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Aspects of the epidemiology of bovine tuberculosis in badgers and cattle. II. The development and use of a typing system for *Mycobacterium bovis*

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SUMMARY

A relatively simple method for the detection of intra-specific variation of *Mycobacterium bovis* is described, based on the ability of strains to utilize or remove amino acids from solution. Using the method, 31 strains of *M. bovis*, isolated from badgers and cattle, have been divided into four major groups with some additional sub-groups and intermediate strains. Preliminary results suggest that the biotypes observed are relatively stable. The method was used in the investigation of an outbreak of bovine tuberculosis in cattle in Gloucestershire where badgers were implicated as a source of infection. The results suggested that transmission of infection had occurred on at least two or three separate occasions. It is suggested that the method could be used in studying the epidemiology of bovine tuberculosis in wild animals, cattle and man.

INTRODUCTION

In the first paper in this series the results of investigations into the prevalence of *Mycobacterium bovis* infection in wild animal populations was reported (Barrow & Gallagher, 1980). A study was made of the role of various wild animal species as reservoirs of infection for cattle on two farms where outbreaks of the disease had recently occurred. The results supported previous findings (Report, 1976, 1977 and 1979) and suggested that, in the areas studied, the badger (*Meles meles*) was the only source of infection for cattle. The existence of a considerable reservoir of *M. bovis* infection in badgers in the south-west of England presents considerable potential threats to both animal and public health. It is generally accepted from epidemiological evidence that some transmission from badgers to cattle has occurred, transfer in the opposite direction being unlikely because so few cases of advanced tuberculosis now occur in cattle. The exact extent of transmission to cattle, however, is unknown and the magnitude of the threat to public health has not yet been assessed. This is largely because of the absence of an adequate bacteriological typing system for *M. bovis*.

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The criteria for a suitable typing system are that the taxon can be divided into a reasonable number of stable classes and that the technique produces reproducible results and is relatively easy to carry out. These have not yet been fulfilled by any of the methods so far applied to the *M. tuberculosis* – *M. bovis* group of organisms. Thus, bacteriophage typing of *M. bovis* has revealed no consistent patterns that could be used in epidemiological investigations (Baess, 1969; Richards, 1974) although limited investigations are possible with the two or three sub-divisions available within *M. tuberculosis* (Redmond, Bates & Engel, 1979). Similarly, patterns of sensitivity to and production of mycobacteriocins have not been exploited. Although Takeya and Tokiwa (1974) have reported several divisions within *M. tuberculosis*, these results have not been substantiated. The possibilities of serological typing have never been investigated in any depth because of the 'rough' nature of the surface of the *M. tuberculosis* – *M. bovis* group.

Recently, however, Grange (1976) described a system for the identification of several species of mycobacteria based on the ability of taxa to remove a variety of amino acids from solution. Sub-specific divisions were observed in several of the species studied, some of which, such as *M. avium* and *M. fortuitum* are known to be heterogeneous. Although in most cases, only a few strains of each species were studied, 20 strains of *M. avium* were examined and a great deal of heterogeneity was observed. It was therefore considered that this method could be employed to study strain variation within *M. bovis*. If the sub-divisions observed were stable enough there were possibilities for the application of the technique to the study of the epidemiology of *M. bovis* infection in badgers and cattle.

MATERIALS AND METHODS

Source of strains

The majority of strains of *Mycobacterium bovis* were originally isolated from either badgers or cattle and were obtained from Mr J. Gallagher, Veterinary Investigation Centre, Gloucester, or Dr K. Birn, Central Veterinary Laboratory, Weybridge. *M. bovis* 19210 was obtained from the American Type Culture Collection. *M. bovis* 16054 and *M. tuberculosis* 16606 were isolated from cases of human tuberculosis and were obtained from Dr P. Jenkins, Tuberculosis Reference Laboratory, Cardiff.

Strains were stored as dense suspensions in Middlebrook 7H11 broth (Difco) at −70 °C and were cultured in 7H11 broth modified by the addition of 10 % bovine serum and 0·5 % lysed sheep blood (Gallagher & Horwill, 1977). For the culture of *M. tuberculosis*, 0·5 % glycerol was included in all media.

Procedure for identification and typing

On receipt, the purity and correct identification of all strains were ascertained. The tests used were those described by Barrow & Gallagher (1980).

For epidemiological typing each strain was cultured for 4 weeks in 20 ml modified 7H11 broth. Bacterial growth was aseptically drawn off and was transferred to a sterile bijou bottle containing a few glass beads. Growth was homo-

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genized for a minute or two using a rotary mixer, The resulting fine suspension was used to inoculate 40 well-dried plates of modified Middlebrook 7H11 agar. The inoculum was spread evenly over the surface of each plate with a sterile cotton wool swab soaked in sterile broth. The plates were placed in a sealed container and were incubated at 37 °C for 28 days. Surface growth was harvested and suspended in sterile phosphate buffered saline, pH 7·4 (PBS). The cells were washed twice in sterile PBS and were finally used to determine their ability to remove amino acids from aqueous solutions using the method described by Grange (1976). The amino acids tested were β-alanine, arginine, asparagine, ethionine, glutamic acid, glycine, histidine, lysine, ornithine, serine, tryptophan, tyrosine and valine. The L-isomers were used except in the case of ornithine where because of availability a mixture of D- and L-isomers was used.

The strain of *M. tuberculosis* was treated similarly except that all Middlebrook media contained 0·5 % glycerol.

RESULTS

Using the tests described by Barrow & Gallagher (1980), the identity of all strains received as *M. bovis* was confirmed.

Pattern of removal of amino acids

A great variety was observed in the patterns of removal of amino acids from solution amongst the cattle and badger strains of *M. bovis* tested (Table 1). Several strains were completely inactive while others removed the majority of the amino acids tested. Many strains lay in an intermediate position between these extremes and apart from strains which were inactive, the strains were grouped with some difficulty. All or most strains in each of the groups formed had one or more reactions in common. A great deal of intra-group variability nevertheless existed.

Group 1 contained six strains which removed no amino acids from solution. A further strain, M 200, removed small amounts of asparagine and has thus been placed as an intermediate.

Group 2 contained five strains all of which were able to remove serine. In addition, four strains removed glutamic acid and two removed arginine. Individual strains removed glycine, histidine and tyrosine.

Group 3 contained nine strains all of which were able to remove valine from solution. Two sub-groups were created to aid classification although the basis of this sub-division was somewhat arbitrary. Sub-group A contained four strains, including the standard strain ATCC 19210, which were less reactive than those of sub-group B and were able to remove arginine in addition to valine. The position of ATCC 19210 was uncertain because, although it removed arginine, it also removed glutamic acid and serine. Sub-group B contained five strains, some of which were able to remove glutamic acid, histidine, serine and tyrosine.

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Table 1. *Patterns of amino acid removal amongst strains of* Mycobacterium bovis *studied*

Strain number	Host source	Geographical source (county in U.K.)	Removal of: β-alanine	arginine	asparagine	ethionine	glutamic acid	glycine	histidine	lysine	ornithine	serine	tryptophan	tyrosine	valine	Group and sub-group
M 733	C[1]	Gloucestershire	−	−	−	−	−	−	−	−	−	−	−	−	−	Group 1
M 877	B[2]	Unknown	−	−	−	−	−	−	−	−	−	−	−	−	−	
AF 281	C	Unknown	−	−	−	−	−	−	−	−	−	−	−	−	−	
AF 800	C	Ayr	−	−	−	−	−	−	−	−	−	−	−	−	−	
AF 891	C	Aberdeen	−	−	−	−	−	−	−	−	−	−	−	−	−	
AF 897	C	Durham	−	−	−	−	−	−	−	−	−	−	−	−	−	
M 200	C	Gloucestershire	−	−	±	−	−	−	−	−	−	−	−	−	−	Intermediate
AF 894	C	Durham	−	−	−	−	−	+	−	−	−	+	−	−	−	Group 2
M 626	B	Cornwall	−	−	−	−	+	−	−	−	−	+	−	+	−	
1712	B	Avon	−	+	−	−	+	−	−	−	−	+	−	−	−	
M 627	B	Gloucestershire	−	+	−	−	+	−	−	−	−	+	−	−	−	
AF 189	C	Cornwall	−	−	−	−	+	−	+	−	−	+	−	−	−	
M 888	B	Dorset	−	+	−	−	−	−	−	−	−	−	−	−	+	Sub-group A
1084	C	Lancashire	−	+	+	−	−	−	−	−	−	−	−	−	+	
AF 895	C	Durham	−	+	+	−	−	+	−	−	−	−	−	−	+	
ATCC 19210	C	Not applicable	−	+	−	−	+	−	−	−	−	+	−	−	+	Group 3

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M 836	B	Not applicable	−	−	−	−	−	−	−	−	−	−	−	+	+	Sub-group B
M 646	B	Cornwall	±	−	−	−	+	−	+	−	−	+	−	+	+	
M 893	C	Dorset	−	−	−	−	+	−	+	−	−	−	−	−	+	
AF 33	C	Cornwall	−	−	−	−	+	−	−	−	−	+	−	−	+	
M 800	B	Dorset	−	−	−	−	−	−	+	−	−	+	−	−	+	
AF 227	C	Cornwall	−	+	−	−	+	+	−	−	−	+	−	−	−	Sub-group A
M 740	B	Gloucestershire	−	±	−	−	+	+	−	−	−	+	−	−	−	
AF 471	C	Gloucestershire	−	−	+	−	+	±	−	−	−	+	+	−	−	
1135	B	Avon	−	−	−	−	+	+	−	−	+	+	+	+	−	
C 13	C	Not applicable	−	+	−	−	+	+	−	−	+	+	+	+	−	
M 857	B	Wiltshire	+	−	−	+	+	+	−	−	+	+	+	−	−	
																Group 4
17	B	Avon	+	+	+	−	+	+	+	−	−	+	−	−	−	
AF 853	C	Gloucestershire	+	+	+	−	+	+	+	−	−	+	+	+	+	Sub-group B
AF 888	C	Durham	+	+	+	−	+	+	−	+	+	−	+	+	+	
AF 896	C	Durham	+	+	+	−	+	+	+	+	+	+	+	+	+	

[1]: Cow, [2]: Badger

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Table 2. *The reproducibility of patterns of amino acid removal of selected strains*

		Removal of												
Strain tested	Test no.	β-alanine	arginine	asparagine	ethionine	glutamic acid	glycine	histidine	lysine	ornithine	serine	tryptophan	tyrosine	valine
M. tuberculosis 16606	1	−	−	−	−	−	−	−	−	−	−	−	−	−
	2	−	−	±	−	−	−	−	−	−	−	−	−	−
	3	−	−	−	−	−	−	−	−	−	−	−	−	−
M. bovis 626	1	−	−	−	−	+	−	−	−	−	+	−	+	−
	2	−	−	−	−	+	−	−	−	−	+	−	+	−
	3	−	−	−	−	−	−	−	−	−	−	−	−	−
M. bovis 627	1	−	+	−	−	+	−	−	−	−	+	−	−	−
	2	−	+	−	−	+	−	−	−	−	+	−	−	−
M. bovis 16054	1	+	−	+	−	+	+	−	−	−	−	−	+	+
	2	−	±	−	−	+	+	−	−	−	+	−	−	+

Group 4 contained ten very reactive strains which were again divided into two sub-groups to aid classification. Members of the two sub-groups, shared attributes and one strain, 17, appeared to be intermediate and its position is uncertain. All strains were able to remove both glutamic acid and glycine from solution and all except strain AF 888 were able to remove serine. The three strains of sub-group B were also able to remove β-alanine, asparagine, tyrosine and valine. Sub-group A contained six strains which were slightly less reactive and were in general unreactive with these four amino acids, although there were exceptions.

Stability and reproducibility (Table 2)

Before a system of sub-specific classification can be used for epidemiological purposes, the stability and reproducibility of the observed types have to be determined. The great deal of variation observed amongst the strains tested here increased this demand.

Three strains of *M. bovis* and one of *M. tuberculosis* were tested on different occasions. *M. tuberculosis* was unreactive on two occasions, but on the third there was a slight removal of asparagine. *M. bovis* 627 showed exactly the same pattern on two separate occasions. *M. bovis* 626 showed identical patterns on two occasions but on the third was completely non-reactive. The human strain of *M. bovis*, 16054, produced a different pattern on two separate occasions, the two patterns differing in reaction with five amino acids, these being β-alanine, arginine, asparagine, serine and tyrosine.

Host and geographical variation

Relatively few strains have been studied but some simple conclusions can be drawn from Table 1.

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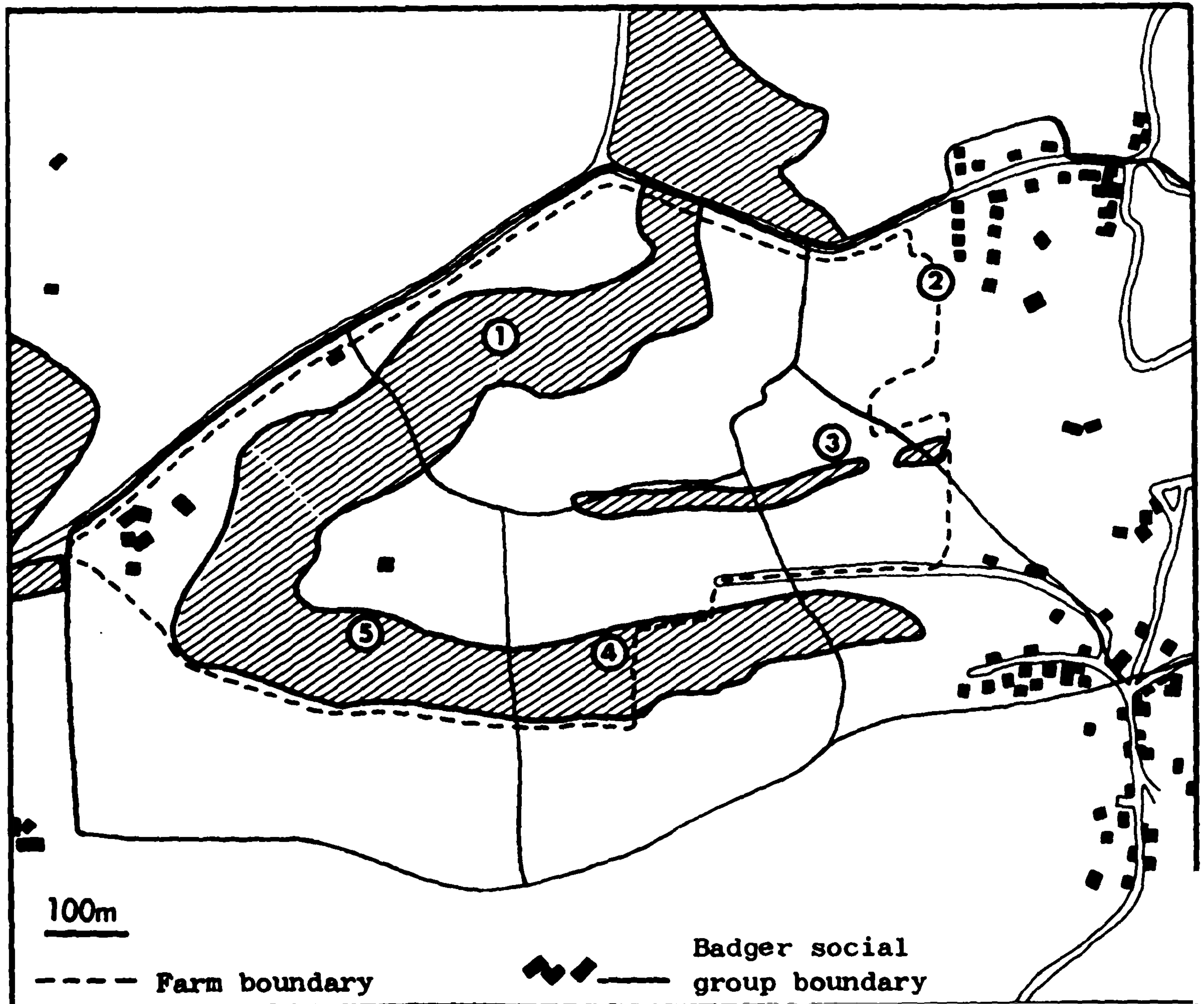

Fig. 1. Sketch map of area studied. Hatching denotes wood areas. Numbered circles represent situation of badger setts. The boundaries of the farm studied and of the badger social groups concerned are marked.

Strains isolated from both badgers and cattle were found in all of the four major groups formed. Rather than arguing for the existence of two discrete populations of *M. bovis* in the two species of host, it suggests that one population exists and that possibly some transfer of *M. bovis* from one host to the other has occurred. The extent of transfer can not be estimated without examination of a larger number of strains.

Strains from the same group were often isolated from different parts of the country. The strains AF 227 and M 740 from sub-group A of group 4 were isolated from Cornwall and Gloucestershire respectively. Translocation of animals is unlikely to be the cause of this because of the frequency with which it has occurred in the present study. It is more likely that strains in different parts of the country continually change often producing phenotypes which are identical.

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Table 3. *Patterns of amino acid removal of strains of* M. bovis *isolated from badgers and cattle from a farm in Gloucestershire*

Strain no.	Removal of: β-alanine	arginine	asparagine	ethionine	glutamic acid	glycine	histidine	lysine	ornithine	serine	tryptophan	tyrosine	valine	Classification (group no.)	Source	Social* group
852	−	−	−	−	−	−	−	−	−	−	−	−	−	1	Cattle	—
848	−	−	−	−	−	−	−	−	−	+	−	−	−	1?	Cattle	—
853	+	+	+	−	+	+	+	−	−	+	+	+	+	4	Cattle	—
441	−	−	−	−	−	−	−	−	−	−	−	−	−	1	Badger	1
434	−	−	−	−	−	−	−	−	−	−	−	−	−	1	Badger	3
453	−	−	−	−	−	−	−	−	−	−	−	−	−	1	Badger	3
442	−	−	−	−	−	−	−	−	−	+	−	−	−	1?	Badger	2
483	−	+	+	−	+	+	−	−	−	+	+	−	−	4	Badger	3

* Badgers only, see Fig. 1.

Application in epidemiology

The great degree of variability amongst strains of *M. bovis* detected by this method combined with a reasonable degree of reproducibility suggested that the system could be applied to epidemiological work. This was attempted with strains isolated from both cattle and badgers from one farm in Gloucestershire. This farm formed part of a larger study area described by Cheeseman *et al.* (in prep.). The farm (Fig. 1) had recently experienced cases of bovine tuberculosis in cattle. Over 30 badgers from 5 social groups were caught within the farm boundary and 12 were found to be tuberculous. Three cattle and five badger strains were examined and identified according to the grouping of Table 1.

The results shown in Table 3 can be interpreted with some difficulty. Three types were found amongst both cattle and badger strains. Types from groups 1 and 4 could be recognized. The true identification of strains 848 and 442 was uncertain as the reproducibility study had shown that the removal of serine was occasionally unpredictable. Thus these strains may have belonged to group 1. Similarly, strains 853 and 483, although both belonging to group 4, appear to fall into different sub-groups, 483 into sub-group A and 853 into sub-group B. At this juncture the significance of this is unclear. Reference to Fig. 1 shows that the strains were isolated from badgers from three adjacent social groups. The isolation of identical types (strains 441, 434 and 453) from two adjacent groups suggests that some transfer of infection has occurred between social groups 1 and 3. The identification of two or possibly three types amongst both badger and cattle strains also suggests that transfer of infection between one species and the other had occurred on two or possibly three occasions. Further investigations involving examination of more strains from badgers on the farm and from social groups further afield may clarify the results.

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DISCUSSION

In many western countries the incidence of bovine tuberculosis in cattle and man is extremely low and in this respect is a dying disease. Nevertheless, in addition to the problem of persistent infection in cattle in parts of the south west of England, cases of *M. bovis* tuberculosis in children still occur where infection cannot be attributed to the usual sources. In cases such as these and in studying the epidemiology of the reservoir of infection in badgers with its threat to animal and public health the possession of an adequate bacterial typing system would be an advantage.

Despite some obvious shortcomings in technique the results presented here are promising. The method was almost identical to that used by Grange (1976) with the exception of the use of an agar culture medium and a smaller range of amino acids. Fewer amino acids were used because some, such as sarcosine and hydroxyproline, produced poor colouring after ninhydrin treatment and were omitted.

The tests used to determine strain stability showed that the patterns of amino acid removal were not completely reproducible. One interesting, incidental result was the absence of any activity in the one strain of *M. tuberculosis* tested. This suggested that this method could assist in species differentiation within the *M. bovis* – *M. tuberculosis* group of organisms and led to a study which will be described elsewhere. There was a reasonable degree of reproducibility among the badger and cattle strains of *M. bovis* tested and this could perhaps be improved by the use of more rigidly standardized procedures and/or the elimination of some of the possible sources of error. These latter include the possibilities of contamination and of phenotypic and genotypic change.

Fungal contamination was a problem during the work, but one which was relatively easily detected by eye and dealt with. Strains showing evidence of this were discarded and the tests subsequently repeated. The possibility of bacterial contamination was not investigated and may thus have been the cause of some of the discrepancies observed in the reproducibility and stability study. This, however, could also be dealt with by the incorporation of antibiotics into the amino acid solution or in the saline used for suspending bacterial growth.

One of the strains tested, M626 showed identical patterns of amino acid removal on two occasions but on the third was completely non-reactive. This suggests the possibility of genotypic change, possibly mutational or resulting from the loss of a lysogenic phage. This emphasizes the need to work with strains as fresh as possible since, presumably, the chance of genotypic changes having occurred increases with the age of the strain. Certainly, in studies of heterogeneity in the *M. tuberculosis* – *M. bovis* group, pyrolysis mass spectrograms of *M. bovis* strains changed with the age of the strain (Wieten *et al.* 1979; Wieten *et al.* 1980). In the present study strains differed greatly in age, some being a matter of months old while others were over eight years old.

The relevance of phenotypic – genotypic change should be investigated further in relation to the present work. In addition, a greater understanding of the biochemical basis of the present method used would assist in assessing the stability

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of the patterns observed and would help in suggesting ways of either combatting or accommodating it. It may well be that, however well standardised procedures are, a certain amount of variability may be observed within a strain. The introduction of some sort of a scheme of 'maximum permissible variation' as is used in the phage typing of *Staphylococcus aureus* (Williams & Rippon, 1952; Parker, 1972) could help in this regard.

The great variability observed between strains in this study produced some difficulty in grouping the patterns observed. The errors mentioned above no doubt contributed to some degree but, nevertheless, there appeared to be a great deal of inherent variability within *M. bovis*. At this stage, as in the case of *S. aureus*, it seemed unwise to resort to defining types. Rather, similar patterns of amino acid removal were grouped together on the basis of similarity in reaction with one or more amino acids. Conceivably, less arbitrary methods such as numerical taxonomy could improve ease in the grouping of strains. The small amounts of variation caused by instability in removal of one or two acids would then carry less weight in the classification than they do with the present method.

There was no clear-cut association between pattern of amino acid removal and geographical or host source. However, relatively few strains have been studied and any relationship may be revealed by examination of a greater number. The absence of such an association does not preclude the method from application in epidemiological investigations. In fact, the absence of such an association is optimistic in that it suggests that one promiscuous rather than two discrete populations of *M. bovis* exists in the two hosts. Similar patterns of removal were found in strains isolated from both badgers and cattle. This could have been the result of mutation and selection in two discrete microbial populations but it is more likely to result from transfer of strains between the two host species or from one to the other.

The results of the incomplete investigation into one case in Gloucestershire can perhaps be interpreted albeit with some difficulty. It was thought that tuberculous badgers had infected several cattle on one farm. The shortcomings of the investigation are that not all isolates of *M. bovis* were examined and the possibility of some instability in serine removal complicated the interpretation of the results. The results suggested that transfer of *M. bovis* from badgers to cattle or vice versa had occurred on two or three occasions. It is not possible to say in which direction transfer occurred. This can only be deduced from a full investigation into the pathology of the disease in the badgers and cattle concerned.

Thus this system, used as a mycobacterial typing system, could assist in investigations into the problem of the reservoir of *M. bovis* infection in badgers in the south-west of England and their role in the infection of cattle. Full pathological and bacteriological investigations (including typing) combined with some knowledge of the local ecology of the relevant badger population, could produce a fairly complete picture of some aspects of the epidemiology of infection in these two hosts. The use of the technique could assist in estimating the magnitude of the threat to animal health and, by including strains of *M. bovis* isolated from man, could help in assessing its significance for public health.

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REFERENCES

BAESS, I. (1969). Sub-division of *M. tuberculosis* by means of bacteriophages. *Acta Pathologica Microbiologica Scandinavica* **76**, 464–74.

BARROW, P. A. & GALLAGHER, J. (1981). Aspects of the epidemiology of bovine tuberculosis in badgers and cattle. 1. The prevalence of infection in two wild animal populations in the south-west of England. *Journal of Hygiene* **86**, 237–45.

GALLAGHER, J. & HORWILL, D. M. (1977). A selective oleic acid albumin agar medium for the cultivation of *Mycobacterium bovis*. *Journal of Hygiene* **79**, 155- 60.

GRANGE, J. M. (1976). Enzymic breakdown of amino acids and related compounds by suspensions of washed mycobacteria. *Journal of Applied Bacteriology* **41**, 425–31.

PARKER, M. T. (1972). Phage-typing of *Staphylococcus aureus*. In *Methods in Microbiology* 7*B*, (ed. J. R. Norris and D. W. Ribbons). London: Academic Press.

REDMOND, W. B., BATES, J. H. & ENGEL, H. W. B. (1979). Methods for bacteriophage typing of mycobacteria. *Methods in Microbiology* **13** (ed. T. Bergan and J. R. Norris). London: Academic Press.

REPORT (1976, 1977 & 1979). Bovine tuberculosis in badgers. Unpublished reports by the Ministry of Agriculture, Fisheries & Food.

RICHARDS, W. D. (1974). Phage susceptibilities of *Mycobacterium bovis* isolates. *Annali Sclavo* **17**, 618–22.

TAKEYA, K. & TOKIWA, H. (1974). Bacteriocin-typing of *Mycobacterium tuberculosis*. *American Review of Respiratory Diseases* **109**, 304–5.

WIETEN, G., HAVERKAMP, J. & MEUZELAAR, H. L. C. (1979). Pyrolysis mass spectrometry in mycobacterial taxonomy and identification. *Proceedings of the VI Conference on Systematics of the Genus* Mycobacterium, *Atlanta, Georgia*.

WIETEN, G., HAVERKAMP, J., MEUZELAAR, H. L. C., ENGEL, H. W. B. & BERWALD, L. G. (1981). Use of pyrolysis mass spectrometry in the routine differentiation of mycobacteria of the 'Tuberculosis complex'. *Journal of Clinical Microbiology*. (In the Press.)

WILLIAMS, R. E. O. & RIPPON, J. E. (1952). Bacteriophage typing of *Staphylococcus aureus*. *Journal of Hygiene* **50**, 320–53.

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