolates (91R, 182, 1209, 10L, 248R, 248L, 833L and 942R) from the ocular samples had DNA sequences homologous to Matsu, suggesting that they were M. bovis isolates, but 5 isolates from heifers and all 9 isolates from Asian face flies were not identified as M. bovis. The isolation frequency of M. bovis for the 2 years is shown in Table 2. Unfortunately, no M. bovis was isolated from the 331 Asian face flies tested (Table 2).

Plasmid profile of M. bovis: To estimate if a clonal M. bovis isolate was distributed on Farm Y or if M. bovis isolates with different characteristics are continually introduced from foreign sources, plasmid profile analysis was done. As shown in Fig. 2, plasmid profiles of the 8 M. bovis isolates from the farm were compared with those of a reference Matsu isolate. All 8 isolates and the reference Matsu strain contained two kinds of plasmids; 35-kb and 4.0-kb in molecular size (Fig. 2). This suggests the possibility that M. bovis isolates with similar characteristic were widespread on Farm Y.

DISCUSSION

On the Y farm, epizootics of IBK have repeatedly occurred every summer, as evidenced by the present 2-year observation for IBK infection during 1991 and 1992 (Table 1). In 1986, 1987, 1988, 1989 and 1990, 11.6%,
24.3%, 17.7%, 15.8% and 16.8% of the heifers on this farm, respectively, were shown to be affected with IBK (unpublished data). Although the occurrence of IBK varied from year to year, IBK appears to be a serious contagious disease on this farm. Asian face flies have long been suspected of playing a role as a vector in the dissemination of IBK. In fact, it was reported that *M. bovis* has indeed been isolated in the U.S.A. from the wild face fly [2, 6, 21], *M. autumnalis*, which is closely related to the Asian face fly. Unfortunately, however, no *M. bovis* was isolated from Asian face flies in the present study. Berkebile et al. [2] reported that less than 1% of

![Fig. 1. Southern blot analysis of homology among chromosomal DNAs from moraxella-like isolates. Chromosomal DNA from each isolate: lanes 1 and 13, Mutsu; lane 2, 91R; lane 3, 1209; lane 4, FL2; lane 5, FL3; lane 6, FL4; lane 7, FM8; lane 8, 248R; lane 9, 833L; lane 10, 248L; lane 11, 10L; lane 12, 10L1; lane 14, 182; lane 15, 253R; lane 16, 487R; lane 17, FL24; lane 18, FL88; lane 19, FL124; lane 20, FL224; lane 21, 942R; lane 22, FM27; lane 23, 111L1; lane 24, 544L. The numbers on the left indicate \( \lambda \)-HindIII digests as a molecular weight marker.](image)

![Fig. 2. Agarose gel electrophoretic profiles of *M. bovis* plasmid DNA. Plasmid DNA from *M. bovis* isolates: lanes 1 and 11, standard plasmid DNA, R100 (100-kb), Rs-a (34-kb), pBR322 (4.3-kb) and pBluescript KS+ (3.0-kb); lane 2, Mutsu; lane 3, 91R; lane 4, 1209; lane 5, 248R; lane 6, 248L; lane 7, 833L; lane 8, 10L; lane 9, 182; lane 10, 942R. The numbers on the left indicate the plasmid DNA molecular weight standards (kb). The numbers with arrows on the right indicate positions and sizes (kb) of the plasmid DNA from the *M. bovis* isolates. Open circular plasmid DNAs (OC1 and OC2) are derived from 35-kb and 4.0-kb plasmids, respectively. The linear form (linear) is derived from the 4.0-kb plasmid. Chro, chromosomal DNA.](image)

<table>
<thead>
<tr>
<th>Source</th>
<th>1991</th>
<th>Month of sampling in 1992</th>
<th>Total (%)</th>
</tr>
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<tr>
<td></td>
<td>8</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>Ocular swabs&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3/46&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0/22</td>
<td>0/6</td>
</tr>
<tr>
<td>Asian face flies</td>
<td>0/38</td>
<td>0/104</td>
<td>0/11</td>
</tr>
</tbody>
</table>

<sup>a</sup> Ocular swabs were obtained from the heifers with IBK.

<sup>b</sup> No. of positive samples/No. of samples tested.

<sup>c</sup> Designation of *M. bovis* isolates.
field-collected face flies were contaminated with *M. bovis*,
even when they were collected from herds of cattle in
which IBK caused by this organism was prevalent. This
failure to isolate *M. bovis* from Asian face flies may be due
to the small numbers of flies tested. Since insecticide-
treated herds with a smaller population of face flies had
fewer isolates of *M. bovis* and fewer clinical cases of IBK
[6], effective fly control with insecticides may be essential
to prevent the spread of IBK within herds.

Plasmid profile analysis has been widely used as an
epidemiological tool for distinguishing isolates within
epidemics of bacterial infection, especially in the family of
*Enterobacteriaceae* [3, 4, 18]. All present isolates of
*M. bovis* commonly contain the same number of plasmids of
identical molecular size (35-kb and 4.0-kb), suggesting the
possibility that these isolates originated in a single source,
in the other words, that clonal *M. bovis* spread on the
farm. In larger herds such as this one where many heifers
were introduced from various dairy farms, plasmid profile
analysis may be of value in tracing the spread of *M. bovis*
infection. However, to confirm if all *M. bovis* isolates
isolated on this farm throughout the years are identical,
other molecular analysis such as restriction enzyme
mapping or DNA sequencing of the plasmids is necessary.

McDonald and Pugh [13] screened 200 *M. bovis* isolates
and reported that those isolates could be classified into
three groups on the basis of the plasmid profile analysis.
The profile pattern (Fig. 2) observed in this study
belonged to group II, a major group in which 60% of the
200 isolates tested were classified [13]. Although the
function of the plasmid carried by *M. bovis* is not known
[22, 23], plasmid profile analysis is thought to be a
practical test for differentiating *M. bovis* isolates in
epidemiological studies.

Acknowledgements. The authors thank Dr. Nobuyuki Tera-
kado for supplying *M. bovis* strains Mutsu and EPP-63. The
authors thank Dr. Koji Horii for his valuable suggestions. Thanks
are also due to Drs. Kenmitsu Horiiuchi and Takashi Kayama for
their sampling help in this survey.

References

1. Baptista, P. J. H. P. 1979. Infectious bovine keratoconjunc-
Field association of female face flies with *Moraxella bovis*,
an etiological agent of bovine pinkeye. *J. Econ. Entomol.*
74: 475–477.
3. Brunner, F., Margadant, A., Peduzzi, R., and Piffaretti,
J.-C. 1983. The plasmid pattern as an epidemiologic tool for
Salmonella typhimurium epidemics: comparison with the
radio labeling DNA restriction endonuclease fragments to
6. Gerhardt, R. R., Allen, J. W., Greene, W. H., and Smith,
P. C. 1976. The role of face flies in an episode of infectious
156–159.
7. Hall, R. D. 1984. Relationship of the face fly (Diptera:
Muscidae) to pinkeye in cattle: A review and synthesis of
Conservation of DNA sequences for plasmid-mediated
165: 324–327.
of infectious bovine keratoconjunctivitis in a beef herd. *J.
Ultraviolet radiation and *Moraxella bovis* in the etiology of
bovine infectious keratoconjunctivitis. *Am. J. Vet. Res.* 26:
1331–1338.
and Berg, D. 1977. Plasmids studied in *Escherichia coli* and
other enteric bacteria. pp. 607–638. In: Insertion Elements,
Plasmids and Episomes (Bukhari, A. I., Shapiro, J. A., and
Adhya, S. L. eds.), Cold Spring Harbor Laboratory, Cold
Spring Harbor, N. Y., U.S.A.
Molecular Cloning: A Laboratory Manual 2nd. ed., Cold
Spring Harbor Laboratory, Cold Spring Harbor, N. Y.,
U.S.A.
keratoconjunctivitis: carrier state of *Moraxella bovis* and
development of preventive measure against disease. *J. Am.
The isolation and characterization of *Moraxella bovis*. *Am.
Use of agarose gel electrophoresis of plasmid deoxyribonucleic
33: 1105–1108.
among DNA fragments separated by gel electrophoresis. *J.
transmissability of *Moraxella bovis* by the face fly. *J. Econ.
Entomol.* 58: 444–446.
bovine keratoconjunctivitis epizootic associated with area-
wide emergence of a new *Moraxella bovis* plus type. *Am.
23. Wilt, G. R., Wu, G., Bird, R. C., and Toivo-Kinnunen, M.
1990. Plasmid content of pillated and nonpillated forms of