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A bacteriological study of endemic tuberculosis in birds

By W. B. SCHAEFER*
Denver, Colorado

J. V. BEER and N. A. WOOD
Wildfowl Trust, Slimbridge

E. BOUGHTON
Central Veterinary Laboratory, Weybridge

P. A. JENKINS and J. MARKS
Tuberculosis Reference Laboratory, Cardiff

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SUMMARY

Typing of *Mycobacterium avium* strains obtained in a study of endemic tuberculosis in a Wildfowl Reserve permitted the recognition of two separate infected groups. The main infection was in Anatidae and was due to *M. avium*, type 1; the other was in chickens used for incubation and brooding and the predominance in it of type 2 agreed with normal experience of birds, pigs and cattle in Britain. Many of the strains isolated from the Anatidae were aberrant and methods used to investigate these are described; two of the strains may belong to a new type. Birds which died from other causes, usually trauma, often had subclinical tuberculosis and 5% of the samples of mud and soil examined yielded *M. avium*.

INTRODUCTION

The Tuberculosis Reference Laboratory was recently invited by the Wildfowl Trust, Slimbridge, to study endemic tuberculosis occurring in their extensive collection of Anatidae (ducks, geese and swans). The causes of the endemic situation are being investigated and, it is hoped, progressively eliminated.

The results of a collaborative investigation into the bacteriological aspects of the subject were unexpected in some respects and are recorded here as an aid to further studies of a similar nature. Two series were examined of birds found dead or moribund and considered tuberculous, the main series comprising Anatidae, the other chickens used for incubation and brooding. A third series consisted of Anatidae found dead, usually from trauma, and judged then to be free from tuberculosis. In each series the preliminary diagnosis was made by macroscopic examination of the viscera and Ziehl–Neelsen films. Finally, a study was made of samples of soil, mud and muddy water from the environment of the birds.

* Requests for reprints to be addressed to Dr W. B. Schaefer, National Jewish Hospital, Denver, Colorado, U.S.A. Dr Schaefer's work was supported by contract no. NIH-70-2079 of the National Institutes of Health.

MATERIALS AND METHODS

The birds were refrigerated until necropsy was possible and a portion of liver was then sent to the Reference Laboratory by post. A further piece was fixed by formalin for section but as this procedure did not prove to be of value it was discontinued after 16 cases.

Microscopical examination

Liver samples were homogenized by grinding in distilled water. The suspensions obtained after coarse fragments had settled were centrifuged (1200 *g* for 20 min.) and films of the deposits made for staining by the Ziehl–Neelsen method. They were graded as scanty, moderate or heavy positives or as negative. Environmental specimens were not subjected to microscopy.

Cultural examination

The centrifuged deposits used for films were each mixed with 2 ml. 5% H_2SO_4 (v/v). Each mixture was transferred to a sterile bottle and held at room temperature for 40 min. It was then diluted with 18 ml. sterile water, centrifuged for 20 min. at 1200 *g* and the deposit obtained after decantation inoculated on two slopes of Lowenstein–Jensen medium in universal containers and into Kirchner medium. Penicillin, 100 units/ml., was included in the Kirchner medium and 60 units/ml. in one of the two slopes.

Soil and mud specimens were shaken mechanically with sterile water and allowed, as were shaken specimens of muddy water, to settle at 4° C. until the supernatant fluid was only faintly turbid (2–4 days). To two 10 ml. volumes of this fluid were added 10 ml. of (*a*) 0·26% and (*b*) 0·06% benzalkonium chloride respectively, each in 10% trisodium phosphate (Jones & Jenkins, 1965). The mixtures were shaken occasionally during 60 min. at room temperature, centrifuged for 20 min. at 1200 *g* and decanted. The deposit was neutralized to phenol red with 0·1 N-HCl and inoculated on Lowenstein–Jensen medium with and without penicillin. The remainder of each specimen was held at 4° C. and if the cultures became contaminated, 2 ml. volumes of similar suspensions were each treated with 2 ml. N-NaOH for 15, 30 and 60 min. respectively in a 37° C. incubator, diluted and centrifuged. The deposits were each inoculated on two slopes of buffered egg medium containing penicillin.

All inoculated media were incubated at 37° C. The slopes were discarded after 6 weeks if negative, the Kirchner media then being checked by subculture on egg medium.

Identification of strains

A limited systematic examination was carried out comprising morphology, the character of growth at 25, 37, 42 and 45° C., pigmentation after incubation in light and in the dark at 37° C., arylsulphatase activity and sensitivity tests on cycloserine and ethionamide. The methods used for these tests were described in detail by Birn *et al.* (1967).

Except for the fast-growing mycobacteria cultured from the environment, all strains were subjected to lipid analysis by thin-layer chromatography as described by Marks, Jenkins & Schaefer (1971). Known strains of *M. avium*, types 1 and 2, were examined in parallel on each plate.

All the strains subjected to lipid analysis were typed by agglutination and if necessary by absorption of agglutinating sera. The serological methods used and the preliminary plating for homogeneity were described by Schaefer (1965).

Passage of aberrant strains

Sixteen strains isolated from Anatidae differed initially in one or more respects from what was regarded as the normal behaviour of *M. avium* (see Results). Each was inoculated intravenously into two fowls at a dose of 0·01 mg. Survivors were killed after approximately 4 months. The liver and spleen of these and of the birds which died were examined histologically and for mycobacteria by microscopy of smears and sections and by culture. The 64 re-isolated strains obtained were subjected to the systematic examination, lipid analysis and serological tests used for the original cultures. Pigmented strains were plated at high dilutions to detect mixtures of organisms – initially on egg medium, but if they still appeared pure a single colony was further plated on oleic acid–albumin agar medium in the manner of Schaefer (1965).

RESULTS

The main investigation consisted of the examination of 78 Anatidae found dead or moribund in the course of 15 months which were considered to be tuberculous on macroscopic examination of the viscera. The results of the cultural examinations made and the classification of the isolates are summarized in Table 1. One culture was lost by contamination. A reasonable assessment of the error of macroscopic diagnosis is provided by the outcome of negative cultures in eight cases – six being negative on filming and two scanty positives. In addition, one of the positive cultures was probably not significant, the film having been a scanty positive and the organism *M. intracellulare* (B 31). Of the other 68 cultures, 55 were finally identified as *M. avium* type 1, eight as *M. avium* type 2, and five were unclassifiable. All the latter had the cultural characters of *M. avium* but lacked the lipids or antigens of known types; two were virulent and the group therefore may contain or comprise a new entity. In one of the virulent strains a serological and lipid relationship to type 3 was suspected on first isolation but not confirmed.

M. avium was isolated from 10 of the 21 dead Anatidae considered non-tuberculous at necropsy but growth was usually scanty and microscopy was negative in six cases. The findings suggest that in most such cases the organism was not causing serious infection.

The tuberculosis in chickens had evidently an independent epidemiology because, of the strains of *M. avium* isolated, eight belonged to type 2, three to type 3 and only one to type 1, a distribution quite different from that found in the Anatidae.

Two strains of *M. avium* type 2 and one of type 1 were isolated from 65 environ-

Table 1. *The isolation of* M. avium *and other mycobacteria from the livers of birds found dead or dying in a Wildfowl Reserve and from their environment*

Series	Results of culture with the number and character of the strains isolated	
Anatidae considered to be tuberculous at necropsy	*M. avium* type 1	48
	M. avium type 2	5
	Negative on culture*	8
	Contaminated	1
	16 aberrant strains; final diagnosis:	
	M. avium type 1	7
	M. avium type 2	3
	M. intracellulare	1
	Uncertain status	5
	Total	78
Anatidae considered not to be tuberculous at necropsy	*M. avium* type 1†	8
	M. avium type 2‡	2
	Negative on culture	11
	Total	21
Chickens used for fostering and considered tuberculous at necropsy	*M. avium* type 1	1
	M. avium type 2	8
	M. avium type 3	3
	Total	12
Soil, mud or muddy water from the Anatidae environment	*M. avium* type 1	1
	M. avium type 2	2
	Free-living mycobacteria	14
	Negative on culture	47
	Contaminated	1
	Total	65

* Six liver homogenates were negative on microscopy at the Reference Laboratory (Ziehl–Neelsen stain) and two were scanty positives.

† Five homogenates negative similarly, three positive. One strain was aberrant, being serologically type 1 but without specific lipid.

‡ One negative similarly, one positive.

mental specimens. As only a small proportion of the considerable area of the Wildfowl Reserve was sampled, the positive findings probably represent a large reservoir of infective material.

Aberrant strains

Of the 69 strains considered to be possibly *M. avium* which were isolated from the main series of Anatid birds, 16 were aberrant in at least one character. In contrast, none of the 12 chicken or 3 environment strains was aberrant. One was aberrant of the 10 strains isolated from Anatidae considered non-tuberculous on macroscopic examination. Each of the aberrant strains was inoculated into two fowls, and when they died or were killed cultures were obtained from spleen and liver. Details of the characters of the original and passaged strains from the main series are given in Table 2. In these 16 cases the final diagnosis was *M. intracellulare* in one and *M. avium* of unknown or uncertain type in five. Ten strains were finally assigned to *M. avium* type 1 or type 2 and in five of these the diagnosis of type would not have been reached without the passage. It appears therefore that when

a strain is rough and lacks specific lipid on initial examination, a second attempt may be successful and save the use of passage. Re-examination will be more effective if the strain is first plated on oleic acid–albumin agar medium and a transparent colony selected. In the case of six typed *M. avium* strains, only one of the two fowls inoculated died in the 4 months observation period and in two other cases (B 23, B 94), neither fowl died. These results illustrate the limitations of a virulence test in the diagnosis of *M. avium* when there is a tendency to roughness.

Five of the unclassified strains, B 19, B 46, B 98, B 120 and B 122, had the cultural characters of *M. avium* but lacked specific lipid and could not be identified serologically. Two of these, B 19 and B 46, each killed the two fowls inoculated but the other three were avirulent. A weak lipid pattern suggestive of *M. avium* type 3 was observed in the initial analysis of strain B 46 and a trace of agglutination obtained with type 3 serum, but these findings were not confirmed in repeat tests, nor was any type-specific character detected after passage.

Three of the primary isolates from birds were pigmented. One proved to be a pure strain of *M. avium* type 1. The second was *M. intracellulare* and the third was a mixture of nonpigmented *M. avium* type 2 and pigmented *M. intracellulare*. The mixture persisted after passage and could not be separated on egg medium although plating on oleic acid–albumin agar medium was successful.

DISCUSSION

The present investigation demonstrates that classification at type level is needed to understand the epidemiology of avian tuberculosis. Its use was essential to the recognition of two separate endemic infections in the Wildfowl Reserve studied. In the Anatidae, 87% of the typable strains of *M. avium* isolated belonged to type 1 compared with only 8% of the strains from tuberculous chickens in the same unit. The distribution of types in the chickens was very similar to that met in unselected sporadic isolates from wild, game and zoo birds and fowls examined in the Reference Laboratory and at Weybridge in recent years (Table 3).

The Reference Laboratory's experience of strains from British Anatidae outside the Wildfowl Reserve totals nine (four since series A), of which three were type 1, five type 2 and one type 3. The two most recent were pigmented type 1 strains isolated from ducks from different counties. Gordon, Garside, Dobson & Reid (1941) record the isolation of rough strains from ducks but it now appears that other aberrancies occur with some frequency amongst Anatidae strains. Three other examples of Anatidae infected with pigmented type 1 *M. avium* have been recorded (Chalquest & Matsuoka, 1962; Schaefer, 1965) making a total of six with those described here. We have also been informed of a similar isolation from a cormorant, which suggests an association between pigmentation and water as a medium of infection.

A striking feature of the present investigation was the difficulty or failure met in typing 14 of the 68 strains in the main series considered to be *M. avium*. The difficulty occurred in lipid analysis in one case, serotyping in another and both techniques together in 12 cases. This experience contrasts with the immediate typing by lipid analysis of all 70 sporadic and chicken isolates examined by the

Table 2. *Examination of* 16 *strains which were not typical* M. avium *on primary isolation*

No.	Aberrant characters on primary isolation	Survival time of 2 inoculated fowls*	Properties after passage through fowls: Serology	Properties after passage through fowls: Lipid pattern	Conclusions about the nature of the organism
B 15	Rough;† spontaneous agglutination	(*a*) 6 weeks (*b*) Survived	*M. avium* type 1	*M. avium* type 1‡	A rough *M. avium* type 1. Smoothness (i.e. proportion of smooth colonies) was improved by passage
B 19	Rough; spontaneous agglutination. No specific lipid	(*a*) 6 weeks (*b*) 10 weeks	All rough; either spont. agglut. or unclassified§	Non-specific	In the absence of recognized type characters, the virulence suggests a new type or variety of *M. avium*
B 23	Rough.‖ No specific specific lipid	(*a*) Survived (*b*) Survived	*M. avium* type 1	*M. avium* type 1¶	A rough *M. avium* type 1 of low virulence. Smoothness was still limited after passage
B 31	Serologically unclassified.§ No specific lipid. Pigmented	(*a*) Survived (*b*) Survived	1 isolate unclass.§ 3 isolates rough and spontaneously agglutinated	Non-specific	*M. intracellulare*. The absence of virulence and of any specific property weighed against *M. avium* which also is hardly ever pigmented
B 38	Pigmented	(*a*) 6 weeks (*b*) 7 weeks	*M. avium* type 1	*M. avium* type 1	A pigmented *M. avium* type 1. Purity established by repeated plating
B 40	Rough; serologically unclassified.§ No specific lipid	(*a*) 8 weeks (*b*) Survived	*M. avium* type 2	(1) *M. avium* type 2. (2) Non-specific (from survivor)	A rough *M. avium*, typable only after passage. Smooth colonies were too few in the second case to give the specific lipid pattern
B 46	Rough (trace of type 3 agglutination). Weak? type 3 lipid pattern	(*a*) 8 weeks (*b*) 8 weeks	Rough and unclassified§	Non-specific	Either a new virulent type of *M. avium* or a variety of type 3. Only rough colonies were found on plating passaged isolates
B 47	Rough; unclassified.§ No *Avium* lipid pattern. Pigmented	(*a*) 10 weeks (*b*) Moribund at 16 weeks	*M. avium* type 2 mixed with rough *M. intracellulare*	*M. avium* type 2 mixed with *M. intracellulare*	The separation of *M. avium* and the pigmented *M. intracellulare* was only achieved by plating on oleic acid–albumin agar medium
B 59	No specific lipid (*M. avium* type 1 on repeat)	(*a*) 6 weeks (*b*) Survived	*M. avium* type 1	*M. avium* type 1	A rough *M. avium* type 1. The use of passage could have been avoided (see text)

Table 2 (*cont.*)

No.	Aberrant characters on primary isolation	Survival time of 2 inoculated fowls*	Properties after passage through fowls		Conclusions about the nature of the organism
			Serology	Lipid pattern	
B 94	Rough; spontaneous agglutination. No specific lipid	(*a*) Survived (*b*) Survived	*M. avium* type 1	*M. avium* type 1	A rough *M. avium* type 1. Even after passage smooth colonies were few; one isolate was all rough and lacked specific lipid
B 98	Rough; spontaneous agglutination. No specific lipid	(*a*) Survived (*b*) Survived	Rough; spontaneous agglutination	Non-specific	Cultural characters of *M. avium* but no support from pathogenicity, serology or lipid analysis
B 106	Rough (a few *Avium* 2 colonies on repeat). No specific lipid. (*Avium* 2 on repeat)	(*a*) 10 weeks (*b*) Survived	3 isolates rough. 1 *M. avium* type 2 after re-examination	*M. avium* type 2	A rough *M. avium* type 2. If the repeat tests had been awaited passage would have been unnecessary
B 107	Rough. No specific lipid	(*a*) 14 weeks (*b*) Survived	3 isolates rough. 1 *M. avium* type 1	*M. avium* type 1 (1 isolate non-specific)	A rough *M. avium* type 1. Smoothness was still limited after passage
B 113	Rough. No specific lipid	(*a*) 12 weeks (*b*) Survived	3 isolates rough. 1 *M. avium* type 1 (from survivor)	Non-specific	A rough *M. avium* type 1. Only one isolate could be identified after passage
B 120	Rough. No specific lipid	(*a*) Survived (*b*) Survived	Rough	Non-specific	As B 98
B 122	Rough; unclassified.§ No *Avium* lipid	(*a*) Survived (*b*) Survived	Rough	Non-specific	As B 98

Rough strains could not be typed by sero-agglutination except in cases where this is indicated.

* Survivors killed after 4 months.

† *M. avium* type 1 serum reduced to half of original titre by absorption.

‡ Same result with primary isolate.

§ No agglutination with any *Avium-intracellulare* serum held.

‖ *M. avium* type 1 serum reduced to 1/16 of original titre by absorption.

¶ With two isolates the preliminary selection of a smooth colony was necessary.

Table 3. *Comparison of the general incidence of* M. avium *types with the Wildfowl Reserve incidence*

	M. avium types		
	1	2	3
A. Unselected isolates from birds			
(1) Reference Laboratory	7	38	11
(2) Weybridge	7	37	8
B. Wildfowl Reserve, chicken cases	1	8	3
C. Wildfowl Reserve, Anatidae cases	55	8	0

Reference Laboratory and their successful serotyping in all 53 cases attempted with only the need for two repeat tests. The findings may reflect a general association of aberrant strains of *M. avium* with tuberculosis in Anatidae but was probably mainly due to a variation or tendency of the endemic type 1 organism towards roughness and loss of specific lipid; this extended well beyond the 14 difficult strains. The variation may perhaps have been caused by infection of or relapse in birds which had residual immunity from previous experience of that type. The isolation of *M. avium* from several birds lacking evidence of overt tuberculosis indicated the prevalence of subclinical infection which would result in such immunity.

Two of the *M. avium* strains met in the present study had the cultural characters of *M. avium* and were highly virulent for fowls but could not be typed by present methods. One gave a trace of agglutination with type 3 serum and initially a weak lipid spot of the type 3 pattern but these findings could not be confirmed on repetition or with the four isolates obtained after passage. It must be left for further investigation to decide whether these two virulent strains represent a new type or variants of a known type as described by Schaefer, Davis & Cohn (1970).

As a result of the experience gained it can now be seen that much labour will be saved in the typing of difficult *M. avium* strains by first plating on oleic acid–albumin agar medium. The selection of a transparent colony for the examination will give the best chance of successful typing by agglutination or lipid analysis. The same procedure should be applied to pigmented strains in order to detect whether a mixture is present; egg medium appears to be unsatisfactory for this purpose and passage may fail to separate the organism (case B 47). Finally, if typing remains impossible, passage may be considered. At least two fowls should be inoculated and at least two different organs cultured at necropsy. It may be necessary to plate the cultures obtained again to find suitable colonies.

Although the present study does not embrace the basic causes of the endemic situation in the Reserve, the recovery of three strains of *M. avium* in its limited examination of the environment appears to be significant. Resistance to tuberculosis may also be affected by the protein and vitamin content of the food supply (Ratcliffe, 1946).

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