Comparison of serological and cultural detection of mycobacterial

infections in bovine, caprine, ovine, and porcine species

by

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## LITERATURE REVIEW

### Introduction and Review of the Disease

Paratuberculosis is an insidious mycobacterial disease of the intestinal mucosa caused by <u>Mycobacterium paratuberculosis</u>. It is characterized by chronic wasting, emaciation, alopecia, and diarrhea. The clinical manifestations are known as Johne's disease (6) and, in cattle, were described first by Jöhne and Frothingham in 1895 (52). Twort and Ingram were the first to isolate and characterize the causal organism, <u>M</u>. <u>paratuberculosis</u>, in 1910. They succeeded in isolation by employing an egg medium containing glycerin and killed tubercle bacilli (139, 140). <u>M</u>. <u>paratuberculosis</u> most commonly causes disease in ruminants; however, it has been isolated from pigs (115, 120), monkeys (110), horses, and other mammals (41, 70, 115). Clinical disease usually does not occur in nonruminants.

### Clinical Manifestations and Histopathology

The clinical manifestations in cattle include emaciation, development of rough, off-colored hair coat (70) or sometimes patches of alopecia, intermittent diarrhea, abortions, mastitis, and infertility (87, 88). In goats, a soft, pasty stool, decreased milk production and chronic wasting have been reported (7, 72, 97, 100). Stockman provided a detailed description of the disease in sheep in 1911 (127). He characterized the mucosa as being coated with a creamy mucus,

but no corrugations as seen in cattle. Scrappings of the mucosa yielded enormous numbers of acid-fast bacilli, mostly arranged in clumps and enclosed in macrophages. This macrophagic enclosure is a classic characteristic of paratuberculosis.

In goats, as in cattle, a similar description was made in 1982 by Morin (97). Clinical and pathological findings from that study paralleled what has been reported from previous studies for goats (7, 28, 31, 40, 70, 72, 74, 80, 97, 100, 121, 144) and sheep (28, 41, 42, 63, 64, 70, 83, 91, 127, 146). He stated, "Gross lesions in the intestinal tract were limited to mild thickening and corrugations of mucosa of lower small intestine, cecum and colon...." Various degrees of thickening and marked corrugations of the mucosa of the small intestine were found in about 40% of 273 infected goats examined in Norway (31). That marked thickening and corrugations of the mucosa of the small intestine was found to be more common in typical cases of Johne's disease in cattle (14, 60); however, caseation necrosis in the mesenteric lymph nodes has been reported once in this species (14). Morin further reported caseation necrosis of Peyer's patches and ulcerations in two of the goats studied and small tuberculoid nodules in the liver of another animal.

M'Fadyean, in 1918, was the first to write a comprehensive description of the histology of paratuberculosis (95). He reported that the infection began and ended with the invasion of the intestines and

associated groups of lymph nodes. He stated, "In tuberculosis the lesion exhibits a power of progressive growth which enables them in many cases to attain to a very large size and almost without exception they soon undergo necrotic and degenerative changes which entail striking alterations in their naked-eye appearance. In Johne's disease, the new tissue which is formed in the parts invaded by the bacilli is very moderate or absolutely meagre in amount, and, at least in the bovine species, the lesions never show to the naked eye any evidence of caseous or calcareous degeneration. Finally, the nodular character of the lesions which is so common in tuberculosis is never seen in Johne's disease."

Although pigs rarely contract infection with M. paratuberculosis (115, 120), they very frequently become infected with organisms of the Mycobacterium avium complex (12, 21, 25, 29, 30, 31, 51, 55, 77, 114, 124, 125, 127, 132, 137, 150).

Merkal et al., in 1970 (88), offered an immunologic explanation for the illness. That explanation implied that the diarrhea, lacrimation, rales, and some hematologic changes result from antigen-antibody reactions in infected intestinal tissue, with concomitant release of histamine by degranulation of mast cells. The fever, prolonged leukopenia and anemia were thought to be delayed hyper-sensitivity reactions.

In that study, desensitization of clinically ill cattle with intra-venously administered johnin and treatment with methotrexate,

antihistamines, and histidine decarboxylase inhibitor usually were
followed by reduction or cessation of diarrhea. The febrile responses
of paratuberculous cattle were eliminated by desensitization or by
methotrexate treatment, but were not affected by treatment with
antihistimines or histidine decarboxylase inhibitor. Other workers
(14) have found degranulation of mast cells to be common in
paratuberculous cattle with diarrhea, also implying that vasoactive
mediators such as histamine are involved in the diarrhea. Antigen-
antibody deposits have been implicated in the glomerular alteration
associated with the proteinuria sometimes present in paratuberculous
animals (99).

Additionally, Buergelt and Duncan (13) reported that milk
production was reduced in cattle infected with M. paratuberculosis.
They also noted with infected cattle lower fertility rates, premature
parturitions, and abortions.

### Economic Effects

In the 1960s, British surveys (71) determined paratuberculosis to
be the most economically important infectious disease of cattle in
England. In the United States, it occurs in all areas that have large
concentrations of cattle (65). It is very important also in dairy
goat herds and in sheep raising areas.

More recently, several surveys employing cultural isolation of M.
paratuberculosis from mesenteric nodes of cull cattle have been

conducted in the United States. Bement and Sanderson (8), in 1974, reported an incidence of about 3% in cull cattle sent to slaughter in California, and Riemann et al. (117) reported an incidence of up to 9% in some areas of California in 1983. Hurley and Arnoldi (50) reported a rate of 11% from cattle sent to slaughter in Wisconsin in 1982, and McPherron et al. (81) estimated that about 12% of Illinois cattle were infected, representing about one-third of the herds in the state. Chiodini (19), culturing from numerous mesenteric lymph nodes and intestinal specimens, reported an incidence of 18% in a limited study of New England cull cattle in 1982. It is believed that perhaps as many as 20% of the cattle in the United States are infected (19).

<div align="center">

Past Serologic and Allergic Tests to Detect

Paratuberculous Animals

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Many tests have been employed in attempts to identify apparently healthy paratuberculous animals. These include assays of cell-mediated immunity such as intradermal skin tests (9, 45, 66, 68, 91, 107, 123, 138, 148), lymphocyte transformation tests (3, 15, 16, 23, 53, 148), and macrophage migration inhibition tests (1, 4, 9, 51, 148), and the tests of humoral immunity such as complement fixation tests (9, 24, 36, 38, 40, 69, 80, 112, 142, 148), hemagglutination tests (38, 40, 67, 83, 92, 113, 148), agar gel immunodiffusion tests (AGID) (9, 38, 40, 73, 75, 83, 92, 121, 122, 148), immunoelectrophoresis (IE) (75, 98), immunoperoxidase (101), fluorescent antibody tests (2, 33,

34, 35, 37, 38, 39, 40, 148), radioimmunoassay (149), and enzyme linked immunosorbent assay (ELISA) (59, 75, 113, 117, 151, 152).

All of these tests had been found to be unsatisfactory as field tests for the identification of subclinically infected animals, due to a high incidence of false-positive and false-negative results (9, 15, 16, 23, 34, 35, 37, 38, 40, 44, 49, 53, 56, 59, 75, 80, 85, 87, 92, 112, 121, 122, 135, 148, 149, 151, 152). Recently, however, increased sensitivity in using agar gel immunodiffusion as the primary diagnostic test for M. paratuberculosis infection in cattle was reported (121, 122).

Precipitin type tests, AGID, IE, and counterimmunoelectrophoresis (CIE) (75, 85, 98, 102, 103, 104, 121, 122), though usually insensitive in the earlier stages of the disease due to the animal's low production of antibodies, frequently remain positive until the death of the animal (83). With methods currently in use, precipitin type tests are far more sensitive in animals such as goats and sheep than in cattle (75, 121, 122). Fluorescent antibody tests, which detect the same surface antigens as the principal precipitin tests, have been reported to be more useful in cattle, however, than precipitin tests because they are more sensitive (2, 33, 34, 35, 37, 38, 39, 40).

The surface antigens detectable by gel diffusion precipitin and fluorescent antibody tests include some shared determinants common to most mycobacteria, nocardia and corynebacteria (22, 33, 34, 36, 38, 43, 44, 46, 57, 66, 67, 80, 82, 85, 94, 105, 106, 109, 116, 123). The

other mycobacterial infections of cattle that may cause cross-reactions are M. bovis and M. fortuitum. Some nocardial mastitis occurs in cattle, and many goats tested have been found to have anti-coryne-bacterial antibodies that cause indistinct bands of precipitate with paratuberculosis antigen (36, 75, 80, 109, 112, 116, 151, 152).

ELISA was first described by Engvall and Perlmann in 1971 (26, 27). Since then, numerous laboratories have applied the procedure (17, 18, 20, 26, 32, 59, 61, 75, 96, 108, 128, 130, 133, 134, 136, 143, 147, 151, 152). It has many advantages over most other serologic diagnostic tests due to its sensitivity, relative ease of operation and rapid processing.

Recently an ELISA has been developed for the detection of anti-M. paratuberculosis antibodies in cattle (151, 152). This latter test employs Mycobacterium phlei absorption of test sera to remove cross-reacting antibodies, as shown to be an effective system by Ankerst, et al. for the removal of cross-reactive antibodies in rubella detection by absorption with Staphylococcus aureus (5). ELISA tests also have been developed for detection of M. avium infections in pigs (129, 131).

## Cultural Diagnosis

Numerous methods for cultural isolation of M. paratuberculosis have been reported (38, 40, 42, 43, 47, 54, 56, 58, 62, 76, 84, 86, 89, 90, 92, 93, 107, 117, 118, 119, 138). Cultural isolation of

M. paratuberculosis from tissue or fecal specimens usually requires 2
to 3 months.  Acids, alkalies, oxidizing agents, and detergents have
been used to decontaminate specimens being prepared for cultural
isolation of M. paratuberculosis.  Most of these procedures have been
nearly as deleterious to the M. paratuberculosis as to the contaminating
organisms present in the specimens (89).  The agents that currently
are most in use for decontamination are oxalic acid (10, 58),
benzalkonium chloride (93), and hexadecylpyridium chloride (145).

### Relationship of M. paratuberculosis to M. avium

Although M. paratuberculosis has been considered to be a distinct
species since the turn of the century, it is very closely related to
the M. avium complex.  The colonies of M. paratuberculosis, though
more slowly growing, look very much like those of M. avium, and the
biochemical reactions of both species are very similar (94, 138).
Also, avian tuberculin can be used interchangeably with johnin in
serologic tests (45).

Usually, M. paratuberculosis does not produce progressive
disease in birds, but mycobactin-requiring M. avium isolated from wood
pigeons (79), subsequently has produced clinical signs of Johne's
disease when inoculated in cattle (78).  Intestinal infection with M.
avium in some animals produces signs of illness indistinguishable from
Johne's disease, and M.paratuberculosis instilled in the lung can
produce tubercles, although the organisms do not proliferate in the
lung (64, 82).

MATERIALS AND METHODS

Serum Sources

## Bovine sera

Serum samples from various sources were obtained from 47 adult

cattle naturally infected with M. paratuberculosis and from 123 adult

non-infected cattle from several herds with no history of

paratuberculosis. These cattle represented seven breeds. M.

paratuberculosis infection of the cattle was determined by cultural

examination of fecal specimens or by culture of tissue samples. When

the blood samples were taken, only five animals expressed clinical

signs of paratuberculosis. Samples from one bleeding per animal were

evaluated.

## Caprine sera

NADC herd    Thirty-one African pygmy goats under study at the

National Animal Disease Center (NADC) in Ames, Iowa, were evaluated

by culture from fecal and tissue specimens and by serologic tests.

Twelve males were infected experimentally, either with the laboratory

adapted NADC strain 18 [American Type Culture Collection (ATCC) 12227],

or with the virulent neotype ATCC strain 19698 M. paratuberculosis

(four, and eight animals, respectively). One additional male goat

acquired the infection by natural exposure to the experimentally

inoculated goats. Eight female goats also acquired the infection in

the same manner (one became infected with strain 18, and seven were infected with strain 19698). The ten remaining goats were culture-negative. Bleedings, skin tests, fecal cultures, and weighings were done at various time intervals over the 112-week period. Twenty goats from NADC, of mixed breed and sex, and non-paratuberculous by culture from feces, were bled and used as negative controls.

NIH herd     Twenty-one mixed breed and sex goats, suspected as paratuberculous, under study at the National Institute of Health (NIH), Bethesda, Maryland, were bled. Fecal and/or tissue samples from these animals were submitted to us for cultural examination.

Ovine sera

In a previous study (83), 45 Corriedale sheep were inoculated with an in vivo-grown strain of M. paratuberculosis, either by oral (O), intravenous (IV), or intratracheal (IT) routes, at two to four weeks of age; sera from eight of these sheep were assessed. In this study, bleedings and skin tests had been done over a 72-week period, and culture of tissue had been done at necropsy. Of this group, three orally inoculated animals were assayed serologically. One of the three had died with clinical paratuberculosis, while the other two were recovering as demonstrated both by culture and by serology. Two additional animals from the IV and IT groups also were assayed.

Animals #146, #151, and #156 each had been inoculated orally with 200 mgs (dry weight basis) of in vivo-grown M. paratuberculosis

suspended in 40 mls of cream. Animals #133 and #163 each had been inoculated intravenously (jugular vein) with 50 mgs (dry weight basis) of in vivo-grown M. paratuberculosis suspended in four mls of saline. Animals #161 and #168 had been inoculated intratracheally. The in vivo-grown organisms were suspended in two mls of saline and were injected into the trachea by means of an atomizer. The purpose of this method was to produce an aerosol within the trachea so the organisms would be evenly distributed in the lungs. Twenty-five, mixed-sex, non-paratuberculous-by-fecal-culture sheep from NADC were bled for test sera, and were used as negative controls.

## Porcine sera

Abattoir pigs     Tuberculous lesions and serum samples were obtained from 255 pigs found at slaughter to have lesions consistent with mycobacteriosis. The lesions were stored at 4°C or less at the abattoir for up to seven days. The blood samples were held at room temperature until the serum had separated from the clot, then were refrigerated. After transfer to NADC, the lesions and serum samples were stored at -70°C.

Arizona pigs     Forty-eight animals under study at the University of Arizona at Tuscon were bled before inoculation and at 4 and 8 weeks post-inoculation (PI). Ten animals received Trudeau Mycobacterial Culture Collection (TMC) M. avium serotype 8. Two of the ten received

10 mgs (wet weight basis) in sterile saline injected into the cranial
vena cava (animals #56 and #59). The remaining eight received an oral
inoculation of 50 mgs (wet weight basis) suspended in milk. Table 1
has details of the age of each animal at inoculation, route of
inoculation, and bleeding data. Twenty-six additional animals of
various ages from a tuberculosis-free herd in Arizona were bled for
test sera, and served as controls.

Table 1. Arizona pigs

| Pig number | Age at inoculation | Bleedings PI | Inoculated | Age at necropsy |
|---|---|---|---|---|
| 56 | 1 week | 4 and 8 weeks | IV | NA[a] |
| 57 | 1 week | 4 and 8 weeks | oral | NA |
| 58 | 1 week | 4 and 8 weeks | oral | NA |
| 59 | 1 week | 4 and 8 weeks | IV | NA |
| 60 | 6 weeks | 4 and 8 weeks | oral | NA |
| 61 | 6 weeks | 4 and 8 weeks | oral | NA |
| 62 | 4 weeks | 4 and 8 weeks | oral | NA |
| 63 | 4 weeks | 4 and 8 weeks | oral | NA |
| 64 | 23 weeks | 4 weeks only | oral | 27 weeks |
| 65 | 23 weeks | 4 weeks only | oral | 27 weeks |

[a]Not applicable.

Pennsylvania pigs    Two pigs under study at the University of
Pennsylvania School of Veterinary Medicine were given 40 mgs (wet
weight basis) TMC M. avium serotype 4, orally. Five months PI they
were bled for test sera, and then were necropsied. M. avium was
recovered by culture from the mesenteric lymph nodes. The serum
samples arrived frozen at NADC and were stored at -14°C.

Canada pigs    Two additional lyophilized serum samples were
obtained from Dr. John Stevens at the Animal Disease Research
Institute at Nepean, Ontario, Canada.  Two bleedings from each animal
(pre-inoculation and PI) were examined.  Each animal was given 5 mgs
(wet weight basis) of TMC M. avium serotype 8 orally at approximately
10 weeks of age.  The animals each were given an additional 2 mgs
(wet weight basis) orally daily for the next four days.  One animal
(9201) was bled at 11 weeks PI.  The second animal (9303) was bled at
16 weeks PI.  Histopathological data are summarized in Table 2.  The
serum samples were rehydrated with sterile distilled water upon arrival
at NADC, and were frozen at -70°C, thereafter.

Table 2.  Canada pigs

| Number | 9201 | 9303 |
|---|---|---|
| Necropsy days PI | 77 | 113 |
| Acid fast bacilli present (AFB) on tissue smear | + | + |
| Culture | Mesenteric | Mandibular |
| Gross lesions | NVL [a] | NVL |
| Microscopic lesions | granulomatous lesions with AFB consistent with mycobacteriosis | NVL |

[a] NVL = No visible lesions.

## Bacteriological Examination

Bacteriological examination by culturing was performed as
described by Merkal (86) on the bovine, caprine, and ovine samples.
For the porcine lesions (Abattoir pigs), the samples were thawed and
inspected for overt tubercles.  When tubercles were found, a 0.5-g
piece was excised and ground in a 15-ml Ten Broeck tissue grinder with
7 mls of 0.3% benzalkonium chloride decontaminating solution.  The
resulting suspensions were decanted into 50-ml round-bottom, screw-
top centrifuge tubes and were further diluted with sufficient
benzalkonium chloride solution to fill the tubes.  The tubes were
stored at room temperature overnight to allow the mycobacteria to
sediment and to allow time for the benzalkonium chloride to kill the
contaminants.  One-tenth ml of the sediment was distributed into each
of four flasks of egg yolk medium (47) and incubated for six weeks
at 37°C.  The cultures were examined and results were recorded after
this incubation period.

## Antigen Preparation

Crude protoplasmic antigen was prepared as previously described
by Merkal (85).  Bacterial strains used were M. paratuberculosis NADC
strain 18 and M. avium TMC 702.  Briefly, three-week-old cultures of
the bacteria grown on Dorset-Henley synthetic liquid medium were
harvested, washed with water, and collected by centrifugation.  The
cells were resuspended in water, ruptured in a refrigerated French

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press at 40,000 psi. and centrifuged at 59,000 X g for 30 minutes to remove unbroken cells and debris. The supernatant fluid was decanted into clean centrifuge tubes and again centrifuged at 59,000 X g for 90 minutes. The resulting supernatant fluid was decanted, dialyzed against tap water overnight, centrifuged at 59,000 X g for another 90 minutes, filter-sterilized through a 0.22-μ apd filter, and then frozen and lyophilized.

## Immunological Tests

AGID and IE were conducted as follows. The serum samples were treated with M. phlei (50 mgs dry weight/ml) suspended in phosphate-buffered-saline (PBS) (pH 7.2, 0.1 M) to remove cross-reacting antibodies. For the AGID, a gel consisting of 1% agarose (Miles Laboratories, Elkhart, IN) in 0.03 M barbital buffer (pH 8.6) was poured, 3-mm deep, into disposable 100 X 15 mm petri dishes, and a 7-hole pattern was cut into each gel. Diameters of these wells were 4-mm (center antigen well) and 5-mm (serum wells); the center of the antigen well was 8 mm from the center of the serum wells.

For the IE assay, 0.03 M barbital buffer (pH 8.6) agar was poured onto microscope slides as a 2-mm deep layer in Gelman frames (Scientific Products, Chicago, IL). A 4-mm diameter hole was cut in the center of the gel with 2 troughs along side of it. The troughs were 65-mm long x 2-mm wide and 3-mm from the center of the antigen well. For both AGID and IE, antigen wells were filled with 35 μl of antigen

(20 mgs/ml). For IE, the gels were migrated by electrophoresis at
400 volts in a Gelman Immunoelectrophoresis apparatus for 45 minutes.
Test serum samples and controls (65 µl each for AGID and 200 µl each
for IE) were added, and the gels were allowed to incubate at room
temperature. IE were allowed to incubate in a humid chamber. This
was not necessary for AGID using covered disposable petri dishes. AGID
results were recorded at 24, 48, 72, and 96 hours. IE results were
recorded 24 and 48 hours after samples were added. The 96 (AGID)
and 48 (IE) hour readings were used for final results.

An ELISA has been described for the detection of bovine anti-M.
paratuberculosis IgG1 (151). The assay was carried out in disposable
polystyrene micro-cuvette trays (Gilford Instrument Laboratories, Inc.,
Oberlin, OH). Each cuvette was filled with 100 µl of a solution
containing 50 µg antigen per ml of 0.05 M carbonate-bicarbonate buffer
(pH 9.6), incubated for three hours at 22°C on a horizontal shaker
and then was allowed to stand overnight at 4°C. The cuvettes then
were washed three times with PBS (pH 7.2, 0.1 M), containing 0.05%
Tween 80 (W-PBS). They were dried and stored in a sealed plastic bag
at 4°C until used.

The sera to be tested were absorbed with 10 volumes of a
suspension of autoclaved, lyophilized M. phlei (50 mg/ml) in PBS
(pH 7.2, 0.1 M); absorption was for 1 hour at 22°C, with gentle shaking
motion on a horizontal shaker. The absorbed test sera then were

centrifuged for 15 minutes at 3,000 rpm, and the supernatant fluid was decanted into a clean test tube. The M. phlei absorption removed cross-reacting antibodies that were present due to exposure to other mycobacteria or related organisms; this absorption also reduced non-specific antibody binding to the surface of the cuvette. The supernatant fluids obtained after centrifugation were further diluted in PBS (pH 7.2, 0.1 M) containing 1.0% Tween 80 and 0.1 M NaCl (D-PBS). Fetal calf serum was added to a 10% final concentration to reduce non-specific protein binding to the cuvette's surface when testing bovine, caprine, and ovine samples. Fetal pig serum obtained from a local abattoir was used at 10% concentration in the D-PBS for the porcine samples. All samples were diluted to a final working concen-tration of 1:150.

Volumes of 100 μl of these 1:150 dilutions of test sera were added to the antigen-coated cuvettes, which then were incubated for 30 minutes at 22°C with gentle motion. The cuvettes were washed three times with W-PBS, then 100 μl of the IgG fraction of rabbit anti-bovine IgG, anti-caprine IgG, anti-ovine IgG, or anti-porcine IgG (Cappel Laboratories, Cochranville, PA), diluted in D-PBS, were added to each cuvette. After block titration to determine appropriate concentrations, rabbit anti-bovine IgG was used at 1:4,000 dilution, anti-caprine IgG and anti-ovine IgG at 1:60 dilution, and anti-porcine IgG at 1:100 dilution. Following incubation at 22°C for 30 minutes, the cuvettes were washed as previously described.

Finally, 100 µl of peroxidase-conjugated IgG fraction from anti-rabbit IgG-Fc fragment specific (Cappel Laboratories, Cochranville, PA), diluted 1:20,000 in D-PBS, were added to each cuvette. The incubations and washings were carried out as previously described. The final PBS wash was followed by one wash with 0.025 M citrate buffer (pH 4.0) to release bound Tween 80 solution. The substrate solution (400 µl volume per cuvette) contained 2.0 mM hydrogen peroxide and 0.2 mM 2,2'-azino-di-(3-ethyl-benzy-thiazoline sulfonic acid) in 0.05 M citrate buffer (pH 4.0).

The optical density was measured at a wavelength of 405 nm with a Gilford PR-50 ELISA processor-reader after 30 minutes of incubation at 22°C. The ELISA indices of the test sera were calculated by averaging optical densities of each test serum, run in quintripulcate, and dividing by the average of negative-control samples, also run in quintuplicate. Figure 1 shows a disposable polystyrene cuvette representing the color reactions of "negative," "suspicious," and "positive" tests. Indices of less than 1.5 were considered to be negative, 1.5 to 2.0 were suspicious, and 2.0 or greater were considered to be positive.

Figure 1.   Disposable polystyrene micro-cuvette used for ELISA.
            Wells 1 through 3 represent positive animals; wells
            4 through 6 represent suspicious animals; and wells
            7 through 10 represent negative animals.

## RESULTS

### Bovine

One hundred and seventy cattle were assayed for the presence of
M. paratuberculosis by culturing of fecal material or tissue samples
obtained at necropsy.  Serum samples taken at the same time were
assayed on AGID, IE, or ELISA.  Of the 47 sera obtained from culture-
positive cattle, 34 (72%) were positive on AGID and 40 (85%) were
positive on ELISA.  Seven (15%) produced "suspicious" indices on ELISA.
Thirty-one were assayed on IE, of which 19 (61%) produced precipitin
bands.  Of the thirteen culture positive animals with negative AGID
results, four were positive in the ELISA; six of the culture-positive
animals with positive AGID results were negative in the ELISA.  None
of the culture-negative cattle that were tested yielded positive ELISA.
Four animals with positive indices on ELISA that were culture negative
were sent to slaughter.  Samples were taken of peripheral blood,
mesenteric lymph nodes and of associated tissues.  Acid fast bacilli,
using Ziehl-Neelsen stain, were found in mesenteric lymph nodes from
all four animals.

The sera of 50 cattle that were culture and skin test negative
were examined by AGID.  Twenty-five of these produced precipitin bands
in AGID that were indistinguishable from specific reactions.  These
50 sera, along with the 34 positive sera from culture-positive animals,
were absorbed with M. phlei and then were lyophilized.  These test sera

were reconstituted to one-third their original volumes with sterile distilled water, and then were assayed in AGID and IE. The 25 "false-positive" sera now were negative, the 25 previously negative sera remained negative, and the reactions of sera from the culture-positive animals were not visibly diminished. Figures 2 and 3 show precipitin bands characteristic of paratuberculous cattle in IE and AGID tests.

## Caprine

One hundred and eight-nine caprine serum samples were examined. One hundred and forty-eight sera were from infected or suspected infected NADC pygmy goats, 21 were from the NIH "suspect" herd and 15 were from goats demonstrated to be non-paratuberculosis by fecal culture analyses. Test bleedings with the NADC herd were done about every eight weeks. The NIH herd and control goats were bled and samples were taken for fecal culture once. Skin tests were performed semi-annually both on the NADC test herd and on the NADC control animals, but they were not done on the NIH herd. Of the total number of bleedings, AGID was performed on all samples, IE on 50, and ELISA on 155.

## NADC herd

Seven male pygmy goats were inoculated orally with strain 19698 M. paratuberculosis at approximately 16 to 32 weeks of age. Four of these goats died from Johne's disease within 112 weeks (Goats #1, #2,

Figure 2.   Precipitin bands of two paratuberculous bovine sera
            migrated by electrophoresis of M. paratuberculosis
            strain 18 antigen are shown.  Such reactions are
            from passive diffusion of treated and concentrated
            serum to electrophorically separated antigens.

Figure 3.   Typical AGID precipitin bands produced by passive
            diffusion of strain 18 antigen (center well) to
            absorbed and concentrated paratuberculous bovine
            sera (outer wells) are shown in Figure 2.
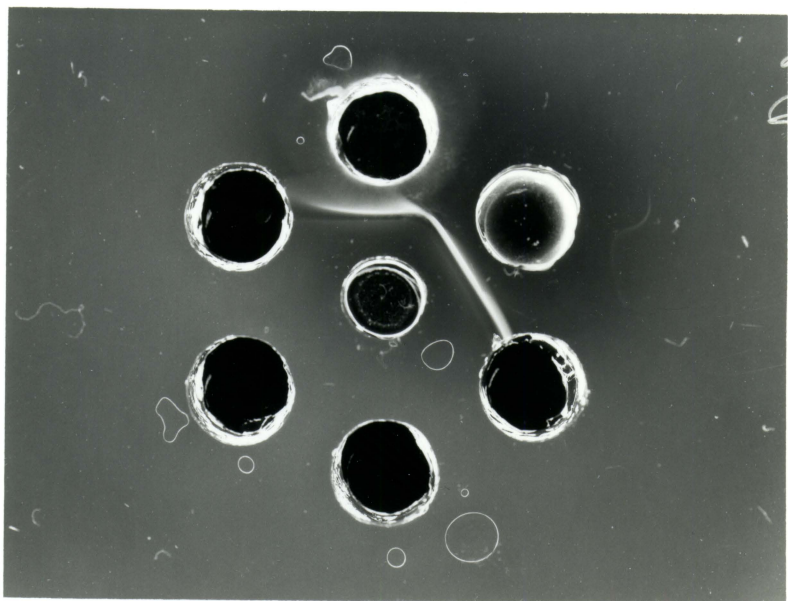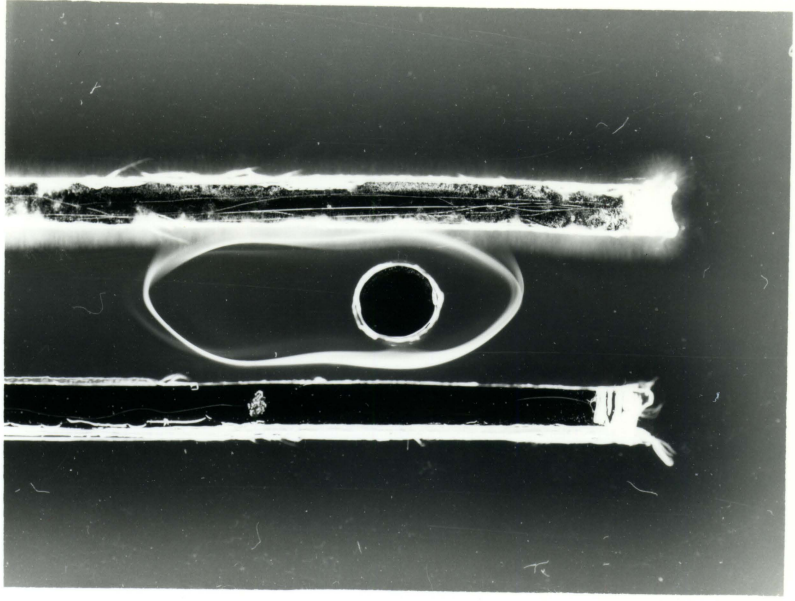
#3, and #4). These four had been dosed with four grams (wet weight basis) of strain 19698 mycobacteria suspended in 40 mls of 11.5% butterfat cream. Three other males were given 100 mgs (dry weight basis) of lyophilized strain 19698 contained in a gelatin capsule. These animals are termed "experimentally inoculated" males. One male (Goat #5) was housed with three of the experimentally inoculated males and acquired the infection by natural exposure. This animal is termed "heavy natural exposure". These animals also were used in chemotherapeutic experiments after high fecal shedding was detected (50). All were fecal-culture-positive after 8 to 20 weeks PI. Seven females acquired strain 19698 M. paratuberculosis infection by natural exposure to an "experimentally inoculated" male. These are termed "light natural exposure". These animals were housed together in an outside pen for 16 weeks. Five additional goats (four males and one female) were fed the laboratory adapted strain 18 in a vaccination experiment. The final ten animals were culturally negative. AGID, IE, and ELISA all were negative on the culturally negative animals. See Table 3 for details.

Goat #1, "experimentally inoculated" with strain 19698, yielded positive fecal culture 12 weeks PI. AGID precipitin bands appeared at 20 weeks PI and increased in number until the animal died 96 weeks PI. IE showed increasing numbers of bands (from one to three), until 56 weeks PI, when the number of bands decreased to one, ELISA gave

Table 3.  NADC goat herd

| Goat number | Sex | Type of inoculation | | Strain 19698 | Strain 18 |
|---|---|---|---|---|---|
| | | Experimental | Natural | | |
| 1 | M | X | | X | |
| 2 | M | X | | X | |
| 3 | M | X | | X | |
| 4 | M | X | | X | |
| 5 | M | | X | X | |
| 6 | M | X | | X | |
| 7 | M | X | | X | |
| 8 | M | X | | X | |
| 9 | M | X | | X | |
| 10 | F | | X | X | |
| 11 | F | | X | X | |
| 12 | F | | X | X | |
| 13 | F | | X | X | |
| 14 | F | | X | X | |
| 15 | F | | X | X | |
| 16 | F | | X | X | |
| 17 | M | X | | | X |
| 18 | M | X | | | X |
| 19 | M | X | | | X |
| 20 | M | X | | | X |
| 21 | F | | X | | X |
| 22* | M | | | | |
| 23* | F | | | | |
| 24* | F | | | | |
| 25* | F | | | | |
| 26* | M | | | | |
| 27* | M | | | | |
| 28* | M | | | | |
| 29* | M | | | | |
| 30* | F | | | | |
| 31* | F | | | | |

*Non-inoculated control animals.

consistently high indices (3 to 4) with a marked decrease at the time
of death (2.5). Culture data were erratic due to the chemotherapeutic
experiments being conducted on the animal; however, there was a
correlation between depressed serologic titers and reduced colony
counts. Intradermal results confirmed what has been demonstrated
previously (83, 91), that the response can be elicited early in the
infection, but, then it becomes negative either because the animal
is getting over the infection, or because it is becoming clinically
ill and is anergic.

Goats #2, #3, and #4, experimentally infected with strain 19698,
yielded similar serologic results. However, positive cultures were
consistently demonstrated before any positive serologic or intra-
dermal responses. The remaining culture-negative animals were used
as controls to set indices for serologic responses assayed. Figures
4, 5, 6, and 7 detail chemotherapies, culture results, body weights,
ELISA indices, and AGID results for Goats #1, #2, #3, and #4. Figure
8 details cultural results, body weights, ELISA indices, and AGID
results for Goat #5 which did not receive chemotherapy.

Figure 9 shows culture, ELISA, and AGID of the pygmy goat herd
at NADC. Figures 10 and 11 show typical positive IE and AGID results.

### Ovine

Sera from orally inoculated sheep #151 were collected at various
times following inoculation and assayed sequentially on AGID and

Figure 4.  Chemotherapies, culture results, body weights, ELISA
indices, and number of AGID bands for "experimentally
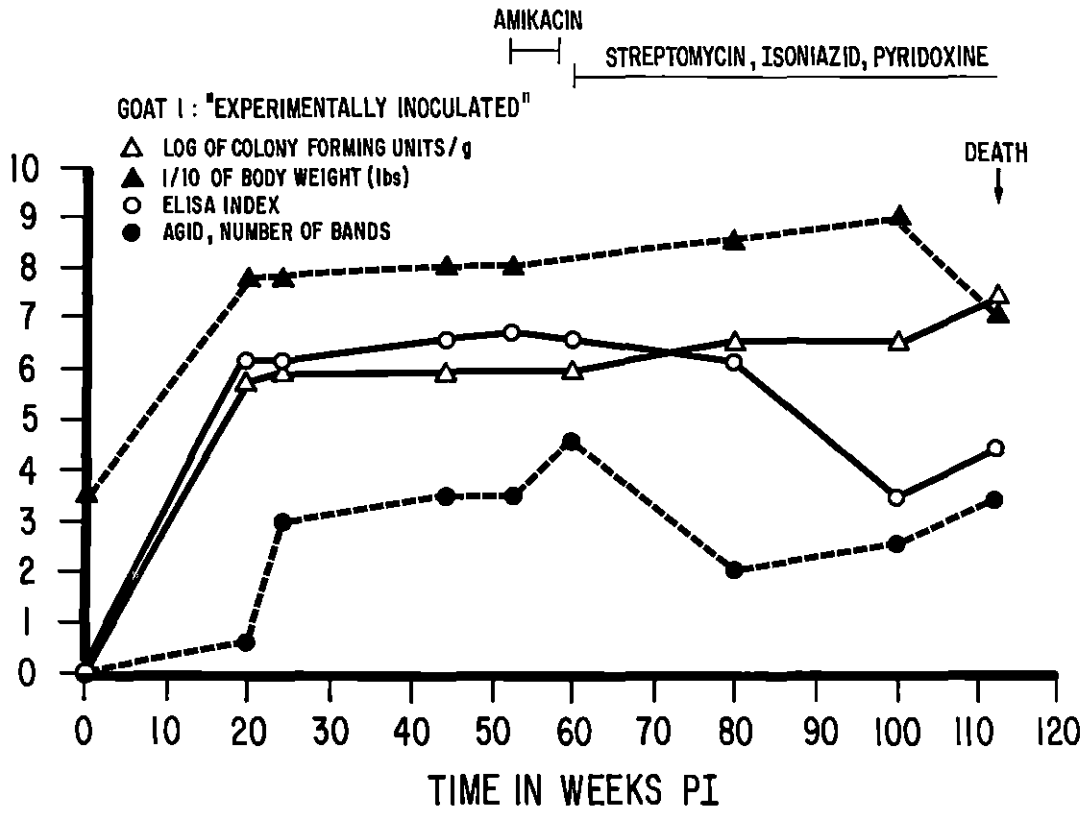inoculated" Goat #1.

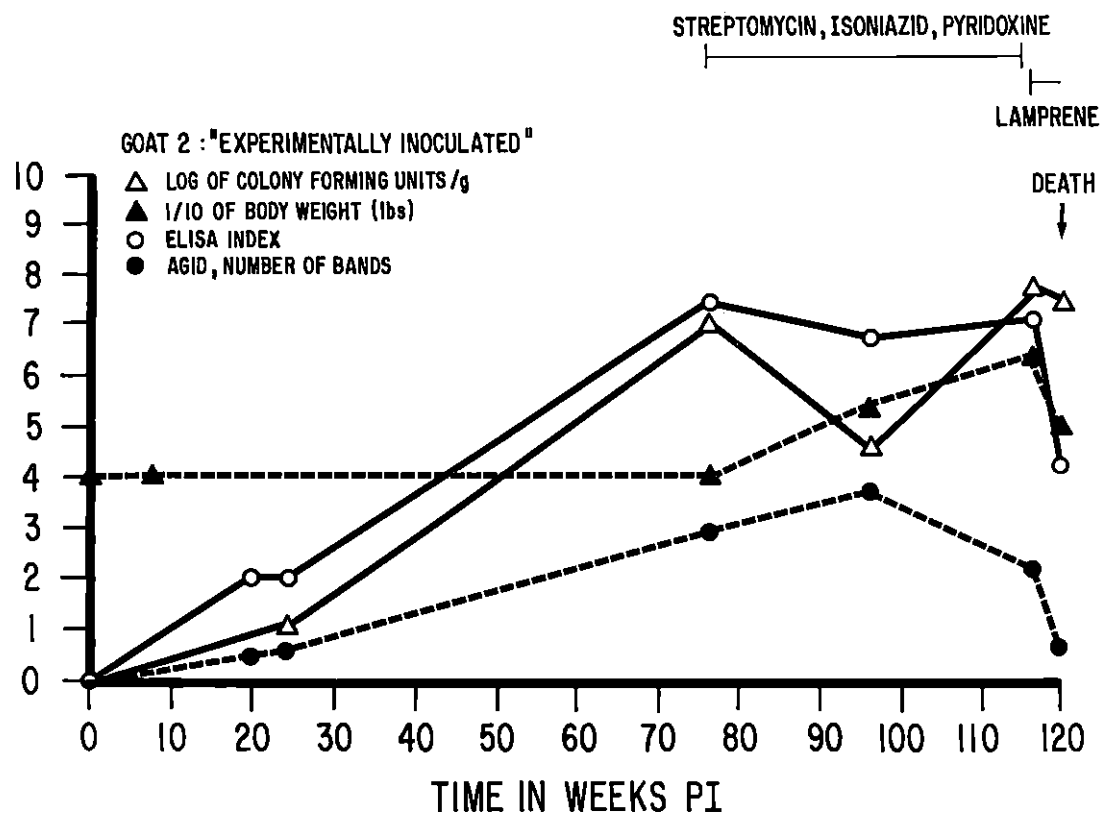AMIKACIN

STREPTOMYCIN , ISONIAZID, PYRIDOXINE

GOAT I : "EXPERIMENTALLY INOCULATED"

△ LOG OF COLONY FORMING UNITS / g
▲ 1/10 OF BODY WEIGHT (lbs)
○ ELISA INDEX
● AGID, NUMBER OF BANDS

DEATH

TIME IN WEEKS PI

Figure 5.  Chemotherapies, culture results, body weights, ELISA
           indices, and number of AGID bands of "experimentally
           inoculated" Goat #2.

31

Figure 6.   Chemotherapies, culture results, body weights, ELISA
            indices, and number of AGID bands of "experimentally
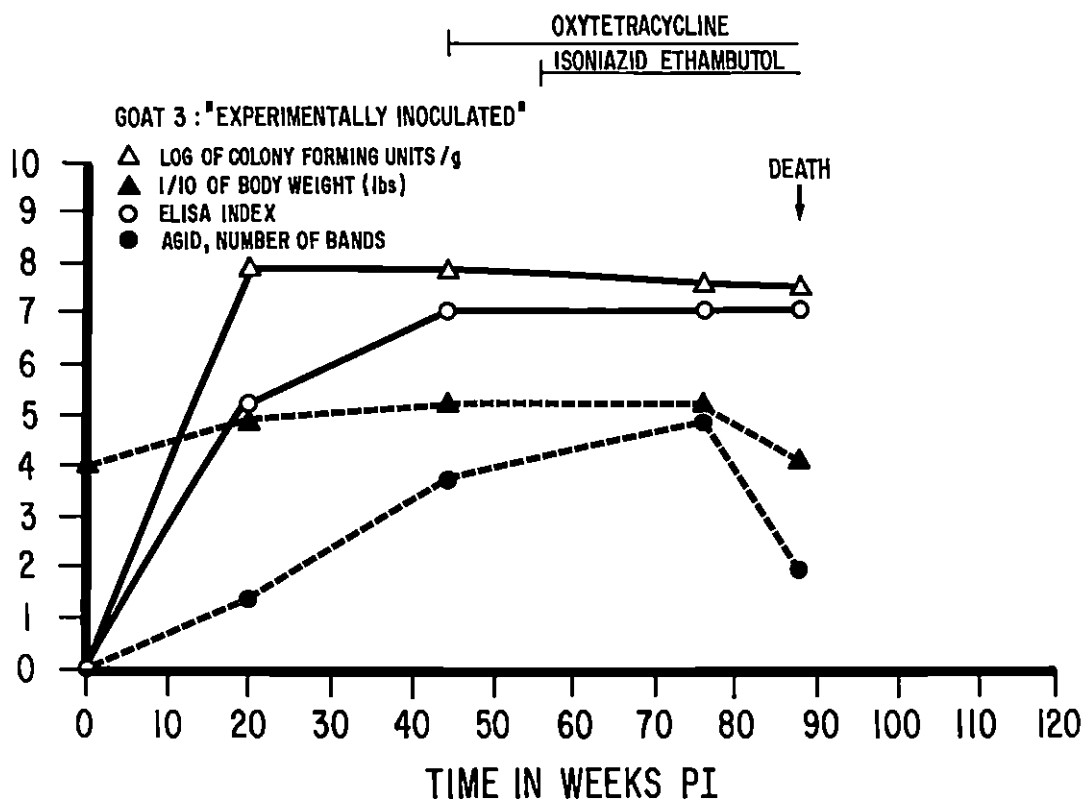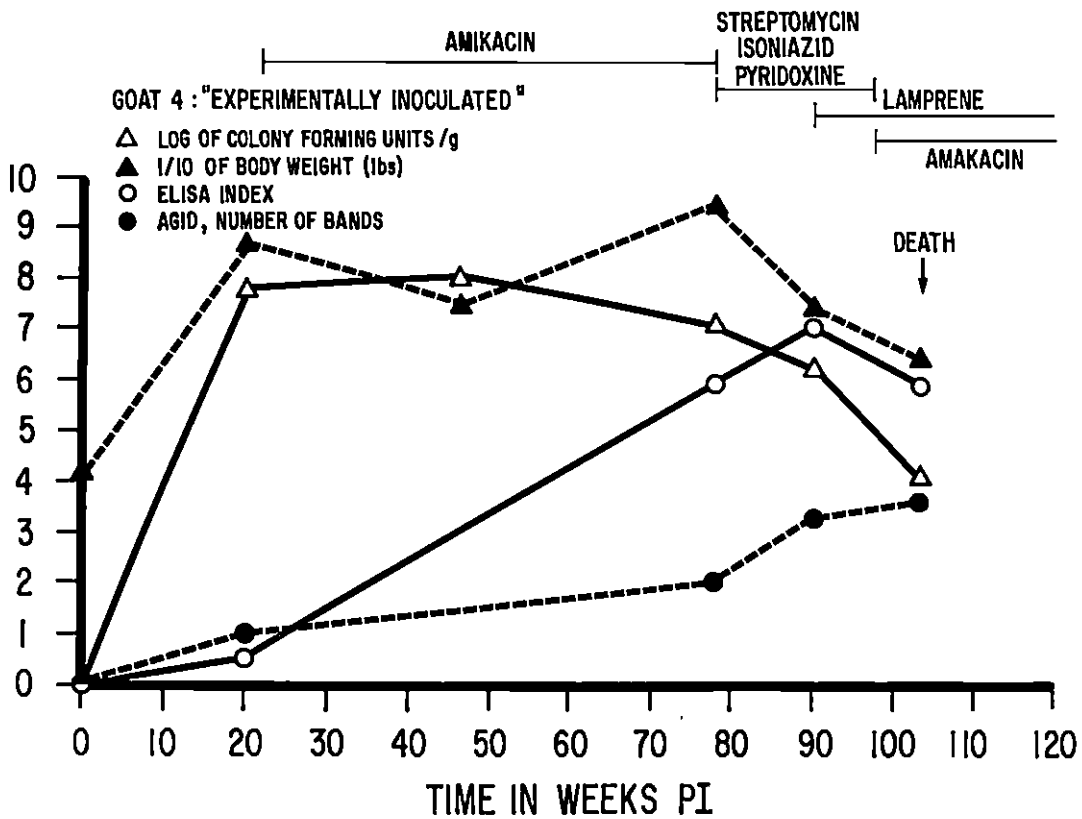            inoculated" Goat #3.

OXYTETRACYCLINE

ISONIAZID ETHAMBUTOL

GOAT 3 : "EXPERIMENTALLY INOCULATED"
△ LOG OF COLONY FORMING UNITS /g
▲ 1/10 OF BODY WEIGHT (lbs)
○ ELISA INDEX
● AGID, NUMBER OF BANDS

DEATH

TIME IN WEEKS PI

Figure 7.    Chemotherapies, culture results, body weights, ELISA indices, and number of AGID bands of "experimentally inoculated" Goat #4.

35



GOAT 4 : "EXPERIMENTALLY INOCULATED"

△ LOG OF COLONY FORMING UNITS /g
▲ 1/10 OF BODY WEIGHT (lbs)
○ ELISA INDEX
● AGID, NUMBER OF BANDS

Figure 8. Culture results, body weights, ELISA indices, and number of AGID bands of "heavy natural exposure" Goat #5. "0" weeks is approximately date of the infection.
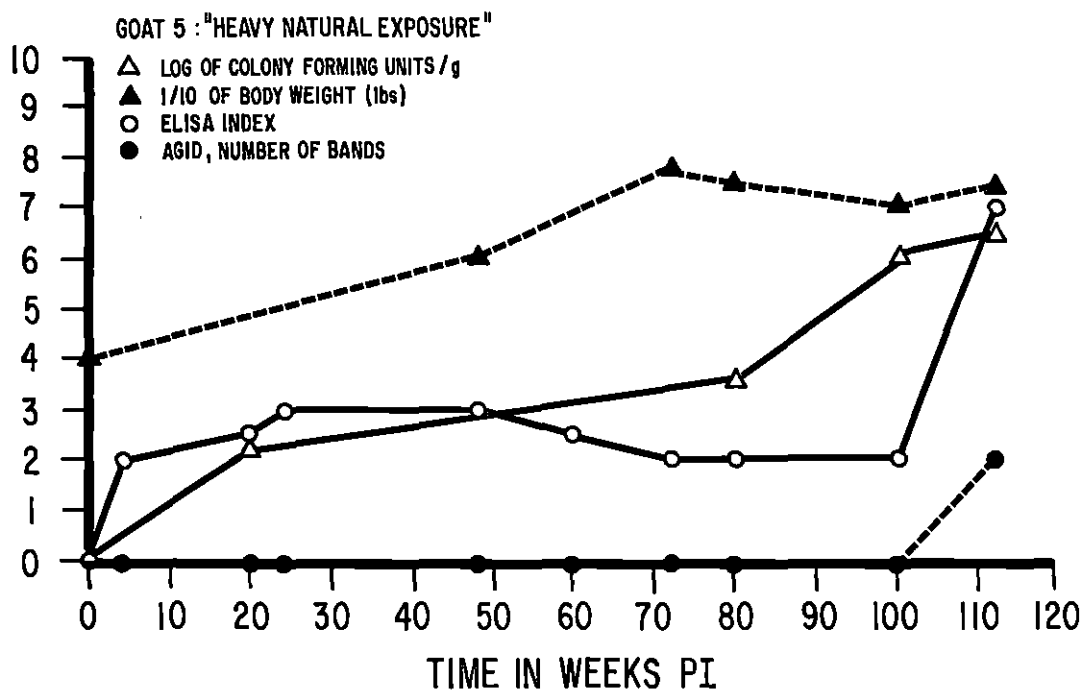
GOAT 5 : "HEAVY NATURAL EXPOSURE"
△  LOG OF COLONY FORMING UNITS / g
▲  1/10 OF BODY WEIGHT (lbs)
○  ELISA INDEX
●  AGID, NUMBER OF BANDS

TIME IN WEEKS PI

Figure 9.  Culture, ELISA, and AGID data of the NADC pygmy goat herd.
"Experimentally inoculated" represent four male goats
orally dosed with M. paratuberculosis. "Light natural
exposure" represent seven female goats which were housed
with one of the "experimentally inoculated" males.
"Heavy natural exposure" represents one male which was
housed with the "experimentally inoculated" males. As
shown, ELISA was positive before AGID with all three
groups. Only with experimental inoculation or heavy
natural exposure did the culturing of feces detect
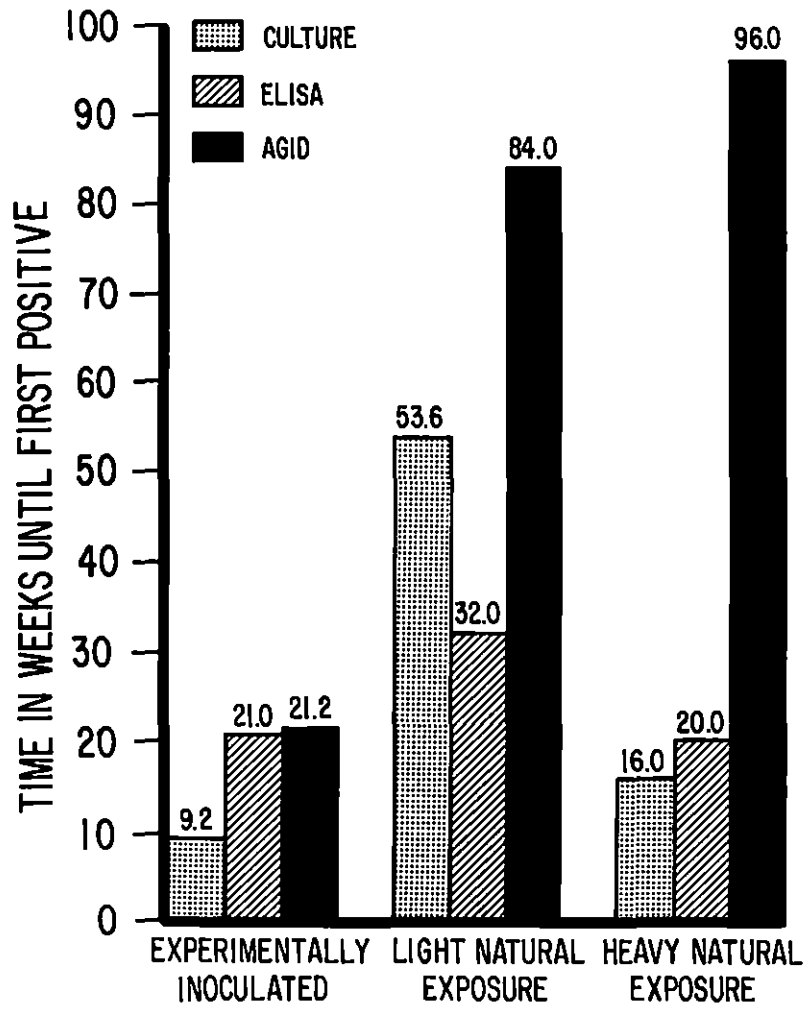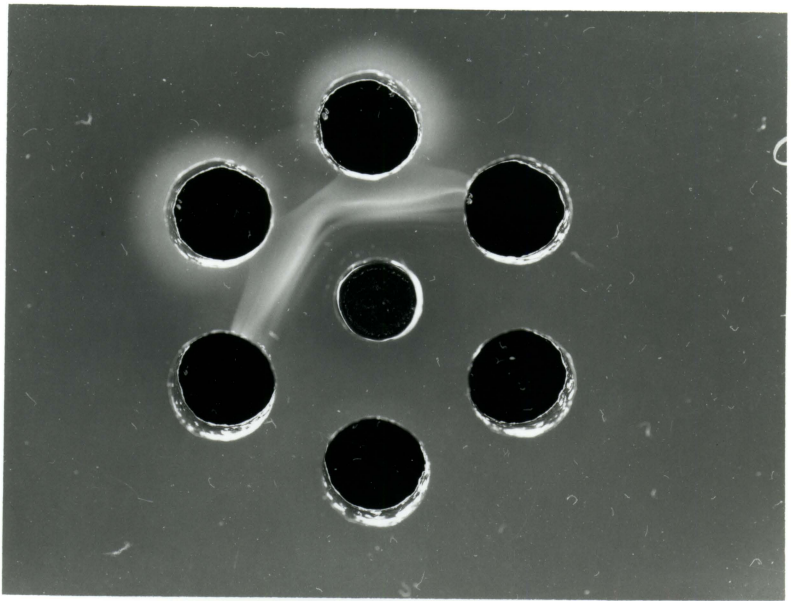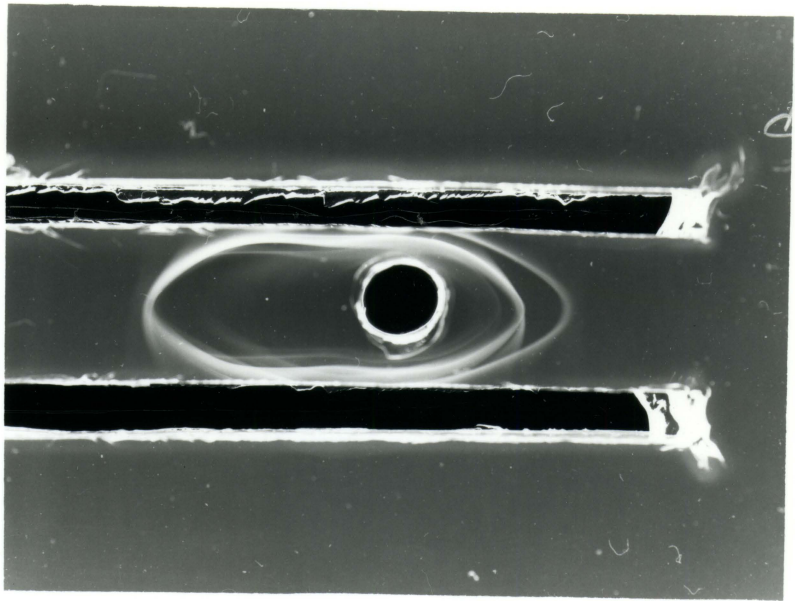animals before serological methods.

Figure 10.   Separation of strain 18 M. paratuberculosis antigen
             by electrophoresis and reactions with sera from two
             paratuberculous goats.

Figure 11.   Typical precipitin bands produced in AGID with strain 18
             antigen and the sera shown in Figure 10.

ELISA. The results are shown in Table 4. Sera from this animal produced a precipitin band at 8 weeks PI; at 10 weeks PI, two lines were visible; and at 16 weeks PI, three precipitin lines were detected. The serum from the animal continued to produce three precipitin lines until 52 weeks PI, when an additional band was detected. The animal died of clinical paratuberculosis (Johne's disease) at 56 weeks PI. ELISA detected antibodies at 4 weeks PI and remained positive until death. Cultures of selected tissues were positive at necropsy; see Table 5.

Sheep #146 produced precipitin lines on AGID at 12 weeks PI. Sheep #156 produced precipitin lines on AGID at 6 weeks PI. Animal #146 had no precipitin bands detected at the end of the 66-week study, and cultures were negative on all tissue samples. Animal #156 died at 64 weeks PI with a reduced detectable precipitin line number, but was culture positive at necropsy. See Table 5.

Animals inoculated IV, #133 and #163, had been necropsied at 36 and 18 weeks PI, respectively. Animal #133 had detectable antibodies by AGID at 4 weeks PI, and the number of bands had increased to three by the time of necropsy as evaluated by AGID. AGID was positive on animal #163 at 4 weeks PI (single band) and had increased by one band at necropsy. In this experiment, ELISA indices paralleled those obtained with AGID. Both test animals were culture positive at necropsy.

Table 4. Serological responses of sheep

| Weeks PI | Sheep number and inoculation | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 146(O) | | 151(O) | | 156(O) | | 133(IV) | | 163(IV) | | 161(IT) | | 168(IT) | |
| | AG | ELA | AG | ELA | AG | ELA | AG | ELA | AG | ELA | AG | ELA | AG | ELA |
| 2 | | | | | | | | | | | 0 | 4 | 0 | 1 |
| 4 | 0 | 2 | 0 | 3 | 0 | 2 | 1 | 2 | 1 | 5 | 0 | 5 | 0 | 1.5 |
| 6 | 0 | 2.5 | 1 | 4 | 1 | 4 | | | 1 | 5 | 3 | 5 | 1 | 2 |
| 8 | 0 | 3 | 1 | 3 | 1 | 4 | | | 1 | 6 | 3 | 5 | 2 | 2 |
| 10 | 0 | 2 | 2 | 3 | 1 | 4 | | | 1 | 5 | | | 3 | 2 |
| 12 | 1 | 2 | 2 | 2 | 1 | 4 | | | 1 | 5 | 2 | 5 | | |
| 14 | 1 | 2 | 2 | 1.5 | 1 | 4 | 3 | 7 | 1 | 8 | 2 | 5 | | |
| 18 | 1 | 2 | 3 | 1.5 | 2 | 4 | | | 2 | 7 | 3 | 4 | | |
| 20 | 1 | 4 | 3 | 2 | 2 | 4 | | | | | 3 | 4 | | |
| 22 | 1 | 4 | 3 | 2 | 2 | 4 | | | | | 4 | 5 | | |
| 24 | 1 | 4 | 3 | 2.5 | 2 | 4 | | | | | 4 | 5 | | |
| 26 | 2 | 4 | 3 | 3 | 2 | 4 | | | | | 4 | 5 | | |
| 28 | 2 | 3 | 3 | 3 | 3 | 4 | | | | | 5 | 6 | 4 | 6 |
| 30 | 2 | 3 | 3 | 3 | 3 | 4 | | | | | 5 | 6 | | |
| 32 | 2 | 3 | 3 | 3 | 3 | 4 | | | | | 5 | 6 | | |
| 34 | 2 | 4 | 3 | 3 | 3 | 3 | | | | | 5 | 6 | | |
| 36 | 2 | 3 | 3 | 3 | 2 | 3 | | | | | 5 | 6 | | |
| 38 | 1 | 3 | 3 | 3 | 2 | 3 | | | | | | | | |
| 40 | 1 | 3 | 3 | 3 | 2 | 3 | | | | | | | | |
| 42 | 1 | 4 | 3 | 3 | 2 | 2 | | | | | | | | |
| 44 | 1 | 3 | 3 | 3 | 2 | 2 | | | | | | | | |
| 46 | 1 | 3 | 3 | 3 | 1 | 2 | | | | | 5 | 6 | | |
| 48 | 1 | 3 | 3 | 3 | 1 | 2 | | | | | | | | |
| 50 | 1 | 3 | 3 | 3 | 1 | 2 | | | | | 5 | 6 | | |
| 52 | 1 | 2 | 3 | 3 | 1 | 2 | | | | | | | | |
| 54 | 1 | 2 | | | 1 | 2 | | | | | | | | |
| 64 | 0 | 2 | | | | | | | | | | | 3 | 3 |
| 66 | | | | | | | | | | | | | | |

AG = AGID bands.

ELA = ELISA indices, less than 1.5 is negative, 1.5 - 2.0 is suspicious, 2.0 or greater is positive.

(O) = Orally inoculated.

(IV) = Intravenously inoculated.

(IT) = Intratracheally inoculated.

Table 5.  Cultural results of sheep*

| Tissue | 146(0) | 151(0) | 156(0) | 133(IV) | 163(IV) | 161(IT) | 168(IT) |
|---|---|---|---|---|---|---|---|
| **Lymphatic** | | | | | | | |
| Abomasol node | 0 | 0 | 3 | 0 | 4 | 0 | 0 |
| Bronchial node | 0 | 0 | 0 | 0 | 4 | 0 | 0 |
| Cecal node | 0 | 0 | 0 | 0 | 4 | 0 | 2 |
| Hepatic node | 0 | 0 | 0 | 0 | 4 | 0 | 0 |
| Iliac node | 0 | 0 | 0 | 0 | 4 | 0 | 0 |
| Inguinal node | 0 | 0 | 0 | 0 | 4 | 0 | 2 |
| Mediastinal node | 0 | 0 | 0 | 0 | 4 | 0 | 0 |
| Mesenteric node | 0 | 0 | 3 | 0 | 4 | 0 | 0 |
| Popliteal node | 0 | 0 | 2 | 0 | 1 | 0 | 1 |
| Prefemoral node | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| Prescapular node | 0 | 0 | 0 | 0 | 3 | 0 | 1 |
| Spleen | 0 | 0 | 0 | 0 | 4 | 0 | 0 |
| **Digestive** | | | | | | | |
| Cecum | 0 | 0 | 0 | 4 | 4 | 0 | 3 |
| Spiral colon | 0 | 4 | 0 | 0 | 4 | 0 | 0 |
| Terminal colon | 0 | 4 | 0 | 0 | 2 | 0 | 0 |
| Duodenum | 0 | 0 | 0 | 4 | 4 | 0 | 3 |
| Ileocecal valve | 0 | 4 | 0 | 3 | 4 | 0 | 0 |
| Ileum | 4 | 0 | 0 | 3 | 4 | 0 | 3 |
| Liver | 0 | 0 | 3 | 0 | 1 | 0 | 0 |
| Rectum | 0 | 1 | 1 | 0 | 3 | 0 | 3 |
| **Respiratory** | 0 | 0 | 0 | 0 | 3 | 0 | 0 |

0 = no growth, 2 = 2 to 5 colonies per tube, 3 = 6 to 20 colonies per tube, 4 = 20 or more colonies per tube.

*Taken from R. S. Merkal (83).

Animals #161 and #168, inoculated IT, were positive by AGID test at 6 weeks PI. Sera from animal #161 produced an increasing number of precipitin bands as assayed by AGID, until necropsy, when five were detected. ELISA indices were positive at 2 weeks PI and increased until necropsy. However, no colonies were produced from tissues cultured for M. paratuberculosis from this animal. Animal #168 produced three bands by AGID test at 10 weeks PI. The same number of bands were detected on serum obtained at necropsy. ELISA indices were positive at 6 weeks PI, and the animal was culture positive at necropsy. Figures 12 and 13 show typical IE and AGID reactions for sera from paratuberculous sheep.

## Porcine

### Abbattoir pigs

Multiple specimens were obtained from some animals; therefore, although 255 animals were examined, a total of 289 specimens were cultured. From these, 151 cultures of M. avium complex isolants, representing 106 animals, were obtained. Typing of biochemical sensitivity or resistance to various drugs was performed according to the protocol outlined in "Laboratory Methods in Veterinary Mycobacteriology" (147). The samples were serotyped according to agglutination of homologous anti-sera. Those samples which were untypeable by serology due to "rough" growth characteristics were submitted to National Jewish Hospital in Denver, CO, for typing by

Figure 12.   Immunoelectrophoresis of strain 18 antigen against
             sera from two paratuberculous sheep.

Figure 13.   Typical precipitin bands of two M. paratuberculosis
             infected sheep to strain 18 antigen..
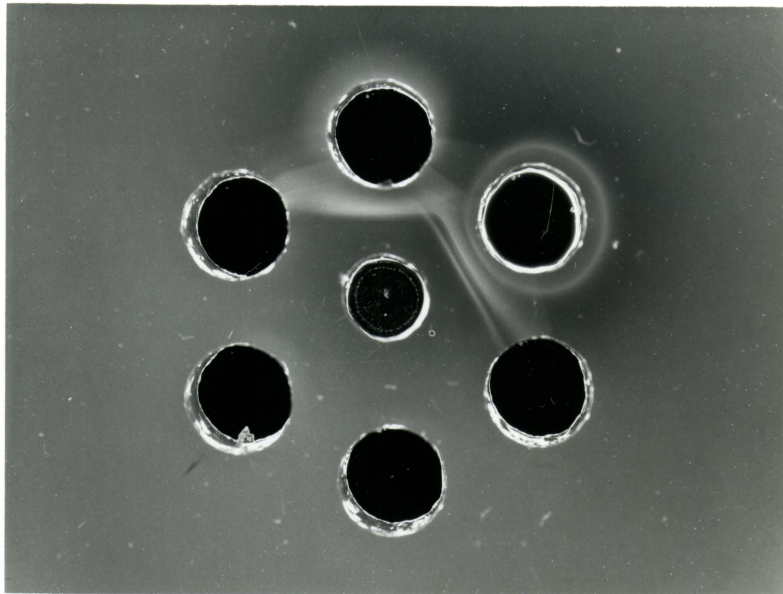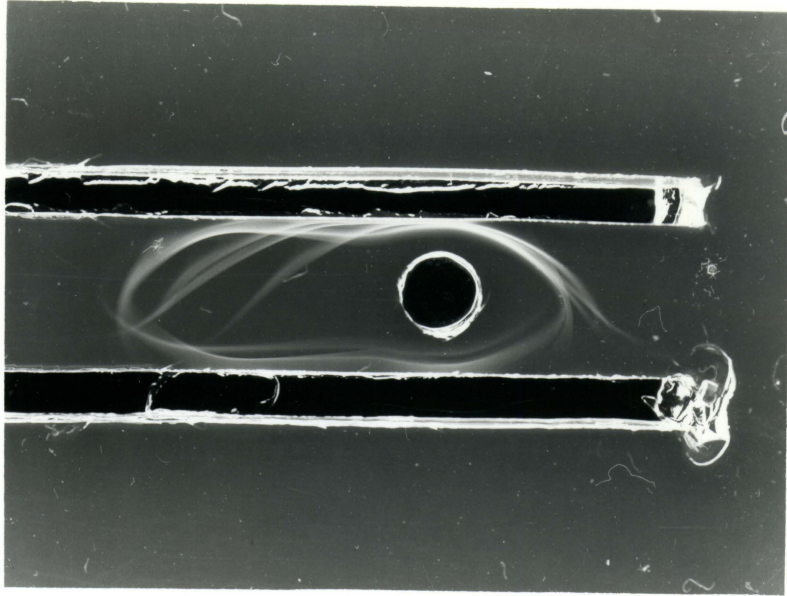
thin layer chromatography. Serovars most often found were 1, 2, 4, 4/9, 6, 8, 9, 10, and 11. Several additional isolants were reported as "untypeable" even by thin layer chromatography.

AGID was run on sera from all of the culture-positive pigs. Sixteen percent of the sera produced precipitin bands to TMC 702 M. avium antigen. ELISA was performed on 30 sera from selected positive-culture animals. The sera from these animals had produced precipitin bands to M. avium antigen in AGID. Ten sera had positive indices, 10 had suspect indices, and 10 were negative. AGID, IE, and ELISA also were run on culture-negative samples. After absorption with M. phlei, no sample produced positive reactions.

## Arizona pigs

Serum samples obtained from the University of Arizona were evaluated. All pre-bleedings of pigs aged 1 week to 120 weeks were negative on AGID and ELISA. IE was not done, since the AGID was negative. Bleedings of these animals at 4 weeks after either IV or oral inoculations yielded positive indices on ELISA. AGID were positive in less than one percent of these sera. Eight weeks PI bleedings of inoculated animals continued to be positive on ELISA, but negative in AGID.

Pennsylvania pigs    Neither serum from the University of Pennsylvania pigs produced precipitin lines with AGID with either strain 18 or TMC 702 antigens. ELISA, however, detected one animal as "positive" and the other as "suspect", with strain 18 antigen.

Canada pigs     AGID and ELISA were negative with the sera from both pigs from pre-inoculation bleedings.  AGID detected both animals as positive using TMC 702 and strain 18 antigens at necropsy bleedings. ELISA indices were classified as "suspect" at the time of necropsy bleeding on both animals.

## DISCUSSION

In this study, comparisons have been made of results of culturing of fecal or tissue specimens with results of AGID, IE, and ELISA of serum samples from bovine, caprine, ovine, and porcine species infected with mycobacteria. This project was justified by the fact that M. paratuberculosis is the primary mycobacterial pathogen of bovine, caprine, and ovine species. Also, members of the M. avium complex are the most frequent mycobacterial pathogens in lesions found at slaughter in pigs (12, 25, 29, 30, 126, 137).

Fecal or tissue examination by culturing is the most reliable diagnostic test for detecting subclinical cases of paratuberculosis, but it is an extremely time-consuming process that requires 6 to 12 weeks for identification of the organism (93). Using lesions observed at slaughter as the method for diagnosing mycobacteriosis in porcine species has a failure rate of up to 70% (11, 12, 21, 25, 77, 111, 114, 124).

A variety of serological techniques, as mentioned previously, had been found to lack specificity, which leads to many false-positive reactions in specimens taken from non-infected animals or lack of sensitivity which led to false-negative reactions in samples from infected animals. For example, Sherman and Gezon (121) and Sherman and Markham (122) demonstrated that with the AGID non-specific bands formed with sera from many non-paratuberculous animals. The problem

of cross-reacting has been solved with the absorption and concentration treatment reported here.

Little research has been directed towards serological detection of mycobacterial infections in swine. Mycobacteria are not an important health problem in swine, partly due to the animal's superior ability to recognize them as a foreign substance and "wall them off" by means of tubercle formation, and eventually to recover from the infection.

Recently, an ELISA for the detection in serum from paratuberculous cattle of antibodies to protoplasmic antigen of $\underline{M}$. paratuberculosis was reported (151). The ELISA results correlated positively with results from the fecal culture test. Additionally, ELISA was positive in a higher percentage of the fecal positive animals than was the AGID. However, cross-reactions in sera from animals exposed to mycobacteria other than $\underline{M}$. paratuberculosis were a problem in this particular ELISA. In a subsequent report (152), Yokomizo and colleagues showed that absorption treatment with $\underline{M}$. phlei reduced cross-reactions due to other species of mycobacteria and other closely related microorganisms such as corynebacteria, nocardia, and rhodococcus. Increased specificity and sensitivity were obtained with no loss of anti-$\underline{M}$. paratuberculosis antibody levels.

The AGID test, in the past, has been unsatisfactory for the detection of paratuberculosis in cattle when using untreated sera.

Cross-reactions with antigens common to other mycobacteria and related species occur, and the test is relatively insensitive.

Several methods have been developed to increase the sensitivity and specificity of the AGID test. These improvements include: 1) pretreatment with M. phlei to reduce non-specific antigen-antibody reactions, and 2) concentration of the test serum, either (a) by lyophilization and rehydration to one-third its original volume, or (b) concentration by the Minicon system (Amicon Corp., Lexington, MA), or (c) concentration by Lyphogel, a dried polyacrylamide gel (Gelman Scientific, Inc., Ann Arbor, MI). Such treatments allowed detection of low levels of antibodies. Each of these methods are relatively inexpensive and are not very time consuming.

Caprine and ovine species are very prolific antibody producers and the primary problems with those animals are non-specific cross-reactions. AGID is a relatively quick and effortless test. Following absorption with M. phlei, and concentration, it also is more sensitive and specific than previously has been reported. IE is even more sensitive than AGID, but it requires more effort and a larger volume of serum.

ELISA has advantages over AGID and IE. One advantage of the ELISA is its ability to detect reactor animals very early after infection. It sometimes has been positive up to two years before any shedding was detected by culture of fecal specimens. It has greater sensitivity,

and it is safe for any laboratory to use, which is an advantage over the radioimmunoassay. Finally, the test can be set up and results can be available in less than six hours from the receipt of serum samples, whereas the AGID and IE tests require a minimum of 96 hours to conduct.

Culture of fecal or tissue samples from animals suspected of being paratuberculous is the most reliable method to date for establishing that an animal is infected to the level of being dangerous to other susceptible animals, but the procedure requires from 6 to 12 weeks to obtain results. It may be necessary to repeat such samplings before positive results are obtained.

Serological methods for detection of mycobacteriosis in bovine, caprine, and ovine species have merit. In these species, ELISA detected infections as much as 24 months before organisms could be cultured from fecal specimens in this study. Lastly, AGID, in most instances, detected infected animals months before culture methods. However, such clear-cut differences were not observed in porcine species. ELISA identified only one-third of the pigs that had de- tectable lesions at slaughter, or that had been experimentally inoculated with large quantities of $\underline{M}$. avium. AGID also was a poor test for detecting circulating, precipitating antibody. These data would given credence to an hypothesis that pigs have efficient immune responses to mycobacteria that they acquire through exposure to their environment. They are able to clear themselves of these organisms,

before sufficient antibody levels are established to be detectable serologically. It would be logical to conclude that evolution has produced an animal that can spend an entire lifetime exposed to many kinds and massive quantities of some mycobacterial pathogens, and still remain reasonably unharmed.

Paratuberculosis has been a difficult disease to diagnose. However, with culture methods now in practice, animals in advanced stages of the disease can be detected. Animals have two defense mechanisms to prevent such infections. The first is the physical barrier of the skin and other epithelial surfaces of the lungs and intestinal tract. The second defense mechanism is the destruction of microbes after they have entered the body, either by cell-mediated immune response or by antibody production. Diagnosticians use the latter two responses to detect infection or they try to detect the infecting organisms in tissues or effluents.

Animals most often become infected with M. paratuberculosis while they are young, usually before weaning. The young calf, kid, or lamb ingests the organisms by nursing on a fecal contaminated teat. The organism, M. paratuberculosis, is easily coated by the butterfat in the milk and passes through the intestinal epithelium as a lipid droplet into the lamina propria and into the associated mesenteric nodes and macrophages of the young animal. At this point no culture method will detect the infection unless an inordinately high number of organisms are ingested.

The largest problem in culturing the organisms from effluents is in the sampling. The chances of being able to culture one organism out of several liters of effluent are very slim. However, when the organisms multiply to a point where several million are in those several liters of effluents, then it is far easier to obtain a sample which has a chance of containing at least one organism that can form a colony when inoculated onto culture medium.

Once the organisms are ingested and have been carried by macrophages into the lymph nodes, they remain quietly multiplying. At some point when the macrophage can no longer keep the bacterial metabolic products membrane bound, the animal is stimulated to elicit cell-mediated responses. T-cells produce lymphokines and other products. This response is early in the infection and can be detected by such tests as intradermal skin tests, macrophage-migration-inhibition and lymphocyte-transformation tests. These responses go away in most animals. This may be due either to recovery from the infection or because the animal is succumbing to the disease and stops being reactive. Eventually the infection becomes heavy enough so that the animal starts producing circulating antibody. Only the most sensitive tests will detect that level of antibody. Such tests as ELISA and radioimmunoassay detect animals at this level of the infection.

By the time the infection has progressed to the point where organisms can be cultured from the fecal material, the least sensitive serologic tests, such as immunoelectrophoresis, agar gel

immunodiffusion, complement fixation, and passive hemagglutination are useful. Animals which react in these tests have little hope of surviving the infection.

Diagnostic tests, therefore, can be used as herd management tools. Animals which produce precipitin bands in IE and AGID should be considered for culling. ELISA also may be used in herds where the infection level is low and the animals are not yet shedding the organisms. Also, it could be especially useful for detecting infected, non-shedding, animals before purchase.

## SUMMARY

Various methods to increase the specificity and sensitivity of three serologic assays were reported. Absorption of test sera with Mycobacterium phlei increased the specificity of the three serologic assays evaluated. These assays were AGID, IE, and ELISA. The absorptions removed non-specific, cross-reacting antibodies. Various methods of concentration were used to increase sensitivity of AGID and IE.

A comparison of culturing fecal or tissue specimens with the results of these three serologic tests were made on sera from bovine, caprine, ovine, and porcine species infected with mycobacteria. Culturing of specimens was the definitive test for the presence of mycobacteria in these animals; however, serological methods have merit for the purposes of improving management of infected herds and preventing the purchase of infected animals by buyers.

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