Evaluation of Two Enzyme-Linked Immunosorbent Assays for Detecting *Salmonella enterica* subsp. *enterica* Serovar Dublin Antibodies in Bulk Milk

J. VELING,1* F. G. VAN ZIJDERVELD,2 A. M. VAN ZIJDERVELD-VAN BEMMEL,2 Y. H. SCHUKKEN,3 AND H. W. BARKEMA1

Animal Health Service, 7400 AA Deventer,1 and Department of Bacteriology, Institute for Animal Science and Health, ID Lelystad, 8200 AB, Lelystad,2 The Netherlands, and Department of Population Medicine and Diagnostic Sciences, Cornell University, Ithaca, New York 148533

Received 20 February 2001/Returned for modification 24 April 2001/Accepted 23 July 2001

Two enzyme-linked immunosorbent assays (ELISAs) for the detecting *Salmonella enterica* subsp. *enterica* serovar Dublin antibodies in bulk milk were developed and evaluated for potential use in control programs. The ELISAs were based on either lipopolysaccharide (LPS ELISA) or flagellar antigen (GP ELISA). Sensitivity was determined with 79 case herds with a wide range of clinical signs. Specificity was determined with 125 Dutch and 200 Swedish control herds. The relation between antibodies in bulk milk, antibodies in serum, and the level of milk production of individual cows was studied with 61 case herds. The optimal optical density (OD) values of the LPS ELISA and the GP ELISA were determined to be 0.2 and 0.5, respectively. The sensitivities of the LPS ELISA and the GP ELISA were 54 and 63%, respectively, with a specificity of 98% for both ELISAs with samples from the Dutch control herds. The specificities for samples from the Swedish herds were 100% for the LPS ELISA and 95% for the GP ELISA. The sensitivity of the combination of tests was 65% when samples were run in parallel, and the specificity was 100% when samples were run in series, irrespective of whether the samples came from Dutch or Swedish control herds. The variance (R^2) in the OD value for bulk milk samples could be explained by the percentage of seropositive lactating cows in a herd with the LPS ELISA for 51% of the samples and with the GP ELISA for 72%. The variance in the OD value was best explained by the combination of the percentage of seropositive lactating cows in the herd and the mean log_{10} serum antibody titer for that herd (R^2 = 62% for the LPS ELISA and R^2 = 75% for the GP ELISA). Case herds more often tested negative by the ELISA with bulk milk when the percentage of seropositive lactating cows was less than 5%. It is concluded that both ELISAs with bulk milk can be used in control programs to distinguish between infected and noninfected herds. Specificity can be increased by using the two tests in combination. Sensitivity was relatively low for both single tests and both tests combined.

Salmonellosis occurs throughout the world and has a great impact on farm economics and public health (11, 27a). *Salmonella enterica* subsp. *enterica* serovar Dublin and serovar Typhimurium appear to be the commonest serovars isolated from cattle. Serovar Dublin infections in dairy herds may cause serious problems in calves and adult cows, such as septicemia, diarrhea, and abortion. In The Netherlands, serovar Dublin is the most frequently isolated serovar and is the second most common cause of enzootic abortion (27).

Screening to distinguish between infected and noninfected herds is important in control programs. The use of tests adapted for use with bulk milk samples is of interest because of the potential cost savings and the possibility for the automation of testing. Tests adapted for use with bulk milk have been developed for several bovine diseases, such as those caused by *Brucella abortus* (22), bovine leukemia virus (19), bovine viral diarrhea virus (15), *Leptospira hardjo* (1), bovine herpesvirus type 1 (24), *Mycobacterium avium* subsp. paratuberculosis (14), bovine corona virus (23), bovine respiratory syncytial virus (2), *Coxiella burnetii* (16), *Ostertagia* spp. (10), and *Staphylococcus aureus* (5).

Enzyme-linked immunosorbent assays (ELISAs) based on lipopolysaccharide (LPS) for serovar Dublin and serovar Typhimurium in milk have been evaluated by Hoorfar et al. (7) and Hoorfar and Wedderkopp (8). A study of serovar Dublin indicated the possibility of identifying serovar Dublin-positive and serovar Dublin-negative herds by an LPS ELISA with bulk milk (7) with a sensitivity of 100% and a specificity of 95%. However, in that study the number of case herds was limited. Wedderkopp (28) evaluated an LPS ELISA on a larger scale and determined a sensitivity of 88% and a specificity of 89%.

Recently, two ELISAs became available for evaluation of bulk milk, one ELISA based on LPS antigen (LPS ELISA) and one ELISA based on flagellar antigen (GP ELISA). The GP ELISA has antigenic code “g,p,” according to the Kauffmann-White scheme for flagellar antigens (17). The fact that the ELISAs are based on different antigens offers the opportunity to increase the specificity of the LPS ELISA by using the ELISAs in combination.

The purpose of this study, therefore, was to evaluate the test characteristics and potential use in control programs of two ELISAs, ELISAs based on LPS and flagellar antigen, for screening of bulk milk for serovar Dublin antibodies. Addi-
tionally, the relationship between the detection of antibodies in bulk milk, on the one hand, and the serology and the level of milk production of individual lactating cows, on the other, was determined.

**MATERIALS AND METHODS**

**Study design. (i) Farms.** The study was performed with samples from 79 known serovar Dublin-infected herds (case herds) and 325 herds without a history of serovar Dublin infection (control herds). The 79 serovar Dublin-infected herds were selected between September 1995 and February 1997 from among herds for which samples or dead animals had been sent to the Animal Health Service (Drachten, The Netherlands) for diagnostic reasons. Clinical signs were confirmed by at least one serovar Dublin-positive culture. All 79 farmers stated that this was the first known Salmonella infection on the farm. This statement was confirmed by the veterinary practitioner and by laboratory information for the farm recorded at the Animal Health Service for a period of at least 3 years before the outbreak. The time of the outbreak (day 0 [D0]) was defined for each farm as the day that the first serovar Dublin-positive culture was sampled. Animals were individually identified by ear tags provided by the Identification & Registration system applied in The Netherlands (13). The mean number of animals per herd was 141 (range, 28 to 370 animals).

The herds without a history of salmonellosis consisted of 125 herds from The Netherlands and 200 herds from the north of Sweden. Dutch herds were selected from dairy farms that had sent in bulk milk samples for bovine herpesvirus type 1 certification. Farmers were asked to participate if the herd had had no history of clinical salmonellosis in the past 3 years. The clinical status of the herd was confirmed by checking laboratory information for the farm recorded at the Animal Health Service. The 200 control herds from the north of Sweden were randomly selected. Infections with serovar Dublin are unknown in this part of Sweden (A. Engvall, personal communication).

(ii) **Data collection.** Blood and bulk milk samples from the case herds were collected on the same day and were immediately transported to the laboratory of the Animal Health Service. Samples were processed within 24 h and were stored at −15°C before analysis at ID Leylest (Leylest, The Netherlands). Samples were collected 2 to 4 months after D0, with an average time of collection of 87.3 days. The time of sampling was chosen according to the expectation that a maximum number of blood samples would be seropositive at the time of sampling. Blood samples were taken from the coccygeal vein. Information about the lactation status (lactating versus nonlactating) of cows was recorded on the day of sampling. The level of milk production of individual animals could be estimated for animals in 61 of the 79 serovar Dublin-infected herds. Excluded from this analysis were 12 herds for which the level of milk production was not regularly recorded and 6 herds for which the interval between sampling and regular recording of the level of milk production was longer than 42 days. Data on the level of milk production were obtained from the Royal Dutch Cattle Syndicate (Arnhem, The Netherlands). Milk samples from the control herds were collected without preservatives and were stored at −15°C before analysis at ID Leylest.

**ELISAs. (i) LPS ELISA and GP ELISA for serum antibodies.** Both the indirect ELISA with LPS (LPS ELISA) and the indirect ELISA with the serovar Dublin flagellin (GP ELISA) for serum antibodies have been described before (26). Briefly, the wells of microtiter plates were coated with 100 µl of a solution of purified LPS or 100 µl of a solution of purified flagellin with antigenic code g.p (hence, GP ELISA) of serovar Dublin, both of which contained 5 with a dilution of 1:100. After incubation for 1 h at 37°C before analysis at 15°C, the plates were washed and the conjugate, an optimal dilution of horseradish peroxidase-labeled monoclonal antibody against immunoglobulin G1 (IgG1), was added. After incubation for 1 h at 37°C the substrate solution with 5-aminosalicylic acid as the chromogen was added. The plates were read after a 2-h incubation at room temperature. Titers are expressed as the number of individuals determined. No serovar Dublin antibodies by the GP ELISA than by the LPS ELISA when samples with comparable OD values in a range from 0.1 to 0.9 were tested. However, more Dutch control herds with an OD value of 0.1 were positive for serovar Dublin antibodies by the GP ELISA. No serovar Dublin antibodies were detected by the LPS ELISA with the Swedish control herds.

ROC curves for the different combinations of tests and control herds are presented in Fig. 1A (LPS ELISA) and Fig. 1B (GP ELISA). The AUCs were 81.8% (95% confidence interval [CI], 78.3 to 85.2%) for the LPS ELISA and the Dutch control herds, 84.2% (95% CI, 80.9 to 87.4%) for the LPS ELISA and the Swedish control herds, 92.4% (95% CI, 90.3 to 94.4%) for the GP ELISA and the Dutch control herds, and 88.0% (95% CI, 85.6 to 90.4%) for the GP ELISA and the Swedish control herds. The AUC for the GP ELISA was greater than that for the LPS ELISA with samples from the Dutch control herds.

The highest DPR by the LPS ELISA was reached with an OD value of 0.1 (DPRs, 59 and 68% for the combination with the Dutch control herds and the Swedish control herds, respectively). The highest DPR by the GP ELISA was reached with an OD value of 0.4 (DPRs, 71 and 65% for the combination with the Dutch control herds and the Swedish control herds, respectively).

With the specificity set at 98%, the cutoff values of the ELISAs were analyzed further. The sensitivities of the LPS was measured at 450 nm with an ELISA reader. Appropriate controls were included on each test plate. The net OD values were used for analysis.

**Analysis and analytical methods.** Bulk milk samples from case and control herds were used to estimate the sensitivities and the specificities of the two ELISAs. Sensitivity was defined as the proportion of bulk milk samples that tested positive (with an OD value equal to or above the cutoff value) among the case herds. Specificity was defined as the proportion of bulk milk samples that tested negative (with an OD value below the cutoff value) among the control herds (12). Cutoff values were evaluated with respect to sensitivity, specificity, and differential positivity rate (DPR) (9). Optimal cutoff values were defined as those with a specificity of at least 98% because a lower specificity would lead to an unacceptable number of false-positive reactions in control programs. Statistical significance was defined at P equal to 0.05. Differences in sensitivities and specificities between the two ELISAs were tested by McNemar’s chi-square test for paired data (3). The sensitivities and specificities of the two ELISAs were further evaluated by receiver operating characteristics (ROC) analysis and by calculation of the area under the ROC curve (AUC) (4).
ELISA were 54.4% (95% CI, 43.5 to 65.4%) for the combination of the Dutch case and the Dutch control herds (OD = 0.2) and 63.3% (95% CI, 52.7 to 73.9%) for the combination of the Dutch case and the Swedish control herds (OD = 0.1). The sensitivities of the GP ELISA were 63.3% (95% CI, 52.7 to 73.9%) for the combination of the Dutch case and the Dutch control herds (OD = 0.5) and 39.2% (95% CI, 28.5 to 50.0%) for the combination of the Dutch case and the Swedish control herds (OD = 0.8). The sensitivity of the LPS ELISA was higher than that of the GP ELISA for samples from the Swedish control herds when the specificity was at least 98%. No difference between the sensitivities of the two ELISAs was found when samples from Dutch control herds were used. OD values of 0.2 (LPS ELISA) and 0.5 (GP ELISA) were chosen as optimal cutoff values for further evaluation of the combination of the ELISAs and of the relation between the results of the ELISAs with bulk milk and the serology of lactating animals. The chosen cutoff values resulted in sensitivities of 54.4 and 63.3% for the LPS ELISA and the GP ELISA with bulk milk, respectively.

Combination of LPS and GP ELISA. The association between the results of the LPS and GP ELISAs with bulk milk is presented for 79 case herds (Fig. 2A) and 125 Dutch control herds (Fig. 2B). The correlation coefficient of the two ELISAs with bulk milk from case herds was 0.94 (P < 0.001). With the cutoff values of the LPS ELISA (OD = 0.2) and the GP ELISA (OD = 0.5) that had been determined, samples from two herds tested positive by each ELISA but not by the two ELISAs in combination (Fig. 2B). The specificity of the combination of the two tests was 96.8% (95% CI, 93.7 to 100%) when samples were tested in parallel. All samples were negative when serial testing was applied (specificity, 100%).

Association between antibodies in bulk milk and serum of lactating cows. The association between OD values for bulk milk and within-herd seroprevalence (percentage of seropositive lactating cows) for 61 herds is presented in Fig. 3A (LPS ELISA) and Fig. 3B (GP ELISA). The correlation coefficients for the bulk milk OD value and the percentage of seropositive lactating cows were 0.72 and 0.84 for the LPS ELISA and the GP ELISA, respectively. The variance in the OD value for bulk milk samples could be explained for 51% (LPS ELISA) and 72% (GP ELISA) by the percentage of seropositive lactating cows (Table 2). The mean proportions of seropositive lactating cows per herd were 15.0% (range, 0 to 55.6%; 95% CI, 11.7 to 18.4%) for the LPS ELISA and 13.7% (range, 0 to 52.8%; 95% CI, 11.0 to 16.3%) for the GP ELISA. The mean proportions of seropositive lactating cows per herd were 19.5% (95% CI, 14.8 to 24.2%) and 9.7% (95% CI, 5.3% to 13.5%) for herds that tested positive and negative by the LPS ELISA with bulk milk, respectively. The mean proportions of seropositive lactating cows per herd were 16.0% (95% CI, 12.3 to 19.8%) and 9.7% (95% CI, 6.8 to 12.5%) for herds that tested positive and negative by the GP ELISA with bulk milk, respectively. Bulk milk samples tested negative by both ELISAs more often when the percentage of seropositive cows was lower than 5% than when the seroprevalence was higher (for the LPS ELISA, chi-square = 8.09 and P = 0.004; for the GP ELISA, chi-square = 3.84 and P = 0.05) (Fig. 4).

The combination of the percentage of seropositive lactating cows and the percentage of milk produced by these cows explained 54% (LPS ELISA) and 72% (GP ELISA) of the variations in the OD values for bulk milk samples. The mean level of milk production per herd was 25.1 kg/cow (standard deviation, 3.7 kg/cow; range, 14.8 to 33.6 kg/cow). Seropositive cows detected by the LPS ELISA and the GP ELISA produced 2.8

| Cutoff value (OD) | LPS ELISA | GP ELISA |
|------------------|-----------|----------|
|                  | Serovar Dublin-infected herds | Control herds, The Netherlands | Control herds, Sweden | Serovar Dublin-infected herds | Control herds, The Netherlands | Control herds, Sweden |
| 0.1              | 54 | 68.4 | 11 | 8.8 | 0 | 0 | 76 | 96.2 | 55 | 44.0 | 115 | 57.5 |
| 0.2              | 43 | 54.4 | 2 | 1.6 | 0 | 0 | 66 | 83.5 | 17 | 13.6 | 59 | 29.5 |
| 0.3              | 35 | 44.3 | 2 | 1.6 | 0 | 0 | 62 | 78.5 | 10 | 8.0 | 32 | 16.0 |
| 0.4              | 30 | 38.0 | 2 | 1.6 | 0 | 0 | 59 | 74.7 | 5 | 4.0 | 20 | 10.0 |
| 0.5              | 28 | 35.4 | 1 | 0.8 | 0 | 0 | 50 | 63.3 | 2 | 1.6 | 11 | 5.5 |
| 0.6              | 27 | 34.2 | 0 | 0 | 0 | 0 | 47 | 59.5 | 1 | 0.8 | 7 | 3.5 |
| 0.7              | 22 | 27.8 | 0 | 0 | 0 | 0 | 40 | 50.6 | 1 | 0.8 | 7 | 3.5 |
| 0.8              | 17 | 21.5 | 0 | 0 | 0 | 0 | 31 | 39.2 | 0 | 0 | 4 | 2.0 |
| 0.9              | 14 | 17.7 | 0 | 0 | 0 | 0 | 29 | 36.7 | 0 | 0 | 3 | 1.5 |
| 1.0              | 13 | 16.5 | 0 | 0 | 0 | 0 | 22 | 27.8 | 0 | 0 | 2 | 1.0 |
| 1.1              | 12 | 15.2 | 0 | 0 | 0 | 0 | 20 | 25.3 | 0 | 0 | 1 | 0.5 |
| 1.2              | 10 | 12.7 | 0 | 0 | 0 | 0 | 16 | 20.3 | 0 | 0 | 0 | 0 |
and 3.5% less milk than seronegative cows of the same herds, respectively.

The combination of the percentage of seropositive lactating cows in the herd and the mean log_{10} serum antibody titer of that herd explained 62% (LPS ELISA) and 75% (GP ELISA) of the variations in the OD values for bulk milk samples. For all seropositive animals, the proportions of animals with serum antibody titers higher than the cutoff value (1:100) were 19.7 and 22.3% for the LPS ELISA and the GP ELISA, respectively. Bulk milk samples tested positive by the LPS ELISA more often when one or more lactating animals in the herd had an antibody titer of 200 or higher (chi-square = 13.46 and P < 0.001). For the GP ELISA, this association tended to be significant (chi-square = 2.92 and P = 0.087).

**DISCUSSION**

The sensitivities of the LPS ELISA and the GP ELISA were 54 and 63%, respectively. Both values are lower than those reported by Hoorfar et al. (7), who found a sensitivity of 100% and a specificity of 95% with 10 case herds and 20 control herds. Wedderkopp (27) also reported a higher sensitivity of 88% and a specificity of 89%. The relatively low sensitivities of the ELISAs used in our study may be the consequence of the selection criteria used for the case herds, the time of sampling, and the cutoff values chosen for the tests. The severities of clinical symptoms and the ages of the diseased animals were not included as selection criteria. The only selection criterion for the case herds in this study was a single bacteriological culture positive for serovar Dublin, irrespective of whether the positive sample came from a lactating animal or a nonlactating animal. Selection of only those herds with lactating cows in which clinical symptoms occurred would certainly influence...
the test results (26a). Sampling of blood and bulk milk sampling occurred 2 to 4 months after the first serovar Dublin-positive sample was detected. It is possible that no antibodies were detected because antibody titers can decrease rapidly in seropositive cows (21, 26) or because seropositive cows were culled shortly after infection. The rate of detection of infected herds therefore can possibly be increased by more frequent sampling (28). The cutoff values of the LPS ELISA and GP ELISA for bulk milk samples were determined with a specificity of at least 98%. This specificity is consistent with the common practice of defining the cutoff value as the mean plus 2 or 3 standard deviations for the negative control group (18). A disadvantage of this approach is that it assumes that the results for the negative control group have a normal distribution, whereas the results for the control herds in our study were not normally distributed. Another disadvantage is that the test results for the case herds are neglected. Selection of the cutoff value by using the DPR has the advantage that it combines the test results for the control and case herds. A disadvantage of this approach is that it aims for the highest value, irrespective of the purpose of the test and of the consequence of false-positive and false-negative reactions. In control programs that emphasize the certification of herds as being free of serovar Dublin, a higher cutoff value can be chosen to increase the specificity, resulting in fewer false-positive reactions. In control programs that emphasize the identification of infected herds, a lower cutoff value can be chosen to increase the sensitivity, resulting in fewer false-negative reactions.

At the same specificity, the cutoff value of the GP ELISA was higher (OD = 0.5) than that of the LPS ELISA (OD = 0.2). The higher cutoff value of the GP ELISA is probably due to the presence of flagellar epitopes common to other Salmonella serovars or other bacteria. The unexpected finding that samples from the Swedish control herds tested positive by the GP ELISA is in line with this assumption. Clinical infections with serovar Dublin are unknown in the part of Sweden where the samples were obtained. These observations support the choice of the LPS ELISA for screening of bulk milk for serovar Dublin.

The LPS ELISA used in the study cannot discriminate between infections with serovar Dublin and serovar Typhimurium (8). It has become possible to distinguish between the two serovars in serum by making use of monoclonal antibodies (6) or modification of the LPS antigen (11). Further research is needed to evaluate the possibility of a test based on the flagellar antigen (GP ELISA) (25, 26) to discriminate between the two serovars in cattle.

The OD values of the two ELISAs for bulk milk were evaluated in relation to individual titers in serum rather than individual titers in milk. The reason for this is that earlier studies found unexpectedly high titers in milk during the first 14 days after parturition (20) and more false-positive reactions in control herds (7). In addition, in control programs for serovar Dublin it is expected that serum will be used more frequently than milk for the detection of carrier animals because carriers are not always lactating. Further research is needed to evaluate the use of ELISAs with bulk milk in relation to the presence of carrier animals.

The variation in the OD values of the two ELISAs with bulk milk could best be explained by the percentage of seropositive cows and the mean log_{10} serum antibody titer for the seropositive animals in the herd. Control programs for Salmonella are especially important if carriers of Salmonella are present. The observation that the LPS ELISA with bulk milk detected positive samples more often when one or more lactating cows had a serum antibody titer higher than the cutoff value is therefore interesting because carriers of serovar Dublin often have a high serum antibody titer (26). Introduction of the percentage of milk produced by seropositive cows did not further increase the percentage of the variance in the OD value that was explained. This may be because the overall difference in the volume of milk produced between seropositive and seronegative cows was small.

This study underlines the potential use of ELISAs with bulk milk for distinguishing between serovar Dublin-infected and serovar Dublin-uninfected herds. The actual use of such ELISAs in a control program depends on the test characteristics of the ELISAs in combination with the true prevalence of

**FIG. 3. Association between percentage of seropositive lactating cows and the OD value for bulk milk samples for 61 Dutch serovar Dublin-infected herds determined by LPS ELISA (A) and GP ELISA (B). A simple regression line is included.**
the infection in a country or region. In The Netherlands, serovar Dublin is estimated to be present in 5 to 10% of the dairy herds (unpublished observations). At this prevalence, the number of false-positive reactions will be relatively high compared with the number of true-positive reactions if the specificity of the test or combination of tests is lower than 100%. We found a specificity of 98.4% for both ELISAs for the Dutch control herds with the cutoff values that had been determined. Use of a combination of the two ELISAs in our study increased the specificity to 100% without a significant decrease in sensitivity, although the number of samples tested was small. With samples from the Swedish control herds, the specificity of the LPS ELISA was 100% and that of the GP ELISA was 94.5%. Use of a combination of the two tests would give a specificity of 100%. It is possible to increase the rate of detection of serovar Dublin-infected herds by using an ELISA with bulk milk in combination with serology for young calves (26a).

In conclusion, an LPS ELISA can best be used to screen bulk milk samples for serovar Dublin because it has a better specificity than the GP ELISA. Use of a combination of the LPS and GP ELISAs can be advantageous to further increase specificity. The OD values for bulk milk samples can best be predicted by the percentage of seropositive cows and the mean log_{10} serum antibody titer for the herd. The amount of milk produced by seropositive cows had no additional effect on the variation in the OD values of the two ELISAs with bulk milk.

### TABLE 2. Univariate linear regression analysis of OD values for bulk milk and serological status of lactating animals in serovar Dublin-infected herds (n = 61)

| ELISA and parameter | LPS ELISA with bulk milk (OD) | GP ELISA with bulk milk (OD) |
|---------------------|-------------------------------|-------------------------------|
|                     | β          | SD (β)   | R²     | β          | SD (β)   | R²     |

- LPS ELISA serum
  - Seropositive cows (%)
    - 0.030 0.004 0.51
  - Cows with positive milk (%)
    - 0.031 0.004 0.53
  - Mean log_{10} serum antibody titer
    - 1.447 0.173 0.54

- GP ELISA serum
  - Seropositive cows (%)
    - 0.038 0.004 0.60
  - Cows with positive milk (%)
    - 0.039 0.004 0.61
  - Mean log_{10} serum antibody titer
    - 1.794 0.181 0.62

- Milk production of all seropositive cows in the herd (in kilograms) divided by total milk volume of the herd (in kilograms).
- Sum of log_{10} serum antibody titer for all seropositive lactating cows in the herd divided by number of lactating cows in the herd.

*a* All univariate linear regression models were significant at \( P < 0.0001 \).

*b* Sum of log_{10} serum antibody titer for all seropositive lactating cows in the herd divided by number of lactating cows in the herd.

---

**FIG. 4.** Relationship between antibodies to serovar Dublin in bulk milk samples and percentage of seropositive lactating cows per herd (titer, \( \geq 1:100 \)) determined by an LPS ELISA (OD \( \geq 0.2 \)) or GP ELISA (OD \( \geq 0.5 \)).
ACKNOWLEDGMENTS

This work was supported by “Diergezondheid in Beweging,” a mutual project organization of the Ministry of Agriculture, Nature Management and Fisheries (grant DIB/97858/FP).

We are indebted to A. Engvall from the National Veterinary Institute of Sweden for providing the bulk milk samples from Sweden. We thank H. J. Stel for collecting the samples and J. v. d. Schans for coordinating the data collection. We also thank J. Verhoef for suggestions regarding the manuscript.

REFERENCES

1. Dom, P. P., P. Haesebroeck, R. Vandermeersch, J. Descamps, and K. Van Omneslaeghe. 1991. Prevalence of Leptospira interrogans serovar hardjo antibodies in milk in Belgian dairy herds. Vet. Q. 13:118–120.
2. Elvander, M., S. Edwards, K. Naslund, and N. Linde. 1995. Evaluation and application of an indirect ELISA for the detection of antibodies to bovine respiratory syncytial virus in milk, bulk milk, and serum. J. Vet. Diagn. Invest. 7:177–182.
3. Fleiss, J. L. 1981. Statistical methods for rates and proportions. John Wiley & Sons, Inc., New York, N.Y.
4. Frankena, K., J. P. T. M. Noordhuizen, J. P. Willeberg, P. F. Voorthuysen, and J. O. Goelma. 1998. EPISCOPE: computer programs in veterinary epidemiology. Vet. Rec. 126:573–576.
5. Grove, T. M., and G. M. Jones. 1992. Use of an enzyme-linked immunosorbent assay to monitor the control of Staphylococcus aureus mastitis. J. Dairy Sci. 75:423–434.
6. Hoorfar, J., N. C. Feld, A. L. Schirmer, V. Bitsch, and P. Lind. 1994. Serodiagnosis of Salmonella dublin infection in Danish dairy herds using O-antigen based ELISA. Can. J. Vet. Res. 58:268–274.
7. Hoorfar, J., P. Lind, and V. Bitsch. 1995. Evaluation of an O antigen enzyme-linked immunosorbent assay for screening of milk samples for Salmonella dublin infection in dairy herds. Can. J. Vet. Res. 59:142–148.
8. Hoorfar, J., and A. Wedderkopp. 1995. Enzyme-linked immunosorbent assay for screening of milk samples for Salmonella typhimurium in dairy herds. Am. J. Vet. Res. 56:1548–1554.
9. Jensen, A. L., and J. S. D. Poulsen. 1992. Evaluation of diagnostic tests using relative operating characteristic (ROC) curves and the differential positive rate. An example using the total serum bile acid concentration and the alanine aminotransferase activity in the diagnosis of canine hepatobiliary diseases. J. Vet. Med. 39:656–668.
10. Kloosterman, A., H. W. Ploeger, E. J. Pieke, T. J. G. M. Lam, and J. Verhoef. 1996. The value of bulk milk ELISA Ostertagia antibody titers as indicators of milk production response to anthelmintic treatment in the dry period. Vet. Parasitol. 64:197–205.
11. Konrad, H., B. P. Smith, G. W. Dilling, and J. K. House. 1994. Production of Salmonella serogroup D (O9)-specific enzyme-linked immunosorbent assay antigen. Am. J. Vet. Res. 55:1647–1651.
12. Martin, S. W., A. H. Meek, and P. Willeberg. 1987. Veterinary epidemiology: principles and methods. Iowa State University Press, Ames.
13. Nielsen, M., F. C. M. Jansen, L. A. van Wuijkhuise, and A. A. Dijkhuizen. 1996. Dutch cattle identification and registration (I&R) system: analysis of its use for controlling an outbreak of foot and mouth disease. Tijdschr. Diergeneesk. 121:576–581.
14. Nielsen, S. S., S. M. Thamsborg, H. Houe, and V. Bitsch. 2000. Bulk-tank milk ELISA antibodies for estimating the prevalence of paratuberculosis in Danish dairy herds. Prev. Vet. Med. 44:1–7.
15. Niskanen, R. 1993. Relationship between the levels of antibodies to bovine viral diarrhea virus in bulk tank milk and the prevalence of cows exposed to the virus. Vet. Rec. 133:341–344.
16. Palha, G. A., L. E. Green, G. Lloyd, D. Patel, and K. L. Morgan. 1999. Prevalence of antibodies to Coxiella burnetii (Q fever) in bulk tank milk in England and Wales. Vet. Rec. 144:519–522.
17. Popoff, M. Y., and L. Le Minor. 1992. Antigenic formulas of the Salmonella serovars. Report of the W. H. O. Collaborating Center for Reference and Research on Salmonella. Institute Pasteur, Paris, France.
18. Richardson, D. M., A. Turner, D. W. Warnock, and P. A. Llewellyn. 1983. Computer-assisted rapid enzyme-linked immunosorbent assay (ELISA) in the serological diagnosis of aspergillosis. J. Immunol. Methods 56:201–207.
19. Sargeant, J. M., D. F. Kelton, S. W. Martin, and D. E. Mann. 1997. Evaluation of a bulk-milk ELISA test for the classification of herd-level bovine leukemia virus status. Prev. Vet. Med. 34:223–230.
20. Smith, B. P., D. G. Oliver, P. Singh, P. A. Marvin, B. P. Ram, L. S. Jang, N. Sarkiv, J. S. Orskoborn, and K. Jackson. 1989. Detection of Salmonella dublin mammary gland infection in carrier cows, using an enzyme-linked immunosorbent assay for antibody in milk or serum. Am. J. Vet. Res. 50:1352–1360.
21. Spier, S. J., B. P. Smith, J. W. Cullor, J. S. Dilling, and L. D. Pfaff. 1989. Use of ELISA for detection of immunoglobulins G and M that recognize Salmonella dublin lipopolysaccharide for prediction of carrier status in cattle. Am. J. Vet. Res. 51:1900–1904.
22. Toen, C. O., C. A. Haas, R. D. Angus, and A. S. Townsend. 1995. Evaluation of a potassium chloride extract of Brucella abortus in an ELISA for detecting Brucella antibodies in bulk tank samples from cows. Vet. Microbiol. 45:185–189.
23. Traven, M., L. Bjornerot, and B. Larsson. 1999. Nationwide survey of antibodies to bovine coronavirus in bulk milk from Swedish dairy herds. Vet. Rec. 144:527–529.
24. van Wuijkhuise, L., J. Bosch, P. Franken, K. Frankena, and A. R. Elbers. 1998. Epidemiological characteristics of bovine herpesvirus 1 infections determined by bulk milk testing of all Dutch dairy herds. Vet. Rec. 142:181–184.
25. van Zijperveld, F. G., A. M. van Zijperveld-van Bemmel, and J. Anakotta. 1992. Comparison of four different enzyme-linked immunosorbent assays for serological diagnosis of Salmonella enteritidis infection in experimentally infected chickens. J. Clin. Microbiol. 30:2560–2566.
26. Veling, J., F. C. van Zijperveld, A. M. van Zijperveld-van Bemmel, H. W. Barkema, and Y. H. Schukken. 2000. Evaluation of three newly developed enzyme-linked immunosorbent assays and two agglutination tests for detecting Salmonella enterica subs. enterica serovar Dublin infections in dairy cattle. J. Clin. Microbiol. 38:4402–4407.
27. Visser, S. C., J. Veling, A. A. Dijkhuizen, and R. M. Huirne. 1997. Economic losses due to Salmonella dublin infection in dairy cattle. P. 143–151. In Symp. Animal Health Mgmt. Econ. Copenhaegen, Denmark.
28. Wedderkopp, A. 2000. Application of serological assay for screening of Salmonella Dublin infection in dairy herds. Ph.D. thesis. Copenhagen University, Copenhagen, Denmark.