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Performance of a Noninvasive Test for Detecting *Mycobacterium bovis* Shedding in European Badger (*Meles meles*) Populations

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The incidence of *Mycobacterium bovis*, the causative agent of bovine tuberculosis, in cattle herds in the United Kingdom is increasing, resulting in substantial economic losses. The European badger (*Meles meles*) is implicated as a wildlife reservoir and is the subject of control measures aimed at reducing the incidence of infection in cattle populations. Understanding the epidemiology of *M. bovis* in badger populations is essential for directing control interventions and understanding disease spread; however, accurate diagnosis in live animals is challenging and currently uses invasive methods. Here we present a noninvasive diagnostic procedure and sampling regimen using field sampling of latrines and detection of *M. bovis* with quantitative PCR tests, the results of which strongly correlate with the results of immunoassays in the field at the social group level. This method allows *M. bovis* infections in badger populations to be monitored without trapping and provides additional information on the quantities of bacterial DNA shed. Therefore, our approach may provide valuable insights into the epidemiology of bovine tuberculosis in badger populations and inform disease control interventions.

Mycobacterium bovis infection in wildlife is an issue of growing importance worldwide, with infections found in a range of species, including buffalo in Africa (1), wild boar in Spain (2), brushtail possums in New Zealand (3), and European badgers in the United Kingdom (4) and the Republic of Ireland (5). In the United Kingdom and the Republic of Ireland, badgers are involved in the transmission of tuberculosis (TB) to cattle (6–8). The incidence of *M. bovis* in cattle herds in the United Kingdom has been increasing for over 30 years (9), resulting in substantial economic losses (10). Once infected, badgers may intermittently shed *M. bovis* cells in sputum, feces, and urine (4), creating an environmental source of potential infection for other badgers and cattle (11, 12). *M. bovis* DNA has been shown to survive outside the host for up to 21 months, and 16S rRNA has been detected in badger setts and latrines (13). In addition, studies have found a 2.5% positivity rate when culturing from badger feces (14), and *M. bovis* has been cultured from cattle feces several months after excretion (15). Furthermore, *M. bovis* that had persisted in soil for over 12 months was able to colonize mice (16). This indicates that at least a proportion of *M. bovis* cells shed in badger feces can remain viable in the environment. Monitoring *M. bovis* infections in badger populations is important for understanding the location and spread of disease and directing control efforts. TB control interventions targeted at badgers are currently based on culling, vaccination, and farm biosecurity (17).

Accurate diagnosis of *M. bovis* infections in live animals is challenging yet essential in order to understand the epidemiology of the disease and its onward spread. Currently, infections in live badgers can be monitored through trapping and diagnosis with immunoassays (gamma interferon [IFN-γ] assay [18] and the Brock Stat-Pak assay [7]) and culture (19). Culture of clinical samples (sputum and feces) has low sensitivity of 8% and specificity of 100% (20). Furthermore, infected badgers may only intermittently excrete *M. bovis*, and culture from noninvasive sources such as feces is challenging due to decontamination methods. Immunoassays are more sensitive than culture but can be affected by animal age and duration of infection (21). The IFN-γ assay involves stimulating lymphocytes in whole blood and then detecting IFN-γ by a sandwich enzyme-linked immunosorbent assay (ELISA) (18). This method has sensitivity values ranging from 57 to 85% in badgers, with lower sensitivity in cubs than adults, and specificity values ranging from 93 to 98% (18, 20, 21). The Stat-Pak assay is a lateral flow serum antibody test with sensitivity values ranging from 50 to 78% (21) in badgers, with reduced sensitivity in the earlier stages of disease, compared to advanced infections, and specificity values ranging from 93 to 97% (20, 21). As no individual test is sufficiently sensitive or specific enough to use alone for diagnosis, the combined application of the IFN-γ and Stat-Pak assays has been recommended (20). Diagnosis through immunoassay and culture of clinical samples also requires that badgers be trapped, which is costly, logistically challenging, and likely to result in limited sample sizes.

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A noninvasive assay for sampling badger populations may offer an opportunity to gather information on the spatiotemporal distribution of *M. bovis* in badger populations over a larger area, more easily and cost-effectively, than by trapping. A noninvasive approach would also remove the ethical concerns related to trapping. Furthermore, immunoassays provide information on prior exposure but do not identify shedding status. Our study was focused on quantifying fecal shedding of *M. bovis* among badgers at the social group level, using an existing quantitative PCR (qPCR) assay developed in our group that quantifies *M. bovis* genome copy number (22, 23). We aimed (i) to determine the correspondence between immunoassay results and fecal qPCR assay results for *M. bovis* infections in live-trapped badgers and (ii) to establish an optimal fecal sampling regimen to maximize detection of shedding in badger populations.

MATERIALS AND METHODS

Sampling and trapping. Fresh fecal samples were obtained from latrines associated with 12 badger social groups in Woodchester Park (Gloucestershire, England) in 2012 and 2013. In 2012, intensive sampling took place during the two periods of peak badger latrine activity (spring and autumn), when up to 10 unique fecal samples were obtained from each social group on alternate days for 10 days. Feces were collected from latrines within the vicinity of the main sett of each social group. In each season, starting 2 days after trapping operations took place in that location, 10 unique fecal samples per day were taken from the latrines identified near each main sett, on two nonconsecutive days. For the purpose of this study, March through May was classified as spring, June through August as summer, September through November as autumn, and December through February as winter.

To determine the relative performance of the qPCR assay, we compared results to those from immunoassays and culture for clinical samples obtained during routine trapping and sampling of the 12 targeted badger social groups. Sputum samples were collected by aspiration of both the esophagus and the trachea using catheters. Collected samples are then flushed into physiological saline. Sputum and feces were cultured on solid medium (24) and identified as *M. bovis* by typical colony morphology followed by spoligotyping. Each social group of badgers was subjected to one trapping event per season, and trapping took place over 2 consecutive days. Badgers were trapped using baited cage traps placed around the main sett of each social group, and individual animals were identified using a unique tattoo applied at the first capture event. Trapped badgers from each of the 12 social groups were tested with the BrockTB Stat-Pak assay, the IFN-γ assay, and culture of clinical samples. To establish the relative sensitivity and specificity of the fecal qPCR assay, compared to immunoassays, at an individual animal level, we collected fecal samples (following administration of an enema) from badgers trapped and tested throughout the Woodchester Park study area. An individual or a social group was deemed *M. bovis* positive if at least one diagnostic test or culture from a clinical sample was positive. All work was approved by the University of Warwick and the Food and Environment Research Agency Ethical Review Committee and was carried out under a license granted by the Home Office under the 1986 Animals (Scientific Procedures) Act.

DNA extraction and qPCR testing. Total community DNA was extracted from 0.1 g (± 0.003 g) of feces using the Fast DNA spin kit for soil (MP Biomedicals), following the manufacturer's instructions. *M. bovis* was detected and quantified using a qPCR assay that targets the RD4 deletion region unique to the *M. bovis* genome. An initial qPCR screen of each sample was performed using an ABI 7500 Fast qPCR system (ABI) with two technical replicates of each sample. Positive controls (8.5×10^2 genome equivalents) and negative controls were also present in duplicate on each plate. PCRs were set up using 900 nM levels of each primer (RD4F, 5′-TGTGAATTCATACAAGCCGTAGTCG-3′; RD4R, 5′-CCCG TAGCGTTACTGAGAAATTGC-3′), 250 nM TaqMan probe (6-carboxyfluorescein [6FAM]-AGCGCAACACTCTTGGAGTGGCCTAC-tetramethylrhodamine [TMR]), 1 mg/ml bovine serum albumin (BSA), 12.5 μl of Environmental Master Mix 2.0 (ABI), and 10 μl of template, made up to 25 μl with molecular biology-grade water (Sigma-Aldrich). PCR cycling conditions were 50°C for 2 min followed by 95°C for 10 min and then 40 cycles of 95°C for 15 s and 58°C for 1 min. Samples exhibiting amplification in one or more technical replicates were taken on to full quantification using three technical replicates per sample, under the same conditions. If one or more of the technical replicates of the quantification assay exhibited amplification, then the sample was deemed positive for *M. bovis*. Serial dilutions of *M. bovis* BCG Danish 1331 genomic DNA were used as standards for this quantification. A previously described inhibition control assay (23) was used to detect the possibility of false-negative results due to inhibition. Each extracted sample was screened as a singlet; if the threshold cycle difference (ΔC_T) was greater than 2.5, then the sample was rescreened as a doublet. If the average ΔC_T was greater than 2.5, then the sample was reextracted from frozen fecal aliquots; if not, then the sample was considered uninhibited The number of *M. bovis* genome equivalents was quantified independently by qPCR at the University of Warwick and the Animal and Plant Health Agency (APHA) Weybridge.

Statistical analysis. All data analyses were performed using the statistical program R. Binomial and gaussian generalized linear models (GLMs) were used to determine differences in fecal sample positivity (as a binary variable) and *M. bovis* genome equivalents shed between social groups and seasons. All GLMs were carried out with the old oak group as the baseline social group, because it had the lowest prevalence of positive fecal samples, and winter as the baseline season against which all other social groups and seasons were compared. One- and two-way analysis of variance (ANOVA) was used to determine differences among social groups in sample numbers and proportions of trapped animals that were positive. Spearman's ranks were calculated to determine whether there was correspondence in the rank order of social groups based on the prevalence estimated by live testing and fecal qPCR assays.

Sensitivity, specificity, positive predictive value (PPV), and negative predicative value (NPV) were calculated for individual animals, as well at the social group level. The confidence intervals (CIs) for these values were calculated using the Wilson score interval. For analysis of data for individuals, a positive result was defined as a positive result from any diagnostic test or culture with clinical samples from a trap event. For analysis on a social group level, a social group was deemed positive if any diagnostic test or culture was positive for any trap event within the group.

False-positive rates were calculated using 68 known negative fecal samples obtained from captive badgers at the APHA, which were routinely tested for bovine TB using the IFN-γ assay, and 49 water samples. Negative samples were prepared in a double-blind manner and randomly introduced into the experiment at both laboratories. As this qPCR assay is highly specific for *M. bovis* (25), all false-positive findings are expected to result from contamination introduced in the laboratory; therefore, negative controls were included at every stage of DNA extraction and quantification.

RESULTS

Correspondence between immunoassays and fecal qPCR assays for trapped badgers. Routine badger trapping at Woodchester Park took place prior to the collection of feces from latrines. Trapped badgers were tested for *M. bovis* infection with immunoassays (IFN-γ and Stat-Pak assays) and culture of clinical samples and qPCR assays of fecal samples. In total, there were 120 trapping events, with 50% found to be positive by any test. The Stat-Pak assay identified a greater number of positive samples than did either the IFN-γ assay or the qPCR assay, which identified similar numbers of positive samples (Fig. 1). No culture-positive results were obtained from feces or sputum samples from trapped badgers. The correlation between tests was low, as follows: Stat-Pak

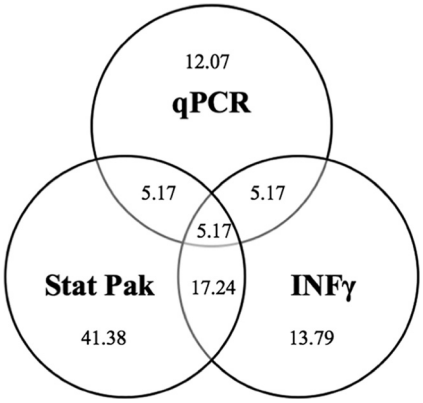

FIG 1 Percentage agreement between positive test results. There were 120 total trappings, with 60 positive trappings for which at least one test gave a positive result.

assay and IFN-γ assay, $r = 0.27$ ($P < 0.05$); Stat-Pak assay and qPCR assay, $r = 0.11$ ($P > 0.05$); IFN-γ assay and qPCR assay, $r = 0.20$ ($P < 0.05$).

As there is no gold standard for diagnosing infections in badgers, sensitivity, specificity, PPV, and NPV calculations were carried out using the Stat-Pak assay and the IFN-γ assay separately as the gold standard and with the two tests combined. The sensitivity of the qPCR assay in comparison with the Stat-Pak and IFN-γ assays, separately or combined, was low, ranging from 14 to 25%, whereas the sensitivity of the Stat-Pak and IFN-γ assays in comparison with one another was higher, at 32% and 59%, respectively (Table 1). The lower sensitivity of qPCR was expected because it is a measure of shedding, rather than infection, and infected badgers may shed *M. bovis* intermittently or not at all.

The relative specificity of qPCR was high, ranging from 91 to 93%, in comparison with the Stat-Pak and IFN-γ assays, which had specificities of 86 and 67%, respectively, in comparison with one another. The PPVs for qPCR ranged from 43 to 64% and from 33 to 59% in comparison with the Stat-Pak and IFN-γ assays, respectively. The high relative specificity of qPCR is due to the definitive detection of the DNA target, which is unique to *M. bovis*.

The NPVs ranged from 54 to 83% for qPCR and from 67 to 86% for the Stat-Pak and IFN-γ assays in comparison with one another. The NPV was lower overall for qPCR than for the immunoassays as the former detects shedding, rather than infected animals, which results in some positive animals being missed.

Historically positive trapping events. As badger populations in Woodchester Park have been extensively studied for over 20 years, trapping information is available for several years prior to this study. In the 120 trapping events discussed above, the captured animal was historically positive by at least one test on 57% of the occasions. Of these historically positive captures, 29% were positive by the Stat-Pak assay only, 25% by the IFN-γ assay only, 43% by the Stat-Pak and IFN-γ assays, and 3% by culture of clinical samples, the IFN-γ assay, and the Stat-Pak assay (see Fig. S1 in the supplemental material). Contemporary (2012) trapping indicated that, in 53% of cases (32 cases), the animal had at least one historical positive test result; 24% were positive by the Stat-Pak assay only, 14% by the IFN-γ assay only, 59% by the Stat-Pak and IFN-γ assays, and 3% by culture and Stat-Pak and IFN-γ assays, but none was positive by fecal culture alone (see Fig. S1 in the supplemental material).

The majority (93%) of animals that were historically Stat-Pak assay positive were also positive with the Stat-Pak assay during contemporary testing. Also, 65% of animals that were historically IFN-γ assay positive were also positive with the IFN-γ assay during contemporary testing (see Fig. S2 in the supplemental material). No captured animals that were historically culture positive were positive by contemporary culture; however, they were all positive by fecal qPCR, indicating that they were still shedding *M. bovis*. Forty-five percent of trapped badgers that were historically positive by both the Stat-Pak and IFN-γ assays were also positive by these tests during contemporary testing. Past diagnostic test results were pooled for all capture events for each badger; therefore, IFN-γ and Stat-Pak assays might have been positive at different capture events rather than simultaneously.

Of the 16 trap events that were positive by qPCR, 12 (75%) were historically positive by at least one of the live tests. Both of the historically culture-positive animals were positive by qPCR.

Contemporary seasonal trapping. Badgers were trapped seasonally throughout 2012, which coincided with the collection of fecal samples from latrines. Trapped badgers were routinely tested using the Stat-Pak assay, the IFN-γ assay, and culture of clinical samples. The numbers of badgers trapped were highly variable among the social groups (5 to 18 animals per group) and seasons (see Tables S1 and S2 in the supplemental material). The numbers of badgers caught per sampling day were also highly variable between seasons, with 2 to 28 badgers being trapped on a given day (see Table S2 in the supplemental material). Greater numbers of badgers were trapped in spring ($t = 4.731$, $P < 0.001$) and summer ($t = 2.880$, $P < 0.05$) than in autumn or winter ($F_{3,44} = 9.421$, $P < 0.001$). There were no differences in the numbers of badgers caught per social group throughout the year ($F_{11,36} = 1,272$, $P > 0.05$) or in the percentages of positive badgers trapped per season ($F_{3,44} = 0.8523$, $P > 0.05$). However, there was significant variation in the percentages of positive animals (as estimated by immunoassay) per social group across the whole year ($F_{11,36} = 3.635$, $P < 0.001$), with the Honeywell ($t = 2.563$, $P < 0.05$), nettle ($t = 2.357$, $P < 0.05$), and septic tank ($t = 2.457$, $P < 0.05$) groups having larger proportions of test-positive badgers than the other groups.

TABLE 1 Sensitivity and specificity of *M. bovis* diagnostics for trapped badgers with Stat-Pak and IFN-γ assays individually and combined as gold standards against qPCR and with Stat-Pak and IFN-γ assays as gold standards against each other

Test(s)	Sensitivity (95% CI) (%)	Specificity (95% CI) (%)	PPV (95% CI) (%)	NPV (95% CI) (%)
Stat-Pak	15.00 (9.41–23.06)	92.19 (85.66–96.07)	54.55 (45.03–83.08)	63.44 (53.91–72.03)
IFN-γ	25.00 (18.08–33.48)	91.40 (84.55–94.97)	42.86 (35.27–52.83)	82.52 (74.71–88.30)
Stat-Pak and IFN-γ	14.29 (8.86–22.24)	92.59 (85.94–96.23)	63.64 (57.81–74.93)	54.35 (44.84–63.56)
Stat-Pak as true positive (IFN-γ)	32.50 (23.89–41.47)	85.71 (77.76–91.14)	59.09 (49.53–68.01)	66.67 (57.21–74.96)
IFN-γ as true positive (Stat-Pak)	59.09 (49.53–68.01)	66.67 (57.21–74.96)	32.50 (24.30–41.94)	85.71 (77.76–91.14)

TABLE 2 Odds of finding *M. bovis*-positive samples each season, with winter as the baseline

Season	Odds ratio (95% CI)	*P*
Spring	1.76 (0.84–3.66)	0.13
Summer	2.72 (1.31–5.64)	0.007
Autumn	1.97 (0.96–4.04)	0.06
Winter	1	

In this study, badger sex was not related to the likelihood of yielding a positive test result (female, odds ratio, 1; male, odds ratio, 0.86 [95% CI, 0.43 to 1.73]; $P > 0.05$). When diagnostic tests were examined individually, neither season nor badger sex was related to the likelihood of a positive Stat-Pak or IFN-γ test result.

Seasonal and social group differences in latrine fecal sampling. The total numbers of fecal samples collected varied between social groups, from 76 to 175 samples across the year (see Table S3 in the supplemental material). On average, more samples were collected per sampling day in the spring, with a mean of 51 samples per day, than in other seasons, which ranged from 23 to 38 samples per day. On each sampling day, the aim was to collect 10 fresh fecal samples; spring and summer sample numbers averaged 9 and 8 samples per day, respectively, with 6 samples per day being collected in winter.

The odds ratios for finding a positive fecal sample were equal across all seasons except for summer, when there was a significantly higher probability (Table 2). There was a significant difference in the number of *M. bovis* genome equivalents shed over the year, with significantly greater numbers of cells being detected in summer and autumn than in winter and spring, although there were no more positive samples in autumn than in winter or spring (Table 2). There was no correlation between the number of badgers trapped and the number of fecal samples collected in the same season per social group ($r = 0.18$, $P > 0.05$) or between the percentage of positive badgers trapped and the percentage of positive fecal samples per social group by season ($r = 0.22$, $P > 0.05$).

Fecal qPCR replication at two centers. A total of 1,090 samples (67% of all samples collected) were subjected to DNA extraction at both the University of Warwick and the APHA Weybridge. Of these, 13% (140 samples) were found to be positive, of which 32% (45 samples) were positive only at the University of Warwick, 29% (41 samples) were positive only at the APHA, and 39% (54 samples) were positive at both centers. There was no statistical difference in whether a sample was positive at the University of Warwick or at the APHA (McNemar test, $\chi^2_{1,1,090} = 0.165$, $P > 0.05$). There was a significant difference in *M. bovis* genome equivalents in samples that were positive at both centers, with fewer genome equivalents being found in samples analyzed at the University of Warwick ($\beta = -2.53$, $P < 0.01$). Although 61% of samples were identified as positive at only one center, the rankings of the social groups (in terms of the proportions of positive samples) were strongly correlated (Spearman's $\rho = 0.750$, $P < 0.05$).

Correspondence between live testing with immunoassays and culture and fecal qPCR testing from latrines. During 2012, 10 of the 12 social groups targeted for the present study were positive by the Stat-Pak and IFN-γ assays, whereas qPCR testing of fecal samples obtained from latrines identified all 12 to be positive (Table 3). The numbers of positive social groups in each season were consistently higher using qPCR testing of fecal samples from latrines, compared with immunoassay results from live-captured animals, for both longitudinal and intensive sampling regimens (Table 3). The Colliers Wood and Wych Elm social groups were negative by contemporary immunoassays for live animals but were found to be positive in three and four seasons, respectively, by qPCR (Table 3). The largest discrepancy between the two diagnostic approaches was in the spring, when live-trapping diagnostics identified 58% fewer social groups as positive, compared to qPCR testing of fecal specimens from latrines. The smallest difference was in the winter, with 8% fewer social groups being identified by immunoassays and culture of clinical samples than by qPCR testing of fecal samples from latrines.

The social groups were ranked according to the proportions of positive test results, using results from live-trapping diagnostics and qPCR testing of fecal samples from latrines. The proportions

TABLE 3 Summary of trapping and fecal qPCR positivity for each social group by season

	Spring			Summer			Autumn			Winter		
Group[a]	Trap positive	qPCR positive	qPCR prevalence (%)	Trap positive	qPCR positive	qPCR prevalence (%)	Trap positive	qPCR positive	qPCR prevalence (%)	Trap positive	qPCR positive	qPCR prevalence (%)
Nettle	−	+	29.17	+	+	50.00	+	+	53.13	−	+	100.00
West	+	+	20.00	+	+	19.44	−	−	0	+	+	7.69
Honeywell	−	+	10.20	+	+	33.33	+	+	4.88	+	−	0
Septic tank	−	−	0	+	+	5.71	+	+	29.63	−	+	15.00
Top	−	+	26.09	+	+	20.00	−	−	0	−	−	0
Wych elm	−	+	15.00	−	+	13.33	−	+	11.11	−	+	20.00
Beech	+	+	8.00	+	+	8.11	+	+	10.45	−	+	11.11
Woodrush	−	+	2.63	+	+	6.06	−	+	11.11	+	−	0
Colliers Wood	−	+	2.56	−	+	5.71	−	+	12.24	−	−	0
Yew	−	+	2.04	+	+	15.63	+	+	3.28	+	+	5.26
Kennel	−	−	0	+	+	21.05	−	−	0	−	−	0
Old oak	−	−	0	+	+	8.57	+	+	2.22	+	−	0

[a] The overall positive proportions were as follows: spring, trap positive, 16.67%; qPCR positive, 75.00%; qPCR prevalence, 9.64%; summer, trap positive, 83.33%; qPCR positive, 100.00%; qPCR prevalence, 17.25%; autumn, trap positive, 50.00%; qPCR positive, 75.00%; qPCR prevalence, 11.50%; winter, trap positive, 41.67%; qPCR positive, 50.00%; qPCR prevalence, 13.26%.

TABLE 4 Sensitivity and specificity of seasonal qPCR results in comparison with all-year trapping data, using Stat-Pak assay, IFN-γ assay, and both Stat-Pak and IFN-γ assays as gold standards

Season and measure	% (95% CI)		
	Stat-Pak assay	IFN-γ assay	Stat-Pak and IFN-γ assays
Spring			
Sensitivity	80.00 (51.73–93.72)	83.33 (55.19– 95.30)	83.33 (55.19–95.30)
Specificity	0.00 (0.00–24.25)	16.67 (4.70–44.81)	16.67 (4.70–44.81)
PPV	80.00 (51.73–93.72)	50.00 (25.38–74.62)	50.00 (25.38–74.62)
NPV	0.00 (0.00–24.25)	50.00 (25.38–74.62)	50.00 (25.38–74.62)
Summer			
Sensitivity	100 (75.75–1)	100 (75.75–100.00)	100 (75.75–100.00)
Specificity	0.00 (0.00–24.25)	0.00 (0.00–24.25)	0.00 (0.00–24.25)
PPV	83.33 (55.19– 95.30)	50.00 (25.38–74.62)	50.00 (25.38–74.62)
NPV	0.00 (0.00–24.25)	0.00 (0.00–24.25)	0.00 (0.00–24.25)
Autumn			
Sensitivity	70.00 (42.07–88.23)	66.67 (39.07–86.19)	66.67 (39.07–86.19)
Specificity	0.00 (0.00–24.25)	16.67 (4.70–44.81)	16.67 (4.70–44.81)
PPV	77.78 (49.49–92.60)	44.44 (2128–70.29)	44.44 (2128–70.29)
NPV	0.00 (0.00–24.25)	33.33 (13.81–60.93)	33.33 (13.81–60.93)
Winter			
Sensitivity	50.00 (25.38–74.62)	66.67 (39.07–86.19)	66.67 (39.07–86.19)
Specificity	100 (75.75–100.00)	66.67 (39.07–86.19)	66.67 (39.07–86.19)
PPV	100 (75.75–100.00)	66.67 (39.07–86.19)	66.67 (39.07–86.19)
NPV	16.67 (4.70–44.81)	66.67 (39.07–86.19)	66.67 (39.07–86.19)

of test-positive samples per group estimated by qPCR testing of fecal samples collected from latrines in June and from live-trapping diagnostics in the summer were highly correlated (Spearman's $\rho = 0.87$, $P < 0.001$). Live-trapping diagnostic results for the whole year correlated strongly with qPCR results for fecal samples collected in June (Spearman's $\rho = 0.71$, $P < 0.05$), with results of fecal sampling for the whole year (Spearman's $\rho = 0.70$, $P < 0.05$), and with results from all longitudinal fecal sampling (Spearman's $\rho = 0.62$, $P < 0.05$). Ranking of social groups on the basis of qPCR results alone correlated well with contemporaneous ranking based on immunoassay and culture results.

Sensitivity and specificity of qPCR tests and immunoassays. Sensitivity, specificity, PPV, and NPV were calculated at the social group level for seasonal qPCR testing of feces from latrines in comparison with the results of live diagnostic tests for a whole year. The group-level sensitivity of qPCR testing was seasonally variable but consistently high, with the highest value being noted in summer, i.e., 100% sensitivity, in comparison with immunoassay results, and the lowest in winter, i.e., 50% (Table 4). Group-level specificity was also highly variable, ranging from 0 to 100%, in comparison with immunoassays. The lower bound of this range is due to no social groups being found negative by both live testing and qPCR. The social groups targeted in this study were all chosen because they were historically positive in live testing; therefore, it is expected that few if any would be negative by both approaches. The PPV of qPCR ranged from 44% to 100%, relative to live testing, as this test has a low false-positive rate. The NPV of qPCR ranged from 0 to 67%. This wide range may be due to the inability of the test to detect the presence of infection in the absence of shedding; therefore, if social groups contain animals that are infected but not shedding *M. bovis* in feces, then they will not be identified by qPCR testing (Table 4).

Comparison of intensive and longitudinal fecal sampling. No differences were observed between intensive and longitudinal sampling in the numbers of *M. bovis* cells shed or the numbers of positive samples, with accounting for season, and this did not vary with social group. When intensive sampling was divided into 2-day sampling periods, to mirror longitudinal sampling, no differences were observed between sampling blocks within intensive periods, in terms of the numbers of positive samples overall or per social group. In the autumn, both intensive sampling and cross-sectional sampling were carried out, with no difference between the sampling approaches in the odds of finding positive samples in each social group.

Level of fecal sampling required to detect positive social groups. Random resampling of fecal samples determined the sampling intensity required to identify, with 95% certainty, positive groups with various proportions of positive samples across a year. The number of samples required varied between 5 for the group with the most positive samples (nettle group) and up to 50 for the group with the least (yew group) (Fig. 2). In the present study, up to 20 fecal samples were collected over 2 days, but more intensive sampling would have been required to collect more. Our results indicate that the number of samples required varies with the season, with the fewest samples being required in early summer. However, sampling in only one season may not detect all positive social groups. In this study, up to 10 social groups were identified as positive by qPCR in a single sampling period. More accurate assessments of the shedding status of a group would require sampling across a whole year.

False-positive rate for fecal qPCR testing. Two (2.9%) of the 68 negative fecal samples tested positive and one (2.1%) of the 46 water samples tested positive, giving a false-positive rate of 2.6%.

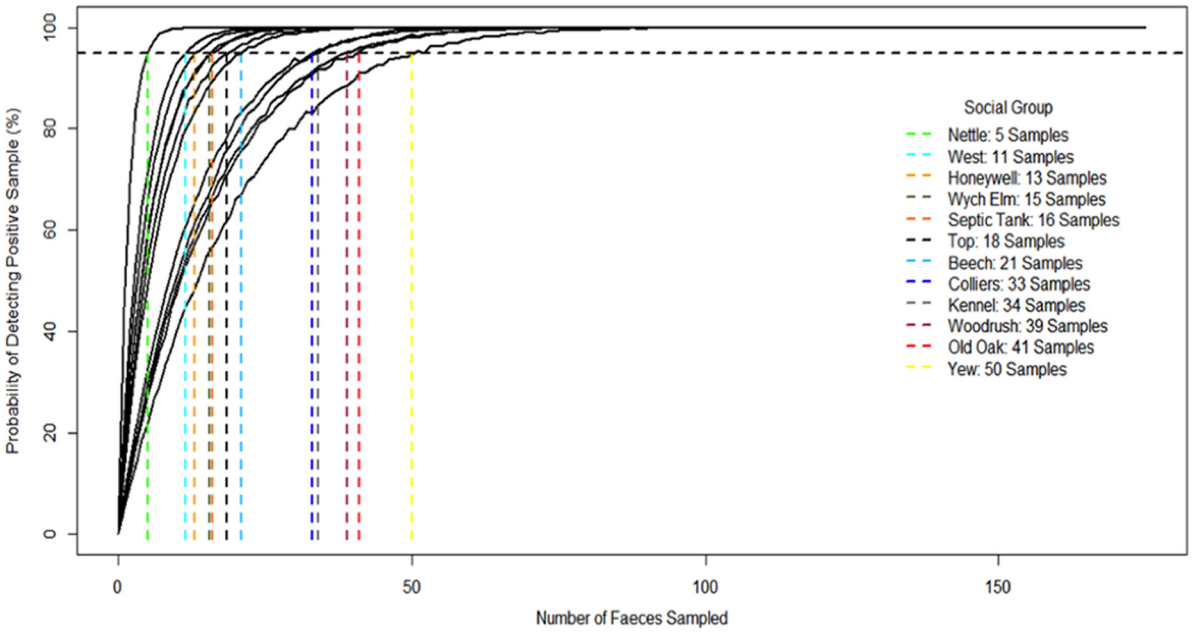

FIG 2 Numbers of fecal samples required to detect a positive social group across a year.

DISCUSSION

The results presented here indicate that qPCR testing of fecal samples from latrines is likely to be as sensitive or more so than live testing in detecting *M. bovis* in badger populations. Therefore, this method provides an alternative or complement to immunoassays and culture of clinical samples, which currently are the only measures of *M. bovis* infections in badger populations that do not involve postmortem examinations but are themselves limited in performance.

When trapped badgers were tested with immunoassays, culture of clinical samples, and qPCR testing of voided feces, there was low correspondence between test results within individuals, as noted in other studies (18). Culture of clinical samples did not produce any positive results, whereas qPCR identified 28% of all immunoassay-positive cases on an individual badger level. In the field, however, where qPCR testing was conducted on feces from latrines, the ranking of social group shedding status inferred from qPCR results correlated strongly with prevalence estimates based on immunoassay results, thus demonstrating the correspondence between approaches. As qPCR quantifies *M. bovis* genome equivalents, it provides a relative measure of the levels of shedding among social groups. Studies in other hosts have found that animals shedding the highest levels of pathogen are responsible for large proportions of transmission events (26, 27). While this has not been explicitly investigated for *M. bovis* in badgers, heterogeneity in individual- and group-level shedding may warrant further research.

Although there was strong correlation between group rankings based on the two diagnostic approaches, immunoassays consistently identified fewer positive social groups than did qPCR testing of fecal samples from latrines per season and across the year. qPCR tests also identified greater differences in the proportions of positive results among social groups than did immunoassays. Notably, the prevalence rates of infections in the west and old oak social groups estimated by immunoassays were similar, but the two groups differed widely with regard to the results of fecal qPCR tests, with the west group being highly positive and the old oak group having the fewest positive fecal samples.

The greater odds of identifying a positive fecal sample from a latrine in summer suggests that this would be the optimal time to sample badger populations. This approach maximized the number of positive samples with the lowest possible sampling intensity. Because the number of fecal samples collected did not differ between seasons, the greater odds of detecting positive samples in summer are due to an increase in positive samples, rather than a greater abundance of fecal samples during this period. The optimal fecal sampling regimen would involve collecting 10 fresh samples per day on 2 nonconsecutive days in early summer, which would detect the top 83.34% of shedding social groups, including those excreting the most *M. bovis* genome equivalents into the environment. This sampling regimen provided qPCR test data indicating relatively high sensitivity and specificity, compared with live testing, and also had greater odds of finding positive samples, compared with all other seasons. Some social groups required more than 20 samples to be collected over a year to detect fecal shedding. If the aim of sampling is to identify groups with the largest proportions of positive samples, then sampling in early summer only may be adequate. In order to identify all positive social groups (as determined by qPCR) in this study, however, two separate sampling sessions would be required, once in early summer and once in late summer, which would be the most cost-effective method for detecting all social groups. The requirement for two sampling sessions to detect all positive groups is most likely due to the intermittent nature of *M. bovis* shedding and the fact that a wider window of sampling is needed to obtain fecal samples from a large proportion of animals in each social group. While the false-positive rate of this qPCR assay is low, the probability of obtaining false-positive results increases if large numbers of samples are tested; therefore, we suggest that positive fecal samples be retested to maintain a low false-positive rate.

In addition to being equally or more sensitive than live-trapping diagnostics, the qPCR assay with latrine samples benefits from being noninvasive and less logistically challenging than live trapping and testing. Our study has identified the potential value of qPCR testing of fecal samples collected from latrines for monitoring *M. bovis* shedding in badger populations at the group level. This may prove to be a valuable adjunct to trapping and live testing in field studies to investigate the epidemiology of *M. bovis* spread in badger populations. However, the approach could be implemented as an alternative to capture and testing when the cost of the latter may be prohibitive for monitoring disease risks over relatively large areas. For example, qPCR testing of latrine fecal samples could be applied at the edges of the areas in which TB is currently endemic in the United Kingdom or throughout high-risk areas, in order to provide spatial information on relative levels of environmental contamination, which may facilitate monitoring of spread and targeting of management. Although our study focuses on badgers, the same approach to noninvasive sampling has the potential to be applied to other pathogens or other wildlife and disease systems, particularly those involving elusive host species or settings in which capture and live testing are challenging.

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H.C.K. was involved in sample collection and processing, undertook statistical analysis, prepared tables and figures, and wrote the manuscript with assistance from E.M.W. and R.J.D. A.M. collected and processed samples and undertook statistical analysis. P.J. collected and processed samples. E.T. advised on the project and data analysis. D.P. collected and processed samples. J.S. coordinated and oversaw processing of samples at the Animal Health and Veterinary Laboratories Agency (AHVLA) Weybridge. J.C. processed samples at AHVLA Weybridge. R.J.D. contributed to the study design, reporting, and data evaluation and organized and oversaw the fieldwork at AHVLA Woodchester. W.G. was involved in designing the original experiment. O.C., in collaboration with E.M.W., was involved in the project design. E.M.W., in collaboration with O.C., devised the experimental setup, supervised all field and practical work, and was responsible for biosafety.

We declare no competing financial interests.

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