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Tuberculosis in East Sussex

III. Comparison of post-mortem and clinical methods for the diagnosis of tuberculosis in badgers

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SUMMARY

Following epidemiological and ecological studies of a defined badger population in an area of East Sussex, removal of all badgers by cage trapping was attempted. Trapping was incomplete due to the activities of protesters. Forty-seven badgers were caught from the eight social groups. All badgers were examined clinically and samples of faeces, urine and tracheal aspirate were taken, together with swabs from any bite wounds, for bacteriological examinations. Forty-five animals were skin tested using whole killed cells of *Mycobacterium bovis* strain AN5, bovine PPD Weybridge and new human tuberculin. Skin test results were recorded after 24 and 72 h. All badgers were killed and subjected to a post-mortem and bacteriological examination.

M. bovis was detected in 10 (21·3%) badgers at post-mortem and in 2 badgers from clinical samples. Four social groups were infected. Positive skin test results were recorded at 72 h with bovine PPD (2 μg and 20 μg/ml), strain AN5 (1 mg/ml) and human tuberculin (2 μg/ml), but not with human tuberculin at 20 μg/ml. Histological sections of the skin test reactions showed the cellular types typical of delayed-type hypersensitivity. The skin test reactions observed were neither sensitive nor specific enough to be of practical value.

INTRODUCTION

The previous paper in this series (Wilesmith *et al.* 1986*b*) described the results of investigations to determine the *Mycobacterium bovis* infection status of badgers in an area of East Sussex and their social group structure. Two previous studies have investigated the ecology and prevalence of *M. bovis* infection in badgers in four areas in south-west England (Cheeseman *et al.* 1981) and in one area in Staffordshire (Cheeseman *et al.* 1985*a*).

This paper describes the attempted removal of a defined badger population from an area of East Sussex to provide further information on the ecology of badgers and the epidemiology of tuberculosis in badger populations, and evaluate some non-destructive methods for the diagnosis of *M. bovis* infection in badgers from naturally infected populations.

MATERIALS AND METHODS

Study area

This was the project area as described previously (Wilesmith *et al.* 1986*a*).

Method of capture

Badgers were caught using cage traps (Cheeseman & Mallinson, 1980). Traps were deployed 21 days before trapping and pre-baited with peanuts for the final 14 days in order to increase trapping efficiency. Saturation trapping was used to achieve the optimal rate of removal. A maximum of 96 traps was deployed at any one time. Trapping commenced in the second week of June 1984. Each badger caught was transferred to a numbered 600 × 350 × 200 mm transportation cage constructed of 10-gauge Twillweld with a mesh size of 25 mm and fitted with a sliding door (Cheeseman *et al.* 1985*a*). Trapping was suspended after three nights due to the activities of protesters. Sticks were then placed in the entrances to all setts, including outliers, and checked for signs of badger activity after 1 week. A second period of trapping on a more limited scale was carried out in the middle of July but was suspended after eight nights. All cage traps were disinfected and steam cleaned before removal from the site.

Laboratory methods

On arrival at the Central Veterinary Laboratory (CVL) each badger was anaesthetized by intramuscular administration of ketamine hydrochloride (Vetalar; Parke, Davis and Co.) (Mackintosh *et al.* 1976). They were identified by inserting plastic ear tags as described by Cheeseman & Harris (1982) and a record made of the sett number they were caught at and their age class, adult or cub. Cubs were defined as animals born in 1984 and identified by means of body weight and size. Each badger was skin tested and clinically examined, then samples of blood, faeces, urine and tracheal aspirate, and swabs from any bite wounds present were taken. The faeces, urine, tracheal aspirates and bite wound swabs were examined culturally and biologically for the presence of *M. bovis* as described in the previous paper (Wilesmith *et al.* 1986*b*).

Skin testing was performed on the clipped flank region after measuring the initial skin thickness with calipers; 0·1 ml of each of the following reagents was injected intradermally using 26-gauge needles; whole killed cells of *M. bovis* strain AN5 1 mg/ml; bovine PPD Weybridge (Lesslie & Hebert, 1975) 20 μg/ml and 2 μg/ml; new human tuberculin 20 μg/ml and 2 μg/ml (kindly supplied by Dr J. Stanford, Middlesex Hospital). The skin-test results were recorded after 24 and 72 h and any badgers with a response were anaesthetized with ketamine so that accurate measurements, and in some cases skin biopsies, could be taken.

The badgers were housed in iron sties in their original social groups, up to five

badgers per sty. One large social group of 11 animals was kept in a converted cow byre with covered mangers for a sleeping area. The badgers were fed on commercial tinned dog food and biscuits, and bedded on barley straw. They had *ad lib.* water, and the cubs had access to sterilized cow's milk.

At intervals varying from 7 to 56 days after arrival at CVL the badgers were anaesthetized with ketamine and 200 ml of blood was collected for subsequent serological testing, the results of which will be reported separately (Mahmood *et al.* in preparation). The badgers were then killed by intravenous administration of pentobarbitone sodium (Expiral, Ceva Ltd) and subjected to a post-mortem examination.

If no visible lesion of tuberculosis was detected a pool of the following tissues was used for culture, separate pools of each tissue being frozen at −70 °C for future reference: pool 1, head lymph nodes; pool 2, broncho-mediastinal lymph nodes and lung; pool 3, carcase lymph nodes; pool 4, liver, spleen, kidney and mesenteric lymph nodes.

The pools of tissues were examined for the presence of *M. bovis* by biological and cultural tests as described by Little *et al.* (1982) and in the previous papers (Wilesmith *et al.* 1986*a*, *b*). If *M. bovis* was isolated from a pool or by biological methods only, the separate tissues were cultured individually in an effort to identify which were infected.

If lesions suspicious of tuberculosis were seen the tissues were all cultured separately, but pooled for biological testing. A portion of each lesion and any skin-test sites showing a response were taken for histological examination, by fixing in 10 % buffered formalin and subsequent staining of sections with haematoxylin and eosin and Ziehl–Neelsen methods.

The sensitivity, specificity and predictive value for the diagnostic methods used were calculated as described by Martin (1977).

RESULTS

The results of the attempted removal of badgers from the eight social groups in the study area are summarized in Table 1. Forty-seven badgers were caught of which 13 were cubs and 34 adults. There was a predominance of females in the adults, 24 being caught. The sex ratio of the cubs was 5 females:8 males. The number of badgers caught from each social group varied between 2 (social group H) and 11 (social group G). The largest number of adults caught was 7 in social group E. The minimum population density was 9 adult badgers per km^2, based on the total territory size (306 ha) recorded previously in 1984 (Wilesmith *et al.* 1986*b*).

M. bovis infection was confirmed in four social groups (B, D, E and F). The crude prevalence of infection was 21·3 %; 9 of the 10 infected badgers had visible lesions typical of tuberculosis on gross examination (Table 2). Two male cubs were infected and both had lesions of tuberculosis, which in one were extensive and miliary in type. The prevalence of infection in adult females was 25 % and in adult males 20 %. Social group E had the highest observed prevalence of infection, with 5 of the 9 badgers infected.

M. bovis was isolated on culture from samples obtained by clinical sampling from

Table 1. *Numbers of badgers trapped by social group and results of post-mortem and bacteriological examination for* M. bovis

Social group		Adults ♀	Adults ♂	Cubs ♀	Cubs ♂	Total ♀	Total ♂	Total ♀+♂
A	Total badgers	2	1	—	—	2	1	3
	Neg. P.M.	2	1	—	—	2	1	3
B	Total badgers	5	1	—	—	5	1	6
	Neg. P.M.	4	1	—	—	4	1	5
	VL *M. bovis*	1	—	—	—	1	—	1
C	Total badgers	3	—	—	—	3	—	3
	Neg. P.M.	3	—	—	—	3	—	3
D	Total badgers	3	2	—	3	3	5	8
	Neg. P.M.	2	—	—	3	2	3	5
	VL *M. bovis*	1	2*	—	—	1	2	3
E	Total badgers	6	1	—	2	6	3	9
	Neg. P.M.	3	1	—	—	3	1	4
	NVL *M. bovis*	1	—	—	—	2	—	1
	VL *M. bovis*	2†	—	—	2	2	2	4
F	Total badgers	1	2	1	1	2	3	5
	Neg. P.M.	—	2	1	1	1	3	4
	VL *M. bovis*	1	—	—	—	1	—	1
G	Total badgers	4	2	3	2	7	4	11
	Neg. P.M.	4	2	3	2	7	4	11
H	Total badgers	—	1	1	—	1	1	2
	Neg. P.M.	—	1	1	—	1	1	2
	Total							
	Total badgers	24	10	5	8	29	18	47
	Neg. P.M.	18	8	5	6	23	14	37
	NVL *M. bovis*	1	—	—	—	1	—	1
	VL *M. bovis*	5	2	—	2	5	4	9

* One badger also found infected on clinical sampling – bite wound.
† One badger also found infected on clinical sampling – faeces and tracheal aspirate.
VL, visible lesions typical of tuberculosis + isolation of *M. bovis*.
NVL, no visible lesions typical of tuberculosis.

only two badgers; from a purulent bite wound in one and from both tracheal aspirate and faeces from the other (Table 2). These samples were also positive in biological tests. A faeces sample taken from badger 87 post-mortem was positive for *M. bovis* on direct culture.

Five of the ten infected badgers were detected only by biological methods and subsequent subculture of the guinea-pigs' tissues. Separate culture of the previously frozen tissues failed to yield *M. bovis*. One badger (no. 81) which had no visible lesion of tuberculosis at post-mortem examination (PME) yielded *M. bovis* on direct culture of the pooled lymph nodes and organs. When these tissues were subsequently cultured separately only the head lymph nodes were culture-positive for *M. bovis*. On direct culture of the lesioned tissues of badger 87 all were positive for *M. bovis* except the kidneys. *M. bovis* was isolated from all the tissues of badger 92 except the spleen and kidneys. *M. bovis* was isolated from the original pool of NVL tissues from badger 95, but could not subsequently be isolated from separate tissues. Badger 97 had the most severe lesions of tuberculosis at PME and *M. bovis* was isolated on direct culture from every tissue examined.

Table 2. *Results of bacteriological examination of clinical samples and post-mortem findings of ten badgers infected with* M. bovis

					Bacteriological results of clinical samples/post-mortem findings					
Social group	Sett no.	Badger No.	Age	Sex	Urine	Faeces	Tracheal aspirate	Wounds	Gross lesions	*M. bovis* isolated
B	2	61	A	F	—	—	—	NV	Miliary lesions in lungs, liver and kidneys	+(B)
D	11+29	56	A	M	—	—	—	NV	Pinhead lesions in lungs	+(B)
D	11+29	85	A	F	—	—	—	NV	Pinhead lesions in lungs	+(B)
D	11+29	87	A	M	—	— (+at PM)	—	+Abscess surrounding right tarsus	Miliary lesions in lungs, pleura, kidney, spleen and associated lymph nodes	+
E	13+14	65	A	F	—	—	—	NV	Miliary lesions in liver, submandibular lymph node enlarged	+(B)
E	13+14	81	A	F	—	—	—	NV	NVL	+
E	13+14	92	A	F	—	+	+	NV	Miliary lesions in lungs. Focal lesions in liver, kidney, spleen and associated lymph nodes	+
E	13+14	95	cub	M	—	—	—	NV	Enlarged bronchial lymph node	+
E	13+14	97	cub	M	—	—	—	NV	Miliary lesions in lungs, pleura, heart, liver, kidneys, spleen and lymph nodes. Submandibular abscess	+
F	10+19	66	A	F	—	—	—	NV	Pinhead lesions in lungs and bronchial lymph nodes. Hepatic lymph node enlarged	+(B)

NVL, no visible lesions of tuberculosis. A, Adult. (B), *M. bovis* detected by biological tests only. PM, post-mortem. NV, none visible.

Table 3. *Analysis of skin-test results and isolation of* M. bovis *in 45 individual badgers of which 10 were infected*

	Sensitivity (%)	Specificity (%)	Predictive value positive	Predictive value negative
All reagents	70	74	44	90
PPD2	10	91	33	78
PPD20	20	88	33	79
T2	10	97	50	79
T20	0	97	0	77
AN5 72 h	30	85	38	81
AN5 ⩾ 7 days	20	91	40	80

PPD2, bovine PPD (Weybridge) 2 μg/ml
PPD20, bovine PPD (Weybridge) 20 μg/ml
T2, new human tuberculin 2 μg/ml
T20, new human tuberculin 20 μg/ml
} All reactions read at 72 h

AN5 72 h, whole killed cells of *M. bovis* strain AN5 1 mg/ml, read at 72 h
AN ⩾ 7 days, whole killed cells of *M. bovis* strain AN5 1 mg/ml, read at 7 days or more

Histological findings

The histological findings in all the tissues examined were typical of 'early lesions' of tuberculosis in badgers as described by Gallagher, Muirhead & Burn (1976). There was typically a mild cellular reaction comprising a small central necrotic focus surrounded by polymorphonuclear leucocytes and epithelioid cells. Outside this were lymphocytes and macrophages and a mild fibroblastic reaction, but no giant cells of Langhans. The cases in this study differed from some of those described by Gallagher, Muirhead & Burn (1976) in that they had not died of tuberculosis, and only very scanty numbers of acid-fast bacilli were seen in all the sections stained by Ziehl–Neelsen's method.

Skin-test results

Any increase in skin thickness together with erythema was recorded as a positive reaction. The optimum time for reading the test was 72 h after inoculation. Any results recorded 1 week or more after testing were those seen at PME and were all at the site of AN5 inoculation. Histological sections of these skin-test reactions showed the cellular types typical of delayed-type hypersensitivity, i.e. initial polymorphonuclear cell infiltration followed by lymphocytes and macrophages and granuloma formation.

The results are summarized in Table 3. Most positive results were obtained using bovine PPD at 20 μg/ml, and AN5 at 1 mg/ml both read at 72 h, and these are included in Table 4 together with a summary of PME and clinical sampling results. Bovine PPD and human tuberculin at 2 μg/ml each detected one infected badger; human tuberculin at 20 μg/ml did not detect any of the infected badgers. A comparison of all the diagnostic methods used to detect *M. bovis* infection is summarized in Table 4.

Table 4. *Comparison of methods of detecting badgers infected with* M. bovis

Social group	Clinical* samples	Faeces† samples	Post-mortem: Visible lesions	Post-mortem: Biological and cultural tests	Skin-test results at 72 h: Number tested	Bovine (20 μg/ml): true +ve	Bovine (20 μg/ml): false +ve	ANS (1 mg/ml): true +ve	ANS (1 mg/ml): false +ve
A	0/6	0/3‡	0/3‡	0/3‡	3	0	2	0	2
B	0/12	0/6	1/6	1/6	6	0	1	0	0
C	0/6	0/3	0/6	0/3	3	0	0	0	2
D	1/16	1/8	3/8	3/8	8	1	0	1	0
E	1/18	1/9	3/9	5/9	9	1	0	2	0
F	0/10	0/5	1/5	1/5	5	0	1	0	0
G	0/22	0/11	0/11	0/11	10	0	0	0	1
H	0/4	0/2	0/2	0/2	1	0	0	0	0

* Urine, tracheal aspirate, bite wound swabs – number of samples *M. bovis*-positive/number of samples examined.

† Taken from the individual badgers.

‡ Number of badgers *M. bovis*-positive/number of badgers examined.

DISCUSSION

The disruption in the trapping unfortunately prevented an examination of the population ecology in the project area. This is apparent from the small numbers of badgers trapped from three social groups, and the badgers were observed in the area after trapping had been stopped. Only an estimate of the minimum population density (9 adults per km^2) was therefore possible, but this was higher than that recorded in Staffordshire (6·2/km^2), Cornwall (4·7/km^2) and Avon (4·9/km^2) and apparently lower than the high densities of 20 adults per km^2 recorded on the Cotswold escarpment in Gloucestershire (Cheeseman *et al.* 1981, 1985*b*).

The disruption also resulted in only an estimate of the prevalence of *M. bovis* infection and an incomplete picture of infection within individual social groups. Bacteriological examination of faeces from these social groups during the spring had been carried out in 1984 and in the previous 3 years (Wilesmith *et al.* 1986*b*). The isolation rate of *M. bovis* from faeces was relatively low in 1984, infection being confirmed in only two social groups (B and F). The other two groups found to be infected as a result of post-mortem examinations (D and E) contained individuals which would appear to have been potential faecal excretors of *M. bovis* during the spring. These animals may have been intermittent excretors (Report, 1983), but the effects of the relatively small numbers of organisms and the presence of contaminant organisms on the isolation rate from faeces indicate the need to examine relatively large numbers of samples over a period of time to establish the distribution of infection in a badger population.

The largest number of badgers was trapped from social group G. This group was infected in 1981 and 1982, but *M. bovis* was not isolated from faeces in 1983 and 1984 (Wilesmith *et al.*, 1986*b*) and *M. bovis* was not detected in any of the 11 badgers. This may be an example of the natural extinction of infection in a social group, but the incompleteness of the trapping necessitates care in interpreting this

finding. This phenomenon has not yet been observed in the prospective study of a naturally infected population described by Cheeseman *et al.* (1985*b*).

The prevalence of infection has been observed to be higher in adult males than in adult females in other areas (Gallagher & Nelson, 1979) and in East Sussex (Wilesmith *et al.* 1986*b*). This difference has been substantiated in badgers trapped in the course of badger removal operations in south-west England to prevent cattle infection (MAFF, unpublished observation). The sex ratio of adult badgers in four studies has been unity (Stubbe, 1965; Kruuk, 1978; Cheeseman *et al.* 1981, 1985*a*), but there was an excess of adult females trapped in this study. This suggests a higher proportion of adult females were caught. The observed crude prevalence of infection is therefore likely to be an underestimate for this population.

The prevalence of infection in cubs was higher than in adults in some areas of south-west England (Cheeseman *et al.* 1981). In a similar study in Staffordshire the prevalence of infected cubs was 7·7% compared with 21·8% of the adults (Cheeseman *et al.* 1985*a*). The observed prevalence was also lower in cubs in this study. The incidence, and therefore importance, of transmission of *M. bovis* from dams to their offspring in their first few months of life is not known. A tuberculous female is known to have produced cubs (Cheeseman & Wilesmith, unpublished observations) and it is possible that the two infected cubs, both from social groups E, had been infected by the tuberculous adult female (92).

Neal (1977) has discussed the difficulties in determining the dates of birth of cubs, but recorded that in southern and south-west England the majority of births occur between mid-January and mid-March, with the peak in the first 3 weeks of February. The cubs caught would therefore have been between 2 and 6 months of age.

Little is known about the incubation period for *M. bovis* in badgers. Faecal excretion of *M. bovis* has been recorded in one badger 95 days after being placed in contact with known infected badgers and in another badger 97 days following experimental infection (Little, Naylor & Wilesmith, 1982). Excretion of *M. bovis* has also been recorded 26 days after intradermal infection (Mahmood *et al.* in preparation). The infected cub with miliary lesions in the lungs and kidneys and a tuberculous abscess in the submandibular lymph node indicates that under natural conditions the incubation period can be less than 6 months.

Biological testing, using guinea-pigs, is the most sensitive method for the isolation of *M. bovis* from cattle tissues (Lesslie, 1959) and from badger faeces (Wilesmith *et al.* 1986*b*). The culture media used proved to be insufficiently sensitive for the isolation of *M. bovis* from tissues and clinical specimens from these badgers; only 5 of the 10 infected badgers were detected by culture. This poor isolation rate on culture media was probably the consequence of the presence of only small numbers of bacilli in the majority of lesions. The preponderance of early lesions in this population is in agreement with the apparent increase in the prevalence of tuberculous badgers observed in the surrounding area during 1984, indicative of a recent increase in the incidence of infection (Wilesmith *et al.* 1986*b*). The freezing of tissues at −70 °C for subsequent isolation of *M. bovis* appears to be contra-indicated. The relative inefficiency of clinical sampling on one occasion to detect infection in badgers has been described previously (Cheeseman *et al.* 1985*a*). However, these authors' findings and those of the present study do

indicate that clinical sampling will detect animals with more advanced lesions, and the probability of such detection is obviously improved by sequential sampling of individuals, as in the prospective study described by Cheeseman *et al.* (1985*b*).

Previously reported attempts to demonstrate a delayed (Type IV)-type hypersensitivity skin response or *in vitro* transformation to bovine PPD in both naturally and experimentally infected badgers have been unsuccessful (Morris *et al.* 1978; Little, Naylor & Wilesmith, 1982). A further attempt to elicit a skin response with bovine PPD in badgers from a naturally infected population resulted in a small increase in skinfold thickness at 2–3 days and histological evidence of a weak delayed hypersensitivity (Higgins, 1985). The cellular reaction to bovine PPD was stronger in infected badgers than in uninfected badgers, but the other aspects of the skin response were not associated with the isolation of *M. bovis*.

This study therefore confirms these recent findings, with badgers exhibiting a delayed hypersensitivity to bovine PPD and whole killed cells of *M. bovis* which was also manifested clinically by a maximum response of oedema, erythema and induration at 72 h. However, these skin tests were neither sensitive nor specific enough to be of practical value. This method of testing also has the disadvantage of having to retain badgers for 72 h.

These results do provide some optimism for a non-destructive diagnostic test, but at present the bacteriological examination of post-mortem tissues remains as the most sensitive diagnostic method for *M. bovis* infection in badgers.

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