Comparison of Six Methods for Isolating Mycobacteria from Swine Lymph Nodes

CHARLES O. THOEN, WILLIAM D. RICHARDS, AND JERALD L. JARNAGIN

Diagnostic Bacteriology Section, Veterinary Services Laboratories, Animal and Plant Health Inspection Service, Ames, Iowa 50010

Received for publication 19 November 1973

Six laboratory methods were compared for isolating acid-fast bacteria. Tuberculous lymph nodes from each of 48 swine as identified by federal meat inspectors were processed by each of the methods. Treated tissue suspensions were inoculated onto each of eight media which were observed at 7-day intervals for 9 weeks. There were no statistically significant differences between the number of Mycobacterium avium complex bacteria isolated by each of the six methods. Rapid tissue preparation methods involving treatment with 2% sodium hydroxide or treatment with 0.2% zephiran required only one-third to one-fourth the processing time as a standard method. There were small differences in the amount of contamination among the six methods, but no detectable differences in the time of first appearance of M. avium complex colonies.

Certain routine mycobacteriological examination methods require elaborate and expensive equipment and are laborious. An increased interest in obtaining rapid diagnostic methods for isolating mycobacteria from animal tissues has resulted from recent changes in Meat and Poultry Inspection Program regulations for handling swine carcasses suspected of being tuberculous. In this report, six different laboratory methods were compared for isolating mycobacteria from tuberculous swine lymph nodes identified by federal meat inspectors at an abattoir in Nebraska.

MATERIALS AND METHODS

Tuberculous lesions in cervical and mesenteric lymph nodes were identified by federal meat inspectors. These were removed entirely or in part from swine carcasses and placed in jars containing a saturated solution of sodium borate for transportation to the laboratory. Upon arrival at the laboratory, each specimen was removed from the sodium borate solution; the fat was removed with sterile instruments and the node was placed in a dilute hypochlorite solution (0.1% NaOCl, wt/vol) for 16 h to control surface contamination.

The following six methods of decontamination were used for preparing tissue suspensions.

Direct inoculation (DI). About 0.2 g of the suspected tuberculous lymph node was removed and submerged in 1.5 ml of 0.2% zephiran for 15 min; it was then transferred to a petri dish and crushed with sterile forceps, and 1.0 ml of nutrient broth was added. Two drops of the tissue suspension was used to inoculate each medium slant.

The remainder of the lymph node was ground in a tightly closed screw-cap blender jar for 2 min, and then 50 ml of nutrient broth (Difco no. 0003-01, Detroit, Mich.) containing 0.4% phenol red indicator was added. Samples of the remaining tissue suspension were processed as follows.

NaOH. Equal volumes of 2% sodium hydroxide and ground tissue suspension (1.5 ml of each) were placed in a sterile screw-cap centrifuge tube (20 by 125 mm) and allowed to stand for 20 min. The suspension was neutralized by adding 1.25 N hydrochloric acid, mixed by hand shaking, and then allowed to sediment for 5 min. Media were then inoculated with 0.2 ml of the supernatant fluid.

NaOH plus centrifugation. This method included the treatment utilized in the NaOH method plus centrifugation of the neutralized suspension at 2,000 rpm in an international model UV centrifuge with a no. 266 rotor (1,000 × g) for 15 min. The supernatant fluid was discarded, and the sediment was suspended in 1 ml of nutrient broth. The suspension (0.2 ml) was used to inoculate each medium slant.

Zephiran treatment (Z). Equal volumes (1.5 ml of each) of ground tissue suspension and 0.2% zephiran solution were placed in a test tube (20 by 125 mm), mixed thoroughly by hand shaking, and allowed to stand for 15 min. Media were inoculated by using 0.2 ml of material from the central portion of this tissue suspension.

Papain-zephiran (PZ). Papain (100 ml; Nutritional Biochemicals Corp., Cleveland, Ohio) was added to the remaining tissue suspension of about 45 ml. Sufficient sodium hydroxide was added to change the indicator from yellow to pink. The suspension with a Teflon stirring bar was placed on a magnetic stirrer at 23 C for 30 min. Equal volumes of this suspension and 0.2% zephiran (1.5 ml of each) were
mixed and allowed to stand for 15 min. The suspension was thoroughly mixed, and each medium slant was inoculated by using about 0.2 ml of the suspension.

Papain-pentane-zephiran (PPZ) (6). The remaining tissue suspension prepared for PZ treatment was allowed to digest for an additional 30 min, 10 ml of pentane was added, and the contents were mixed by hand and allowed to stand for 30 min. The pentane layer was removed with a sterile pipette and filtered through sterile muslin in a funnel. The filtrate was then centrifuged for 20 min at 1,000 \textit{g}. Nutrient broth (2 ml) was added to the sediment and the contents were shaken vigorously. Equal volumes of 0.2% zephiran and the suspension were mixed and allowed to stand for 15 min. Each medium slant was inoculated with about 0.2 ml for the suspension.

Eight medium slants, including two tubes of Lowenstein-Jensen medium, two tubes of Stonebrink medium, one tube of Herrold egg yolk medium, one tube of Herrold egg yolk medium with malachite green, one tube of Herrold egg yolk medium with mycobactin (2 µg/ml), and one tube of Middlebrook 7H10 medium were inoculated with material prepared by each of the tissue preparation methods.

The media were incubated at 37°C and examined at 7-day intervals for 9 weeks. Smears of colonies were stained with carbol-fuchsin for detection of acid-fast bacilli.

Acid-fast bacilli were identified by their growth rate, colonial characteristics, and susceptibility to the following compounds (6): thiophene-2-carboxylic acid hydrazide, 10 µg/ml; streptomycin, 2 µg/ml; isonicotinic acid-hydrazide, 10 µg/ml; neotetrazolium chloride, 25 µg/ml; rifampin, 0.25 µg/ml. Rapidly growing mycobacteria were identified by their growth rate on subculture (1 to 6 days) and by their growth on Lowenstein-Jensen media containing 5% sodium chloride at 28 days (5). Serological identification of the mycobacteria was done by the agglutination method of Schaefer (7).

**RESULTS**

The results of mycobacteriological examinations are presented in Table 1. Colonies were initially rounded and white and usually appeared buff colored after 5 weeks of growth. Cultures of slowly growing nonphotocromogenic mycobacteria were isolated from 36 of the 48 specimens. These cultures were subsequently identified as Mycobacterium \textit{avium} complex bacteria (8, 9) by their resistance to chemical compounds. Serological tests on these 36 strains revealed that 12 were serotype 1, 19 were serotype 2, 3 were serotype 3, 1 was serotype 13, and 1 was serotype 17. Rapidly growing mycobacteria were isolated from 8 of the 48 specimens from which no other acid-fast bacilli were isolated.

A comparison of the efficacy of each of the six methods for isolating \textit{M. avium} complex from swine lymph nodes and the time required for processing specimens by each of the methods are shown in Table 2. The number of \textit{M. avium} complex isolations made varied from 31 to 34 for each of the six methods. These numbers were not significantly different as determined by the chi-square test \((P = 0.75)\). In no instance were all 36 of the \textit{M. avium} complex isolations made by any one method.

The time required for processing tissue specimens by each of three rapid methods in order of decreasing efficiency was: (i) DI, (ii) Z, and (iii) NaOH. The time saved by the rapid methods as compared to the PPZ method was 72% for the DI method, 66% for Z method, and 60% by the NaOH method.

As a group, the three most rapid methods (NaOH, Z, and DI) had significantly fewer contaminated media slants than tissue suspensions prepared by the PPZ method (Table 3). The computed chi-square value (1 degree of freedom) was 9.49, indicating that a real difference was highly probable. Herrold egg yolk medium slants were generally contaminated.

### Table 1. Mycobacterial isolations from tuberculous lymph nodes of 48 swine

| Mycobacteria isolated | No. |
|-----------------------|-----|
| \textit{M. avium} complex |     |
| Serotype 1 | 12 |
| Serotype 2 | 19 |
| Serotype 3 | 3  |
| Serotype 13 | 1  |
| Serotype 17 | 1  |
| Rapid growers | 8  |

### Table 2. Comparison of number of isolations and time required for six tissue preparation methods

| Method          | No. of \textit{M. avium} complex isolated | Average time required for processing one specimen (min) | Time saved* (min) |
|-----------------|------------------------------------------|------------------------------------------------------|------------------|
| DI              | 31                                       | 8.0                                                  | 21.5 (72)        |
| NaOH            | 34                                       | 11.7                                                 | 17.8 (60)        |
| NaOH + centrifugation | 32                                       | 16.0                                                 | 13.5 (46)        |
| Z               | 34                                       | 10.0                                                 | 19.5 (66)        |
| PZ              | 33                                       | 17.0                                                 | 12.5 (42)        |
| PPZ             | 34                                       | 29.5                                                 | 0 (0)            |

* A total of 36 Mycobacterium \textit{avium} complex isolations were obtained by using all six methods.

* Comparison to PPZ technique. Numbers in parentheses are percentages.
These benefits include PPZ 20 medium; Hg, Herrold egg yolk medium; NaOH + centrifugation. Malachite green; Hm, Herrold egg yolk medium plus malachite green plus mycobactin.

More frequent than other media, but considerable variation among all media was apparent.

Isolation failures for the different culture media and processing methods are presented in Table 4. Isolation failures occurred more frequently on Middlebrook 7H10 medium than on the other media. The computed chi-square value was 20.8, which can occur by chance less than 5 times per 1,000. Isolation failures among the other five methods fluctuate within chance variation. The largest number of M. avium complex isolation failures (47/66) recorded on 7H10 medium was for specimens treated with zephiran. Isolation failures were greater for the PZ method than for the other five methods. However, there was not sufficient evidence in these data to establish a true difference among the six methods studied.

The time of first appearance of the M. avium complex colonies ranged from 2 to 9 weeks for the six different methods (Table 5). Five weeks after inoculation, 97% of the specimens treated by the NaOH method were culture positive, 93% of the specimens treated with the Z method were positive, and 89% of the specimens processed by the DI method were positive. The observed differences in colony appearance at 5 weeks are within the limits of random variation, as determined by a chi-square test.

**DISCUSSION**

The rapid methods described in this report provide a marked reduction in processing time, varying from 42 to 72% of that of PPZ method currently in use in this laboratory. These benefits were achieved with no detectable increase in contamination or significant reduction in isolation rate. The mycobacterial isolation rates from tuberculous swine lymph nodes for each of the six different processing methods were in close agreement with reports using other accepted procedures (1–3).

Differences have been reported between the total number of mycobacterial isolations obtained on egg medium as compared to agar medium when mycobacteriologic specimens are treated with zephiran (4). Isolation failures on agar medium have been attributed to a carry-over of traces of zephiran in the inoculum which is inactivated by the lecithin present in egg medium. The data presented herein comparing the number of M. avium complex isolation failures on Middlebrook 7H10 media with those observed on egg containing media support this finding.

Only small amounts of tissue (0.2 g) or tissue suspension (1.5 ml) were processed by rapid methods in comparison to the amount of tissue suspension (40 ml) used in the PPZ method, yet the isolation rates for the rapid methods were not significantly decreased as compared to the rate for the PPZ method.

**TABLE 3. Comparison of the number of contaminated media slants for six different tissue preparation methods**

| Method       | Culture medium* | | | | |
|--------------|-----------------|---|---|---|---|
|              | LP              | ST | 7H10 | Hg | Hm | Hy |
| DI           | 2               | 1  | 2    | 4  | 2  | 5  |
| NaOH         | 2               | 2  | 3    | 2  | 3  | 9  |
| NaOH + centrifugation | 1   | 1  | 3    | 2  | 2  | 6  |
| Z            | 5               | 4  | 1    | 1  | 5  | 4  |
| PZ           | 2               | 0  | 0    | 2  | 1  | 3  |
| PPZ          | 10              | 3  | 5    | 7  | 6  | 6  |

* Total number of trials was 1,728. LP, Lowenstein-Jensen medium; ST, Stonebrink medium; 7H10, Middlebrook 7H10 medium; Hg, Herrold egg yolk medium; Hm, Herrold egg yolk medium plus malachite green; Hy, Herrold egg yolk medium plus malachite green plus mycobactin.

* Mean value for two tubes.

**TABLE 4. Comparison of the number of isolation failures for M. avium complex on six different media by six different tissue preparation methods**

| Method       | Culture medium* | | | | |
|--------------|-----------------|---|---|---|---|
|              | LP              | ST | 7H10 | Hg | Hm | Hy |
| DI           | 4               | 5  | 5    | 4  | 4  | 4  |
| NaOH         | 5               | 5  | 5    | 6  | 7  | 7  |
| NaOH + centrifugation | 8   | 7  | 7    | 6  | 5  | 9  |
| Z            | 5               | 4  | 4    | 17 | 6  | 4  |
| PZ           | 7               | 9  | 22   | 6  | 2  | 7  |
| PPZ          | 10              | 8  | 8    | 5  | 8  | 7  |

* Total number of trials was 1,728. For abbreviations, see footnote a, Table 3.

* Average value for two tubes.

**TABLE 5. Comparison of the first appearance of M. avium complex colonies on media for six different tissue preparation methods**

| Method       | Appearance time (days) |
|--------------|------------------------|
|              | 14 21 28 35 42 49 56 63 |
| DI           | 0 11 10 0 5 0 0 0       |
| NaOH         | 0 13 12 0 1 0 0       |
| NaOH + centrifugation | 1 11 6 7 2 2 1 2    |
| Z            | 2 12 7 10 0 2 0 1       |
| PZ           | 3 11 7 4 3 5 0 0       |
| PPZ          | 2 13 6 7 0 4 1 1       |
It is apparent from the data obtained in this study that a substantial financial savings can be realized by using one of the rapid techniques. Furthermore, it should be emphasized that the rapid methods described may be implemented in a diagnostic laboratory without the addition of expensive equipment.

ACKNOWLEDGMENTS

We are grateful to Merrill R. Swanson for the statistical analysis and to Margaret Champion and Wilma Gene Eacret for technical assistance. We also acknowledge the assistance provided by inspectors of the Meat and Poultry Inspection Program for collection of the tissue specimens.

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