J. Hyg., Camb. (1976), **76**, 407
Printed in Great Britain

Isolation of mycobacteria from dairy creamery effluent sludge

By P. R. J. MATTHEWS, P. COLLINS and P. W. JONES
Institute for Research on Animal Diseases, Compton, Newbury, Berkshire

(*Received* 10 *September* 1975)

SUMMARY

Sixty three samples of dairy creamery effluent were examined for the presence of mycobacteria. Thirty two strains were isolated from 27 samples. These were classified as follows: *M. fortuitum* (13), *M. peregrinum* (6), *M. gordonae* (5), *M. marianum* (*scrofulaceum*) (4), unidentified (4).

Ten strains, representative of the groups isolated, were tested for their effect on experimental animals. None were pathogenic for guinea pigs or mice but a number produced a minimal amount of skin sensitization in guinea pigs to avian and mammalian tuberculin.

INTRODUCTION

Waste materials produced during the manufacture of dairy products are treated to reduce their pollutive capacity and the more solid fraction (sludge) removed from treatment plants is commonly spread on farm land. The possibility that this material contains mycobacteria and could subsequently cause disease or sensitize cattle grazing on treated pastures was investigated. This paper describes the isolation of mycobacteria from dairy waste, the identification of the strains, the study of their potential pathogenic effects in guinea pigs and mice, and their ability to induce sensitization to avian and mammalian tuberculins.

METHODS

Dairy effluent sludge

Sixty three samples of effluent sludge were collected at the point of discharge from dairy factories. These samples had previously been examined for the presence of salmonellas and brucellas by Jones, Bew & Gammack (1975).

Isolation of mycobacteria

On receipt, samples were centrifuged at 3000 rev./min. for 15 min. and 1 g. of the deposit was added to 10 ml. of 0·26 % benzalkonium chloride in 10 % trisodium phosphate. This suspension was filtered through muslin and then incubated in a 37° C. water bath for 30 min. After centrifugation the deposit was resuspended in 2 ml. sterile isotonic saline containing amphotericin B* 1000 μg./ml. and neutralized with N-HCl. The suspension was distributed on five slopes of isolation

* Amphotericin B. Fungizone E.R. Squibb & Sons Inc., New York.

medium and five slopes of isolation medium with the addition of 0·16% crude mycobactin (Stuart, 1965). The slopes were incubated at 37° C. and examined at regular periods for up to six months.

Isolation medium

The medium used was similar to that described by Stuart (1965). To 96 ml. Stuart's basal medium were added – 100 units/ml. penicillin,* 50 μg./ml. amphotericin B, 10 ml. egg yolk emulsion (Oxoid SR 47) and 4 ml. 9% bovine albumin (Armour Laboratories).

Identification of strains isolated

Cultural and biochemical methods. Strains were initially classified into Runyon groups (Runyon, 1959), on the basis of pigmentation and rate of growth. Systematic identification procedures were then carried out.

All strains were tested for growth at 25, 37, 42, 45 and 52° C. observed over a period of 3 weeks, arylsulphatase production in 5 days (Wayne, 1961), nitrate reductase (Virtanen, 1960) catalase activity (Cowan & Steel, 1970), and utilization of the carbohydrates shown in Tables 1 and 2 (Gordon & Smith, 1953). The sensitivity of strains to thiosemicarbazone (10 μg./ml.), cycloserine (320 μg./ml.), ethionamide (160 μg./ml.) and tellurite (300 μg./ml.) was studied.

In addition, non-chromogenic strains were tested for growth on MacConkey agar with or without crystal violet as described by Kubica & Vitvitsky (1974). Tests for starch hydrolysis, casein digestion and reaction in litmus milk were based on the methods of Cowan & Steel (1970). Chromogenic strains were tested for hydrolysis of Tween 80 (Wayne, 1962) and utilization of citrate (method I of Cowan & Steel, 1970).

Serological identification of non-chromogenic strains. Antigen suspensions for agglutination tests and preparation of antisera were prepared according to Schaeffer (1965). Strains used to produce antisera in rabbits were *M. fortuitum* (575), *M. peregrinum* (NCTC 10264) and *M. smegmatis* (NCTC 10265). The serum of each was absorbed with the other two strains to give a specific homologous serum. Strains were tested for agglutination against the three specific sera.

Animal pathogenicity and sensitization. Two strains representative of each of the five groups of mycobacteria isolated were each inoculated into two guinea pigs and seven mice (5 BSVS, 2 C57). The guinea pigs received 1 ml. of a 1·0 mg./ml. suspension and the mice 0·5 ml. of the same suspension by intraperitoneal inoculation. The animals were kept under observation for 12 weeks when they were killed. Comparative tuberculin tests were carried out on guinea pigs at 6 and 12 weeks after inoculation by the standard intra-dermal test with avian and mammalian PPD tuberculins.† The reactions were measured at 48 hr. At the termination of the experiment each animal was examined and the liver and spleen cultured for mycobacteria.

* Benzylpenicillin (Sodium) B.P. Crystapen Glaxo Laboratories, Greenford, England.
† Supplied by the Central Veterinary Laboratory, New Haw, Weybridge, Surrey.

Table 1. *Characteristics of non-chromogens isolated from dairy effluent sludge*

Strain numbers	Growth at temperature (° C.) of				Growth in 7 days	Growth on MacConkey		Enzymic activity					Sensitivity to				Saccharolytic activity (acid production) at 30° C.			Litmus milk (Acid)	Litmus milk (Clot)	Strain resemblance to
	25	37	42	45		Crystal violet		Arylsulphatase	Casein digestion	Catalase	Nitrate reductase	Starch hydrolysis	Cycloserine	Ethionamide	Tellurite	Thiosemicarbizone	Arabinose	Fructose	Mannitol			
						with	without															
11, 13, 23	+	+	−	−	+	+		+	NT	+	+	NT	R	S	R	R	−	+	+	NT	NT	*M. peregrinum*
1, 7	+	+	−	−	+	+		+	NT	+	+	NT	S	S	R	R	−	+	+	NT	NT	
21	+	+	−	−	+	+		+	NT	+	+	NT	R	R	R	R	−	+	+	NT	NT	
2, 3, 5, 12 14, 15, 17	+	+	−	−	+	+		+	NT	+	+	NT	R	S	R	R	−	+	−	NT	NT	*M. fortuitum*
4	+	+	−	−	+	+		+	NT	+	+	NT	S	S	R	R	−	+	−	NT	NT	
6, 10, 20	+	+	−	−	+	+		+	NT	+	+	NT	R	R	R	R	−	+	−	NT	NT	
9, 18	+	+	−	−	+	+		+	NT	+	+	NT	R	S	R	R	−	+	−	NT	NT	
19	+	+	−	−	+	+		+	−	+	+	+	R	R	R	R	+	+	+	+	−	Unidentified
16	+	+	−	−	+	−		−	−	−	−	−	S	S	R	R	−	−	−	−	−	
8	+	+	−	−	+	+		+	+	+	+	+	R	S	R	R	+	+	+	+	+	
22	+	+	−	−	+	+		+	−	+	+	−	R	S	R	R	−	+	−	−	−	

(+), Positive; (−), negative; (S), sensitive; (R), resistant; (NT), not tested.

https://doi.org/10.1017/S0022172400055339 Published online by Cambridge University Press

Table 2. *Characteristics of scotochromogenic strains from dairy creamery effluent sludge*

Strain number	Growth at temperature (° C.) of					Growth in 7 days	Enzymic activity					Sensitivity to				Saccharolytic activity (acid production) at 30° C.				Strain resemblance to
	25	37	42	45	52		Arylsulphatase	Catalase	Nitrate reductase	Tween hydrolysis	Citrate	Cycloserine	Ethionamide	Tellurite	Thiosemicarbizone	Glucose	Mannose	Trehalose	Others*	
24	+	+	−	−	−	+	−	+	−	+	−	S	S	R	R	+	+	−	−	*M. gordonae*
25	+	+	−	−	−	+	−	+	−	+	−	S	S	S	R	+	−	−	−	
26	+	+	−	−	−	+	−	+	−	+	−	S	S	R	R	+	−	+	−	
29	+	+	−	−	−	+	−	+	−	+	−	S	R	R	R	−	−	−	−	
31	+	+	−	−	−	+	−	+	−	+	−	S	R	S	R	−	−	−	−	
27	+	+	−	−	−	−	−	+	−	−	−	S	R	S	R	−	−	−	−	*M. marianum* (*scrofulaceum*)
28	+	+	−	−	−	−	−	±	−	−	−	S	S	R	R	+	−	−	−	
30	+	+	−	−	−	−	−	±	−	−	−	S	R	R	R	−	−	−	−	
32	+	+	−	−	−	−	−	+	−	−	−	S	R	S	R	−	−	−	−	

(+), Positive; (±), weak positive; (−), negative; (S), sensitive; (R), resistant.
* Arabinose, Dulcitol, Erythritol, Galactose, Inositol, Mannitol, Raffinose, Rhamnose, Sorbitol, Xylose.

https://doi.org/10.1017/S0022172400055339 Published online by Cambridge University Press

Table 3. *Reactions to avian and mammalian tuberculins induced in guinea pigs following the inoculation of* 10 *representative strains of mycobacteria isolated from dairy creamery effluent sludge*

Strain number	Designated species	Millimetres of erythema*	
		Avian PPD	Mammalian PPD
2	*M. fortuitum*	8·0	4·0
4		10·0	7·0
1	*M. peregrinum*	10·0	10·0
13		9·0	8·0
16	Unidentified	6·0	4·0
22		4·0	8·0
25	*M. gordonae*	9·0	10·0
29		9·0	9·0
27	*M. marianum*	10·0	6·0
32	(*scrofulaceum*)	6·0	9·0

* Mean skin reaction of 2 guinea pigs per strain.

RESULTS

Thirty two isolations of mycobacteria were obtained from 27 samples over a period of 3 weeks incubation. Five of the samples contained two species of mycobacteria. Cultures were observed for a total period of 6 months but no further isolations were made.

The isolates were divided into two groups on their rate of growth. Those strains reaching maturity by 7 days were classified as rapid growers and those strains taking more than 7 days as slow growers. These two groups were further divided on their pigmentation into chromogens and non-chromogens.

From the selective methods employed for the classification of the non-chromogens (Table 1) six strains were identified as *M. peregrinum,* 13 as *M. fortuitum* and 4 were of uncertain identity. Serological tests confirmed the biochemical identification of 9 strains. Six strains gave cross-reactions with both *M. peregrinum* and *M. fortuitum* antisera and 5 strains agglutinated spontaneously in saline. The scotochromogenic strains were identified as *M. gordonae* (5) and *M. marianum* (*scrofulaceum*) (4) (Table 2).

The representative strains from each group of strains isolated failed to kill guinea pigs or mice. Organisms were recovered from the BSVS and C57 mice inoculated with *M. marianum* (strain 32) after 12 weeks. Macroscopic lesions were not observed. All strains produced varying degrees of skin erythema in guinea pigs injected with avian and mammalian tuberculins (Table 3).

DISCUSSION

The isolation of mycobacteria from material with a heavy and varied microbial flora has long been a problem. During the course of studies on the evaluation of the methods employed in this work, the contamination rate was reduced to less than 1 % without apparent detrimental effects on the isolation of the mycobacteria. Successful isolations of *M. avium* and *M. paratuberculosis* had previously been obtained from faecal material employing the procedures used for dairy sludge. By this method, mycobacteria including atypical avian strains have been isolated from one gram of deposit obtained by centrifugation of dairy effluent sludge containing less than $5{\cdot}0 \times 10^3$ organisms (P. R. J. Matthews, unpublished data). Mycobacteria were recovered from 43 % of the sludge samples examined but *M. avium*, *M. bovis* and *M. paratuberculosis* were not found and it is believed that they were not present or that their numbers were so few that they were undetectable by the methods used.

The groups of mycobacteria which were isolated are commonly encountered in the environment, but are generally difficult to classify. Two of the unidentified strains (Nos 8 and 19) resemble *M. smegmatis* but failed to grow at 45° C. These strains produced antigens which were unstable and therefore unsuitable for serological identification. The ability of *M. peregrinum* to ferment mannitol is the main significant differentiating factor from *M. fortuitum* (Table 1). The nonchromogens grew well on MacConkey agar with or without crystal violet with the exception of the unidentified strain No. 16 which failed to grow on either. Only one *M. peregrinum* strain gave a type specific reaction with absorbed sera, while three strains cross-reacted with *M. fortuitum* serum suggesting that the strains were closely related. Eight *M. fortuitum* strains reacted specifically with absorbed sera and 3 cross-reacted with *M. peregrinum*. One of the unidentified strains (No. 22) was not agglutinated by any of the available sera. The other strains agglutinated spontaneously in isotonic saline.

The scotochromogenic strains isolated from dairy effluent sludge were identified on two major characteristics. *M. gordonae* was distinguished from *M. marianum* by its rapid growth and hydrolysis of Tween 80.

None of the representative strains isolated were pathogenic for guinea pigs or mice although *M. marianum* (strain 32) was recovered from mice after twelve weeks. Sensitization of guinea pigs to avian and mammalian tuberculins occurred with these strains. However, the reactions were minimal when compared with those obtained in guinea pigs infected with avian and mammalian strains. In the majority of cases the strongest reaction was to avian tuberculin.

As cattle are also readily sensitized, it is possible that these strains of mycobacteria could produce a subsequent reaction in this species to the comparative tuberculin test. This possible sensitization of cattle by environmental mycobacteria is of importance to exporters of pedigree stock, who may find their cattle rejected on these grounds.

Any potential hazard from mycobacteria in creamery effluent is dependent on the survival of the organisms on pasture and the time lapse before stock are

allowed to graze. However, it must be emphasized that the species of mycobacteria isolated in this study commonly occur in soil (Jones & Jenkins, 1965; Reznikov & Leggo, 1974) and are therefore probably available normally to grazing cattle from this source. Unfortunately, no figures on the actual concentration of organisms in soil seem to be available.

Of the various species isolated *M. marianum* and *M. fortuitum* may be considered of possible clinical significance in man (Marks, 1972). Mycobacteria, similar to those encountered in this study, have also been found in raw milk (Jones, Jenkins & Hsu, 1966).

The presence of mycobacteria in raw milk or dairy sludge may on occasions be due to environmental contamination. There is little indication that dairy effluent sludge is of significance in the epidemiology of mycobacterial infections.

We wish to express our appreciation to Mrs A. Brown for technical assistance and to Mr J. Fogerty for the preparation of media used. We are grateful to Dr J. Marks of the Public Health Laboratory, Cardiff, for the supply of strains used in the preparation of antisera.

REFERENCES

Cowan, S. T. & Steel, K. J. (1970). *Manual for the Identification of Medical Bacteria.* Cambridge University Press.

Gordon, R. E. & Smith, M. M. (1953). Rapidly growing acid-fast bacteria i. Species descriptions of *Mycobacterium phlei* Lehmann and Neumann and *Mycobacterium smegmatis* (Trevisan) Lehmann and Neumann. *Journal of Bacteriology* **66**, 41.

Jones, P. W., Bew, J. & Gammack, D. B. (1975). The potential animal health hazard of dairy effluent sludge. *Journal of Hygiene* **75**, 143.

Jones, R. J. & Jenkins, D. E. (1965). Mycobacteria isolated from soil. *Canadian Journal of Microbiology* **11**, 127.

Jones, R. J., Jenkins, D. E. & Hsu, K. H. K. (1966). Raw milk as a source of mycobacteria *Canadian Journal of Microbiology* **12**, 979.

Kubica, G. P. & Vitvitsky, J. (1974). Comparison of two commercial formulations of the MacConkey agar test for mycobacteria. *Applied Microbiology* **27**, 917.

Marks, J. (1972). Classification of the mycobacteria in relation to clinical significance. *Tubercle* **53**, 259.

Reznikov, M. & Leggo, J. H. (1974). Examination of soil in the Brisbane area for organisms of the *Mycobacterium avium – intracellulare-scrofulaceum* complex. *Pathology* **6**, 269.

Runyon, E. H. (1959). Anonymous mycobacteria in pulmonary disease. *Medical Clinics of North America* **43**, 273.

Schaeffer, W. B. (1965). Serologic identification and classification of atypical mycobacteria by agglutination. *American Review of Respiratory Diseases* **92**, 85.

Stuart, P. (1965). Vaccination against Johne's disease in cattle exposed to experimental infection. *British Veterinary Journal* **121**, 289.

Virtanen, S. (1960). A study of Nitrate Reduction by Mycobacteria. The use of Nitrate Reduction Test in the identification of mycobacteria. *Acta tuberculosea scandinavica* **48**, suppl.

Wayne, L. G. (1961). Recognition of *Mycobacterium fortuitum* by means of a three day phenolphthalein sulfatase test. *American Journal of Clinical Pathology* **36**, 185.

Wayne, L. G. (1962). Differentiation of mycobacteria by their effect on Tween 80. *American Review of Respiratory Diseases* **86**, 579.