Unclassified Mycobacteria Isolated from Human Suspect Tuberculosis Cases in Newfoundland:

Preliminary Studies on Fifteen Strains

R. W. BUTLER, M.Sc., Dip.Bact.* and
J. E. JOSEPHSON, M.D., C.M., B.Sc.(Med.), F.A.C.P.,†
St. John's, Nfld.

ABSTRACT

Unclassified mycobacteria were isolated from 36 of 35,555 clinical specimens cultured for *M. tuberculosis*. The majority of isolations were from patients suspected of having tuberculosis and from whom repeat attempts at culture failed to yield typical tubercle bacilli. Fifteen strains thus far studied were not capable of causing generalized tuberculosis in guinea pigs, and all were highly resistant to the commonly employed antituberculous therapeutic agents. Eleven of the 15 strains were resistant to 100 or more μg./ml. of streptomycin; 12 strains were resistant to 25 or more μg./ml. of para-aminosalicylic acid; and all 15 showed growth in the presence of 50 or more μg./ml. of isoniazid. All strains were niacin-negative and catalase-positive. In the absence of other cultural findings, isolation of anonymous mycobacteria poses a major problem, especially in those cases in which the clinical and radiographic findings are typically those of tuberculosis.

IN THE past 10 years, acid-fast organisms now designated as unclassified mycobacteria have been detected with greater apparent frequency in cultures from human sources. Considerable interest has been aroused in this group, especially in regard to their exact relation to true human tubercle bacilli, their pathogenicity, and their sensitivity to antituberculous therapeutic agents. Scores of reports dealing with this poorly defined group of mycobacteria have already appeared in the current literature, and a variety of terms have been employed for them, including "atypical", "anonymous", "unclassified", "chromogenic", "nontuberculous acid-fast bacilli", "yellow bacillus" and "unidentified mycobacteria". Since most of the strains involved are pigment producers, a suggested method for classification and recognition is based on the ability of strains to produce pigment under certain conditions. Runyon,[1] who based his system of classification on pigment production and growth rate, introduced the terms *photochromogen, scotochromogen, nonphotochromogen,* and *rapid growers.* Alternatively he also used the designation "Group I", "II", "III" and "IV", respectively, in reference to the four types noted.

In addition to pigment production, other criteria that have been suggested thus far to aid in the differentiation of these unclassified mycobacteria from tubercle bacilli include various cultural characteristics and growth requirements, several biochemical tests, animal pathogenicity tests, sensitivity to therapeutic agents, immunological studies, mycobacteriophages, and special staining procedures. Many of the suggested tests give inconclusive variable results, and thus far we lack adequate methods for the rapid recognition of all strains within this unclassified group of mycobacteria.

Of primary importance is the fact that certain members of this poorly defined group can cause tuberculous-like disease in humans. A positive diagnosis of such infection, however, is most difficult because similar indistinguishable strains are often reported to be isolated from healthy and symptomless persons. With scanty evidence to suggest cross-infection among contacts, human infections caused by these organisms are generally not considered communicable.

Reports indicate a world-wide distribution of these organisms, and in certain areas the incidence of isolation has posed serious problems. Lewis *et al.*[2] reported the isolation of atypical mycobacteria from the sputum of approximately 2% of admissions to tuberculosis hospitals in the state of Florida.

Canadian reports concerning anonymous mycobacteria of human origin are relatively rare, but this does not necessarily mean that these organisms are uncommon throughout Canada. Information regarding anonymous mycobacteria isolated in Canada from clinical specimens has been included in the reports of Prissick and Masson,[3-5] Magnus,[6] Hnatko,[7] Mankiewicz,[8] Boyd, Roy and Craig,[9] and Butler and Josephson.[10]

Chromogenic mycobacteria were first isolated in Newfoundland during 1953. In that year three strains were encountered, one from an abscess, one from an infected cervical lymph node, and one from a specimen of cerebrospinal fluid. All three cultures were observed to produce orange-

Presented in part at the 29th Annual Meeting of the Canadian Public Health Association, Laboratory Section, Toronto, Ont., December 5, 1961.

*Chief Bacteriologist, Laboratories, Department of Health, Newfoundland.

†Director of Laboratories, Department of Health, Newfoundland.

pigmented colonies when grown on Lowenstein-Jensen medium, and were non-pathogenic for guinea pigs.

Our interest was stimulated again during 1957 when four additional isolations were made from clinical specimens. In the four-year period 1957-1960, a total of 35,555 clinical specimens were cultured for *M. tuberculosis* in this laboratory. Of this total 1681 (4.7%) were positive, but an additional 36 (0.1%) yielded chromogens. These 36 isolations were made from 36 patients (Table I).

TABLE I.

Year	*Number of specimens cultured for M. tuberculosis*	*Number of specimens positive for typical M. tuberculosis*	*Number of specimens yielding growth of chromogenic mycobacteria*
1957.....	7888	373	4
1958.....	8832	514	10
1959.....	8942	417	7
1960.....	9893	377	15
	35,555	1681 (4.73%)	36 (0.10%)

The types of specimens from which chromogens were isolated are indicated in Table II.

TABLE II.—TYPES OF SPECIMENS YIELDING GROWTH OF CHROMOGENIC BACTERIA

Year	*Sputum*	*Urine*	*Gastric washing*	*Total*
1957.....	1	3	0	4
1958.....	6	3	1	10
1959.....	3	4	0	7
1960.....	13	2	0	15
	23*	12†	1‡	36

*Total sputa cultured during four years—24,560 (1124 positive for typical *M. tuberculosis*).

†Total urines cultured during four years—7185 (386 positive for typical *M. tuberculosis*).

‡Total gastric washings cultured during four years—615 (48 positive for typical *M. tuberculosis*).

During 1960, which covers the period of this study, 15 chromogens were isolated, 13 being obtained from sputum and two from urine. All 15 specimens were negative for acid-fast bacilli on primary microscopic examination.

The method employed in our tuberculosis laboratory is digestion of specimens with equal parts of 4% sodium hydroxide. Two tubes of Lowenstein-Jensen medium are each inoculated with two drops of concentrated specimen, using a Pasteur pipette. Inoculated media are first incubated at 37° C. for 48 hours in a slanted position and then for eight weeks in an upright position, and cultural readings are made weekly.

The rate of growth on primary culture was very similar to that normally observed for typical *M. tuberculosis*. Not one of the 15 strains of chromogens showed visible growth in less than four weeks, and in the case of five of the strains growth was not detected until the end of the eighth week of incubation. Unlike *M. tuberculosis*, colonies of all chromogens were smooth with entire edges. Growth was also easily suspended in saline or broth. Morphologically, the chromogens were indistinguishable from true tubercle bacilli.

Pigment production on Lowenstein-Jensen medium (37° C.) was of three varieties. One strain produced a brilliant lemon-yellow pigment when grown in either the light or in the dark. Colonies of 12 strains were buff-orange at first, followed by a deepening of the colour to orange with ageing of the cultures. Light did not appear to change the degree of colour. The third variety consisted of two strains which were also buff but did not change to orange with ageing.

When subcultured to Dubos serum broth, all strains grew within four days at 28° C., and within two days at 37° C. With one exception all subcultures to this medium grew within one day at 45° C. Strain No. 1, the lemon-yellow coloured strain, grew slowly at 45° C. Growth at 52° C. occurred with seven of the 15 strains. At this temperature the rate of growth, where occurring, was considerably slower than at lower temperatures (Table III).

To distinguish between "unclassified" mycobacteria and saprophytic mycobacteria, Tarshis[11, 12] suggested the use of thioglycollate medium without indicator (Brewer modified). In this medium saprophytic mycobacteria grow rapidly and luxuriantly within one day, with formation of a characteristic pellicle, whereas the unclassified mycobacteria grow slowly. None of our 15 strains grew rapidly, and none showed the characteristic pellicle described by Tarshis as typical for saprophytes. Our most rapid growth occurred in four days, the majority of strains showing growth only after prolonged periods of incubation. The slowest-growing strain grew in 45 days. Thioglycollate medium will not support the growth of *M. tuberculosis*.

Additional media employed to determine growth requirements and cultural characteristics of the chromogens included Hartley's broth, Loeffler's medium, infusion agar, blood agar and glycerol agar (nutrient agar with 7% glycerin). Results showed that 11 strains grew in Hartley's broth, five on Loeffler's medium and five on infusion agar. All 15 chromogens grew on blood agar within five days and on glycerol agar within 10 days (Table III).

Results for the peroxidase test,[12] the catalase test, the niacin test,[13] the serpentine cord test[12] and for animal pathogenicity, with a notation of the clinical status of the patients concerned, are given in Table IV.

Sensitivity tests were performed using Sauton fluid medium, the therapeutic agents being sterilized by filtration before being added to the sterile medium. As indicated in Table V, all 15 strains possessed marked resistance to one or more of the three antituberculous therapeutic agents employed.

TABLE III.—CULTURAL CHARACTERISTICS OF CHROMOGENIC MYCOBACTERIA

Culture No.	Source sputum (S), urine (U)	First appearance primary growth on L-J medium 37°C.	Pigment on L-J medium 37°C.		Growth in Dubos serum broth*				No. of days required for at least 1 + growth of subcultures at 37°C.					
			Grown in light	Grown in dark	28°C.	37°C.	45°C.	52°C.	Thioglycollate medium (Brewer modified)	Hartley's broth	Loeffler's	Infusion agar	Blood agar	Glycerol agar
1	S	8 weeks	LY	LY	3	2	15	—	19	11	29	—		
2	S	7 "	B-LO	B-LO	3	1	1	11	19	11	—	—		
3	S	8 "	B-LO	B-LO	3	1	1	—	45	11	—	—	All	All
4	S	6 "	O	B-LO	4	1	1	—	19	11	—	25	good	good
5	S	8 "	B-LO	B-LO	4	1	1	—	19	—	—	17	growth	growth
6	S	6 "	B-LO	B-LO	3	1	1	—	24	—	—	—	in	in
7	S	5 "	B-LO	S-LO	3	1	1	11	19	11	—	—	five	six
8	S	5 "	B-LO	B-LO	4	1	1	—	17	29	—	—	days	to
9	S	5 "	B-LO	B-LO	4	1	1	—	10	10	10	18	or	ten
10	S	5 "	B-LO	S-O	3	1	1	—	11	17	—	—	less	days
11	S	4 "	B	B	3	1	1	6	10	10	10	18		
12	S	8 "	O	B-LO	3	1	1	6	36	—	—	—		
13	U	8 "	B-LO	B-LO	3	1	1	6	36	—	—	—		
14	U	5 "	B-LO	S-O	2	1	1	17	36	29	18	—		
15	S	4 "	B	B	2	1	1	17	4	5	10	10		

Colour code: LY—Brilliant lemon yellow; B-LO—Buff-light orange; O-Orange; S-LO—Salmon-light orange; B—Buff.
Negative (—) indicates no growth in four weeks.
*Figures indicate the day growth was observed.

TABLE IV.—CHROMOGENIC MYCOBACTERIA ISOLATED FROM CLINICAL SPECIMENS

Culture No.	Peroxidase test	Catalase test	Niacin test	Serpentine cord test	Pathogenicity for guinea pig	Clinical status of patient
1	+	+	—	—	—	Bilateral bronchiectasis; left lower lobectomy March 1960
2	—	+	—	—	—	Bilateral moderately advanced pulmonary tuberculosis
3	—	+	—	—	—	No evidence of active tuberculosis (died May 1960—"heart condition")
4	—	+	—	sl. +	—	Moderately advanced pulmonary tuberculosis (chronic, stable)
5	—	+	—	+	*Abscess in omentum	Pneumonia January 1960. No evidence of pulmonary tuberculosis
6	—	+	—	—	†Abscess site of injection, omentum and diaphragm	Bilateral moderately advanced pulmonary tuberculosis (active, chronic)
7	—	+	—	+	—	Old inactive primary tuberculosis
8	—	+	—	—	—	Suspected tuberculosis by x-ray
9	—	+	—	sl. +	—	No evidence of active tuberculosis; healed primary, left side
10	—	+	—	—	†Abscess site of injection	Bilateral moderately advanced pulmonary tuberculosis (lobectomy 1957)
11	—	+	—	—	†Abscess site of injection and omentum	History not available
12	—	+	—	—	—	Tuberculosis-like lesion on x-ray examination
13	—	+	—	—	—	History not available
14	—	+	—	—	†Abscess in omentum	History not available ("chest neg." 1960)
15	—	+	—	—	—	Bilateral moderately advanced pulmonary tuberculosis, active

*AFB seen in smear. Organism recovered on culture.
†AFB seen in smear. Organism failed to grow on culture.

TABLE V.—CHROMOGENIC MYCOBACTERIA: RESULTS OF SENSITIVITY TESTS USING STREPTOMYCIN, PAS AND INH

Resistant to μg./ml. (Sauton medium)

Strain No.	*Streptomycin*								*PAS*							*INH*			
	5	*10*	*25*	*50*	*100*	*250*	*500*	*>750*	*10*	*25*	*50*	*100*	*200*	*300*	*>400*	*25*	*50*	*100*	*>200*
1		X							X								X		
2								X		X								X	
3					X					X								X	
4			X							X								X	
5					X					X									X
6			X							X									X
7					X					X									X
8					X					X									X
9								X				X							X
10	X								X									X	
11						X							X						X
12						X					X								X
13					X				X									X	
14					X					X									X
15								X							X				X

Eleven of the 15 strains (73%) were resistant to 100 or more μg./ml. of streptomycin. All 15 chromogens were resistant to levels of 10 or more μg. of para-aminosalicylic acid (P.A.S.)/ml. Exceedingly high levels of resistance were found toward isonicotinic acid hydrazide (INH), 14 of the strains showing growth in the presence of 100 or more μg./ml. of this therapeutic agent.

CONCLUSIONS

The isolation of anonymous mycobacteria from only 0.1% of all clinical specimens cultured for *M. tuberculosis* during the past four years suggests a rather limited distribution of these organisms in the province of Newfoundland. Thus far we have isolated members of the unclassified mycobacteria from a variety of human sources including a swab from an abscess, aspiration from an infected cervical lymph gland, cerebrospinal fluid, urine, sputum and gastric washings. In the current study attempts to reisolate the organism from repeat specimens from the 15 patients involved have been, with one exception, unsuccessful. This fact coupled with the observation of very light growth obtained in all primary cultures tends to weaken the evidence that these organisms are really the causative agent of a disease process. However, in the absence of the cultural findings, isolation of anonymous mycobacteria poses a major problem, especially in those cases in which the clinical and radiographic findings are typically those of tuberculosis.

Five of the 15 cases studied have had true *M. tuberculosis* isolated within the past 10 years, but in none of these 15 cases has a positive culture of *M. tuberculosis* been obtained during the past two years.

The overall significant yearly increase in the number of anonymous mycobacteria being reported may in part be attributed to recent alertness on the part of bacteriologists. We have no doubt that certain anonymous mycobacteria in the past were reported as *M. tuberculosis*, especially when the strains obtained were poorly pigmented and showed slightly rough colonies. Some such strains have recently been encountered in our laboratories and will be reported in a separate communication. There is no doubt either that other acid-fast chromogens of the deep yellow-orange varieties were discarded as mere saprophytes.

In practice, routine procedures should include investigative studies on all acid-fast bacilli that differ in their cultural and other characteristics from those of typical *M. tuberculosis*. Where facilities and experience with such organisms are limited, cultures should be submitted to a suitable reference laboratory.

REFERENCES

1. RUNYON, E. H.: *Med. Clin. N. Amer.*, **43**: 273, 1959.
2. LEWIS, A. G., JR. *et al.*: *Ann. Intern. Med.*, **53**: 273, 1960.
3. PRISSICK, F. AND MASSON, A. M.: *Canad. J. Public Health*, **43**: 34, 1952 (abstract).
4. *Idem*: *Canad. Med. Ass. J.*, **75**: 798, 1956.
5. *Idem*: *Canad. J. Microbiol.*, **3**: 91, 1957.
6. MAGNUS, M.: *Canad. J. Public Health*, **47**: 43, 1956 (abstract).
7. HNATKO, S. I.: *Canad. J. Microbiol.*, **2**: 39, 1956.
8. MANKIEWICZ, E.: *Ibid.*, **4**: 565, 1958.
9. BOYD, G. L., ROY, T. E. AND CRAIG, G. M.: *Amer. Rev. Resp. Dis.*, **84**: 123, 1961.
10. BUTLER, R. W. AND JOSEPHSON, J. E.: *Canad. J. Public Health*, **53**: 35, 1962 (abstract).
11. TARSHIS, M. S.: *J. Lab. Clin. Med.*, **54**: 630, 1959.
12. *Idem*: *Ibid.*, **57**: 480, 1961.
13. KONNO, K. AND SBARRA, A. J.: *Amer. Rev. Tuberc.*, **79**: 810, 1959.

INDEX TO VOLUME 87

Reprints of the Index to Volume 87, July-December 1962, published in the issue of December 29, 1962, are available for the convenience of readers who desire a separate copy. Requests should be addressed to: The Canadian Medical Association Journal, 150 St. George Street, Toronto 5, Ontario.