NOTES

Modification of Schaefer’s Procedure for Serotyping of Organisms of the *Mycobacterium avium*-M. *intracellulare*-M. *scrofulaceum* complex

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Modifications to the tube-agglutination procedure which Schaefer developed for serotyping of organisms of the *Mycobacterium avium*-M. *intracellulare*-M. *scrofulaceum* complex are proposed.

Recent work (1, 3, 4, 6–9, 11, 12) suggests that the agglutination procedure with which Schaefer (10) discovered distinct serotypes among organisms of the *Mycobacterium avium*-M. *intracellulare*-M. *scrofulaceum* complex is the key to better understanding of the ecology of these organisms and the epidemiology of tuberculosis-like disease they cause in animals and man. Despite its potential, however, Schaefer’s procedure is not in widespread use. The reason for this is not clear, but one of the deterrents must be the fact that the procedure is involved, laborious, and expensive. We have found that minor modifications to Schaefer’s procedure reduce from 44 to 22 the number of hyperimmune rabbit antisera that need be prepared, reduce by 80% the expenditure of antisera and of bacterial suspensions of strains being identified, yet leave its capacity to distinguish the 22 serotypes of the *M. avium*-M. *intracellulare*-M. *scrofulaceum* complex almost unimpaired. This note describes the modified procedure and compares its effectiveness with the original.

One antiserum (instead of the 2 required by the original procedure) is prepared for each of the 22 serotypes. Rabbits are hyperimmunized with reference strains as described by Schaefer (10), but the bacterial suspensions that are used are prepared according to Yoder and Schaefer (13).

To determine the agglutinin titer, 0.1-ml volumes of progressive dilutions of antisera in phenolized phosphate-buffered saline (13) and equal volumes of homologous bacterial suspensions prepared in the same way as those with which rabbits are immunized are mixed in scratch-free tubes (7 by 50 mm) and incubated at 35 C for 20 hr. (The 0.1-ml volumes are one-fifth of the volumes used by Schaefer.) Agglutination is read in oblique fluorescent light against a black background in a darkened room. Agglutination that is complete or almost complete and is scored as 4 or 3+ on the scale used by Schaefer (10) is recorded as +; agglutination that is 2+ on Schaefer’s scale is recorded as (+); and the absence or virtual absence of agglutination scored as 0 or 1+ on Schaefer’s scale is recorded as –. The use of racks holding 25 agglutination tubes per row, 2 rows per rack, allows agglutination to be read at a glance.

Patterns of cross-agglutination that the antisera are likely to display when used at concentrations corresponding to four times their titer are mapped by screening the antisera at this concentration with bacterial suspensions of heterologous reference strains. The agglutination test is performed, read, and scored as described above. The antisera agglutinating heterologous strains with an intensity scored as + are then absorbed with the cross-reacting strains as outlined by Jenkins et al. (2). The antisera so treated usually become type specific at concentrations corresponding to four or eight times their titer prior to absorption.
Bacterial suspensions of strains to be identified are prepared in the same way as those of reference strains. Suspensions are first screened with unabsorbed antisera at concentrations corresponding to four times their titer. Strains that are agglutinated by two or more antisera with an intensity scored as + are then retested with the appropriately absorbed antisera at concentrations corresponding to four or eight times their titer prior to absorption. Agglutination tests are performed, read, and scored as described above.

To confirm the effectiveness of the modified procedure, we arranged that 80 strains previously serotyped by Schaefer’s procedure be presented to us as coded “unknowns” to be serotyped by the modified procedure. All known serotypes with the exception of serotype 3 were represented. Designations of strains that were included among the 80, their

### Table 1. Designation, code number, and serotype of strains used to verify ability of modified procedure to distinguish serotypes of the Mycobacterium avium-M. intracellulare-M. scrofulaceum complex

| Strains          | Serotype*   |
|------------------|-------------|
| Clancy*14(3), O’Neili25(34), ATCC15769(36) | 1           |
| McKenna*6(58), 16960-3309(70) | 2           |
| 17752-372(31), 14141-1395(44), Kirchberg*7 | 2           |
| Borne Iowa*16(26), 290-152*43 | 3           |
| 14604-1610*13, Keas*19, 1418-1426*60 | 4           |
| Cheltenham 13528-1072(44), Warsik*25 | 5           |
| 3259-685*19, 5668-49*52 | 5           |
| Russell 916*13, Peterson 17673*56 | 6           |
| 12315*67, Sweetman*77 | 6           |
| QingP22*3, P21P2*4, Manten 157*28, QP*10*50, Tolbert AT21*66 | 7           |
| QP244*11, Flannigan*5, 2219*29, Altmann | 7           |
| QP*4*35, Melnick*61 | 7           |
| Newberry*20, Findley*26, 6450-204*75 | 7           |
| QP190*1, P39P2, J2970*45, P57*45 | 7           |
| QP*22*64 | 7           |
| Lynn H*6* | 7           |
| QingP234*1,3, Huntley*27, WS52*41 | 8           |
| P23*1, P21P2*7, Stacey*37, 14658-1680*59, S.J. Bull No. 2*70, Mafgen*58 | 8           |
| P42*15, QP*10*24, Kovacs 4549*47 | 8           |
| Kerr*1, T501*51, 12305-406*69. | 8           |
| 6450-204*75 | 8           |

| Dunstan*1*30, Prasser*1*53, Connell*53 | 9           |
| Mayo*11*11, Gamoule*16, Matschloske*58 | 9           |
| Yandle*12 | 9           |
| Laidlaw*12, Retchford*17, Smith*132 | 9           |
| Guise*40, Brooks*71, Eckhardt*74 | 9           |
| Young*6, Le Rue*21, Lunning*55 | 9           |
| Bridge*57, P29*60 | 9           |
| McKenzie*10, QP10*25, Strong*49 | 9           |
| Cox*68, P58*79 | 9           |

* Serotype as determined by the procedure described by Schaefer (10).

* Strain from the collection of the Tuberculosis Section, Laboratory of Pathology and Microbiology, Queensland Department of Health, Brisbane.

* Number in parentheses is the code number by which strains were known while their identification by the modified procedure was in progress.

* Strain from the collection of W. B. Schaefer, National Jewish Hospital and Research Center, Denver, Colo.

* Strain isolated by the senior author from lymph nodes of pigs.

* Strain serotyped by us using the procedure Schaefer described and antisera he provided; the other 67 not marked with this symbol were serotyped by Schaefer.

* Strain from the collection of H. Saito, Hiroshima University School of Medicine, Hiroshima.

* Nontypable.

### Table 2. Patterns of cross-agglutination displayed by antisera prepared and serotype of strains with which absorption of the cross-agglutinating antisera was made

| Antiserums | Reference strains against which antisera were prepared | Serotype of heterologous strains agglutinated by antisera | Serotype of strains with which antisera were absorbed |
|------------|------------------------------------------------------|--------------------------------------------------------|-----------------------------------------------------|
| 1          | McKenna*6(36)                                       | 2, Gause*1                                           | 2, Gause*1                                         |
| 2          | 17752-372                                           | 1                                                     | 1                                                     |
| IIIb       | Borne Iowa*16                                        | 1                                                     | 1                                                     |
| IV         | Waisik                                               | 1                                                     | 1                                                     |
| V          | 3259-685                                             | 1                                                     | 1                                                     |
| VII        | Manten 157                                           | Chance                                                | Chance                                               |
| Altman     | Lunning, Scrofulaceum                                | Lunning, Scrofulaceum                                 | Lunning, Scrofulaceum                                 |
| Davis      | S.J. Bull No. 2                                     | 1                                                     | 1                                                     |
| Darden     | WS52                                                 | 1                                                     | 1                                                     |
| Wilson     | P42                                                  | Chance                                                | Chance                                               |
| Yandle     | Wilson                                               | Wilson                                                | Wilson                                               |
| GAUSE      | Guise                                                | 1                                                     | 1                                                     |
| LANDING    | La Rue                                               | 1                                                     | 1                                                     |
| SJ. BULL   | 12305-406                                           | 1                                                     | 1                                                     |
|橋       | 6450-204                                             | 1                                                     | 1                                                     |

* At concentrations corresponding to four times their titer.

* Strain isolated at the Tuberculosis Section, Laboratory of Microbiology and Pathology, Queensland Department of Health, Brisbane, and serotyped by W. B. Schaefer, National Jewish Hospital and Research Center, Denver, Colo.; the other 20 not marked with this symbol were from the collection of W. B. Schaefer.
### Table 3. Agglutination* of test strains by unabsorbed antisera

| Test strains | Antisera at concentrations corresponding to four times their titer | Serotype |
|--------------|---------------------------------------------------------------|----------|
|              | 1 | 2 | IIIa | IIIb | IV | V | VI | VII | Altmann | Boone | Chance | Darden | Davis | Howell | Watson | Wilson | Yandle | Gause | Lunning | Scrofulaceum |
| 12, 17, 32, 34, 40, 71, 74, 76 | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 1 or Gause |
| 31, 44, 46 | + | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 1 or 2 |
| 26, 43 | - | - | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | IIIa |
| 13, 18, 60 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | IIIb |
| 42, 65 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | IV |
| 19, 52 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | V |
| 56, 77 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | VI |
| 33, 67 | (+) | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | VII or Chance |
| 38, 50, 66 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | VII |
| 3, 4, 47 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | VII or Chance |
| 1, 9, 28, 35, 61 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | Altmann |
| 8, 21 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | Arnold or Lunning |
| 20, 39, 57, 62 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | Arnold or Scrofulaceum |
| 5, 29, 45, 48, 64 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | Boone |
| 15 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | Chance or Howell |
| 6 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | Chance or Howell |
| 23, 27, 41 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | Chance or Howell |
| 2, 7, 37, 59, 70, 78 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | Darden |
| 24 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | Davis |
| 22, 51, 69, 75 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | Howell |
| 30, 53, 80 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | Watson |
| 11, 73 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | Wilson |
| 16, 58, 72 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | Wilson or Yandle |
| 55 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | Yandle |
| 10, 25, 49, 68, 79 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | Lunning |

* Symbols: +, (+), and − indicate complete or almost complete agglutination, intermediate agglutination, and absence or virtual absence of agglutination, respectively.

* Nontypable.
### Table 4. Agglutination of cross-reacting test strains by absorbed antisera

| Test strains | Absorbed antisera at concentrations corresponding to four or eight times their titer prior to absorption | Control | Serotype |
|--------------|----------------------------------------------------------------------------------------------------|---------|----------|
|              | Gause (1)* | 1 (Gause) | 2 (1) | VII | Change (Howell) | Howell | Lunning | Arnold and Lunning | Arnold (Scrofulaceum) | Wilson (Yandle) | Yandle (Wilson) |
| 12, 17, 32, 40, 71, 74 | + | - | | | | | | | | | |
| 34, 76 | - | + | | | | | | | | | |
| 31, 44, 46 | - | - | + | - | | | | | | | |
| 3, 4, 47 | - | - | + | - | | | | | | | |
| 6 | - | - | - | + | - | | | | | |
| 15 | - | - | - | - | | | | | | |
| 8, 21 | - | - | - | - | | | | | | |
| 20, 39 | - | - | - | - | | | | | | |
| 57, 62 | - | - | - | - | | | | | | |
| 73 | - | - | - | - | | | | | | |
| 11 | - | - | - | - | | | | | | |

* Symbols: + and – indicate complete or almost complete agglutination, and absence or virtual absence of agglutination, respectively.
* Names and numbers in parentheses refer to the serotype of the strains with which the antisera were absorbed.
* The control consists of a 0.1-ml volume of bacterial suspension of test strain and an equal volume of phenolized phosphate-buffered saline.
serotype, and the code number by which they were known while serotyping was in progress are shown in Table 1.

Designations of reference strains against which antisera were prepared, patterns of cross-agglutination that the antisera displayed, and the serotype of strains with which absorption was made are shown in Table 2. Our failure to obtain a reference strain for the recently discovered and rare serotype 3 (5) meant that no antiserum against this serotype could be prepared.

The agglutinability of 76 strains that remained in stable suspension is shown in Tables 3 and 4. (Strains 14, 36, 54, and 63 could not be serotyped since they were autoagglutinable.)

Comparison of results obtained by the modified procedure (Tables 3 and 4) with those previously obtained by the procedure described by Schaefer (Table 1) reveals only one discrepancy: we found strain 47 to belong to serotype VII, yet this strain designated Kovacs 4549 was received from Schaefer as a reference strain for serotype Howell.

The failure of the modified procedure to identify correctly 1 of the 76 strains that were serotyped appears to be a low price to pay for the concomitant saving in effort, time, and money.

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ADDITION IN PROOF

Strain Kovacs 4549, which we received from W. B. Schaefer as a reference strain for serotype Howell, has recently been re-examined by him and found to be serotype VII. The results obtained by the modified procedure are, therefore, in complete agreement with those of the original.

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