Analysis of Possible Factors Affecting the Specificity of the Gamma Interferon Test in Tuberculosis-Free Cattle Herds

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Bovine tuberculosis (TB) is still a zoonotic problem in the world. Despite the fact that eradication programs for bovine TB are being implemented in many countries, it remains a public health problem. These programs are mainly based on a single intradermal tuberculin test using bovine tuberculin purified protein derivative (PPD), isolation, and slaughtering of infected animals. The aim of this study was to assess the specificity of the gamma interferon (IFN-γ) test in TB-free cattle herds, by using not only Australian tuberculins but also tuberculins produced at our institute, and to correlate the response with the type of production (beef cattle, dairy cattle, and a dual-purpose breed), the housing system, and the age of the animals. We studied 800 animals selected from 20 TB- and paratuberculosis-free herds. The animals were tested in parallel, after stimulation with Australian tuberculins and tuberculins produced at our institute, by using the skin test and two IFN-γ assays. The results of this trial showed that the specificity of the IFN-γ test is higher than that of the skin test (96.8%) and ranges from 97.3% (using only Australian tuberculins) to 98.6% (using tuberculins produced at our institute). We found that different categories of cattle could influence the specificity of the skin test but that these differences tended to be reduced in the IFN-γ assay, especially when Italian PPDs were used.

Materials and Methods

Animals. Eight hundred cows were selected from 20 officially certified TB-free herds in the Region of Umbria (Italy), into which no cows from other herds had been introduced in the previous 2 years. The animals were found to be free of paratuberculosis, as confirmed by serological tests and by the absence of clinical signs over the years. The animals were tested in parallel by use of a skin test and two IFN-γ assays, one with Australian PPDs and one with PPDs produced at our institute.

Experimental design. All the animals involved in the trial were divided according to the type of production (beef cattle, dairy cattle, or dual-purpose breed), the housing system, and age. They were analyzed to ascertain their TB infections there may be no clinical signs of the disease. The specificity of clinical signs is low, and in less-advanced infections there may be no clinical signs of the disease. Eradication programs for bovine TB are being implemented in many countries; these are mainly based on the single intradermal tuberculin test using bovine tuberculin purified protein derivative (PPD). If the strategy that has allowed the disease to be kept under control in Italy is based on the use of the skin test (5), isolation, and slaughtering of infected animals. The prevalence of the disease in Italy has decreased to less than 1% (2). In order to eliminate the disease, further diagnostic tests are needed, to be used in vivo together with the tuberculin skin test in order to increase sensitivity and specificity (4, 15). Indeed, in some geographical areas where the disease is becoming relatively scarce, exposure to environmental mycobacteria (such as those in bird feces and natural vehicles of infection) could induce a nonspecific reaction to the TB skin test and hinder the eradication of TB (31).

Many studies of serological assays that detect circulating antibodies to Mycobacterium bovis have been carried out. None have shown adequate sensitivity or specificity (10, 16, 17, 21, 23, 25). Their failure can be attributed to the nature of the immunological response generated in M. bovis-infected cattle: it has been shown that most infected cattle have an effective cell-mediated immune response (CMI), with minor involvement of the humoral immune response (20). CMI has been widely recognized as the main factor involved in the containment of the infection (3, 26). According to these findings, diagnosis of TB should also be based on the evaluation of CMI. A promising enzyme-linked immunosorbent assay (ELISA), which may be employed as a useful ancillary test, has been proposed lately (1). The gamma interferon (IFN-γ) test has turned out to be the best, because it measures the in vitro proliferation of stimulated T cells from M. bovis-infected animals on the basis of production of the cytokine IFN-γ (29), which is predominantly released by T cells after antigenic stimulation. IFN-γ is thought to be involved in immunity to mycobacterial infections and is released in vitro in quantities that are readily measurable by enzyme immunoassay (32). Indeed, the IFN-γ assay, as other authors have already demonstrated (15, 28, 31), has proved extremely useful as an in vitro supportive test for the skin test, because it has high sensitivity and specificity (8, 24, 30). For that reason, the IFN-γ test has been used in Northern Italy and has made a valuable contribution to the eradication of TB in cattle (2). In order to better assess the efficacy of the IFN-γ test, we carried out a trial using animals from TB-free herds, considering different parameters which could modify the test’s specificity.
TABLE 1. Results of the skin test and IFN-γ assays

| Test | No. of animals testing | M. bovis negative | M. bovis false positive | Specificity (LC; CI) |
|------|------------------------|-------------------|-------------------------|---------------------|
| Skin test | 775 | 25 a | 96.8 (95; 35–97.93) |
| IFN-γ assay | | | | |
| With Australian antigen | 779 | 21 ab | 97.34 (95; 95.9–98.3) |
| With Italian antigen | 789 | 11 b | 98.6 (95; 97.5–99.3) |

a A total of 800 animals were tested.
b Categories were compared; values with different letters are statistically different from each other (P < 0.05).
c Specificity, LC, and CI are expressed as percentages.

status by using the skin and IFN-γ tests as described below. The specificity of the diagnostic test was evaluated for each group.

Tuberculin skin test. A single comparative intradermal skin test was performed by using bovine (50,000 IU/ml) and avian (25,000 IU/ml) tuberculins produced at our licensed laboratory. Italian bovine tuberculins are produced only at our authorized laboratory (Istituto Zooprofilattico Umbria e Marche) and another laboratory (Istituto Zooprofilattico Abruzzo e Molise). The avian tuberculin is produced only at our authorized laboratory. We sell the tuberculins only to Veterinary Health Units. The same Ministerial protocol for the production of tuberculins is followed at both institutes. Ministerial authorities oversee the process and ensure standardization of the products at both institutes according to the criteria reported in the manual of the Office International des Epizooties (18).

The potency of our tuberculins (50,000 IU/ml) is estimated by comparison, in previously sensitized guinea pigs, with bovine and avian PPDs prepared according to the Weybridge Reference Standard.

The skin test involves intradermal injection of the tuberculins, and the interpretation is based on observation and the recorded increase in skin thickness. In the single intradermal test (a single injection of bovine tuberculin), the animals were considered negative if only limited swelling was observed, with an increase of no more than 2 mm in skin thickness, and with no clinical signs.

The animals were considered dubious if no clinical signs were observed or if the increase in skin thickness was more than 2 mm and less than 4 mm.

The animals were considered positive if clinical signs were observed or if there was an increase of 4 mm or more in skin thickness.

Animals that were positive by the single intradermal test were subjected to the comparative intradermal test (injections of bovine and avian tuberculins).

In the interpretation of this kind of test, animals were considered to be positive if the bovine reaction was positive and more than 4 mm greater than the avian reaction. Animals were considered dubious if the bovine reaction was positive and 1 to 4 mm greater than the avian reaction.

The animals were considered negative if the bovine reaction was negative or if the bovine reaction was positive but equal to or less than a positive avian reaction. Animals that tested bovine tuberculin positive by the single intradermal test, dubious by the comparative intradermal test, and negative by tests carried out after slaughtering were considered false positive.

IFN-γ test. A heparinized blood sample (10 ml) was collected from each animal just before the tuberculin skin test was carried out. The samples were delivered to the lab within 8 to 10 h of blood collection. Each sample (1.5 ml) was divided into several aliquots, and each aliquot was stimulated, not only with 30 μg of Australian bovine PPD and 30 μg of Australian avian PPD (CSL Ltd., Melbourne, Australia), as suggested in the manual for the Bovigam ELISA (CSL Ltd.), but also with 10 μg of bovine PPD and 10 μg of avian PPD produced at our institute by the method described previously (6, 12). The concentrations of the Australian and Italian tuberculins were determined on the basis of the results obtained by trials described previously (11, 25).

Italian researchers have demonstrated that good IFN-γ production is obtained, in blood samples of skin test-positive animals, by using a low concentration (10 μg/ml) of Italian PPDs.

A solution of phosphate-buffered saline (PBS) (0.01 M; pH 7.2) was used to estimate the basal value of IFN-γ. Blood samples were incubated at 37°C in a humidified atmosphere (5% CO₂) for 16 to 24 h. After incubation, the plasma of each sample was collected by centrifuging the blood cultures at 500 × g for 10 min at room temperature (22 ± 5°C); this plasma was tested with the Bovigam ELISA (CSL Ltd.) by following the instructions included in the kit. The optical density (OD) of the results was measured with a spectrophotometer (450 nm). Because the basal OD of the animals tested in Italy was higher than 0.05 (7), in order to obtain a superior cutoff (29), we have modified the Australian method of identifying bovine TB-positive animals (B > N + 0.05 and B ≥ A; where B is M. bovis positive, N is negative, 0.05 is the cutoff OD, and A is Mycobacterium avium positive) according to the following criteria described by other authors (6, 7, 11). (i) Samples were considered unfit if the basal OD was higher than 0.150. (ii) Samples were considered negative when the ODs of samples stimulated with avian PPD and bovine PPD were less than twice the OD obtained for PBS-stimulated (negative) cells. (iii) Samples were considered M. bovis positive when the ODs of samples stimulated with bovine PPD were both more than or equal to twice the OD obtained for PBS-stimulated cells and the ODs of samples stimulated with avian PPD were less than or equal to twice the OD obtained for PBS-stimulated cells. (iv) Samples were considered M. avium positive when the ODs of samples stimulated with avian PPD were more than or equal to twice the OD obtained for PBS-stimulated cells and the ratio between the ODs of bovine PPD- and avian PPD-stimulated cells was higher than 1.1. (v) Samples were considered M. avium positive when the ODs of samples stimulated with avian PPD and samples stimulated with bovine PPD were both more than twice the OD obtained for PBS-stimulated cells, and the ratio between the ODs of bovine PPD- and avian PPD-stimulated cells was higher than 1.1. (vi) Samples were considered M. avium positive when the ODs of samples stimulated with avian PPD and samples stimulated with bovine PPD were both more than twice the OD obtained for PBS-stimulated cells, and the ratio between the ODs of bovine PPD- and avian PPD-stimulated cells was in the 0.9-to-1.1 range.

Animals testing positive for bovine TB by the IFN-γ test but negative by the tests carried out after slaughtering were considered false positive.

Statistical analysis. Specificity was measured as the percentage of negative results in the total population and was calculated by the following formula: true negatives × 100/(true negatives + false positives). The confidence limits (LC) were calculated by using the normal distribution for infinite populations. The confidence level was considered to be 95% (27). The statistical significances of several differences found in the analysis and the grouping of data were also calculated. EPI INFO 2000, freeware distributed by the Centers for Disease Control and Prevention, Atlanta, Ga., was used as an electronic support for statistical calculations.

TABLE 2. Results of IFN-γ assays according to the type of production

| Production type | No. of animals tested | M. bovis false positive | M. bovis negative | M. avium positive | M. avium negative | M. avium positive | M. avium negative |
|----------------|-----------------------|-------------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| Beef          | 550                   | 540                     | 10 a              | 5                 | 124               | 8 b               | 18                |
| Dual purpose  | 150                   | 145                     | 5 ab              | 18                | 127               | 5 b               | 18                |
| Dairy         | 100                   | 90                      | 10 b              | 21                | 69                | 3 b               | 28                |

a Categories were compared; values with different letters are statistically different from each other (P ≤ 0.05).
TABLE 3. Results of the IFN-γ assays according to the housing system

| Housing system category | No. of animals tested | Skin test | IFN-γ assay |
|-------------------------|-----------------------|-----------|-------------|
|                         |                       |           | With Australian antigen | With Italian antigen |
|                         |                       | M. bovis false positive<sup>a</sup> | M. avium positive<sup>a</sup> | M. bovis false positive<sup>a</sup> | M. avium positive<sup>a</sup> |
| Tie stall               | 350                   | 345       | 5 a         | 349         | 0 a         | 1           | 349         | 0 a         | 1           |
| Mixed housing           | 300                   | 290       | 10 ab       | 270         | 10 b        | 20          | 287         | 5 b         | 28          |
| Free housing            | 150                   | 140       | 10 b        | 116         | 11 b        | 23          | 109         | 6 b         | 35          |

<sup>a</sup> Categories were compared; numbers with different letters are statistically different (P ≤ 0.05)

RESULTS

Results of the skin test and the two IFN-γ assays. Out of 800 animals tested, 25 tested positive by the single intradermal test, 21 were found reactive to M. bovis by the IFN-γ assay using Australian PPDs, and 11 were found reactive to M. bovis by the IFN-γ assay using PPDs produced at our institute. The animals that were found to be M. bovis reactors in the IFN-γ assays were the same as those that were positive by the skin test.

Animals with positive results by these tests were slaughtered, and we carried out a postmortem examination and classical microbiological isolation, performed on their mediastinic and retropharyngeal lymph nodes. All the slaughtered animals tested negative by the postmortem exams and were considered false-positive animals (Table 1). We compared the results obtained by using different tuberculins in the IFN-γ assay, and then we compared these results with those obtained by the skin test. Statistical analysis showed that in the IFN-γ assay, the use of Australian PPDs or PPDs produced at our institute did not give statistically different results (P = 0.108; χ² = 2.58), but the difference between the IFN-γ assay using PPDs produced at our institute and the skin test was significant (P = 0.02; χ² = 4.80), while the difference between the IFN-γ assay using Australian PPDs and the skin test was not significant (P = 0.65 χ² = 0.20) (a P value of ≤0.05 was considered significant).

Results classified by the different categories of animals. In order to assess whether different categories of animals respond differently to the diagnostic tests, we divided the tested animals according to the type of production (beef cattle, dairy cattle, or dual-purpose breed), the housing system, and age.

Type of production. The results of the IFN-γ assay demonstrated that fattening farms, containing only beef cattle, showed fewer reactors to M. bovis than breeding farms containing dairy cattle (Table 2). These differences were confirmed by the P values (P < 0.0005) of the χ² test using 2 degrees of freedom. The use of Italian or Australian PPDs showed similar trends, although PPDs produced at our institute showed fewer reactors in all categories. According to the results of the skin test, it is possible to estimate that animals raised for beef production are three times less likely to show false-positive reactions than animals sampled in farms containing dairy cattle (odds ratio, 3.30; 95% confidence interval [95% CI], 1.50 to 7.24; LC, 95% [P < 0.005]).

Housing system. Another parameter utilized to evaluate different specificities was the housing system (Table 3). When results were clustered by the type of housing system, we found that with the IFN-γ assay, the tie stall system of housing showed fewer animals falsely positive to M. bovis, while more animals reared in a free-housing system or under mixed conditions reacted positively to M. bovis, especially when both our PPDs and Australian PPDs were used (P < 0.05 by the χ² test using 2 degrees of freedom).

According to the results of the skin test, the risk of animals reacting positively to M. bovis was three times higher with a free- or mixed-housing system than with a tie stall system (odds ratio, 3.11; 95% CI, 1.18 to 8.21; LC, 95% [P < 0.005]).

Age. We divided the animals into three groups according to age. When the animals were analyzed by the skin test, the statistical analysis clearly showed that animals in the 2- to 3-year age group had the highest reactivity relative to animals in the 4- to 5-year and 6- to 7-year age group (Table 4). This information was not confirmed when we used the IFN-γ assay. There were no statistically significant differences (P > 0.05 by the χ² test using 2 degrees of freedom).

DISCUSSION

The aim of the present study was to evaluate the specificity of the diagnostic tests used in the TB eradication programs. Our findings showed that the IFN-γ assay has a higher specificity than the skin test in TB-free herds. In fact, specificities

TABLE 4. Results of IFN-γ assays according to age

| Age (yr) | No. of animals tested | Skin test | IFN-γ assay |
|---------|-----------------------|-----------|-------------|
|         |                       |           | With Australian antigen | With Italian antigen |
|         |                       | M. bovis false positive<sup>a</sup> | M. avium positive<sup>a</sup> | M. bovis false positive<sup>a</sup> | M. avium positive<sup>a</sup> |
| 2-3     | 150                   | 142       | 8 ab        | 140         | 4 a         | 6           | 140         | 1 a         | 9           |
| 4-5     | 500                   | 483       | 17 a        | 471         | 15 a        | 14          | 466         | 9 a         | 25          |
| 6-7     | 150                   | 150       | 0 b         | 124         | 2 a         | 24          | 119         | 1 a         | 30          |

<sup>a</sup> Categories were compared; numbers with different letters are statistically different (P ≤ 0.05).
were 96.8% with the skin test, 97.3% with the IFN-γ assay using Australian PPDs, and 98.6% with the IFN-γ assay using PPDs produced at our institute; these values are higher than those reported by other authors (6). It is noteworthy that the Italian tuberculins had a lower concentration of PPD than the Australian tuberculins while achieving comparable results. This suggests that the biological effect of PPD does not depend on its protein content. The use of tuberculins produced at our institute had a lower concentration of PPD than those reported by other authors (6). It is noteworthy that the PPDs produced at our institute; these values are higher than the concentration of PPD in the test results. The IFN-γ assay tends to reduce these influences, especially when our PPDs are used to stimulate blood lymphocytes. We found that the number of M. bovis reactors was different when the animals were assayed with the skin test versus the IFN-γ assay. The skin test specificities recorded were lower than the specificities of the IFN-γ assay using PPD produced at our institute, as confirmed by the χ² test. These results strongly suggest that a possible way to improve diagnostic procedures, in order to better control the spread of TB, might be based on the right choice of diagnostic test, focusing particular attention on the characteristics of the PPDs. Field trials have been conducted in different countries, and the results obtained suggest that the IFN-γ assay is more specific than the skin test (32). We found that the housing system and the type of production could influence the specificity of the TB diagnostic tests. Even though our results do not throw light on the reason for this, we can suppose that these effects result from exposure to environmental mycobacteria, which can sensitize the animals. Indeed, other authors (9, 14, 15, 22) have advanced the hypothesis that the contamination of feed with environmental mycobacteria, present in bird feces, and possible contact with other natural vehicles of infection, could induce a nonspecific reaction in the TB skin test. With regard to age, we noted that the risk of sensitization is higher for cows 2 to 3 and 4 to 5 years old than for cows 6 to 7 years old.

The reason for this, as other authors have reported (15), is the complex antigenic structure of bovine tuberculin, some particular components of which could induce an immunosuppressive response after repeated intradermal injections. This hypothesis has also been suggested by authors who found a similar phenomenon when using tuberculin on humans (3). On the basis of the results obtained from our research, especially regarding the high level of specificity (98.6%) achieved with the IFN-γ assay using the tuberculin produced at our institute, we can conclude that the IFN-γ assay would be a valid diagnostic aid, together with the skin test, in the final phases of eradication programs.

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