Serological Diagnosis of Bovine, Caprine, and Ovine Mastitis Caused by *Listeria monocytogenes* by Using an Enzyme-Linked Immunosorbent Assay

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An enzyme-linked immunosorbent assay for detecting *Listeria monocytogenes* antibodies in bovine (n = 35), caprine (n = 27), and ovine (n = 30) milk samples was evaluated by comparison with bacteriological examination. Microtiter plates were coated with proteins obtained from culture supernatant, and antibodies were revealed with a monoclonal antibody able to react with the immunoglobulins belonging to the three animal species. The arithmetic mean optical density (OD) of milk samples infected with *L. monocytogenes* was above that of uninfected milk samples or milk samples infected with pathogens other than *L. monocytogenes*.

With an OD threshold of 0.2 for goat and ewe milk samples, the sensitivity and specificity of the test were 100 and 88%, respectively. The choice of a different OD threshold (0.5) for cows allowed the discrimination of all of the infected cows and yielded no false positives, and both sensitivity and specificity were 100%.

Although listerial intramammary infections (IMI) are rare, identification of infected animals is necessary for the dairy industry since contaminated milk and milk products have been involved in several outbreaks of listeriosis (5, 10). This kind of IMI is also difficult to eliminate because antibiotic treatments are, in most cases, inefficient (2, 4, 9, 13) so that infected animals have to be culled from the herd (4).

When *Listeria monocytogenes* is detected in several successive samplings of bulk milk, bacteriological examination of milk from each animal in the herd is performed to identify the animal responsible for the contamination. The bacteriological methods used are expensive and time consuming, and milk samples should be taken aseptically. Moreover, these methods could be inappropriate and, thus, some listerial IMI may not be identified (4, 7, 12). Because the level of *L. monocytogenes* excretion in milk is variable, with less than 10 CFU/ml of milk in some cases (2), identification of infected animals by direct plating is difficult or even impossible and an enrichment step for bacteriological examination is necessary. Analysis of a milk sample from each animal in a herd could indicate those whose milk has an elevated specific antibody concentration. These animals should then be considered suspect, and bacteriological examination of only their milk samples should be performed.

The work presented here describes an evaluation of the ability of an enzyme-linked immunosorbent assay (ELISA) to differentiate cows, goats, and ewes with listerial IMI from uninfected animals or animals infected with pathogens other than *L. monocytogenes*.

**Milk and whey samples and bacteriological examination.** Composite milk samples were obtained from four cows with IMI that were experimentally infected with *L. monocytogenes* (2) and from seven natural infections observed in different herds. The four cows experimentally infected were inoculated in two quarters with *L. monocytogenes* from either serotype 1/2a or 4b. Whey samples from nine ewes with experimental mastitis were kindly provided by F. Schelcher (National Veterinary School, Toulouse, France). All of the ewes were experimentally infected with serotype 4b *L. monocytogenes*. Composite milk samples from goats with listerial IMI were collected from six different herds, and serotypes were not determined.

Other composite milk samples from cows, goats, and ewes were collected aseptically for bacteriological examination. A 25-μl volume of each milk sample was plated on esculin sheep blood agar medium. All of the cultures were incubated aerobically for 18 to 24 h at 37°C before being examined for identification of bacterial colonies.

According to bacteriological examination, samples were divided into three groups: uninfected animals (group A), animals with IMI caused by pathogens other than *L. monocytogenes* (group B), and animals with IMI caused by *L. monocytogenes* (group C) (Table 1).

**ELISA.** Flat-bottom 96-well microtiter plates were coated with a preparation of proteins (2.5 μg/ml) from *L. monocytogenes* 4b culture supernatant fluid. This preparation was obtained after a single step of adsorption chromatography (1). The presence of listeriolysin and p60 in the antigenic preparation was confirmed by immunoblotting with an anti-LLO monoclonal antibody (MAb) and anti-p60 serum (1), and the presence of phosphatidylcholine phospholipase C is suggested by a band at 29 kDa (6) in the electrophoretic profile.

The procedure for the ELISA was described elsewhere (1), except for the use of a peroxidase-conjugated MAb that was provided for the test by Vetoquinol Biotechnology (Lure, France). The MAb, directed against bovine immunoglobulin G (IgG), is able to react with caprine and ovine immunoglobulins. Moreover, instead of 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), revelation of the ELISA was performed with tetramethylbenzidine.

Briefly, 100-μl composite milk or whey samples were assayed in duplicate. Each composite milk or whey sample consisted of a mixture from the four quarters (cows) or the two half udders (goats and ewes). After 1 h at 37°C, each well was washed, 100 μl of MAb peroxidase conjugate was added, and the plate was incubated for 1 h at 37°C. The microtiter plate was then washed, and revelation was performed with tetramethylbenzidine for 30 min under shaking in the dark. The optical density (OD) at 450 nm was read with an automated microplate reader.
TABLE 1. Results of bacteriological examinations of aseptic milk samples

<table>
<thead>
<tr>
<th>Pathogen(s) isolated</th>
<th>Group</th>
<th>No. of milk samples from:</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cows</td>
<td>Goats</td>
</tr>
<tr>
<td>None</td>
<td>A</td>
<td>13</td>
<td>8</td>
</tr>
<tr>
<td>Pathogens other than</td>
<td>B</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Listeria monocytogenes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coagulase-negative</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Staphylococci</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Streptococcus uberis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>11</td>
<td>6</td>
</tr>
<tr>
<td>Listeria monocytogenes</td>
<td>C</td>
<td>11</td>
<td>13</td>
</tr>
</tbody>
</table>

(Titertek Multiscan Spectrophotometer; Flow Laboratories, Helsinki, Finland).

The relationship between the infection status of the mammary gland and the ELISA result is shown in Table 2. For the three animal species, the arithmetic mean ODs calculated for milk samples of groups A and B were significantly lower ($P < 0.01$) than the mean of OD of milk samples infected with *L. monocytogenes* (group C). All of the OD values for cow and goat milk samples from groups A and B (uninfected or infected with pathogens other than *L. monocytogenes*) were below the lowest OD value recorded for milk samples infected with *L. monocytogenes*. By contrast, four ewe milk samples infected with coagulase-negative staphylococci (data not shown) had OD values included in the range of values recorded for ewe milk samples infected with *L. monocytogenes*. Previous results have indicated that the increase in milk of antibodies directed against the antigenic preparation used in this test generally becomes significant 3 weeks after experimental infection of cows (1) and also experimental infection of ewes (data not shown). Moreover, the titers of antibody, mainly IgG, in milk remained elevated during the 6 months of infection of cows (1). It was impossible to determine the persistence of antibodies in milk after spontaneous recovery, since this event was not recorded during the study (2).

Because all of the milk samples from animals infected with *L. monocytogenes* had an OD of $>0.200$, this value was taken to discriminate samples infected by *L. monocytogenes* from uninfected samples or samples infected with another pathogen (Table 3). With this OD threshold value, nine cow milk samples and four ewe milk samples were positive by ELISA but negative for *L. monocytogenes* by bacteriological examination. Thus, for three animal species ($n = 92$), the sensitivity and specificity of the test were 100 and 77.9%, respectively. However, for goats and ewes only ($n = 57$), the sensitivity and the specificity were 100 and 88.5%, respectively, with a threshold value of 0.2, and with another threshold value of 0.5 for cows ($n = 35$), both sensitivity and specificity reached 100%.

The choice of threshold is important for the value of the test. With a higher threshold value, milk samples could be considered negative by ELISA but positive after bacteriological examination. These results could be explained by recent infection of the animal. As already described for cows experimentally infected (1), the increase of antibodies in milk appeared at least 3 weeks postinfection.

Although the arbitrary threshold value of 0.2 gave a sensitivity of 100% and a specificity of 88.5% for goat and ewe milk samples, it was inappropriate for the test with cow milk samples. As shown in Table 2, the maximum OD value observed for uninfected cow milk samples or milk samples from cows with IMI caused by a pathogen other than *L. monocytogenes* was 0.435. Each milk sample from cows with listerial mastitis showed an OD of $>0.5$. Thus, a threshold value at 0.5 more efficiently discriminated cows infected with *L. monocytogenes* from uninfected cows or those infected with other pathogens, since both sensitivity and specificity were 100%. Whatever the group of samples, the OD values of bovine milk samples were generally higher than those of caprine or ovine milk samples. These results were probably due to the use of a MAB conjugate that is initially directed against bovine IgG but reacts with IgG from the other two animal species. The choice of different threshold values, one for cows and the other for small ruminants, seems necessary at this step of the study.

In some cases, ELISA and bacteriological examination results differed. Although most listerial IMI are persistent and spontaneous, cure is not frequent (2, 13); samples which were positive by ELISA but negative for culture of *L. monocytogenes* could be explained by the presence of antibodies resulting from previous spontaneously cured infections or from a high level of “natural” antibodies directed against *L. monocytogenes* resulting from a listerial infection other than mastitis. It is likely that IMI caused by pathogens other than *L. monocytogenes* induce exudation of plasma proteins from blood to the mammary gland (3) and, concurrently, exudation of natural antibodies directed against *L. monocytogenes*. The difference between ELISA and bacteriological results could also be ex-

<table>
<thead>
<tr>
<th>Group</th>
<th>Pathogen(s) isolated</th>
<th>Mean OD at 450 nm (range) of milk samples from:</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Cows</td>
</tr>
<tr>
<td>A</td>
<td>None</td>
<td>0.177 (0.012–0.474)</td>
</tr>
<tr>
<td>B</td>
<td>Pathogens except <em>L. monocytogenes</em></td>
<td>0.175 (0.001–0.435)</td>
</tr>
<tr>
<td>C</td>
<td><em>L. monocytogenes</em></td>
<td>1.202 (0.562–1.846)</td>
</tr>
</tbody>
</table>

### Notes

1. It was impossible to determine the persistence of antibodies in milk after spontaneous recovery, since this event was not recorded during the study (2).
2. The ELISA was considered positive if the OD was $>0.2$.
3. The values shown are the numbers of samples positive or negative followed by the total numbers of samples.
plained by intermittent shedding or a low level of *L. monocytogenes* in milk, making isolation of *L. monocytogenes* by direct plating on sheep blood agar impossible. Intermittent shedding of *L. monocytogenes* has already been described for goats and ewes without clinical signs of listeriosis (8, 12). Moreover, there was no relationship between the false positives registered by ELISA and the pathogen species isolated from the udder because the four false-positive ewe milk samples were infected with coagulase-negative staphylococci and among the nine false-positive cow milk samples, four were uninfected, one was infected with coagulase-negative staphylococci, two were infected with *Staphylococcus aureus*, and two were infected with *Streptococcus uberis*.

This preliminary study was conducted with a small number of milk samples. The test is being evaluated by using a larger number of animals belonging to the three species. For instance, the test has already been used for a herd of more than 100 goats in whose bulk tank milk *L. monocytogenes* has been isolated several times. Samples from three goats had ODs of >0.4 (unpublished observation). On bacteriological examination of milk samples from these goats, one of them turned out to be positive for *L. monocytogenes*.

This kind of evaluation should allow us to specify both the OD threshold value suitable for each animal species and the procedures for the use of the test. With this ELISA, it will be possible to detect animals (cows, goats, and ewes) which have IMI caused by *L. monocytogenes*. This method is rapid and less expensive than the bacteriological methods currently used and could be applied to all animals of the herd from whose bulk tank milk *L. monocytogenes* has been isolated. The test should permit reduction of the number of bacteriological tests of animals with high levels of antibodies in their milk, which would minimize the cost for detection of an infected animal.

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REFERENCES