Papers

Geographical association between the genotype of bovine tuberculosis in found dead badgers and in cattle herds

A. V. Goodchild, G. H. Watkins, A. R. Sayers, J. R. Jones, R. S. Clifton-Hadley

In a survey, 457 badgers that had been found dead in Wales were postmortem-examined, and samples were examined by histology and by extended culture (for up to 12 weeks). *Mycobacterium bovis* was cultured from 55 badgers (12.0 per cent), and the histology typical of *M bovis* infection was seen in a further six (1.3 per cent). The prevalence in badgers in each of 10 geographical areas varied between 0 and 26 per cent (P<0.001), and was associated with the incidence of confirmed *M bovis* infection in cattle herds in the same areas (P<0.01). In northern Wales, bTB was rare in both hosts. An infected badger was 12.3 times more likely to be within 5 km of a confirmed cattle bTB breakdown than an uninfected badger. The *M bovis* isolates from badgers belonged to one of four genotypes defined by spoligotype and variable number tandem repeat type. These genotypes were also found in 290 concurrent confirmed herd breakdowns, and tended to be similar to the genotypes in badgers in the same geographical areas. When badgers and cattle no more than 30 km apart were compared, the genotype diversity was greater in cattle than in badgers (P=0.016), suggesting that the movement of cattle plays a greater part in the spatial distribution of *M bovis* than the movement of badgers.

THE number of cattle herds confirmed to have bovine tuberculosis (bTB) in Wales has doubled every 4.3 years between 1998 and 2009. This rate of increase is greater than was seen in Great Britain as a whole, which had experienced a doubling time of 6.2 years over the same period (Defra 2010). The number of confirmed herd breakdowns (CHB) of bTB disclosed in Wales reached 513 in the year 2009 (Defra 2010). The geographical distribution of bTB in cattle in Wales is heterogeneous; the great majority of cases are in the south-west (Pembrokeshire and Carmarthenshire) and in the east and south-east (close to the border with England, in Powys and Monmouthshire). Fewer cases are disclosed in north and north-west Wales (Defra 2010).

In Great Britain, cattle and badgers are involved in a shared epidemic of bTB (Jenkins and others 2007). The probability that cattle become infected with bTB by badgers is associated with the density of badger setts (Wilesmith 1983, Cheeseman and others 1989), the prevalence of the disease in nearby badgers (Woodroffe and others 2005) and herd management and animal behaviour (Gallagher and Clifton-Hadley 2000). There was, however, no simple relationship between the badger population density in a national survey and the prevalence of bTB in badger removal operations (BROs) (Cheeseman and others 1989). In the randomised badger controlled trial (RBCT), bTB in cattle herds during the first proactive cull was more reliably predicted by the prevalence of bTB in nearby culled badgers than by the number of infected badgers per square kilometre (Donnelly and Hone 2010) or the number of badgers found during culling (Vial and others 2011).

Animal Health and Veterinary Laboratories Agency (AHVLA) has historic data for the prevalence of bTB in badgers that were found dead after road traffic accidents (RTA), culled in BROs or proactively removed in the RBCT in England. The weighted mean prevalence of bTB in 110,271 RTA badgers in 1972 to 1999 was 19.0 per cent, slightly lower than the matched prevalence of bTB in 105,868 BRO badgers removed in the same year and 10 × 10 km map square, which was 21.4 per cent. This was not unexpected, as BROs tended to target areas with high prevalence. Later data that compared 18,536 RTA badgers with 24,446 RBCT badgers in 1997 to 2005 found that the weighted prevalence was 22.1 per cent in RTA badgers and 19.5 per cent in RBCT badgers. The authors concluded that RTA badgers are informative indicators of the prevalence of bTB in the badger population; since the majority of found dead badgers are killed in road accidents, they could fill the same role.

Identification and molecular typing (genotyping) of *Mycobacterium bovis* isolates by spacer oligonucleotide typing (spoligotyping) and variable number tandem repeat (VNTR) typing is routinely performed in Great Britain and other countries (Smith and others 2003). Genotypes of *M bovis* tend to be geographically localised (Smith and others 2003, Woodroffe and others 2005, Jenkins and others 2007), and genotype data can be used to trace the probable geographical origin of infection in herds that have purchased cattle (Gopal and others 2006). Localised correlations between the prevalence in badgers and the incidence in cattle have long been recorded (Abernethy and others 2003), and as expected the proportions of *M bovis* of each genotype found in cattle

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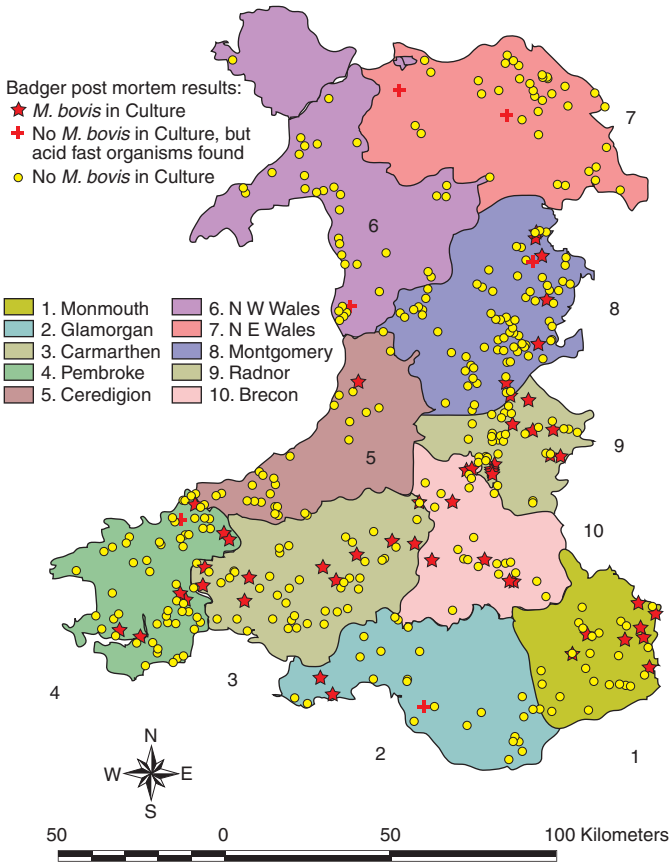

FIG 1: Location and *M bovis* status of 457 found dead badgers postmortem-examined in Wales between October 26, 2005, and May 31, 2006, in 10 geographical areas

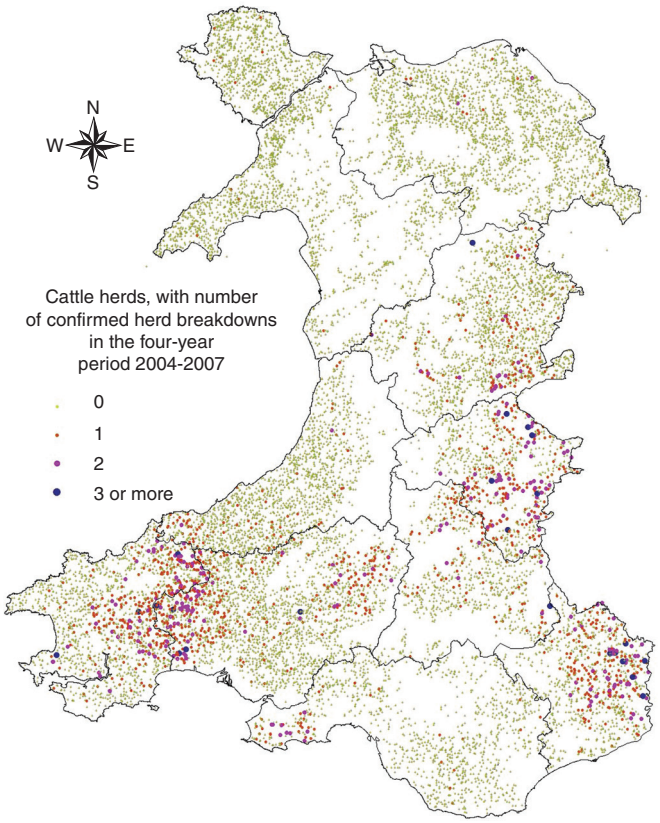

FIG 2: Cattle herds: the locations and numbers of confirmed herd breakdowns (CHB) disclosed between January 1, 2004, and December 31, 2007

TABLE 1: Frequency of genotypes of *M bovis* and the prevalence of tuberculosis in found dead badgers in 10 geographical areas of Wales between October 26, 2005, and May 31, 2006

		Number of postmortem-examined badger numbers with each *M bovis* genotype or AFOs, and prevalence							
							Badgers with *M bovis* or AFOs		Two-tailed P=95% binomial CI
Geographical area label (as used in Fig 1)	Number of badgers postmortem-examined	9;b	9;c	17;a	22;a	AFOs	Number	Prevalence (%)*	
1. Monmouth	31	0	1	0	**7**†	0	8	26%	(12-45%)‡
2. Glamorgan	30	**2**	0	0	0	1	3	10%	(2-27%)
3. Carmarthen	55	**6**	0	3	0	0	9	16%	(8-29%)
4. Pembroke	63	**6**	0	2	0	1	9	14%	(7-25%)
5. Ceredigion	31	0	0	**1**	0	0	1	3%	(0.1-17%)
6. NW Wales	38	0	0	0	0	2	2	5%	(0.6-18%)
7. NE Wales	38	0	0	0	0	1	1	3%	(0.1-14%)
8. Montgomery	84	0	0	**5**	0	1	6	7%	(6-15%)
9. Radnor	50	0	**13**	0	0	0	13	26%	(15-40%)
10. Brecon	37	0	**6**	0	3	0	9	24%	(12-41%)
Total	457	14	20	11	10	6	61	13.3%	(10.4-16.8%)

*The prevalence of *M bovis* infection detected by culture alone averaged 12.0 per cent and varied significantly by geographical area (χ^2=37.46, 9 d.f., P<0.0001. Prevalence defined by the presence of *M bovis* or AFOs also varied significantly by geographical area (χ^2=23.25, 9 d.f., P=0.006)
†In each geographical area, the number of badgers having the most prevalent molecular types are in bold
‡Pezzullo 2010b
AFOs Acid-fast organisms

and badgers in RBCT reactive culling operations closely resembled one another (Woodroffe and others 2009). There was, however, no significant evidence that cattle movements affected this (Woodroffe and others 2009).

A report on the prevalence of bTB in badgers that were found dead in Wales (VLA 2007) was commissioned by the Welsh Assembly Government and was based on data for badgers collected between October 2005 and May 2006. The present study follows on from this report with a more detailed description of the methods and results, highlighting the geographical distribution of four genotypes of *M bovis* in the badgers and in contemporaneously culled cattle.

The authors examined found dead badgers for *M bovis* infection by postmortem examination, histology, extended culture and genotyping. The study was designed to map the distribution of prevalence of *M bovis* and its genotypes in found dead badgers in Wales, and to describe the spatial relationship between the prevalence of *M bovis* and its genotypes in badgers and the incidence of *M bovis* and its genotypes in cattle. Although the study was limited to a seven-month period by the resources available, it was possible to calculate (from data on 57 690 badgers examined between 1972 and 2006 by AHVLA) the size of the likely bias in the estimate of average prevalence. A preliminary calculation of the mean prevalence was 13 per cent, slightly less than the mean prevalence of bTB in the south-west and west Midlands of England in both a seven-county survey of road-killed badgers in 2002 to 2005 (Independent Scientific Group on Cattle TB 2007), and the RBCT (Woodroffe and others 2009).

TABLE 2: Number of herds, of herds with confirmed new bovine TB herd breakdowns and annual confirmed herd incidence in 10 geographical areas in Wales for two periods of time

		Period in which the infection was first detected in cattle herds					
		During the Found Dead Badger Survey, ie, October 26, 2005, to May 31, 2006 (218 days)			From January 1, 2004, to December 31, 2007 (1461 days)		
		Cattle herds			Cattle herds		
Geographical area label (as used in Fig 1)	Prevalence in badgers (From Table 1)	Number of herds in existence	Number of CHB disclosed	Annual confirmed incidence*	Number of herds in existence	Number of CHB disclosed	Annual confirmed incidence*
1. Monmouth	25.8%	1038	40	6.81%	1026	247	6.52%
2. Glamorgan	10.0%	1167	12	1.74%	1156	58	1.27%
3. Carmarthen	16.4%	2404	51	3.75%	2404	387	4.27%
4. Pembroke	14.3%	1542	63	7.53%	1542	365	6.54%
5. Ceredigion	3.2%	1437	17	2.04%	1442	65	1.16%
6. NW Wales	5.3%	2201	2	0.15%	2206	13	0.15%
7. NE Wales	2.6%	2196	1	0.08%	2193	19	0.22%
8. Montgomery	7.1%	1552	46	5.13%	1554	215	3.57%
9. Radnor	26.0%	744	43	10.44%	743	250	9.11%
10. Brecon	24.3%	728	25	6.02%	720	120	4.35%
Total or mean	13.3%	15,009	300	3.47%	14,986	1739	3.02%
Correlation between annual confirmed incidence in herds and prevalence in badgers (Spearman's ρ)				+0.830 (P=0.0030)			+0.879 (P=0.0008)

*The denominator for incidence is the average number of herds that were not under movement restriction for the control of confirmed or unconfirmed bovine TB

Materials and methods

Locating and collecting badgers

Local authorities, countryside organisations and individuals in Wales found dead badgers and reported their locations to Animal Health, who recorded map references and collected potentially suitable carcases between October 26, 2005, and May 31, 2006. Animal Health delivered these carcases to one of three Veterinary Laboratories Agency (VLA) Regional Laboratories, where they were stored at between 2°C and 8°C for no more than three days before postmortem examination.

Postmortem examination and sampling

Of 727 badger carcases reported to Animal Health, 549 were collected and 457 (63 per cent of reported carcases) were suitable for postmortem examination. Unsuitable carcases comprised those with punctured body cavities, or those that were distended with gas, invaded by maggots or flattened by vehicles. The prevalence of bTB was calculated for suitable carcases only. There was no evidence that suitable carcases differed from unsuitable or uncollected carcases in terms of sex, tooth wear or body length. They were examined at one of three VLA Regional Laboratories by the procedure later reported by Jenkins and others (2007) as modified by Crawshaw and others (2008). The pooled sample of lymph nodes taken for bacteriological culture from each carcase consisted of halves of the retropharyngeal, bronchial, mediastinal and hepatic lymph nodes (or as many as were detectable), and was preserved in 15 ml of 1 per cent aqueous cetylpyridinium chloride. Any visible lesions typical of bTB in the remaining halves of these lymph nodes were preserved in buffered 10 per cent formaldehyde solution for histology. A further 14 organs and lymph nodes were also incised and examined; if any gross internal lesions typical of bTB were found, portions were added to the pool for bacteriology, and (if sufficient) to the sample for histology. If bite wounds were found, they were excised, sampled for bacteriology and histology, and placed in additional containers.

Culture, molecular typing and histological examination

The samples for bacteriology were sent to a fourth VLA Laboratory (in Truro), usually arriving on the day after the postmortem examination. They were washed in sterile 0.85 per cent saline solution, homogenised by standard methods, inoculated onto 12 modified Middlebrook 7H11 agar slopes (Atlas and Snyder 1995) and incubated at 37°C. The slopes were examined weekly from the end of week 2 for a maximum of 12 weeks, and were harvested when colonial growth was sufficient for genotyping. Incubation for more than six weeks, if necessary, was a modification of the conventional procedure recommended by Crawshaw and others (2008).

Genotyping was performed at the VLA Weybridge Laboratory using spoligotyping (Kamerbeek and others 1997) and VNTR typing (Exact Tandem Repeat loci A to F, Frothingham and Meeker-O'Connell 1998), and served to confirm that the organisms isolated were *M bovis*. Genotypes of *M bovis* were labelled according to the current VLA convention, using numbers to represent spoligotypes and lower case letters to represent the VNTR pattern within each spoligotype. These labels could be translated into the combinations of international spoligotype numbers (Smith and Hilscher 2009) and VNTR patterns that were found in Great Britain. The same genotyping methods were applied to the cultures of *M bovis* from cattle slaughtered as part of the national bTB control programme of Great Britain.

If gross internal lesions suggestive of tuberculosis or bite wounds were seen from which *M bovis* could not be cultured, the samples of formalin-preserved samples of the internal tissues or bite wounds, if available, were sectioned, stained by the Ziehl-Neelsen method, and examined for acid-fast organisms (AFO) (Sheehan and Hrapchak 1980). Finding AFOs was taken as evidence of mycobacterial infection, but did not allow the identification at species level or differentiation from *Nocardia* species.

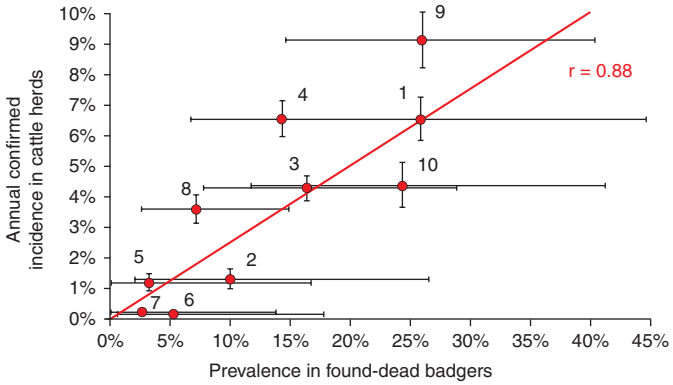

FIG 3: Scatter diagram of confirmed new incidence of bovine TB in cattle herds during the four-year period 2004 to 2007 against the prevalence of bTB (as *M bovis* or acid-fast organisms) in found dead badgers for the 10 geographical areas, with 95 per cent binomial CI for both variables and the line of best fit (weighted by the number of badgers). Slope=0.25 (95 per cent CI 0.15 to 0.35, P<0.001). The intercept was not significant (t=1.21, P=0.26). The quadratic coefficient was not significant (t=0.23, P=0.83)

Interpretation of cattle statistics

The location (map reference) of cattle herds was taken from the download from the Animal Health database VetNet nearest to the middle of the badger collection period (February 12, 2006). This was a reference to the location of the herd rather than to the location of the holding.

TABLE 3: Number of herds, of herds with unconfirmed new bovine TB breakdowns and annual unconfirmed herd incidence in 10 geographical areas in Wales for two periods of time

		Period in which the infection was first detected in cattle herds			
		During the Found Dead Badger Survey, ie, October 26, 2005, to May 31, 2006 (218 days)		From January 1, 2004, to December 31, 2007 (1461 days)	
		Cattle herds		Cattle herds	
Geographical area label (as used in Fig 1)	Prevalence in badgers (from Table 1)	Number of unconfirmed breakdowns*	Annual unconfirmed incidence†	Number of unconfirmed breakdowns*	Annual unconfirmed incidence†
1. Monmouth	25.8%	24	3.9%	166	4.0%
2. Glamorgan	10.0%	1	0.3%	44	1.0%
3. Carmarthen	16.4%	30	2.1%	334	3.5%
4. Pembroke	14.3%	26	2.9%	308	5.0%
5. Ceredigion	3.2%	24	2.8%	152	2.6%
6. NW Wales	5.3%	4	0.3%	15	0.2%
7. NE Wales	2.6%	8	0.6%	45	0.5%
8. Montgomery	7.1%	22	2.4%	124	2.0%
9. Radnor	26.0%	11	2.5%	86	2.9%
10. Brecon	24.3%	12	3.0%	76	2.6%
Total or mean	13.3%	165	1.84%	1350	2.3%
Correlation between annual unconfirmed incidence in herds and prevalence in badgers (Spearman's ρ)			+0.527 (P=0.12)		+0.709 (P=0.022)

*Includes breakdowns described as unconfirmed and unclassified
†The denominator for incidence is the average number of herds that were not under movement restriction for the control of confirmed or unconfirmed bovine TB

TABLE 4: Association between the presence of at least one confirmed breakdown in cattle within 5 km of a found dead badger and the infection status of the badger: univariable analysis

Culture or microscopy result	Risk factor: number of CHB disclosed in cattle herds within 5 km of the badger	
	At least one CHB	No CHB
Badger with *M bovis* in culture or AFOs	60	1
Badger-negative for *M bovis*	319	77
Proportion of badgers with *M bovis* in culture or AFOs	15.8%	1.3%

The difference is significant at P<0.001 by Fisher's Exact two-tailed test. The odds ratio is 14.5 (95 per cent CI 2.5 to 83.9) and the relative risk is 0.158/0.013=12.3 (95 per cent CI 2.3 to 70.6) (Pezzullo 2010a)
AFOs Acid-fast organisms, CHB Cattle herd breakdowns

Confirmed heard incidence was calculated from the Animal Health database VetNet-TBiC (TB in Cattle) as the number of CHB divided by the number of years that the herds were not under movement restriction for bTB. The start date of a CHB in a herd is the date of a test (skin test or abattoir surveillance) that leads to a period of movement restrictions because of the suspicion of bTB, if the infection is confirmed. This confirmation requires a lesion typical of bTB to be found in a skin test reactor, or *M bovis* to be cultured from a tissue sample, at any time during the breakdown. Confirmed breakdowns may be confirmed at any time during the breakdown, and are not normally closed until two successive skin tests reveal no reactors.

Unconfirmed breakdowns are periods of movement restriction due to suspicion of bTB in which the infection may or may not be present but is not confirmed. Unconfirmed incidence was calculated with the same denominator as confirmed incidence.

Two estimates were made of the incidence of confirmed bTB in herds: one for herds first detected to have bTB between October 26, 2005, and May 31, 2006 (with 300 CHB disclosed), and one for herds first detected in the four-year period January 1, 2004, to December 31, 2007 (with 1739 CHB disclosed). The first of these periods coincided with 218 days over which found dead badgers were collected; and the second was long enough for all herds to receive at least one herd test.

Seasonal and spatial analysis of bTB prevalence

Estimation of seasonal bias in bTB prevalence in badgers

The possibility of bias in the estimation of prevalence caused by the uneven distribution of months in which the badgers were collected was examined by comparison with VLA-held databases. The effect of month was calculated by logistic regression from the results of 31,100 necropsies of badgers taken in BROs and 15,600 necropsies of road-killed badgers between 1972 and 1999 in Great Britain, classified by year and source of data. The reference category for month was the month with the largest number of observations, July. The pattern of prevalence calculated from these data was used to estimate the probable effect on average prevalence caused by the actual numbers of badgers collected each month.

Analysis by geographical area

The 10 geographical areas used for the spatial analyses were local authorities or groups of them (Fig 1, online Appendix Table 1). Each geographical area was the source of at least 30 postmortem-examined badgers. For a group of 30 badgers of which none was culture-positive, the 95 per cent binary-distribution CI for prevalence is between 0 and 11.6 per cent (Pezzullo 2010b). The relationship between badger prevalence and confirmed cattle incidence was calculated as a Spearman non-parametric correlation coefficient.

Analysis by distance between badgers and cattle

A case-control approach evaluated potential risk factors that might influence the proportion of found dead badgers that were infected with bTB. The location of each herd was identified by the map reference of the herd, rather than the map reference of the parent holding, if this was different.

In the first of two analyses, the risk factor was simply the presence of one or more infected cattle herd within 5 km of the found dead badger, and the outcome was the result of culture or microscopic examination for AFOs. The resulting 2 × 2 contingency table was tested by Fisher's Exact test and the result expressed as an odds ratio (OR) and relative risk (Pezzullo 2010a).

In the second analysis, the potential risk factors for infection in the found dead badgers were evaluated using a stepwise logistic regression method in which the outcome variable was the infection status of the badger. These factors included the number of bTB-infected cattle herds within 5 km of the badger, whether the badger had a bite wound, the number of active herds within 5 km of the badger, Animal Health Office collecting the badger, and the month of receipt of the badger. Continuous variables were included, but because of their skewed distributions their logarithmically transformed values (log [n+1]) were also included. The authors retained the risk factors in the regression model if they reduced Akaike's Information Criterion (AIC) (ie, if they improved the precision of prediction without including too many variables). After stepwise selection of variables for the model, curvilinear responses to numerical variables and interactions between variables were tested for possible inclusion. Finally, previously excluded variables were reconsidered on the basis of their AIC. The goodness-of-fit between candidate models and the data was tested with the statistic of Hosmer and Lemeshow (1989); P>0.05 indicated a satisfactory model.

TABLE 5: Relationship between the presence of bovine tuberculosis (bTB) in badgers and the number of confirmed new cattle herd breakdowns (CHBs) within 5 km, the presence of bite wounds in badgers and the month in which they were collected (by logistic regression)

Factor*	Odds ratio and 95% CI	Probability
Number of confirmed new breakdowns within 5 km of the badger (CHB)		
Log (number of CHB+1)	6.25 (2.73 to 14.32)	<0.001
Presence (versus absence) of bite wounds in the badger		
Wounds seen	5.04 (2.59 to 9.80)	<0.001
Number of active herds (regardless of bTB infection status) within 5 km of the badger		
Number	0.984 (0.968 to 1.001)	0.062

Number of observations=457; χ^2 for logistic regression (9 d.f.) =86.7, P<0.0001; Hosmer-Lemeshow χ^2 for lack of fit (8 d.f.)=7.55 (P=0.48)
*Two further factors that were not statistically significant (P>0.10) but reduced the Akaike's Information Criterion (AIC) were Animal Health Office responsible for collecting the badger and the month of receipt of the badger. The number of badgers received before February 2006 was 19, and the numbers in February, March, April and May were 150, 138, 107 and 43, respectively

TABLE 6: Genotypes of *M bovis* found in individual cattle in Wales between October 26, 2005, and May 31, 2006

	Genotypes found in cattle samples*					
Geographical area label (as used in Fig 1)	Genotypes that were also seen in badgers† 9;b	9;c	17;a	22;a	Genotypes not seen in badgers‡	Total
1. Monmouth		18	3	37	9;a, 10;a (2), 11;a, 13;a, 22;f, 25;a	65
2. Glamorgan	18	1	3		9;a (2)	24
3. Carmarthen	64	8	25		9;a, 15;g, 17;c, 17;l, 25;a	102
4. Pembroke	104		20		9;a, 9;d, 9;g, 17;l, 21;a	128
5. Ceredigion	12		3		9;g (2), 25;a	18
6. North-west Wales			2		15;a	3
7. North-east Wales				1	25;a	2
8. Montgomery		8	63		19;a, 20;a, 35;a	74
9. Radnor	2	70	11		35;a	84
10. Brecon		26	5	10	15;a	42
Grand total	200	131	135	48	28	542

*Nine cattle for which only the spoligotype was determined are omitted from the table
†Within geographical area, Spearman's coefficient of rank correlation (ρ) between the frequency distribution of genotypes seen in badgers with the distribution of the same genotypes in cattle increased with the number of different genotypes seen in badgers (Table 1). Where two genotypes were found in badgers in a geographical area (ie, in Monmouth, Carmarthen, Pembroke and Brecon), the value of ρ was between 0.8 and 1.0 (see the text)
‡Although these genotypes were not seen in badgers, the proportion of them found in cattle (28/542=5.2 per cent) was not significantly greater than the proportion found in badgers (0/55)

Cattle and badgers: analysis by genotype

Associations by geographical area

The spatial density of cattle confirmed herd breakdowns disclosed per square kilometre per year between 2004 and 2007 (for each of the four genotypes of *M bovis* that were also found in badgers) was mapped in ArcView GIS 3.3 (Environmental Systems Research Institute 2002) using Spatial Analyst kernel smoothing. The position of the infected badgers is shown on the same map.

In each of the eight geographical areas in which the badger genotypes had been determined, the authors calculated the associations between the frequency distribution of the genotypes in badgers and the frequency distribution in cattle. In order to prevent ties, frequencies were adjusted by replacing them with their deviations from expected values. The expected values for each host species were calculated as (geographical_area_subtotal) × (genotype_subtotal)/(grand_total). A Spearman rank correlation between the ranks of genotypes in badgers and their ranks in cattle was then calculated for each geographical area.

Variogram analysis: effects of distance on dissimilarity of genotypes

As the distance between observations (badgers to badgers, CHBs to CHBs and badgers to CHBs) increases, the frequency distribution of any given attribute(s) is expected to approach randomness. In the present calculations, the distances were grouped as ranges of distances (eg, in 10, 20 or 30 km wide classes), the comparisons between badgers and/or herds were between all possible pairs, the attributes in question were genotypes, and the frequency distribution was calculated for the proportion of the pairs of genotypes that differed from one another. From all possible pairs of badgers that were in the ranges >0 to 30, >30 to 60, >60 to 90 and >90 km apart, the authors calculated the proportion of pairs that had dissimilar genotypes. The standard errors of the proportions were calculated using 16 random 50 per cent subsets of the raw data. The analyses were repeated for all pairs of herds, and for all pairs consisting of one badger and one herd. When more than one different genotype had been isolated from the same herd, the authors used the most frequent genotype (the few herds with ties were omitted); cattle herds having none of the four genotypes that were found in badgers were also ignored. Similar analyses were performed for the pairs of badgers and/or herds >0 to 10, >10 to 20, >110 km apart and >0 to 20, >20 to 40, >100 km apart.

Results

Distribution of tuberculosis in badgers found dead in Wales

All except a few (<10) of the found dead badgers were reported to be close to the roads and had lesions typical of a vehicle impact. Although some carcases were rejected, the reasons for doing so reflected damage or decomposition (as specified in Methods) rather than signs likely to be associated with infection.

Of the cultures made from the 457 postmortem-examined badgers, *M bovis* was isolated from 55 badgers (12.0 per cent). The cultured samples from 88 badgers having gross internal lesions considered to be suggestive of bTB infection yielded *M bovis* in only 21 (23.9 per cent) of the badgers, whereas the samples from 369 carcases that had no suggestive lesions yielded *M bovis* in 34 (9.2 per cent). Bite wounds, seen in 101 badgers, were culture-positive in eight animals (7.9 per cent), but in seven of these, samples of internal tissues were also culture-positive.

Histological examination of the samples from 117 culture-negative badgers, 43 of which had gross internal lesions, 64 had bite wounds and 10 had both types of lesion, revealed AFO in samples from six badgers (two in gross internal lesions and four in bite wounds). These six badgers represented a prevalence of 1.3 per cent, giving a total prevalence (*M bovis* and AFO) of 13.3 per cent. The proportion of lesions confirmed by culture was marginally greater in samples from gross internal lesions than from bite wounds (P=0.06 by Fisher's Exact test).

Postmortem examinations were performed between Monday and Thursday, and less frequently on Fridays. The day of the working week on which the postmortem examination was performed was weakly associated with the proportion of badgers yielding *M bovis* on culture (chi-square statistic [χ^2]=9.75, 4 degrees of freedom [d.f.], P=0.045). The proportion of animals that were culture-positive tended to increase between Monday and Friday (5.4, 12.7, 8.5, 18.2 and 16.7 per cent on successive days), but the rate of increase was not significant (r=+0.82, P=0.087), suggesting that delaying the postmortem examination of carcases until after a weekend may not have been the sole source of variation of culture positivity.

Geographical distribution of badgers, herds and bTB

The largest prevalence of *M bovis* infection in the found dead badgers was seen close to the central and southern parts of the border with England and in a band stretching from Radnorshire to

Pembrokeshire (Fig 1). The prevalence of tuberculosis confirmed by culture varied significantly between the 10 geographical areas (χ^2 =32.71 with 9 d.f.; P<0.001, Table 1). The geographical areas used in these analyses are defined in online Appendix Table 1.

Registered cattle herds in Wales are mapped as shown in Fig 2, with symbols indicating the number of CHBs, if any, that were disclosed in the four years between January 1, 2004, and December 31, 2007.

The confirmed incidence of bTB in cattle herds was estimated for both the survey period (218 days) and the four years 2004 to 2007 in each geographical area (Table 2). The confirmed incidence in cattle herds in both periods was significantly correlated (P=0.003 and 0.0008 by Spearman's ρ) with bTB prevalence in the found dead badgers by geographical area. The plot of means and binomial CI of confirmed herd incidence in the four-year period and badger prevalence (Fig 3) suggests that there may have been outliers. For example, the confirmed herd incidence was higher than expected (or the badger prevalence lower than expected) in the point labelled '9' (Radnor). The reverse may be true in north-west Wales (labelled '6'), if it was true that AFOs represent bTB infection. When the confirmed herd incidence plotted against badger prevalence was calculated for the 218-day period (Table 2), the chart (not shown) differed little from that shown in Fig 3.

The incidence of unconfirmed bTB was compared with the prevalence in badgers in a similar manner (Table 3). The significance levels of the correlations were several times weaker than the correlations seen with confirmed incidence. Part of the reason for these weaker correlations may be that the number of unconfirmed breakdowns was smaller.

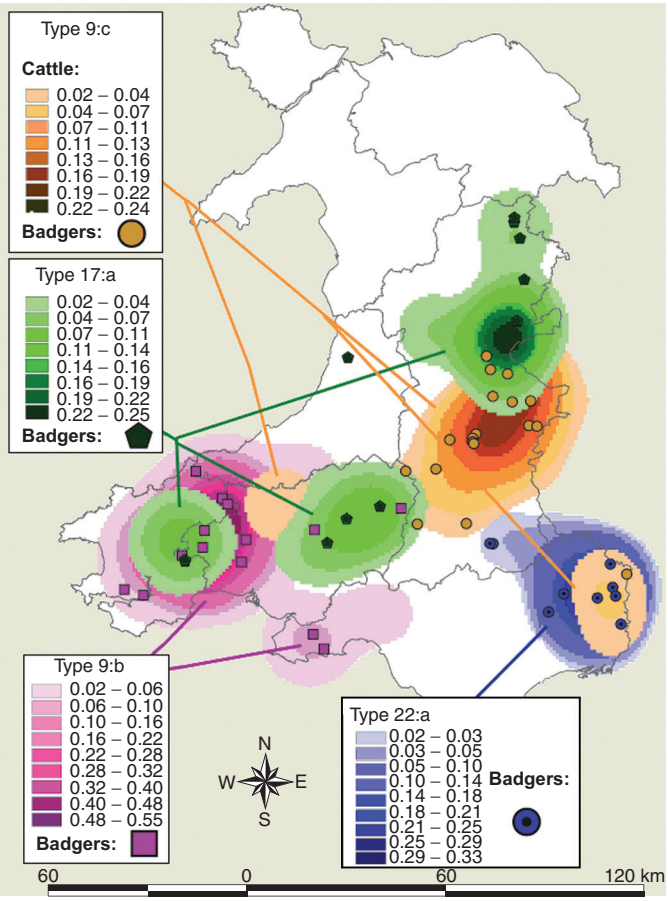

FIG 4: Geographical distribution of genotypes of *M bovis* in postmortem-examined cattle and found dead badgers in Wales. Locations and genotypes of individual badgers found to have *M bovis* are shown as coloured symbols. The relative incidence per square kilometre of *M bovis* genotypes in cattle herds has been kernel-smoothed using a 25 km bandwidth. Note that the kernels for genotype 17;a and genotype 9;c partly obscure other kernels

Analysis by 5 km radii around each found dead badger

There was a strong association (P<0.001) between the infection status (by culture or histology) of a found dead badger and whether any CHB was disclosed within 5 km of the badger in the four-year period 2004 to 2007 (Table 4). The OR was 14.5 (95 per cent CI, 2.5 to 83.9) and the relative risk was 12.3 (Pezzullo 2010a).

Logistic regression analysis

Logistic regression confirmed the association between the infection status of the badgers and the existence of CHBs within 5 km. Stepwise logistic regression chose logarithm of the number of bTB-infected cattle herds within 5 km of the badger, and having a bite wound as the only significant (P<0.05) factor, although the number of herds within 5 km was almost significant and would be protective (P=0.06). The OR were 6.3, 5.0 and 0.984, respectively (Table 5). Two of the other non-significant variables improved the fit of the regression (as measured by a reduction in AIC) and were retained in the model. The factors in the final model comprised two continuous variables based on the cattle within 5 km (the numbers of CHB and of herds), and three categorical variables describing the badger (whether it had a bite wound, the Animal Health Office responsible for collecting it, and the month of receipt of the badger).

Although the prevalence of bTB in badgers varied with month in the logistic regression, univariable analysis showed a peak in May (P=0.0075 by Fisher's Exact test), with less variability in the other months (χ^2=10.19, 3 d.f., P=0.017).

Seasonal bias in collection of data

Badger carcases were not collected throughout the whole year; therefore, the results presented may not be an unbiased estimate of the average prevalence during the year. The authors calculated that the distribution of the months in which the badgers were collected could have caused the mean estimate of the prevalence to be inflated 1.04-fold. The inflation factors for the various geographical areas ranged between 1.02-fold and 1.07-fold and did not differ significantly from the mean (P>0.05).

Comparison of genotypes of *M bovis* in badgers and cattle

Four distinct *M bovis* genotypes were found in badgers. There were three spoligotypes (SB0140, SB0263 and SB0673), and within SB0140 there were two different VNTR patterns. The genotypes have been given the labels 9;b, 9;c, 17;a and 22;a, where

9;b denotes spoligotype SB0140, VNTR 7-5-5-5*-3-2.1;
9;c denotes spoligotype SB0140, VNTR 7-5-2-4*-3-3.1;
17;a denotes spoligotype SB0263, VNTR 7-5-5-5*-3-3.1;
22;a denotes spoligotype SB0673, VNTR 7-5-2-4*-3-3.1.

TABLE 7: Proportion of members of all possible pairs of animals found to have different genotypes of *M bovis*, with 95% CI, between October 26, 2005, and May 31, 2006

Comparison	Distance between the members of pair			
	>0 to 30 km	>30 to 60 km	>60 to 90 km	>90 km
Badger-with-badger	0.245 ± 0.109*	0.679 ± 0.078	0.876 ± 0.057	0.950 ± 0.040
Badger-with-cattle†	0.352 ± 0.033	0.668 ± 0.018	0.863 ± 0.019	0.948 ± 0.010
Cattle-with-cattle†	0.387 ± 0.035	0.649 ± 0.031	0.859 ± 0.019	0.941 ± 0.011
Difference between badger-with-badger and cattle-with-cattle	0.142 ± 0.114 (P=0.016)	0.03 ± 0.084 (P=0.48)	0.017 ± 0.060 (P=0.57)	0.009 ± 0.041 (P=0.67)

*95 per cent CI, calculated from 16 analyses using random 50 per cent subsamples of animals
†Each herd was represented by one animal with a typical genotype. Cattle that had a genotype not found in badgers were ignored (see the footnote shown in Table 6)

The numbers of found dead badgers from which the listed genotypes were isolated were 14, 20, 11 and 10, respectively (Table 1). In the six geographical areas in which four or more badgers were infected, the relative frequency of each genotype varied significantly by geographical area (χ^2=90.25, 15 d.f., P<0.0001). In the seven badgers in which the genotypes were identified in both internal tissues and bite wounds, the genotypes were identical.

M bovis isolates with the same four genotypes were found in samples taken from 514 bTB-suspect cattle from 299 herds in Wales during the 218-day period in which badgers were collected and were labelled according to the above convention. Samples from 28 other cattle slaughtered at the same time revealed 15 further genotypes of *M bovis* that were not found in badgers. These represented 28/514 (5.2 per cent) of all genotyped cattle samples, but this proportion was not significantly different from the proportion (0/55) found in the badgers (P=0.098 by Fisher's Exact test; Pezzullo 2010a). Isolates from most of the herds were genotyped; for example, of 300 herds in which *M bovis* infection was first detected in the 218-day period, 290 (96.7 per cent) of herds had at least one sample genotyped. An average of 1.89 samples per herd was genotyped, and a single genotype was found in 89 (84.0 per cent) of the 106 CHB in which more than one isolate was genotyped.

The genotypes of *M bovis* identified in the cattle in Wales in the 218-day period are tabulated by geographical area as shown in Table 6 and mapped using kernel smoothing as shown in Fig 4. Genotypes 22;a and 9;b appear to form discrete groups, genotype 9;c has one main group and two smaller ones, and genotype 17;a appears in three groups (Fig 4). The geographical distribution of the four genotypes in the badgers is broadly similar to their distribution in the cattle (Table 1). In the four geographical areas in which two different genotypes were found in badgers, the Spearman rank correlation coefficient (ρ) between the adjusted ranking in badgers (based on Table 1) and the adjusted ranking in cattle (based on Table 6) averaged +0.9 (range +0.8 to +1.0). In the four geographical areas in which only one genotype was found in the badgers, the corresponding ρ averaged +0.8 (range +0.4 to +1.0). The d.f. for these estimates were too small for their statistical significance to be calculated, but the consistently positive sign of all 8 estimates of ρ suggests a real relationship. In the remaining two geographical areas, no *M bovis* sample was genotyped in the badgers; hence, ρ could not be calculated.

In badgers, some genotypes seem to be found outside the observed ranges of the same genotypes in cattle. For example, the three northernmost badgers with genotype 17;a appear further north than the majority of cattle with the same genotype, and two badgers in Carmarthen with genotype 9;b appear in one of the areas dominated by genotype 17;a (Fig 4). More data will be needed before the statistical or biological significance of these apparently outlying badgers can be established.

Variogram analysis of genotype of *M bovis* in badger and cattle isolates

Each cattle herd has been represented by the genotype of one typical animal in the herd. The variograms described in the present study represent, for badger–badger, cattle–cattle and badger–cattle pairs, the way in which the proportion of pairs with dissimilar genotypes varies with the distance apart of the members of these pairs. The results for 10 and 20 km intervals were not statistically significant and are not shown, but the results for 30 km intervals are given in Table 7. When the distance between the members of a pair was between 0 and 30 km, the proportion of badger–badger pairs having different genotypes was significantly (P<0.05) lower than the proportion of cattle–badger or cattle–cattle pairs. At distances greater than 30 km, there was no significant effect of animal species, where a large proportion (86 per cent or more) of the pairs already had different genotypes.

An alternative measure of dissimilarity is Simpson's Index of Diversity (SID; Simpson 1949), which tends to be greatest when genotypes are mixed in equal proportions. The SID behaved similarly to the variogram described above, but since the SID depends on less information than the variogram, the differences between badgers and cattle at given distances were not significant.

Discussion

Assessment of materials and methods

Adequacy of sample

Determining disease prevalence in a population ideally requires a sensitive, specific test applied to an adequately sized random sample. The series of the found dead badgers described here was subjected to detailed postmortem examination, was moderate in size, but was not a truly random sample. The study examined 457 badgers, which is intermediate between the numbers in two other recent studies: 277 in Northern Ireland (Abernethy and others 2003) and 3238 in seven English counties (Independent Scientific Group on Cattle TB 2007).

In the period during which the badgers were collected (218 days), the genotypes of *M bovis* in cattle were determined. On average, 1.89 cattle samples from each of 290 CHB were genotyped and the genotypes were not determined for only 10 further CHB. The number of cattle samples genotyped was therefore more than 10 times greater than the number of badger samples genotyped. Although samples were not taken from all cattle with evidence of infection, the genotypes in the majority of herds from which more than one animal was sampled were similar to one another. The authors therefore consider that the sample of genotypes in cattle herds was adequate for comparison with the genotypes in the badgers.

Age, sex and time pattern of the badgers sampled

The badgers were sampled in a 218-day period beginning in October and ending in May in the present study, a shorter period than for the other two studies mentioned above (3.6 or 4.0 years). According to the monthly distribution of badger prevalence between 1972 and 1999 described above, the uneven distribution of samples throughout the year in this study did not bias the estimate of prevalence significantly. It should also be noted that month did not affect the multivariable relationship between the prevalence in badgers and the confirmed cattle herd breakdowns within 5 km (Table 5), except for an almost significant rise in May.

The rise in the prevalence in badgers in May in the current study had not been seen in the pattern of prevalence in road-killed badgers between 1973 and 1999 (VLA data); therefore the authors examined demographic factors. Tooth wear, a proxy for age, did not significantly contribute to the logistic regression mentioned above (Table 5), although cub carcases were collected only in the later months of the study period (as in Rogers and others 1997). In male badgers, however, the prevalence of bTB was greater than in females, as found by Cheeseman and others (1989) and Woodroffe and others (2009). Because the proportion that were male increased between April and May (P=0.035 by Fisher's Exact test), it would have contributed a part of the rise in prevalence in May. Overall, the proportion of the found dead badgers that were male was significantly greater than half (59 per cent, with a 95 per cent CI of 55 to 64 per cent), possibly a result of males' greater dispersal behaviour (Gallagher and Clifton-Hadley 2000).

Collection of badgers

An examination of the map reveals that the found dead badgers tended to be reported near to main roads, but the number collected per square kilometre in each geographical area did not vary with the incidence of confirmed bTB in cattle herds (P=0.28). This was unexpected, as the population density of the badgers is known to vary geographically (Woodroffe and others 2005, Vial and others 2011), and reporting of the found dead badgers had been expected to reflect the public perception of the importance of badgers in bTB transmission (Cheeseman and others 1989).

Comparability of laboratory results with other studies

The estimates of bTB prevalence in the study are likely to have been affected by postmortem examination protocol and culture technique. In the present study, cultures were incubated for up to 12 weeks, twice the conventional incubation time, which has been shown to increase the detection of *M bovis* by a factor of about 1.3 (Crawshaw and others 2008). In addition to the *M bovis* found in 55 badgers by culture, AFOs were found in a further six badgers by histology. Because some of the mycobacteria identified by culture in the study

were *M avium* (Fig 1), and *Nocardia* species also are AFOs, the identification of these AFOs as *M bovis* was less certain than identification by culture.

Comparison of prevalence and incidence with other published estimates

Prevalence in badgers

There are no other recent studies in Wales with which to compare the results of the current survey, but the average prevalences of *M bovis* infection in several studies in the United Kingdom were of similar orders of magnitude. In comparison with 13.3 per cent (95 per cent CI 10.4 to 16.8 per cent) in the present study (if one includes AFOs), the results include 17.7 per cent (Abernethy and others 2003) and 15.0 per cent (Independent Scientific Group on Cattle TB 2007). The present study differed from the other two by using a more sensitive culture technique and having a possible upward bias due to the sex ratio, but on the other hand it included badgers from areas in which the confirmed cattle herd incidence was less than 1.0 per cent (Table 2).

Incidence in cattle

bTB incidence in cattle herds in Wales was lowest in north-west and north-east Wales (about 0.2 per cent) and highest in Monmouth, Pembroke and Radnor (over 6.5 per cent). This wide range of incidence is comparable with the difference in the incidence between the north and west regions of England (Defra 2010, county data for 2006); hence, the results may be of relevance to bTB epidemiology in England.

Relative proportions of badgers, cattle herds and cattle with evident infection

The proportion of individual badgers with evidence of infection was on average 3.5 times greater than the proportion of cattle herds sustaining a confirmed breakdown each year (Table 2). Since only a minority of cattle in confirmed herd breakdowns are found to be infected (Wales regional data for 2006, Defra 2010), the prevalence of bTB infection in badgers was much larger than the prevalence in cattle. This should not be interpreted as evidence that the predominant direction of transmission is from badger to cattle, because the pathogenesis, immunology and latency of bTB differ between badgers and cattle (Gallagher and Clifton-Hadley 2000).

Geographical comparisons

Correlation between badger prevalence and cattle incidence

There was variation in the prevalence of *M bovis* in found dead badgers and in confirmed and unconfirmed incidence of bTB in cattle herds between parts of Wales, in particular between the northern and southern halves of Wales. The strength of the correlation between confirmed incidence in cattle herds and badger prevalence (Table 2) may be attributable to the large differences in badger prevalence between the 10 geographical areas of Wales. The authors also found a weaker but sometimes significant association between unconfirmed herd incidence and badger prevalence (Table 3). Donnelly and others (2007, supplementary material) found that confirmed herd incidence rather than unconfirmed herd incidence was reduced after proactive badger culling. The authors have found that unconfirmed incidence in cattle herds in a locality tends to increase with confirmed incidence, but not in direct proportion. The role of badger prevalence in whether a cattle herd breakdown is confirmed is not clear.

Multivariable logistic regression analysis confirmed the association between *M bovis* infection in the found dead badgers and the measures of bTB in cattle herds within 5 km. Association between confirmed herd incidence and badger prevalence was seen at a 1 to 2 km scale in the RBCT, which was conducted in areas with appreciable confirmed bTB incidence in cattle (Woodroffe and others 2005). In Northern Ireland, an association was not seen, but the geographical distribution of bTB in cattle was more uniform than in the present study (Abernethy and others 2003). The current study highlights the fact that the prevalence of bTB in badgers can be low in areas where the confirmed incidence of bTB in herds is also low, without implying the direction of transmission.

Geographical distribution of genotypes

The four genotypes identified in badgers were also found in cattle, and tended to have distinct spatial distributions in both of the animal species. Dissimilarity of genotypes tended to increase with distance apart. Alongside this, there was greater dissimilarity of genotypes in cattle than in badgers over the first 30 km, but at greater distances the dissimilarity tended to be universally large. Cheeseman and others (1988) observed that the spread of infection between badger social groups in a longitudinal study was slow and restricted. Dissimilarity in cattle was probably inflated by farmers introducing cattle with genotypes of *M bovis* that were not typical for their locality (Gilbert and others 2005, Gopal and others 2006). Additional dissimilarity in cattle was seen within herds; in 16 per cent of the herds from which more than one isolate was genotyped, more than one different genotype was found. But a greater dissimilarity in cattle than in badgers has not always been shown by others (Woodroffe and others 2009).

In conclusion, the present study, although short in duration, has shown that the prevalence and genotype of *M bovis* infection in found dead badgers in Wales is geographically associated with confirmed incidence and genotypes in cattle herds. There is also evidence of greater genotype dissimilarity in cattle herds than in badgers within a 30 km range. Whether this association is maintained chiefly by infection of cattle by badgers or chiefly by infection of badgers by cattle cannot be established from the data, but since genotype dissimilarity was greater in cattle than in badgers (at least over short distances), there was evidence that cattle were exposed to other sources of infection. This result points to the importance of controlling the introduction of bTB into herds through cattle movements, although the results do not establish the relative proportion of infection introduced by cattle movements and the proportion introduced by badgers.

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Geographical association between the genotype of bovine tuberculosis in found dead badgers and in cattle herds

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Notes