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Differentiation of *M. scrofulaceum* from Related Mycobacterial Species by Double–Diffusion Technique

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ABSTRACT

Isolation and differentiation of various species of mycobacteria are predominantly based on conventional, cultural and biochemical tests. Among the non-tuberculous mycobacteria, these tests are unable to differentiate *M. scrofulaceum* from *M. avium-intracellulare* (MAI) because of their identical biochemical characteristics. For this reason, one hundred mycobacterial strains suspected to be *M. scrofulaceum,* which had identical preliminary cultural and biochemical tests, were investigated by immunodiffusion technique. The standard sonicated antigens of *M. scrofulaceum* (22, 159, 1281) were used to sensitize rabbits and to produce specific antisera. The method was performed in plastic petri dishes containing agar and sodium azide. Antiserum was placed in the central well of the agar plate and the homologous ultrasonicates were in the peripheral wells. The results were observed one week later and the mycobacterial strains were divided according to the number of produced precipitation lines. Four different groups were identified on the basis of identical reaction with three antisera (three groups were *M. scrofulaceum*). Group 1 produced identical precipitation lines with antisera 22 and 1281, group 2 with antisera 22, 159 and 1281 and group 3 with antiserum 1281. The present study showed that the technique was able to differentiate *M. scrofulaceum* from MAI and was also able to identify the bacterium as distinct species. Moreover, by using different specific antisera, it is possible to determine different serotypes of *M. scrofulaceum*. *Iran. Biomed. J. 4: 113-116, 2000*

Keywords: Double diffusion, lymphadenitis, *M. scrofulaceum, M. avium* complex

INTRODUCTION

Environmental mycobacteria were ignored by medical microbiologists in the past because they had little virulence for guinea pigs and rabbits, which were used experimentally to differentiate virulent mycobacteria from saprophytes [1]. During the past 40 years, it has become evident that tuberculosis-like diseases due to mycobacteria other than tubercle bacilli occur more frequently than earlier assumed [2]. Members of *M. Mycobacterium avium, M. intracellulare* and *M. scrofulaceum* are among the most common non-tuberculous mycobacteria recovered from human diseases [3, 4]. *M. scrofulaceum* is a slow-growing, pigmented scotochromogen that causes cervical lymphadenitis in children and also pulmonary diseases [5, 6]. In recent years, there have been some reports of *M. scrofulaceum* isolates from lymphadenitis in children and adults [7].

M. scrofulaceum is phenotypically similar to strains of *M. avium* and *M. intracellulare* [8]. Some researchers suggested that *M. scrofulaceum* could be classified as a member of *M. avium* complex because of the variable pigmentation of the species, the similarity of biochemical and cultural properties and the surface antigens [9]. In addition, study of mycobacterial lipids by thin-layer chromatography led to the conclusion that organisms classified as *M. scrofulaceum* should be regarded as the pigmented types of *M. intracellulare* [10]. On the other hand, *M. avium* and *M. scrofulaceum* were found to have distinguishing species specific antigens by immunodiffusion analysis [11, 12]. This technique was used for differentiation of *M. avium-M. intracellulare* strains of diverse origins [13]. The method was able to separate the strains into three groups based on antigenic relationship. Immunodiffusion has a considerable value in species discrimination within the genus. Because of the difficulty in differentiating between *M.*

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scrofulaceum and *M. avium* [9, 14], we used immunodiffusion technique to discriminate these two species.

MATERIALS AND METHODS

Strains. We have used one hundred strains of non-tuberculous mycobacteria suspected to be *M. scrofulaceum*. The strains had different origins and had isolated from patients with lymphadenitis, HlV and Crohn's diseases referred to University Hospitals of London and Cardiff, Wales. The isolates were kept frozen.

Preliminary identification. Most of the strains were identified as *M. scrofulaceum* based on preliminary cultural and biochemical screening and identification tests according to protocol in use at TB reference unit, PHLS, Dulwich Hospital, London [15]. According to these tests, 11 tests were found to be non-*M. scrofulaceum* strains and eliminated from the study. Thin layer chromatography of lipids and pigments were performed later on according to the previously described procedure [10].

Preparation of sonicated antigen. *M. scrofulaceum* was grown on Sauton's medium solidified with 1% agar at 37^{o}C for 3-4 weeks. Harvested organisms were suspended in PBS (pH 7.4) and sonicated in a 100-watt ultrasonic disintegrator for 15 minutes with the wave peak distance set at 6-8 um. The sonicated antigens were stored at –20^{o}C.

Immunizing. Reference strains of *M. scrofulaceum* were used for immunization of rabbits. These were strains 41 (*scrofulaceum*), 42 (*lunning*) and 43 (*gause*) and renamed as 22, 159 and 1281 respectively according to the identification list of UCL department of Microbiology Culture Collection, where the strains were selected. About 6 ml of each standard concentrated sonicated antigen was emulsified with an equal volume of Freund's incomplete adjuvant. One ml of emulsion was injected intramuscularly into each hind leg of 2 rabbits weekly up to 6 weeks. The rabbits were bled two weeks after the last injection. The level of the antiserum was assessed in an immunodiffusion test.

Double diffusion test. The immunodiffusion test was performed according to the procedures described [16, 17]. Briefly, a petri dish containing a layer of 4-5 mm of 1% agar + 1% sodium azide in distilled water (25 ml in each plate) was used. The pH of the agar was 7.0 to 7.2. Wells were cut with a Shandon No. 1812 agar-gel cutter (central well with 6 peripheral wells).

Anti-serum was placed in the central well and the homologous standard ultrasonicates were placed in the top, lower right and lower left wells. Ultrasonicates of the strains under examination were placed in the remaining three wells, so that heterologous sonicated antigens alternated with homologous sonicates and a direct comparison could be made between the precipitation patterns of the known and the unknown strains. Plates were left at room temperature overnight (without lids) for drying and then diffusion was allowed to take place in a moist chamber at room temperature for 7 days. The number of precipitation lines formed by each strain was then observed with a bench light. The plates were photographed against a dark background. The results were analysed and the reactions between antisera and different sonicated antigens were demonstrated.

RESULTS

The mycobacterial strains were divided according to the number of produced precipitation lines. Most of the lines were shared by the majority of the strains. All strains were found to contain at least 5-6 demonstrable antigens when tested against antisera raised to themselves or to the strains of the same species. Four different groups were recognized on the basis of the reaction with three antisera which were tested (3 groups were *M. scrofulaceum* strains). The majority of the strains produced identical precipitation lines with antisera 22 and 1281 (group 1, Table1). Table 1 illustrates the different patterns of precipitation of strains belong to group 2, which showed identical precipitation patterns with antisera 22, 159 and 1281. Group 3 consisted of strains that only showed an identical pattern to antiserum 1281. Group 4 consisted of strains that were not identical with any of the other groups, and proved to be of other species.

Strains 22 (Fig. 1) had 5-6 common antigens with serotype 159 and scotochromogen groups and also to some of the *M. avium* complex group. Strain 159 was shown to have 5-6 common antigens with all *M. scrofulaceum* strains plus 2-3 common antigens with other scotochromogens and a few atypical

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strains (Fig.2). Strain 1281 showed the similar precipitation pattern as 22 (Fig. 3).

Table 1. Response of different strains to antisera against reference strains 22, 159 and 1281. Groups 1, 2 and 3 are *M. scrofulaceum* strains and group 4 is non-*M. scrofulaceum* strain.

Sonicated antigens	Anti-sera			Number of strains
	22	159	1281	
group 1	+	-	+	54
group 2	+	+	+	3
group 3	-	-	+	2
group 4	-	-	+	30

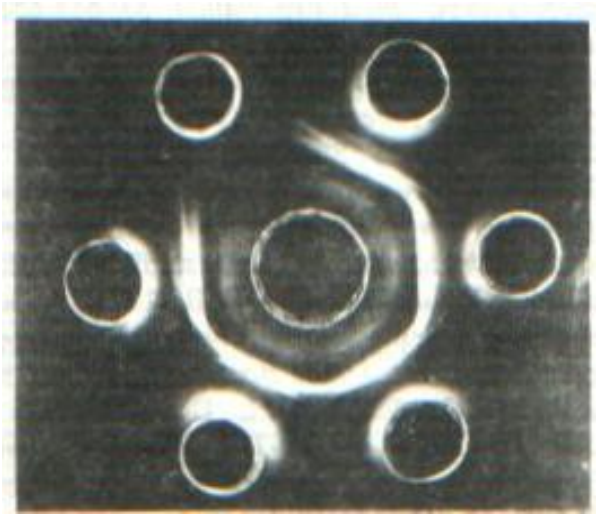


Fig. 1. Double diffusion precipitation test of antiserum to *M. scrofulaceum* strain 22 with homologous extract (top, lower right and lower left wells). Extracts of some test strains belongs to cervical lymphadenitis' patients are located in the rest of the wells.

DISCUSSION

Most of the strains under examination had identical pigmentation, cultural and biochemical identification patterns and thin layer chromatography of lipids and pigments could not significantly make boundary between these close species. So, double diffusion in agar was used for the final conventional identification of *M. scrofulaceum.* Results showed that the majority of strains fell into the group 1, which comprised 92.5% of the total numbers of *M. scrofulaceum* in this study. Group 2 and 3 represented minor groups of

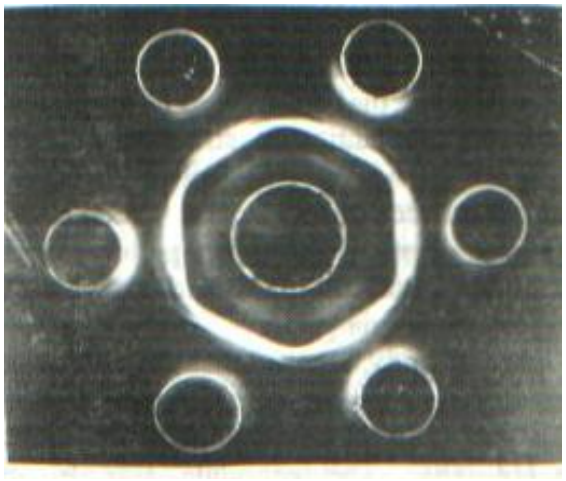


Fig. 2. Double diffusion precipitation test of antiserum to *M. scrofulaceum* strain 159 with homologous extract (top, lower right and lower left wells). Extracts of some test strains isolated from HIV seropositive patients are located in the rest of the wells. Note that the extract in the upper left well belongs to a non-*M. scrofulaceum* strain.

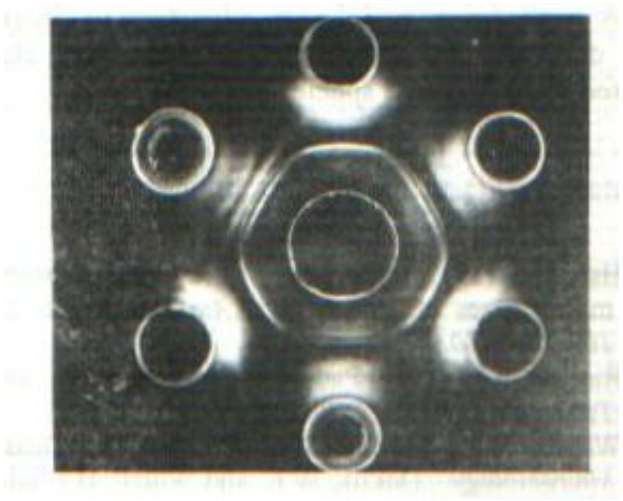


Fig. 3. Double diffusion precipitation test of antiserum to *M. scrofulaceum* strain 1281 with homologous extract (top, lower right and lower left wells). Extracts of some test strains isolated from Crohn's disease patients are located in the rest of the wells.

bacteria which consisted of about 4% and 3.5% of the total numbers of *M. scrofulaceum* respectively. Double diffusion enabled all *M. scrofulaceum* strains to be identified and this is due to the high sensitivity of the method.

The results confirmed that each of the three reference strains of *M. scrofulaceum* examined had a distinctive and well-defined antigenic pattern. There was a close similarity in antigenic structure

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between the different strains within each group. No difference could be demonstrated in serological patterns and in cultures that had been maintained in the collection for many years, freeze-dried strains or recently isolated cultures of the same species. There was no evidence that differences of origin had any influence on the antigenic structure of different strains of *M. scrofulaceum.* Strains isolated from cervical lymphadenitis, HIV and Crohn's disease patients gave identical serological patterns that were in agreement with the antigenic analysis of the reported mycobacteria [17].

The present study, showed that the technique was able to differentiate *M. scrofulaceum* from MAI complex and this was in agreement with previous studies [11, 18]. The technique was also able to identify *M. scrofulaceum* as a clear-cut separate species. Although at the time of study, there were no specific primers available for *M. scrofulaceum*, we have used PCR-RFLP technique for confirmation of the results, which the results of fingerprinting were in agreement to double diffusion analysis [15].

Application of further modern techniques such as DNA sequencing will be of advantage to clearing the difficulty of differentiating these very close related mycobacterial species.

REFERENCES

1. Hsu, H.S. (1971) The fate of *M. tuberculosis* within macrophages of guinea pigs. *Am. Rev. Respir. Dis. 103: 607-610.*
2. Runyon, E.H. (1965) Pathogenic mycobacteria. *Adv. Tuberculosis Res. 14: 235-289.*
3. Willett, H.P. (1992) Mycobacterium. In: *Zinsser Microbiology*. (Joklik, W.K. and Willett, H.P. eds.), 20. N.Y.: Appleton and Lange. pp. 497-525.
4. Fogan, L. (1970) Atypical mycobacteria: their clinical, laboratory and epidemiologic significance. *Medicine (Baltimore*). *49: 243-253.*
5. Prissick, F.H. and Masson, A.M. (1956) Cervical lymphadenitis in children caused by chromogenic mycobacteria. *Canad. M.A.J. 75: 798-803.*
6. Prissick, F.H. and Masson, A.M. (1957) Yellow- pigmented pathogenic mycobacteria from cervical lymphadenitis. *Can. J. Microbiol. 3: 91-100.*
7. Pang, S. (1992) Mycobacterial lymphadenitis in western Australia. *Tuber. Lung. Dis. 73: 637-649.*
8. Hawkins, J.E. (1977) Scotochromogenic mycobacteria which appear intermediate between *Mycobacterium avium-intracellulare* and *Mycobacterium scrofulaceum. Am. Rev. Respir. Dis. 116: 963-964.*
9. Wolinsky, E. (1979) Non-tuberculosis mycobacteria and associated diseases. *Am. Rev. Respir. Dis. 119: 107-159.*
10. Jenkins, P.A. and Marks, J. (1972) Thin-layer chromatography of mycobacterial lipids as an aid to classification: the scotochromogenic mycobacteria including *Mycobacterium scrofulaceum, M. xenopi, M. aquae, M. gordonae* and *M. flavescens. Tubercle 53: 118-127.*
11. Gunthorpe, W.J. and Stanford, J.L. (1972) A study of *Mycobacterium gordonae* and *M. marianum (scrofulaceum*). *Br. J. Exp. Pathol. 53: 665-671.*
12. Stanford, J.L. and Grange, J.M. (1974) The meaning and structure of species and applied to mycobacteria. *Tubercle 55: 143-152.*
13. McIntyre, G. and Stanford, J.L. (1986) The relationship between immunodiffusion serotypes of *Mycobacterium avium* and *Mycobacterium intracellulare. Eur. J. Respir. Dis. 69: 135-141.*
14. Wolinsky, E. and Schaefer, W.B. (1973) Proposed numbering scheme for mycobacterial serotypes by agglutination. *Int. J. Syst. Bacteriol. 23: 182-183.*
15. Khosravi, A.D., Stanford, J.L., Donoghue, H.D. and Rook, G.A.W. (1997) Variation within *Mycobacterium scrofulaceum. J. App. Microbiol. 83: 596-602.*
16. Stanford, J.L. and Gunthorpe, W. J. (1971) A study of some fast-growing scotochromogenic mycobacteria. *Br. J. Exp. Pathol. 52: 627-637.*
17. Stanford, J.L. and Beck, A. (1968) An antigenic analysis on the mycobacteria: *M. fortuitum, M. kansasii, M. phlei, M. smegmatis* and *M. tuberculosis. J. Patho. Bacteriol. 95: 131-139.*
18. Stanford, J.L. (1969) The double diffusion precipitation test in the classification of mycobacteria. [M.D.] London, UK.

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