LEPTOSPIRA INTERROGANS SEROVAR HARDJO INFECTION OF CATTLE

L. M. GORDON

Regional Veterinary Laboratory, Department of Agriculture, P.O. Box 406, Hamilton, Victoria, 3300

SUMMARY: A survey of normal cattle in the Southern Victorian statistical divisions revealed that microscopic agglutination titres to L. hardjo occur at high frequency and are distributed throughout the cattle population. These titres are difficult to interpret as they may represent recent or old exposure, with or without disease.

L. hardjo infection of dairy cattle was studied in 4 herds using the microscopic agglutination and complement-fixation tests. Statistical comparisons of individual titres obtained indicated that the sensitivity of the complement-fixation test was satisfactory for diagnostic purposes, but the test was unable to differentiate between current or past infections.

Introduction

In the 6-year-period, 1970 to 1975, microscopic agglutination (MA) testing at the Veterinary Research Institute, Parkville, Victoria revealed 15,229 reactors (with a titre to Leptospira hardjo of at least 100) from 31,364 samples (T. E. Jones personal communication). Most of these serum samples were from cattle, and had been submitted for disease diagnosis. However the high reaction rate indicated a high level of exposure to infection with this serovar in the cattle population.

The present study was undertaken to estimate the prevalence of L. hardjo infection in Southern Victorian cattle, and to determine the results of infection in 4 representative herds.

As Hodges (1973) suggested the possible use of the complement-fixation (CF) test to detect recent or active infections this test was used in parallel with the MA test for these investigations. In addition, the sensitivity of the CF test for the diagnosis of bovine leptospirosis was investigated by statistical comparison with the MA test in clinically infected, and in clinically normal herds.

Materials and Methods

Serology

The MA test was performed with microtitre equipment according to the procedure described by Cole et al. (1973). Antigen was prepared from 2-day growth of leptospires in Ellinghausen medium, (EMJH)*, the opacity being adjusted to 0.05 units at 420 μm on a 'Bausch and Lomb Spectronic 20' spectrophotometer with uninoculated medium. The antigen was mixed with a vortex mixer before use. Urinary agglutinating antibody was estimated by the MA test after 10-fold concentration of urine in an AMICON B15 ultra-filtration unit.†

* EMJH Difco Cat. No. 0794,0795
† Amicon Corporation, Lexington, Mass., 02173, U.S.A.

Challenge Experiments with Herd A: hardjo Isolates

Call Challenge: Two 2-day-old heifers were inoculated with 2 x 10⁸ and 4 x 10⁹ leptospires/ml. by the subcutaneous and intravenous routes respectively. Each culture had been passed twice through artificial culture medium.
Cow Challenge: Three 4-year-old lactating dairy cows were inoculated with $1 \times 10^7$ leptospires/ml. Two of the cows were inoculated by the intramammary route, and the third by the intravenous route. Each culture had been passed once through artificial culture medium. In addition, a 6-year-old lactating dairy cow was inoculated each day for 5 days by both conjunctival and subcutaneous routes with infected urine taken directly from a cow excreting leptospires.

Survey Studies

A random sample of 10% of each of a number of herds submitted in the Brucellosis eradication campaign was selected to represent the normal cattle population.

The first study in March 1976 was to investigate the incidence of *L. hardjo* infection in Southern Victoria. Of 15,310 serums, 1,488 equally representing all 8 Southern Victorian statistical divisions, were examined by the MA test. The lowest serum dilution used was 1/100.

The second study in June, 1977 was to determine whether leptospiral CF reactors occur in the normal cattle population. Of 7,018 serums from the South Western Division 675 were tested by both MA and CF procedures. The lowest serum dilutions used were MA 1/50 and CF1/4.

Herd Histories

Herd A: In early September 1975, 36 of 110 dairy cows developed fever, depression, sunken eyes and rough coats over 10 weeks. All cattle in the herd were vaccinated with hardjo/pomona vaccine in October and November 1975. Initial studies made on this herd until January 1976 described an illness described as intermittent fever, depression, sunken eyes and rough coats over weeks 5 and 6.

Herd B: In mid January 1977, a dairy farmer consulted his physician regarding an illness described as intermittent fever, weakness. A serum sample taken from him at this time was found to contain *L. hardjo* antibodies. Further investigation revealed that his herd was undergoing a mastitis outbreak with 65 of 140 cattle affected. Blood and milk samples were obtained 2 weeks after onset and the herd was subsequently bled at weeks 8 and 21.

Herd C: In mid October 1975, 15 of 62 milking cows were affected over a 3-week-period with fever accompanied by a sudden decrease in milk production. There were no clinical signs of udder infection, the milk was normal in colour but contained small clots. Milk samples were taken from clinical cases in weeks 3 and 4 after onset. The entire herd was bled and urine samples were taken during week 6. The herd was bled again at week 82.

Herd D: In early October 1975, a mastitis outbreak occurred in a dairy herd grazing steeply undulating country containing swampy areas. Twelve of 84 milking cows were affected in the first week after recognition of the initial cases. The udders of affected cows were flaccid in all quarters and the milk contained yellow-orange clots which were sometimes blood-flecked. Blood samples were taken from all affected cows 1 and 4 weeks after onset. In the eighth week the entire herd was bled, and milk and urine samples were taken.

Results

Survey Studies

March 1976 Survey: In this survey, the number of serums reacting to the MA test at titre $\geq 100$ in individual statistical divisions varied between 20% and 57%, while the overall figure for Southern Victoria was 39%.

June 1977 Survey: In this survey, the relationship between MA and CF titres in normal serums drawn from South Western Victoria was found to be as follows; MA $\geq 50$, and CF $< 4$ (16.5%), both MA $> 50$, and CF $\geq 4$ (14.5%), CF $> 4$, and MA $< 50$ (2.5%). Thus, 31% of serums reacted to the MA test at $> 50$ while 17% of serums reacted to the CF test at $> 4$.

Herd Studies

Tables 1 and 2 summarise the results of serological examinations of herds A, B, C and D.

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### TABLE 1

**Titre Distribution in Herd A Following Outbreak of Mastitis**

<table>
<thead>
<tr>
<th>Weeks Following Outbreak</th>
<th>Percent of Reactors at MA* Titre</th>
<th>Percent of Reactors at CF+ Titre</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>no titre 100 200 400 800 1600 3200 6400 12,800 25,600</td>
<td>no titre 4 8 16 32 64 128 256 512 1024</td>
</tr>
<tr>
<td>1</td>
<td>61 24 11 4 - - - -</td>
<td>53 27 10 8 1 - 1 - - -</td>
</tr>
<tr>
<td>7</td>
<td>47 3 11 11 10 12 4 2 -</td>
<td>42 2 13 13 13 8 9 - - -</td>
</tr>
<tr>
<td>12</td>
<td>14 9 9 15 14 25 6 6 - 2</td>
<td>22 9 28 23 9 3 6 - - -</td>
</tr>
<tr>
<td>12</td>
<td>19 19 11 14 14 18 4 1 -</td>
<td>24 11 26 24 8 3 4 - - -</td>
</tr>
<tr>
<td>60</td>
<td>66 24 7 3 - - - -</td>
<td>40 26 28 5 1 - - - -</td>
</tr>
</tbody>
</table>

* Microscopic Agglutination Test
+ Complement Fixation Test

Arthur Webster Pty. Ltd., Northmead, New South Wales, 2152.
The appearance of "agglutination balls" of leptospires in dark-field examination of urine after the third week is shown by the vertical dividing line. This phenomenon occurred just before there was detectable urinary agglutinating antibody. Dark field examination of urine was continued for 6 months, but leptospirosis did not recur. Fifteen of 16 direct urine cultures were positive during the urinary excretion phase. The identity of one of these isolates was confirmed as *L. hardjo* by cross agglutination studies.

A one-day-old calf fostered to the infected cow during week 2 never gave a serological response to *L. hardjo*. A normal calf was born to the cow after week 33. Titres of the cow and her calf at first week after the birth were MA 50, CF 4, and MA 100, CF 8 respectively.

**Herd B:** Cultural examination of milk samples taken at week 2 indicated that there was concurrent infection with normal mastitis pathogens in

### TABLE 3

**Results of Regression Analysis of log (MA + 10) on log (CF + 10)**

<table>
<thead>
<tr>
<th>Herd</th>
<th>Weeks Following Outbreak</th>
<th>Percentage of Reactors*</th>
<th>Multiple R</th>
<th>P</th>
<th>Degree of Equation Fitted+</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1</td>
<td>49</td>
<td>-‡</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(110 cows)</td>
<td></td>
<td>7</td>
<td>60</td>
<td>0.87</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>87</td>
<td>0.83</td>
<td>&lt;0.01</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>85</td>
<td>0.66</td>
<td>&lt;0.01</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>65</td>
<td>0.34</td>
<td>&lt;0.05</td>
<td>1</td>
</tr>
<tr>
<td>B</td>
<td>10</td>
<td>49</td>
<td>0.75</td>
<td>&lt;0.01</td>
<td>4</td>
</tr>
<tr>
<td>(140 cows)</td>
<td></td>
<td>8</td>
<td>38</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>17</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C</td>
<td>6</td>
<td>92</td>
<td>0.50</td>
<td>&lt;0.01</td>
<td>1</td>
</tr>
<tr>
<td>(62 cows)</td>
<td></td>
<td>82</td>
<td>23</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D</td>
<td>8</td>
<td>95</td>
<td>0.67</td>
<td>&lt;0.01</td>
<td>4</td>
</tr>
<tr>
<td>(84 cows)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* An overall figure combining all CF and MA reactors at each test
† Where 4th degree equations were fitted the x^2 and/or x^3 terms were generally tolerance rejected, that is, too closely associated with x^4 to be fitted
‡ Equations could not always be fitted because of restricted titre range and/or insufficient reactors
Weeks post onset

Clinical mastitis

\[
\begin{array}{c|c|c|c|c|c|c|c}
0 & 1 & 2 & 3 & 4 & 5 & 6 & 7 & 8 \\
\hline
\end{array}
\]

Leptospirosis (culture positive)

Leptospirosis (dark-field positive)

Urinary agglutinating antibody

\[
\text{titre}
\]

Serum agglutinating antibody

\[
\begin{array}{c|c|c|c|c|c|c|c}
& 800 & 400 & 200 & 100 & 128 & 64 & 32 & 16 & 8 & 4 \\
\hline
\end{array}
\]

Serum complement fixing antibody titre

\[
\begin{array}{c|c|c|c|c|c|c|c}
\text{titre} & 128 & 64 & 32 & 16 & 8 & 4 \\
\hline
\end{array}
\]

Figure 1. Phases of a naturally acquired L. hardjo infection.

some affected cows. A serum sample taken from the dairy farmer at this time gave titres to L. hardjo of MA 200, CF 32. The overall serological reaction rate decreased rapidly after the initial test, and further spread did not occur within the herd.

Herd C: Cultural examination of milk samples taken during weeks 2 and 4 indicated that normal mastitis pathogens were absent. Leptospires were detected in 7 out of 62 urine samples by direct examination at week 6. Titres generally fell to low levels by week 82 and there was no further clinical evidence of mastitis by this time.

Herd D: Normal mastitis pathogens were absent from milk samples taken at week 8, and leptospires were not detected in any urine sample taken.

Results of Challenge Experiments with Herd A Isolates

Attempts to reproduce leptospiral mastitis in serologically negative milking dairy cows using infected urine and low passage isolates from the acquired cow by intramuscular and intramammary routes were unsuccessful.

Previous attempts to infect 2-day-old calves with other isolates from this herd were also unsuccessful. On each occasion, blood, urine, milk samples and rectal temperatures were regularly taken for at least 3 weeks, but there were no significant findings.

Discussion

This work has demonstrated the potential use of the CF test in the laboratory diagnosis of leptospirosis in cattle. The sensitivity of the tests and general agreement of CF and MA titres were confirmed in individual animals in infected herds by appraisal of the tabular values given and the multiple correlation coefficients obtained.

The high reactor rates found in the surveys may be attributed to the sub-clinical nature of L. hardjo infection in the cattle population (Hoare and Claxton 1972), and the longevity of the immune response in individual animals. Although the microscopic agglutination (MA) test can at best be regarded as serogroup specific (Turner 1968), serovar hardjo is the sole member of the Hebdomadis serogroup to be isolated from Victorian cattle to date (Gordon 1977). The identification of this isolate was confirmed by the World Health Organization Reference Laboratory at Brisbane. This indicates that the widespread titres observed in the present study were due to infection with L. hardjo.

Twigg et al (1972) in the United Kingdom suggested that infection rates of cattle with leptospires may be related to wildlife in the area. The feral hosts for L. hardjo have yet to be identified in Australia (Sullivan 1974).

In New South Wales, Hoare and Claxton (1972) reported that herds could be infected without showing clinical signs, and that leptospirosis occurred where there was serological evidence of infection in the absence of clinical signs.

A comparison of the numbers of cows exhibiting clinical signs of 'atypical mastitis' against numbers of cows becoming serologically positive could be made with the herd records obtained for 3 of the 4 herds involved in this study. Thus
gested that the higher concentration and per-
on the characterisation of the antibodies detected
bovines, although smaller amounts of IgM are re-
time.
may lead to the microscopic agglutination test
agglutinating antibodies the CF test could be
sistence of IgG I produced in bovine leptospirosis
as complement-fixing antibodies could be detected
complement-fixation test to serological diagnosis
Sharma
Gulasekharam
various authors include haemagglutination (Negi
dard reference procedure for leptospiral diagnosis
in Australian outbreaks.
pathogenesis of serovar hardjo mastitis are re-
required as this is the most common clinical finding
in Australian outbreaks.
The MA test has long been regarded as the stan-
ad man and animals (WHO 1972). Other sero-
which have been proposed by various authors include haemagglutination (Negi et al 1971; Imamura et al 1972; Palit and Gulasekharam 1973), complement fixation (York 1952; Hodges 1973), and immunofluorescence (Torten et al 1966).
Robertson and Boulanger (1962), Palit and Sharma (1971), and Hodges (1973) have made preliminary studies on the application of a complement-fixation test to serological diagnosis of leptospirosis in animals. Hodges suggested that as complement-fixing antibodies could be detected for a short period of time compared with agglutinating antibodies the CF test could be useful in identifying active leptospiral infections.
Morris and Hussaini (1974) found that IgM and IgG1 are responsible for agglutinating activity in bovines, although smaller amounts of IgM are required for agglutination than IgG1. They suggested that the higher concentration and persistence of IgG1 produced in bovine leptospirosis may lead to the microscopic agglutination test detecting residual antibodies for long periods of time.

There appears to be no published information on the characterisation of the antibodies detected by the CF test for leptospirosis, but the results of the present studies indicate that it also detects residual immunoglobulins and is not a satisfactory test for the identification of active leptospiral infections. This was demonstrated by the presence of CF titres in the survey populations, demonstrable titres after one year in the herd studies, and the highly correlated MA and CF titres in the herd studies.
The leptospiral CF has advantages in a diagnostic laboratory doing routine CF tests. Many serovars can be tested for as their antigens are stable and can be standardised between laboratories. The test is simple to perform, sufficiently sensitive, and is useful as a screening test in the zoonoses group with ‘Q’ fever and brucellosis. The main disadvantage with the CF test is that other serovars will sometimes cross react to low titres. While this was not a problem in the present work it could lead to identification problems with single serum samples.

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References

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