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Joseph Paul Truant

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A SURVEY OF TYPICAL AND ATYPICAL MYCOBACTERIA ISOLATED FROM HUMAN SPECIMENS

JOSEPH PAUL TRUANT, PH.D.

THE RE-DISCOVERY of the "atypical" *Mycobacteria* as causative agents of tuberculosis in man has presented several interesting facets. This topic has stimulated many clinicians to re-appraise the thesis that acid-fast organisms represent contaminants.[1, 2] This situation has been especially true in the instances wherein these so-called "unclassified organisms" have been isolated from several specimens[3] or from the same specimens by different laboratories.[2] Although the organisms are usually nonpathogenic for guinea pigs, definite evidence has been accumulated which demonstrates their ability to produce tuberculosis in man.[4-7] Additional emphasis has been placed on this disease entity by the clinicians because this group of acid-fast organisms is usually resistant *in vitro* and *in vivo* to the chemotherapeutic agents commonly used for the treatment of *Mycobacterium tuberculosis*.[3,8]

The interest of the bacteriologists obviously has paralleled the clinicians' interest in the occurrence and significance of these "atypical" acid-fast isolates.[3,9] The term "atypical" was used for these organisms to indicate that they had bacteriological characteristics (e.g. cultural) unlike *Mycobacterium tuberculosis*[9]. The relationship between the two is still uncertain. The author is in complete agreement with the views of those investigators who believe these organisms are human pathogens when two or more isolations of the same organism are obtained from a single patient.[10] This fact together with positive clinical findings constitutes significant evidence that the strains are pathogenic for man even though the isolates may be nonpathogenic for laboratory animals—even though Koch's Postulates are not fulfilled. This thesis as it applies to bacterial disease entities has been discussed at greater length in one of our more recent publications.[11]

MATERIALS AND METHODS

The survey has been in progress during the past two years. During this time we have examined approximately 7000 specimens. There have been 260 acid-fast cultures isolated, of which 18 atypicals (7.0 per cent) have been definitely proven by either two or three groups

* Department of Laboratories.

Table I

SPECIAL BBL MEDIA FOR CULTIVATION OF AFB*

1. Dubos Broth Base without Polysorbate 80	Fluid
2. Dubos Broth Base without Polysorbate 80 + OAC	Fluid
3. Dubos Oleic Acid Agar Base Agar concentration 1.5%	Solid
4. Dubos Oleic Acid Agar Base + OAC Agar concentration 1.5%	Solid
5. MD Medium with added glycerol + OAC Agar concentration 1.0%	Solid
6. TB Broth Base without Polysorbate 80 with added glycerol + OAC	Fluid
7. Middlebrook 7H10 Agar with added glycerol + OADC	Solid
8. TB Broth Base without Polysorbate 80 with added glycerol	Fluid
9. Middlebrook 7H10 Agar with added glycerol + OAC	Solid
10. Middlebrook 7H9 Broth with added glycerol + ADC	Fluid
11. Middlebrook 7H9 Broth with added glycerol + ADC	Fluid
12. Middlebrook 7H9 Broth with added glycerol, 1.5% agar	Solid
13. Middlebrook 7H9 Broth with added glycerol	Fluid

OAC is Oleic Albumin Complex BBL 03-671T
ADC is Middlebrook ADC Enrichment BBL 03-673T
OADC is Middlebrook OADC Enrichment BBL 03-672T
O = Oleic Acid
A = Albumin
D = Dextrose
C = Catalase (Beef)

*Dr. Harriet Vera, Baltimore Laboratory, Baltimore 18, Maryland, kindly supplied the special media (listed above) for culturing acid-fast organisms.

of investigators*. The methodology used by us in the isolation, identification and confirmation of the typical and atypical strains is reviewed in several references.[10, 12, 13]

The routine susceptibility tests were performed on Lowenstein-Jensen medium with the following drug concentrations incorporated: 0.2, 1,5 and 25 mcg. of isonicotinic acid hydrazide; 1.0,10 and 100 mcg. of paraminosalicylic acid; 10.0, 50 and 100 mcg. of streptomycin. Special tube dilutions series with other tuberculostatic drugs such as cycloserine and viomycin were performed on request. The amount of growth on the drug-containing medium was compared to the growth on the two control tubes (non-drug containing media). The reports were reported cn a chart (see Table VII) during a four week growth period.

Special enrichment media were prepared by Dr. Hariette D. Vera, Director of Quality Control Products, Becton, Dickinson and Company, Baltimore Biological Laboratory, Baltimore 18, Maryland (see Table I). Dr. Vera very kindly supplied the details for methods of preparation, etc. The media was incubated overnight as a check on sterility for rapid-growing bacterial strains. The growth promoting characteristics of these media were evaluated with eleven strains of typical and "atypical" *Mycobacteria* as well as *Nocardia*. Smears of the test cultures were also performed to check on the possibility of slow-growing contaminants which could not be recognized by macroscopic procedures.

Both guinea pigs and swiss mice (CF 100) were used for pathogenicity studies for many of the isolates under discussion. Our method consisted of subcutaneous injection 0.6 to 1.0 ml. of 5 mg./ml. into the inguinal area for the guinea pigs and intraperitoneal and intravenous 0.1 ml injections for the mice. Dr. Mallman's procedure[13] consisted of two guinea pigs which received intradermal injections of 0.1 ml. (1.0 mg. wet weight per ml. of Dubos-1 per cent dextrose broth) on the dorsal central surface. Each of two guinea pigs received 0.1 ml. intramuscularly in the left rear leg

* Dr. Virginia Mallman, Department of Microbiology, Michiagn State University and Dr. John Roberts of the Michigan Department of Health have given us very valuable assistance in the identification and/or confirmation of many of the typical and atypical acid-fast isolates.

The acid-fast organisms were inoculated into three different media in duplicate and growth patterns at incubation temperatures of 25°C and 37°C were compared. Dr. Mallman[13] conducted a more detailed survey and evaluated our atypical strains at the following incubation temperatures: 22°C, 30°C, 35°C and 45°C. The usual procedures[12] for the determination of growth rate and pigment production were observed. The cellular morphology was usually observed by the fluorescent technique[12] and occasionally by the Ziehl-Neelsen procedure[12].

The cultures were tested for aryl-sulfatase and niacin production by Dr. Mallman[13] and for catalase and neutral red by us using the methods described by Kubrica[10].

Results

A total of 3699 clinical specimens was submitted during 1962 for examination of acid-fast organisms. A significant number were positive in smears (7.4 per cent) and cultures (6 per cent) as shown in Table II. It can be seen that a larger percentage of positives were demonstrated by the auramine-rhodamine (A-R) technique (3.2 per cent) as compared to the culture technique (1.9 per cent).

Table II

ACID-FAST ISOLATES OBTAINED IN 1962

Total No. of Specimens	AFB positive by A — R*	AFB positive by culture**	Culture + A-R —	A-R + Culture —
3699	274	225	73	121
per cent	7.4%	6.0%	1.9%	3.2%

*A — R — Auramine-Rhodamine Technique[12]
**10 of the patients submitted specimens which yielded 37 (16.4%) "atypical" acid-fast cultures.

It should be emphasized that both methods should be employed since either procedure may fail to identify the acid-fast organisms as is amply demonstrated in Table III. Herein, one can see that there is a wide variation in the number of positives — ranging from 0-100 per cent by either smear and/or culture procedures. It was frequently necessary to examine multiple cultures (see No. 18, Table III) in order to firmly establish the bacteriologic diagnosis of "atypical" tuberculosis. This same occurence was occasionally necessary for the identification of typical acid-fast organisms such as *Mycobacterium tuberculosis*. It is virtually impossible to establish any degree of confidence when only one specimen is positive by one of the two methods (see Table III) even though multiple specimens have been submitted. Occasionally the diagnosis of tuberculosis due to "atypical" organisms is made much easier by the identification of the organisms in smears and cultures of several specimens (see No. 10, Table III).

The "atypical" strains of *Mycobacteria* resembled the typical tubercle bacilli in smears prepared by the auramine-rhodamine (A-R) or Ziehl Neelsen (ZN) techniques. The former frequently grow at the same rate as *M. tuberculosis* in primary cultures but differ in that the colonies were usually smooth and possibly pigmented on American Trudeau Society medium, Lowenstein-Jensen and Petragnani.

An attempt to find a medium which would be superior to those discussed above included an evaluation of the media listed in Table 1. A cross-section of the results obtained with five "atypical" *Mycobacteria* is shown in Table IV with one of these

Table III

SUMMARY OF "ATYPICAL" ACID-FAST ORGANISMS IDENTIFIED BY SMEARS AND CULTURES (1961, 1962)

No.	Name	Lab. No.	Specimen	Total No. of Specimens	Number positive by A-R Smear	Culture
1.	H.K.	2-2-2	Sputum	1	1	1
2.	R.W.	6-10-1	Lymph node	5	0*	2**
3.	J.M.	10-13-9	Sputum	4	1	2
4.	N.W.	9-13-6	Urine	9	0	1
5.	E.J.	6-5-8	Urine	3	3	1
6.	R.B.	12-20-7	Sputum	4	0	1
7.	E.P.	12-9-4	Sputum	10	2	1
8.	J.J.	12-28-6	Sputum	6	3	4
9.	F.B.	8-14-2	Urine	7	0	6
10.	C.P.	1-4-11	Spleen	5	3	5
11.	C.G.	9-3-4	Urine	1	0	1
12.	L.C.	6-24-1	Urine	3	0	1
13.	L.B.	7-2-10	Urine	4	0	1
14.	A.N.	6-16-11	Urine	2	0	1
15.	E.G.	6-20-8	Urine	7	0	1
16.	T.S.	5-10-20	Sputum	16	4	1
17.	H.N.	7-13-3	Urine	8	0	2
18.	E.S.	8-10-13	Sputum	17	3	5
				112	20	37

*The smears of all 5 specimens were negative.
**There were 2 of 5 specimens culturally positive.

Table IV

Growth pattern of an "atypical" MYCOBACTERIA*

Identification of Media**	Culture data in days 1	2	3	6	14	21
1.	—	±	±	±	±	±
2.	—	±	1+[2]	1+	2+	2+
3.	—	—	—	—	2 col.	2 col.
4.	—	—	—	18 col.	1+	1+
5.	—	1 col.[1]	2 col.	25 col.	4+	4+
6.	—	—	±	2+	3+	3+
7.	—	—	—	1+	4+	4+
8.	—	—	±	2+	4+	4+
9.	—	1 col.	1 col.	1+	3+	3+
10.	—	—	1+	1+	2+	2+
11.	—	±	1+	2+	2+	2+
12.	—	2 col.	3 col.	15 col.	3+	4+
13.	—	—	—	±	±	1+
14.[A]	—	6 col.	6 col.	1+	2+	3+

1. Col. = Colony. 2. 1+ = 50-200 col.;
2+ = More 200 col; 3+ and 4+ = Moderate to numerous (confluent).
A. Lowenstein-Jensen medium used as control on the test media.
*The organism was a group II isolated from patient designated as T.S. No. 16 in Table 3.
**See Table No. 1 for description of media according to number.

strains. They usually required 6-14 days to show 1+ to 4+ growth. A single strain of *M. fortuitum* grew well in 2 days on most of these media. Four strains of *Nocardia* grew better and in a shorter period of time (within 1-3 days) than the "atypical" Mycobacteria usuing the subcultures. The "test media" were not examined with primary specimens. No experience with these media has been obtained with the typical Mycobacteria.

The "atypical" strains tested by our group in guinea pigs showed no virulence which was comparable to the *Mycobacterium tuberculosis*. No lesions were found in guinea pigs' kidney, lung, liver or spleen with the former whereas the latter usually produce massive lesions and usually death. The mice were not sufficiently susceptible to the "atypical" organisms to warrant their routine use for virulence testing.

Of the 18 patients whose specimens contained "atypical" acid-fast organisms—one yielded group 1, four patients had group 2, and five were found to have group 3 and

Table V

CHARACTERISTICS OF "ATYPICAL" ACID-FAST ISOLATES

No.	Lab. No.	Growth 22	30	45	37	Pigment Dark	Lite	Colony on Lowenstein	Broth Dubos Dext.	Cells	Group Probable Identification
1.	2-2-2	±	+	—	6 da.	Y[1]	Y	Smooth, Moist Convex	Clumps. yellow bottom	Long, slender beaded rods	II
2.	6-10-1	—	+	—	6 da.	Y	Y	Smooth, Moist Convex	Mucoid, yellow	Lon, slender beaded	II
3.	10-13-9	+	+	—	6 da.	Y	Y	Smooth, Moist Convex	dispersed yellow	Typical A F rods	II
4.	9-13-6	+	+	—	4 da.	B[2]	B	Dry Convex rough	Clumps, no pigment bottom	Pleomorphic long and Coccoid rods	IV
5.	6-5-8	—	±	—	6 da.	B	Y	Smooth, Moist Convex	Dispersed no pigment	Long, slender beaded rods	III
6.	12-20-7	+	+	—	2 da.	B	B	Avian-like	White, Smooth	Long AF rods amorphous	IV
7.	12-9-4	+	+	—	6 da.	B	B	Avian-like	White, Smooth	Short AF rods amorphous	III
8.	12-28-6	—	—	—	11 da.	B	Y	Avian-like	White, some clumps	Long AF rods lloose cords	I

* See Table 3 for type of Specimen.
1 — Y = Yellow, 2 — B = Buff.

an equal number to have group 4. Two patients in this study were shown to have *M. fortuitum* (see Table VI). Dr. Mallman supplied us with the information in Tables V and VI; it should be noted that the reports of Dr. Roberts and our studies on these organisms agreed essentially with the identification given in these tables. The isolants were tentatively identified by each of four categories[13] — (1) the growth characteristics, (2) cytochemical reaction, (3) experimental animal infectivity, (4) the specific hypersensitivity induced.

The *in vitre* susceptibility studies to the three commonly used tuberculostatic agents (INH, PAS and streptomycin) showed no apprreciable inhibitory effect. Some strains showed partial susceptibility to one or more drugs (see Table VII), but usually were not classified as very susceptible to any of the above agents. Experience has shown that inspissation during preparation of medium will partially inactivate some of the drugs. Cycloserine media must be fresh; furthermore degradation of this drug at 37°C is rapid and must be taken into account when reading the susceptibility tests over an extended period of time.

Table VI

CHARACTERISTICS OF "ATYPICAL" ACID-FAST ISOLATES

No.	Lab. No.*	Growth 22	30	45	37	Pigment Dark	photo	Lite	Colony on Lowenstein	Broth Dubos Dext.	Group Probable Identification
9.	8-14-2	+	+	—	3 da.	NP[1]	NC[2]	NC	PP	White bottom smooth	IV
10.	1-4-11	—	+	—	7 da.	NP	NC	NC	PP, Convex	White bottom clumps	III
11.	9-3-4	—	+	—	6 da.	NP	NC	NC	PP, dry heaped	White bottom clumps	III
12.	6-24-1	+	+	—	3 da.	neg.[3]	neg.	neg.	Avian-like	neg.	IV
13.	7-2-10	+	+	—	3 da.	neg.	neg.	neg.	Avian-like	neg.	M. fortuitum
1.	6-16-11	+	+	—	3 da.	neg.	neg.	neg.	Avian-like	neg.	M. fortuitum
15.	6-20-8	+	+	—	3 da.	neg.	neg.	neg.	Avian-like	neg.	IV
16.	5-10-20	—	+	—	10 da.	Y[4]	Y	Y	Avian-like	Y	II
17.	7-13-3	+	+	+	3 da.	neg.	neg.	neg.	Avian-like	neg.	IV
18.	8-10-13	—	—	—	12 da.	neg.	neg.	neg.	Avian-like	neg.	III

* See Table 3 for type of specimen.

1. NP = no pigment. 2. NC = No change. 3. Neg = negative. 4. Y = yellow.

Table VII

SUSCEPTIBILITY PATTERN OF AN "ATYPICAL" *MYCOBACTERIA**

Concentration of antibiotic		Growth in Days 7	14	21
Streptomycin	10	15 col.	1+	4+
	50	— **	—	—
	100	—	—	—
Para-amino salicylic acid	1	4+	4+	4+
	10	4+	4+	4+
	100	3+	4+	4+
Isoniazid Hydrazide	0.2	3+	4+	4+
	1.0	3+	4+	4+
	5.0	1+	4+	4+
	25.00	0	1+	3+
Control		1+	3+	4+

*See patient No. 12 (L. C.) on Table 3.

**Note partial susceptibility to streptomycin and resistant pattern for all concentrations of the other two agents.

Table VIII

A SCHEME FOR THE SEPARATION OF MEDICALLY SIGNIFICANT STRAINS OF UNCLASSIFIED ACID-FAST BACILLI HAS BEEN PRESENTED BY RUNYON.*

Group I.	Photochromogens (yellow bacillus; M. kanaisii). Produces severe pulmonary disease in man; most common in northern midwest and south western U.S.A.
Group II.	Scotochromogens (orange bacillus). Aside from a few isolated cases of pulmonary pathology and occasional lymphadenitis, most strains are thought not to be disease producers! . . . ?
Group III.	Nonphotochromogens (Battey bacillus). Common in southeastern U.S. Produces pulmonary disease which is very difficult to treat.
Group IV.	Rapid Growers. Although exact relationship to disease is not understood, these strains have been isolated both from superficial and deep disease.
	If properly identified, *M. fortuitum* should be so named and not placed in this group.

*Reference No. 7 discusses each group in considerable detail.

Discussion

The data presented herein demonstrates that the "atypical" acid-fast organisms are not infrequently isolated from human specimens. Since the organisms are not easily distinguished from the typical *Mycobacterium tuberculosis* var. *hominis* strains by either smear techniques or in young primary cultures, it is necessary to examine all acid-fast isolates with extreme care. Several initial observations based on laboratory may give the bacteriologist some indication that he may be dealing with an "atypical" strain of *Mycobacteria.*

The suggestions the author would like to put forward are as follows: (1) The macroscopic colony characteristics should be closely examined for differences in dryness (typical strain) and smoothness (atypical except for *M. balnei*). The colony color frequently is of some help as shown by Table 8, (2) The neutral red test should be performed and if positive it gives the investigator an indication of *M. tuberculosis*. A negative neutral red suggests the possibility of *M. balnei* or the "atypical" strains. (3) A very strong catalase activity (4+) is usually demonstrated by the "atypical" whereas the *M. tuberculosis* strains produce a weaker catalase reaction (2+ to 3+). (4) The "atypicals" usually grow, even though slowly, at room temperature. (5) The "atypicals" are usually not pathogenic for the guinea pig as is *M. tuberculosis* var. *hominis*. (6) The "atypicals" isolated previous to patient therapy usually show considerable resistance to the common tuberculostatic drugs such as INH, PAS and streptomycin Other tests which may be used have been described by various investigators[7, 10, 13].

The results shown in Tables V and VI show that a variety of groups I, II, III and IV were isolated from different specimens. Groups III and IV occurred most frequently in our series. These organisms as well as *M. tuberculosis* grew in shorter periods of time on Lowenstein-Jenesen and the American Trudeau Society media than on Petragnani. The thirteen types of synthetic media (see Table 1) did not show any better or earlier growth than that which was observed on the "control slants" containing Lowenstein-Jensen media. These results apply to the evaluation of all the synthetic media using a variety of "atypicals" and *Nocardia* subcultures, not primary specimens.

Sufficient evidence has been accumulated to demonstrate pathogenicity of various members of the four groups of "atypical" organisms. Therefore the author strongly urges a very conscientious bacteriological and clinical evaluation whenever one specimen yields one of these organisms. Since guinea pigs fail to demonstrate pathogenicity for this group, this is an additional reason to examine multiple specimens by smear and culture techniques. Identification of all acid-fast isolates, especially on initial isolation from a suspicious case of tuberculosis should be definitive by using the methods described by Kubica[10] and others.[2, 3, 7]

Although reports from many investigators consistently demonstrate greater resistance by the "atypical" acid-fast organisms to anti-tuberculosis agents than is the case with the typical species of *M. tuberculosis*, one should be aware of the many variations of partial susceptibility which do occur. This point is stressed since *in vitro* susceptibility studies are of prime importance in selection of drugs for therapy.

Runyon[7] states that "some of the newer drugs which are effective against photochromogens are as follows: 3 ethyl isonicotinyl thiomide "1314", cycloserine, amithiozone, thiocarbanilide, thiocarbanidin and kanamycin". He stresses the fact that animal and human trials are necessary before confirming the effectiveness of these agents.

One can anticipate from the resistance *in vitro* patterns that the *in vivo* response would be poor. Various papers[2, 3, 7] have shown that patients afflicted with "atypical"

tuberculosis strains have not responded to therapy with INH, PAS or streptomycin. There is no good controlled—parallel—series to evaluate the efficacy of therapy regimens which have been used.

Runyon[7] and others indicate that patients who have not been adequately treated with drugs are known to have died of progressive pulmonary disease due to "atypical" AFB organisms. He also suggests as others have that resectional surgery whenever possible together with chemotherapy results in a more favorable prognosis.

SUMMARY

1. Eighteen strains of "atypical" *Mycobacteria* consisting of all the four "Runyon groups" have been isolated and positively identified from a variety of clinical specimens.

2. Both fluorescent stained smears and cultures of multiple specimns were helpful in establishing the diagnosis of tuberculosis.

3. Guinea pig inoculations were not helpful for primary specimens but local lesions may be produced on re-passage.

4. Certain of the strains isolated show resemblance to the avian tubercle bacillus.

5. The "atypical" *Mycobacteria* were frequently partially or completely resistant to tuberculostatic agents (ie. INH, PAS and streptomycin) by *in vitro* methods.

6. Neither the bacteriologist or the clinician should discard acid-fast organisms isolated from patients' specimens without serious bacteriological and clinical evaluation. Because the organism fails to fit the culture or virulence pattern of *M. tuberculosis* does not mean that the isolate is non-pathogenic.

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