Non-invasive monitoring of environmental *Mycobacterium bovis* shedding in wild European badger (*Meles meles*) populations

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Declaration

In accordance with the University of Warwick regulations for the degree of Doctor of Philosophy, I certify that this thesis has been written solely by me. The work contained in this thesis is my own unless otherwise stated. No aspect of this work has been submitted to any other institution for another degree or award.

Summary

The herd-level incidence of *Mycobacterium bovis* has been increasing in the United Kingdom (UK) and Republic or Ireland (RoI) for the past thirty years, resulting in substantial economic and animal welfare issues. Failure to control this pathogen in cattle is in part due to European badgers (*Meles meles*), a wildlife reservoir that are responsible for a proportion of transmission of *M. bovis* to cattle. Monitoring infection in badger populations is currently limited due to the need to trap badgers, which requires highly trained field staff and is expensive. In addition, although contact with infected badger faeces is a potential transmission route to cattle, very little is known about the extent and variability of the environmental pool of *M. bovis* shed by badgers. In this project we evaluated the suitability of using environmental badger faeces and a quantitative PCR (qPCR) assay to diagnose and monitor *M. bovis* in badger populations and described the extent of this environmental pool of potential infection.

The first study identified that intensive environmental faecal sampling and analysis with qPCR is at least, if not more, sensitive at diagnosing *M. bovis* in badger populations than the currently used immunoassays. This study also identified that even within a high prevalence population, the levels of shedding of *M. bovis* in faeces are highly variable between groups and between seasons, suggesting that there may be heterogeneity in transmission risk throughout the year. Using this non-invasive qPCR method to monitor the first field trial of oral BCG vaccination identified a trend of decreasing levels of *M. bovis* in faeces with increasing vaccination levels however, these results failed to reach statistical significance, highlighting the importance of adequate sample sizes when implementing this method. Finally, characterisation of the gut and faecal microbiota from animals shedding *M. bovis* in faeces confirmed that the source of faecal *M. bovis* is most likely sputum that has been expelled from the lungs, and not from colonisation of the gut.

The work presented here suggests that this non-invasive monitoring method can be applied to examine the variable pool of *M. bovis* over periods of time and large areas, providing an epidemiological tool which has the potential to be implemented to monitor infection in badger populations and disease intervention strategies.

Abbreviations

ANOSIM	Analysis of similarity
ANOVA	Analysis of variance
APHA	Animal and Plant Health Agency
BCG	Bacillus Calmette-Gurérin
BSA	Bovine albumin serum
CFU	Colony forming unit
CI	Confidence interval
DNA	Deoxyribonucleic acid
EU	European union
FAP	Four areas project
GIT	Gastrointestinal tract
GLM	Generalized linear model
IBS	Irritable bowel syndrome
IFNγ	Interferon gamma
NPV	Negative predicative values
OTU	Operational taxonomic unit
PCoA	Principal coordinates analysis
PCR	Polymerase chain reaction
PPV	Positive predicative values
QPCR	Quantitative polymerase chain reaction
RBCT	Randomised badger culling trials
RD4	Region of difference four
RoI	Republic of Ireland

rRNA	Ribosomal ribonucleic acid
SE	Standard error
SICCT	Single intradermal comparative cervical tuberculin test
SNP	Single nucleotide polymorphism
Stat Pak	Brock TB Stat-Pak™
TB	Tuberculosis
UK	United Kingdom
UCD	University College Dublin
WGS	Whole genome sequencing

1. General Introduction

1.1 Tuberculosis in UK and RoI cattle herds

The herd-level incidence of *Mycobacterium bovis*, the causative agent of bovine tuberculosis, has been increasing in cattle herds in the United Kingdom (UK) for over 30 years (Garnett et al. 2003). In the majority of developed countries this disease has been effectively controlled using a test and slaughter program where any animals that appear infected using the tuberculin skin test are slaughtered and the farmer compensated. However, in the UK and Republic of Ireland (RoI) the failure to eradicate this disease is likely in part due to the presence of European badgers (*Meles meles*) as wildlife reservoirs (Waters et al. 2012). Once implemented in the UK in the 1950's (Krebs et al. 1997), test and slaughter had reduced incidence to 0.05% by the late 1970's (Garnett *et al.* 2003). Since then, incidence has increased to 1.4% in 1996 and then further to 4.7% of herds in 2012 (Defra 2015). Over the last ten years this has cost the taxpayer £500 million and this is predicted to double in the next decade if continued increases are observed (Defra 2014a).

Currently, cattle are tested using the single intradermal comparative cervical tuberculin (SICCT) test (Defra 2014), which compares the reaction to *M. bovis* and *Mycobacterium avium* antigens (Monaghan et al. 1994) and has a sensitivity ranging from 70-90% (Brooks-Pollock et al. 2014). If an animal fails this test, the herd is placed under restricted movement until it has passed up to two tests given at approximately 60 day intervals. The herd will then be tested twice more in the next year. Cases in England are heavily clustered to the South-West and Midlands where herd-level incidence is currently ~9%, and herds are tested annually (Defra 2014a, Figure 1.1, Defra 2014a). In the edge area (the boundary

between the high incidence area and the rest of England where incidence is increasing yearly) incidence is currently at 1% of herds, compared to the rest of the country which has an incidence of 0.01% where herds are tested every four years (Defra 2014). While herd levels of *M. bovis* in RoI are similar to the UK, unlike the UK, herd incidence has decreased from 9.6% in 1995 (Defra 2014a) to 4.2% in 2012 (Department of Agriculture Food and the Marine 2013).

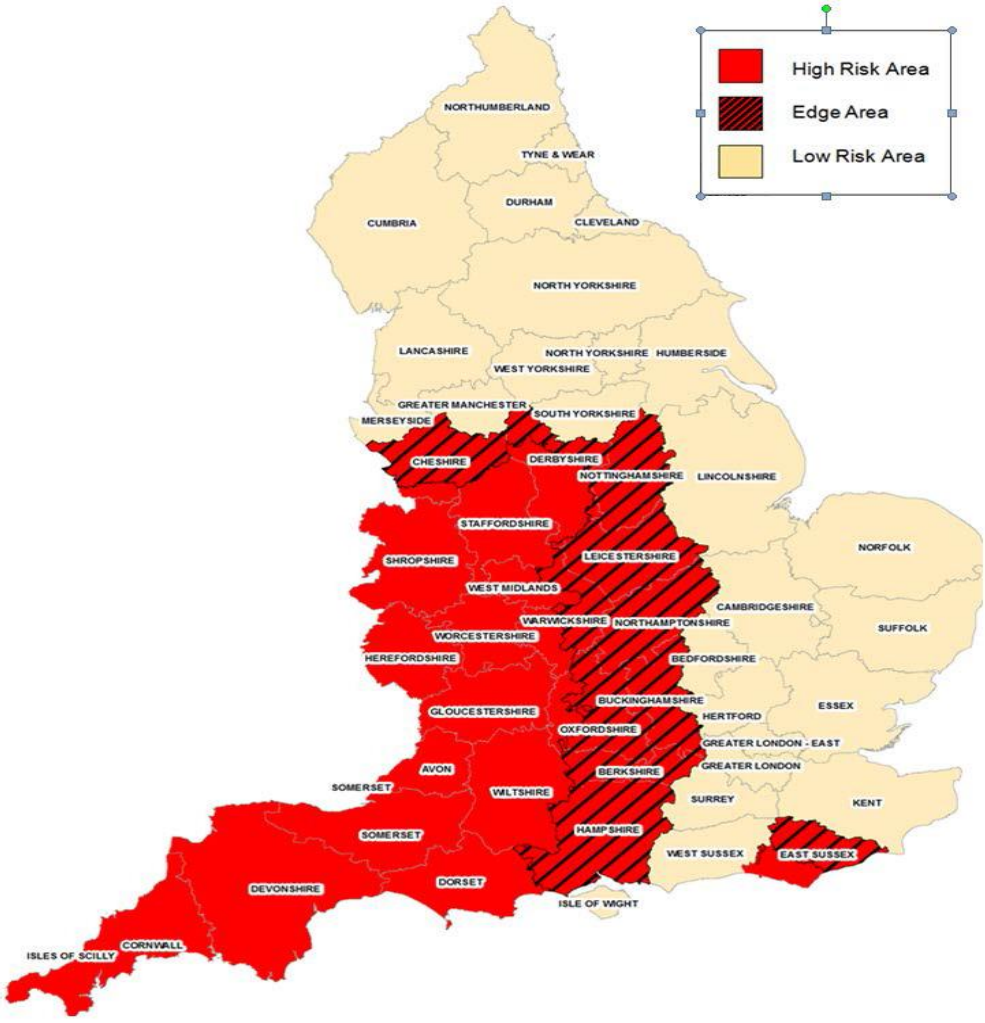

Figure 1.1 The location of the high incidence, low incidence and edge areas in England (Defra 2014a).

1.2 Transmission of *M. bovis* to cattle

The main route of disease transmission within cattle farms is an area of debate. A recent model suggested that the majority of within-farm transmission is through the environment, which may be contaminated pasture or infected wildlife (Brooks-Pollock et al. 2014) in contrast, other studies suggest the main route is respiratory during direct contact between animals (Menzies & Neill 2000). Cattle movement is estimated to be responsible for up to 84% of all cases between farms (Brooks-Pollock et al. 2014). A recent model suggests that the majority of within-farm transmission is through the environment, which may be contaminated pasture or infected wildlife (Brooks-Pollock et al. 2014). A proportion of transmission to cattle is from European badgers (*Meles meles*), (C. Donnelly et al. 2003; J M Griffin et al. 2005). Badgers are estimated to be responsible for approximately 50% of cases in cattle in high incidence areas, 5.7% of which are contracted directly and the rest as a result of subsequent cattle-cattle spread (Donnelly & Nouvellet 2013). Although, several wildlife and domestic animals have been found to be infected with *M. bovis* in the UK and RoI including deer (Delahay and Smith, et al. 2002), pigs (Bailey et al. 2013) and sheep (Defra 2014a), these animals are not thought to be a significant source of *M. bovis* for cattle.

1.3 *M. bovis* infection in badgers

Badgers are mostly nocturnal, social animals that live underground in setts with other animals of mixed age and sex (Roper 2010b). Several animals living together in one or more setts within a defined territory form a social group. The density of badger populations varies within the UK between 2.2 animals per km^2

to 44 per km^2 (O'Connor et al. 2012), ranging from 2 to over 30 individuals per social group with the highest densities found in South-West England (Roper 2010b). Populations are at lower density in the RoI with 2.1 per km^2 (O'Connor et al. 2012) and an average of 2.5-3 individuals per group (Sleeman & Mulacahy 1993).

Transmission between badgers can occur as a result of aerosol transmission via the respiratory system (Cheeseman et al. 1989) and by bite wounding (Clifton-Hadley et al. 1993). Infection in badgers occurs primarily in the lungs as a result of aerosol transmission via the respiratory system (Cheeseman et al. 1989) however, lesions are also observed on the kidneys, liver, lymph nodes and more rarely in the gastrointestinal tract (GIT) (Table 1.1, Corner et al. 2011). The severity of disease varies between animals from the most common form, latent infection (Murphy et al. 2010), to clinical signs of disease which vary from small lesions to severe disease with several lesions at potentially multiple sites (Corner et al. 2011), although not all lesions are caused by *M. bovis* (Emma Travis, In preparation).

Table 1.1 The distribution of Mycobacterium bovis infection and visible tuberculouse lesions in 78 naturally-infected badgers. Corner et al. 2011.

Pool	Culture positive	Visible lesion present
Head	36	6
Carcase	36	8
Abdomen	21	4
Thorax	40	14

When infected, animals may intermittently shed *M. bovis* in their urine, faeces and sputum (Clifton Hadley et al. 1993), creating an environmental pool of

potentially infectious material. Sixty-five percent of animals with visible pulmonary lesions have been found by culture to excrete in faeces; as lesions in the GIT are rare, *M. bovis* in faeces most likely occurs from swallowing infected sputum that has been expelled from the lungs (Corner et al. 2011). Using quantitative PCR (qPCR) all badgers shedding *M. bovis* in their trachea (n= 7) also shed *M. bovis* in faeces (Figure 1.2, (Travis et al. 2015)), which supports the hypothesis that faecal *M. bovis* arises from swallowed sputum that has been expelled from the lungs. Infection without shedding does not appear to reduce the lifespan of badgers however, shedding animals have increased mortality, with males being more severely affected than females (Wilkinson et al. 2000).

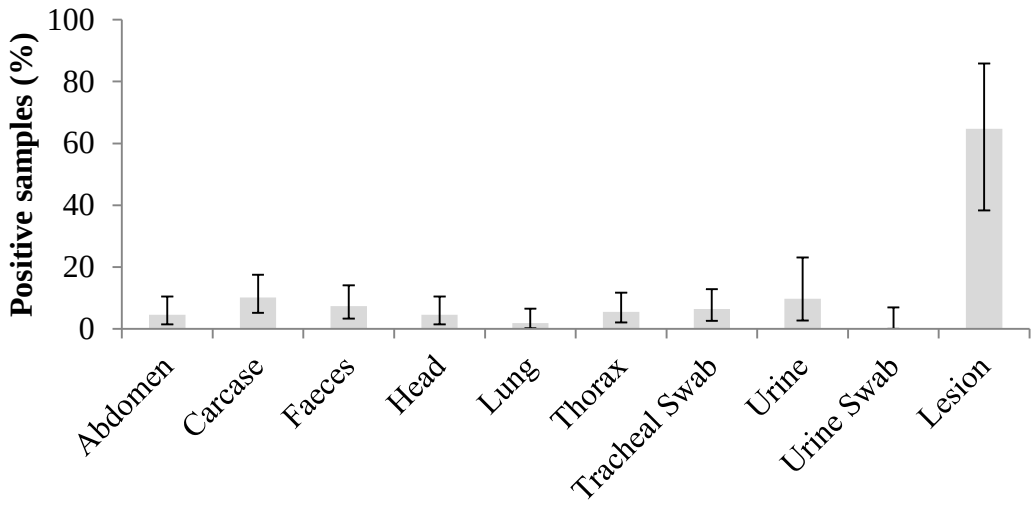

Figure 1.2 Percentage *M. bovis* positivity by qPCR of clinical and excretory samples from 108 badgers from across RoI. For all samples (n=108) with the exception of urine (n=41), urine swabs (n=51) and lesions (n=17). 95% binomial confidence intervals are shown (Travis et al. In preparation).

The majority of transmission between badgers is thought to be aerosol, particularly taking place underground where close contact between individuals facilitates transmission (Delahay et al. 2000). Disease is also transmitted via bite

wounding, the majority of which takes place between males and during the breeding season (Gallagher & Clifton-Hadley 2000; Clifton Hadley et al. 1993). Infection may be transmitted either when an animal excreting *M. bovis* in its saliva bites an uninfected animal or when an uninfected animal bites through the lymph nodes of an infected animal (Jenkins et al. 2012).

Infected animals have been observed to have altered behaviour including increased movement (Garnett et al. 2002) and occupancy of outlying setts (Cheeseman & Mallinson 1981; Weber et al. 2013). The home ranges of infected individuals are on average 50% larger than uninfected animals and they range more widely in their own territory (Garnett et al. 2005). In addition, infected animals forage an average of 65% further away from their own main sett and their ranges extended further into neighbouring territories than uninfected animals (Garnett et al. 2005). Infected animals do not differ in their habitat usage, suggesting they are not utilising different types of food, but rather they may be less competitive and so have to forage further, or infection may increase energy or nutrient demands making it necessary to forage further (Garnett et al. 2005). Alternatively, animals that naturally range more widely may be more likely to contract *M. bovis* through increased contact with sources of infection (Garnett et al. 2005). There is some evidence to suggest that as disease progresses behavioural patterns become increasingly abnormal, with tuberculous badgers being observed residing in cattle sheds and appear more frequently in daylight and with no fear of humans (Cheeseman & Mallinson 1981).

1.4 Transmission of *M. bovis* from badgers to cattle

There is strong evidence to suggest that badgers transmit *M. bovis* to cattle. Firstly, in laboratory studies uninfected cattle housed in proximity to infected badgers become infected (Little et al. 1982). In addition, areas with a high incidence of herd breakdowns also have a high prevalence of disease in the badger population with the same strain types found in badgers and cattle (Young 1997; Olea-popelka et al. 2005; Woodroffe et al. 2005). Furthermore, whole genome sequencing (WGS) of cattle isolates and those from neighbouring badgers found at most, four single nucleotide polymorphisms (SNP) differences between the strains, demonstrating recent transmission, although the direction of this transmission could not be established (Biek et al. 2012). However, the strongest evidence comes from badger culling trials where a decrease in the badger population led to a decrease in incidence of tuberculosis in cattle (Donnelly et al. 2003; Griffin et al. 2005).

The main route of transmission between badgers and cattle is currently unknown. Direct contact between these species is rare (Krebs et al. 1997; Drewe et al. 2013) and badgers appear to avoid contact with cattle (Sleeman & Mulacahy 1993), making direct transmission unlikely. However, behavioural changes observed in the later stages of disease (Cheeseman & Mallinson 1981) may provide opportunity for transmission, as contact would not need to be frequent if the badgers were heavily infected, shedding and behaving abnormally. Observational studies have identified that badgers keep a distance of between 10-15m from cattle when grazing on pasture (Benham & Broom 1989). Badgers have been observed entering farm buildings in order to eat cattle feed (Benham

1993) and badgers found dead on farms four times more likely to have tuberculosis than those found dead at the roadside (Cheeseman & Mallinson 1981).

Indirect contact with infected badger excreta on pasture is another potential route of transmission (Figure 1.3). Badger urine contains up to 300,000 bacilli per ml and faeces 75,000 per gram (Gallagher & Clifton-Hadley 2000). Badger setts and latrines are often located on pasture, where badgers also frequently forage (Kruuk 1978). Badgers also enter farm buildings, where they have been observed defecating in cattle feed (Garnett et al. 2002). The potential for environmental transmission is recognised in other diseases through contamination of animal feed by excreta (Daniels et al. 2003). In addition, modelling has indicated that when each potential transmission route is considered separately, the environment is the single largest contributing factor driving transmission, accounting for 15% of cases (Brooks-Pollock et al. 2014). The role of direct contact and environmental transmission of *M. bovis* could not be detangled in this model however, the short half life of 34 days (Brooks-Pollock et al. 2014) suggests this is bacteria surviving in the environment, as infected badgers may remain infected for several years.

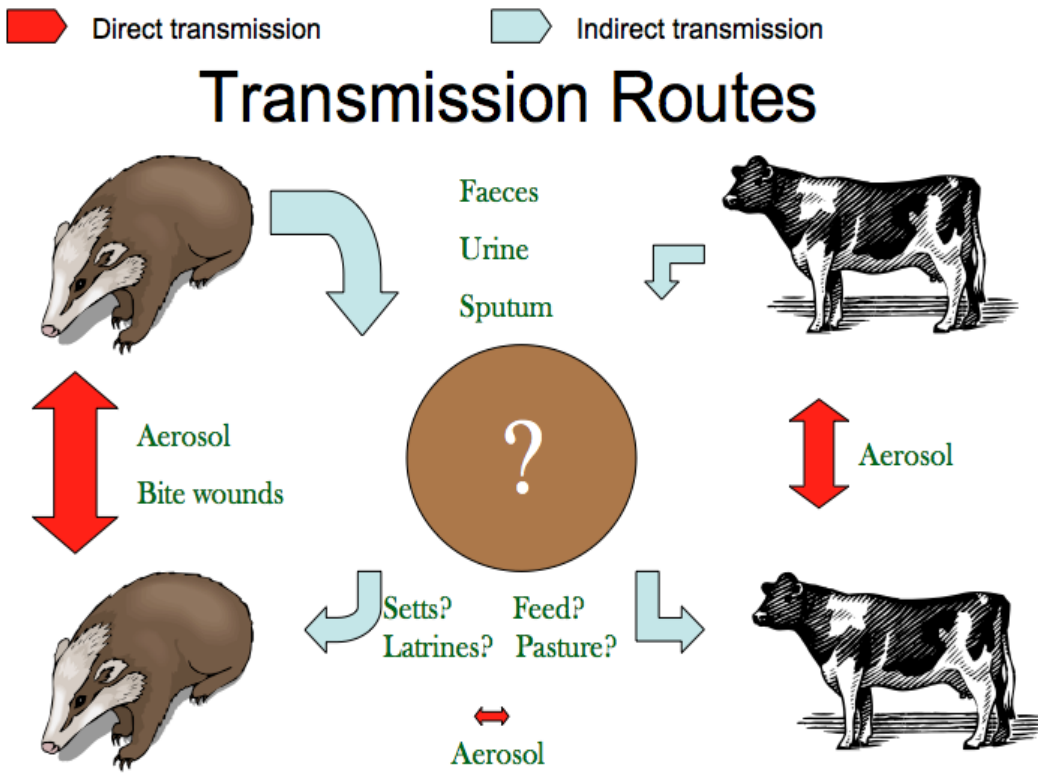

Figure 1.3 *M. bovis* transmission routes within and between cattle and badger populations. Brown circle represents the environment. Arrows represent transmission routes. Size of arrows not an indication of relative importance of the transmission route.

The potential for environmental transmission depends in part on the behaviour of cattle towards badger excreta. Some observational studies have observed cattle avoiding grass contaminated with badger faeces and urine except when grass is scare however, the more unselective individuals would inhale more frequently and graze closely to badger excreta (Benham 1993). Recent work has found that cattle do not avoid grazing near badger faeces, but rather they investigate it by sniffing at close range and are more likely to interact with badger faeces than empty pits or bare ground (Davies 2015). Similar studies have recorded cattle investigating badger faeces on pasture by sniffing and flehman response (Moses 2015), creating further potential for exposure. One study has concluded that, although the probability of infection per cattle contact with badger excreta is low,

the levels of investigation and grazing contact with badger urine and faeces observed could account for disease prevalence in the South-West of England (Hutchings & Harris 1999).

1.5 The environment as a potential source of infection

The possibility of transmission from the environment to cattle depends not only on the response of cattle to badger excreta but also on the viability, persistence and virulence of environmental *M. bovis*. Although badgers are known to shed *M. bovis*, knowledge of the extent and location of the environmental pool of this pathogen is limited, in part due to the use of culture based methods, which are insensitive due to the slow growth rate of *M. bovis* and due to the harsh decontamination methods required to remove competing bacteria. Studies have cultured *M. bovis* from naturally infected badger faeces, demonstrating viability in the environment, and have found up to 5% of faeces are positive (Cheeseman et al. 1985; Wilesmith et al. 1986; Hewson & Simpson 1987) however, Hewson and Simpson concluded that the rate of false negatives were high and that sampling over large areas for long periods of time is required to reduce their occurrence.

While culture studies demonstrate that a proportion of environmental *M. bovis* remain viable in badger faeces, the length of time the cells are viable for will affect the possibility for transmission to cattle. Environmental survival studies produce variable results; with some recording survival up to seven hundred days post seeding and some failing to detect *M. bovis* after less than four weeks (Courtenay & Wellington 2008). It is also possible to capture whole cells from

naturally infected badger faeces by immunomagnetic capture which can be then be grown in culture (Sweeney et al. 2006). Some of the variability in viability and survival is likely explained by the use of culture based methods. Using molecular methods, *M. bovis* DNA has been detected in the environment after twenty one months (Young et al. 2005). Although the presence of DNA does not necessarily demonstrate the presence of viable cells, soil microcosm work has found that *M. bovis* DNA from dead cells does not survive for longer than ten days under optimal conditions (Young et al. 2005). Moreover, viability and survival have been demonstrated by detection of *M. bovis* 16S rRNA, which is rapidly degraded in the environment, in badger faeces after fifteen months in the environment (Young et al. 2005). In addition, *M. bovis* that had been persisting in soil microcosms for months after seeding is able to colonise mice that have been fed the infected soil (Ghodbane et al. 2014). Several studies have also reported infection in cattle after grazing on naturally or experimentally contaminated pasture (Courtenay et al. 2006).

1.6 Diagnosis of *M. bovis* in badger populations

Whichever the method of transmission from badgers to cattle, accurate diagnosis in badgers is required to effectively direct control interventions and to understand the epidemiology of this disease. The current methods of diagnosing *M. bovis* in live badgers require capturing animals and carrying out culture of clinical samples such as sputum and faeces, and testing with the immunoassays IFNγ and Brock TB Stat-Pak™ (Stat Pak). In addition to being expensive, requiring trained individuals and raising ethical issues, the need to capture badgers limits the proportion of animals that can be tested, both logistically and also because

trappability varies between individuals and populations (Tuyttens et al. 1999). In particular, trappability has been found to vary between areas, seasons, years and between cubs and adults. For example, trappability of cubs is higher than adults at Woodchester Park and Nibley but the opposite pattern was observed at Wytham (Tuyttens et al. 1999). The trappability of cubs also varies significantly with season in areas of RoI (Byrne et al. 2012). Furthermore, the method of capture affects trapping efficiency with an estimated 35 – 85% of the population being captured where only cage traps are used (Smith & Cheeseman 2007) and 34-51% in RoI (Byrne *et al.* 2012) where stop restraints are also used.

In addition to the limitations of trapping, each diagnostic test itself has limitations of sensitivity and specificity (Dalley et al. 2008; Chambers et al. 2009). It has been suggested that to improve accuracy of diagnosis that the results of multiple testing methods be interpreted in parallel (Drewe et al. 2010) however, as each test detects different groups of infected individuals, this may result in a proportion of infected individuals being undetected.

1.7 Controlling *M. bovis* in badger populations

As badgers are responsible for up to 50% of *M. bovis* cases in cattle in some areas (Donnelly & Nouvellet 2013), it is important to direct control interventions at badger populations. Control is currently carried out using a combination of culling, vaccination and farm husbandry techniques and methods differs between the UK and RoI.

1.71 Farm husbandry

Farm husbandry involves attempting to separate cattle from sources of infection. Securing farms may include measures such as fencing off badger setts and latrines, raising troughs and securing feed (Garnett et al. 2003; Judge et al. 2011). A study on a small number of farms has found that measures such as sheet metal fencing, securing feed bins and electric fencing to be 100% successful at keeping badgers out of farm buildings when properly used and maintained and also reduce the number times badgers visit farms (Judge et al. 2011).

Farmer compliance with farm husbandry methods is highly variable, with installed badger exclusion mechanisms used on average 59% of nights and one farm using retractable electric fencing on only 7% of nights (Judge et al. 2011). In another study, half of farmers questioned felt that farm husbandry practices were too impractical to invest in and others said they would only invest if grants were given towards the cost (Ward et al. 2006). The reluctance of farmers to adopt these types of measures may be because few feel that badger access to farm buildings is a problem and possibly due to a lack of confidence in official guidance (Ward et al. 2006). If farmers are not confident in, and compliant with farm husbandry, they will not effectively decrease transmission from badgers to cattle.

1.72 Badger Culling

Badger culling trials in the UK and RoI have produced conflicting results and in the UK culls can be controversial with the public. Although small culling trials appeared to have been successful in decreasing herd incidence in the UK (Krebs

et al. 1997), the Randomised Badger Culling Trial (RBCT), which compared reactive and proactive culling to survey only areas, found that while incidence decreased by 19% in proactively culled zones, it increased by 29% in areas surrounding culled zones (Donnelly et al. 2006) as well as increasing by 27% in reactively culled areas (Donnelly et al. 2003). Studies in the RoI, including the Four Areas Project (FAP), have found significantly reduced odds of cattle cases in proactively compared to reactively culled areas (Griffin et al. 2005) and a reductive effect of culling on the risk of future breakdowns (Olea-Popelka et al. 2009).

Increased incidence in cattle herds as a result of culling badgers may be due to perturbation of badger social structures, causing them to range more widely, allowing them to contact more sources of infection and to spread infection more widely themselves (Woodroffe et al. 2005; Pope et al. 2007). After culling, badgers have disrupted social structures (Riordan et al. 2011; Cheeseman et al. 1993), travel distances greater than usual (Cheeseman et al. 1993), range more widely and have territories that overlap with other groups (Tuyttens et al. 2000). Furthermore, these differences were not restricted to groups involved in culling but also extended to adjacent groups and one - two groups away from removal groups (Tuyttens et al. 2000). In addition, an observational study in the RoI suggest that culling 50% of the badger population may result in increased contact between badgers and cattle based on the number of farms visited by social groups and the number of badger groups visiting each farm after culling (O'Corry-Crowe et al. 1996).

The difference in the results of RBCT and trials in the RoI such as FAP may be due to several factors including; the difference in trapping methods used, site selection, which in the RoI included barriers to badger dispersal (Donnelly et al. 2003) and the length and consistency of culling (O'Connor et al. 2012). Despite contradictory results, reactive badger culling has formed part of the national control strategy in the RoI since 2004 (Bhuachalla et al. 2015) and proactive culling was introduced to two areas of the UK, Somerset and Gloucestershire, in 2013 (Defra 2014b).

1.73 Badger Vaccination

As culling is scientifically contentious and controversial with the public, vaccination may offer an alternative control strategy. Vaccinating badgers intramuscularly and orally with Bacillus Calmette-Guérin (BCG) vaccine is protective in the laboratory and reduces the number and severity of lesions (Chambers et al. 2011; Corner et al. 2008; Lesellier et al. 2011; Corner et al. 2010; Murphy et al. 2014). Intramuscular vaccination has been demonstrated to be protective in field studies and can confer herd immunity (Carter et al. 2012). When at least one third of adults in a sett are vaccinated, cubs are 79% less likely to become infected (Carter *et al.* 2012). Although vaccination offers the potential of a less contentious and potentially efficacious method of control, it is currently expensive, with the cost of intravenous vaccination estimated as £2000 – 4000 per km^2 (Chambers *et al.* 2014). Intramuscular vaccination requires animals to be captured which, as discussed previously, may result in uneven coverage across populations (Tuyttens et al. 1999). In addition, trapping animals logistically limits the proportion of the populations that can be vaccinated.

Simulation modelling suggests that a minimum of 40% of the healthy badger population needs to be vaccinated annually to eradicate *M. bovis* from badger populations (Wilkinson et al. 2004). With over 30% of animals infected in some locations this would require a large proportion of all badgers in a given population to be trapped and vaccinated, which may prove challenging given variations in trappability and the need for highly skilled staff. However, eradication of *M. bovis* in badgers may not be required to substantially reduce transmission from badgers to cattle. There is greater potential to vaccinate large proportions of the badger population using vaccine placed in an oral food bait however, although oral vaccine is efficacious in laboratory studies, the first field study of oral vaccine delivery is on-going, and the efficacy of this method is currently unknown.

1.8 Monitoring environmental *M. bovis*

The ability to monitor this disease in badger populations across large areas over time is limited by the cost, time and expertise involved in capturing and taking samples from badgers. As badger faeces are a potential source of infection for cattle, monitoring the levels of *M. bovis* present in badger faeces also monitors the relative potential infection risk as well as shedding and disease in the badger population. Non-invasive monitoring through sampling *M. bovis* in the environment is restricted by insensitive culture techniques that are also not able to accurately enumerate *M. bovis*. *M. bovis* is a member of the *Mycobacterium tuberculosis* complex, the members of which have identical 16S rRNA sequences (Boddinghaus et al. 1990) and whose genomes are 99.9% similar at the

nucleotide level (Sreevatsan et al. 1997). The members of this complex can be distinguished by the presence or absence of a range of genomic regions termed 'regions of difference' (Brosch et al. 2002). The region of difference 4 (RD4) has been deleted in *M. bovis* (Brosch et al. 2002) and therefore the presence of *M. bovis* can be established by detecting the unique sequence region remaining where RD4 would be present in other *M. tuberculosis* complex members. Previous work within our group developed and validated a qPCR assay to quantify the number of *M. bovis* genome equivalents using the region of difference four (RD4) (Sweeney *et al.* 2007; Travis *et al.* 2011). An inhibition control assay was designed to detect inhibition of amplification of the RD4 region in environmental samples such as faeces and soil (Pontiroli *et al.* 2011), which often contain PCR inhibitors in their DNA extracts. This assay has been demonstrated to detect *M. bovis* in badger faeces and sett soil (Travis et al. 2011) but had not previously been used as a tool to detect infection and monitor shedding in a population over time or during an intervention strategy.

Detecting *M. bovis* in faeces is an ideal method for monitoring as it is non-invasive and there are few limitations on the number of samples that can be analysed. Badgers defecate in latrines which are formed of clusters of pits (Figure 1.4), making their faeces simple to find and distinguishable from those of other species. The number of pits or latrines present is strongly correlated with the size of the adult population of the social groups to whom the latrine or pits belong (Tuyttens et al. 2001). Latrines are located both within the territory of the social group (hinterland latrines) and also frequently at the territory boundaries (Delahay et al. 2000).

Figure 1.4 A badger latrine formed of pits (A) and latrine pit containing badger faeces (B).

Faeces are thought to convey messages through scent marking, such as information about group membership (Roper 2010a). All animals of a social group visit latrines within their territory however, use of boundary latrines differs from hinterland latrines, with use of the latter peaking in spring and autumn (Kruuk 1978) which coincides with female oestrus, and being used more frequently by males (Roper 2010a). Latrine use typically lasts between 20-90 seconds and the frequency of use has been observed to be highest in early spring (February-May) (Kruuk 1978). Observational studies have not identified differences in in the number of faeces produced per night between males and females but have noted seasonal differences in the number of faeces deposited,

with faeces from cubs less likely to be found over summer and autumn and other age classes showing reduced deposition of faeces at particular times of year (Brown et al. 1992). A molecular study of hinterland latrines identified equal use between males and females as well as cubs and adults (Wilson et al. 2003). Over ten consecutive days, individuals defecated in latrines on between one and six nights, with the majority defecating on one or two nights over the study (Wilson et al. 2003). When visiting a latrine on 50% of occasions badgers do not defecate at, rather they urinate, deposit scent gland secretions or sniff at the contents of the latrine (Roper 2010a), creating opportunity for environmental transmission (defined as transmission of *M. bovis* from an environmental source such as faeces, urine, feed or soil without direct contact between animals).

1.9 Hypotheses

Although a potential source of infection, little is currently known about the extent and variability of the environmental pool of *M. bovis* shed by badgers, or how the size of this pool relates to levels of infection in the population. In addition, changes in environmental shedding with BCG vaccination have not previously been examined. Furthermore, it is assumed faecal *M. bovis* arises from swallowed sputum due to the rarity of GIT lesions however; infection often does not produce lesions and changes in the the composition of the gut microbiota with disease has not been determined.

1. That testing environmental faecal samples for *M. bovis* by qPCR can detect and monitor changes in infection at the population level.

2. That faecal shedding of *M. bovis* is not an indicator of gut colonisation, and therefore disease does not alter the gut microbiota.

The aims of this project were:

i) To determine the potential of the RD4 qPCR assay as a tool for diagnosing *M. bovis* and monitoring control interventions in badger populations.

ii) To establish and quantify the environmental pool of *M. bovis* shed in badger faeces over time.

iii) To establish whether disease caused by *M. bovis* alters the gut microbial community and whether this corresponds to changes in faecal communities.

The work presented here was undertaken in two contrasting badger populations in Woodchester Park, Gloucestershire, UK in collaboration with the Animal and Plant Health Agency (APHA) and in County Kilkenny, the RoI in collaboration with Eamonn Gormley and Leigh Corner at University College Dublin (UCD). The population at Woodchester Park is high density, with up to 27 badgers per social group (Rogers et al. 1997) in a 7km^2 area and has a prevalence of between 10.3-17.7% (Delahay et al. 2000). The populations is intensively studied and detailed information is available on social groups and individuals. The Kilkenny population is low density and has a predicted prevalence of infection in the badger population of 30% (Aznar et al. 2011) in a 755km^2 area. Previous work within our research group had taken place at both sites, with qPCR validation

taking place at Woodchester, Park and studies into the correspondence between qPCR measures and post mortem at UCD.

The work presented in chapter two evaluates the sensitivity and specificity of qPCR on faeces compared with immunoassays, for diagnosing *M. bovis* at the individual and social group level in a high-density population. The results of these analyses are used to design optimal faecal sampling strategies to maximise the detection of infected social groups. Using the same dataset as chapter two, data chapter three uses qPCR on latrine faeces to determine variability in the environmental reservoir of *M. bovis* that is shed between groups and across seasons. Chapter four evaluates the use of qPCR on latrine faeces in a low-density population over a large area, in addition to assessing the impact of oral BCG vaccination on the environmental reservoir of *M. bovis*. Finally, chapter five assesses the similarity of the microbial communities present in badger gut and faecal communities and establishes changes in these microbial communities with *M. bovis* infection, the presence of lesions in the lungs and faecal shedding.

2. Performance of a Non-Invasive Test for Detecting *M. bovis* Shedding in European Badger Populations

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Author Contributions

H.C.K was involved with 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 and prepared figure 2.4. E.T advised on the project and data analysis. D.P collected and processed samples. J.S coordinated and oversaw processing of samples at APHA Weybridge. J.C processed samples at APHA Weybridge. R.J.D contributed to the study design, reporting and data evaluation, organised and oversaw the fieldwork at APHA Woodchester. W.G was involved with designing the original experiment. O.C in collaboration with E.M.W was involved in the project design. EMW in collaboration with O.C devised the experimental set up and supervised all field and practical work and was responsible for biosafety.

2.1 Abstract

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 incidence in cattle populations. Understanding the epidemiology of *M. bovis* in badger populations is essential to direct control interventions and understand disease spread; however, accurate diagnosis in live animals is challenging and currently uses invasive methods. Here we present a non-invasive diagnostic procedure and sampling regime using field sampling of latrines and detection of *M. bovis* with qPCR, the results of which strongly correlate with the results of immunoassay testing in the field at the social group level. This method allows *M. bovis* infection in badger populations to be monitored without trapping and provides additional information on the quantity of bacterial DNA shed. Our approach may therefore provide valuable insights into the epidemiology of bovine tuberculosis in badger populations and inform disease control interventions.

2.2 Introduction

Mycobacterium bovis infection in wildlife is an issue of growing importance worldwide, with infection found in a range of species including buffalo in Africa (Renwick *et al*. 2007), wild boar in Spain (Aranaz *et al*. 2004), brushtail possums in New Zealand (Coleman & Cooker 2001) and European badgers in the United Kingdom (UK) (Clifton Hadley *et al*. 1993) and Republic of Ireland (RoI) (Gormley & Collins 2000). In the UK and RoI badgers are involved in the transmission of tuberculosis (TB) to cattle (Donnelly *et al*. 2003; Chambers et al. 2008; Aznar *et al*. 2011). The incidence of *M. bovis* has been increasing in UK cattle herds for over thirty years (Defra 2014) resulting in substantial economic losses (Defra 2013).

Once infected, badgers may intermittently shed *M. bovis* cells in sputum, faeces and urine (Clifton Hadley *et al*. 1993), creating an environmental source of potential infection for other badgers and cattle (Hutchings & Harris 1999; Benahm & Broom 1991). *M. bovis* DNA has been shown to survive outside the host for up to twenty-one months and 16S rRNA for has been detected in badger setts and latrines (Young *et al*. 2005). In addition, studies have found a 2.5% positivity rate when culturing from badger faeces (Wilesmith 1986) and *M. bovis* has been cultured from cattle faeces several months after excretion (Courtenay & Wellington 2008). Furthermore, *M. bovis* that had persisted in soil for over twelve months was able to colonise mice (Ghodbane *et al*. 2014). This indicates that at least a proportion of *M. bovis* cells shed in badger faeces can remain viable in the environment. Monitoring *M. bovis* infection 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 (Defra 2014).

Accurate diagnosis of *M. bovis* infection in live animals is challenging yet essential in order to understand the epidemiology of the disease and its onward spread. Currently, infection in live badgers can be monitored through trapping and diagnosis with immunoassays (IFN**γ)** (Dalley *et al*. 2008) and Brock**™** Stat-Pak (Chambers *et al*. 2008)) and culture (Delahay et al. 2000). Culture of clinical samples (sputum and faeces) has a low sensitivity of 8% and a specificity of 99.8% (Drewe *et al*. 2010). Furthermore, infected badgers may only intermittently excrete *M. bovis* and culture from non-invasive sources such as faeces is challenging due to decontamination methods. Immunoassays are more sensitive than culture but can be affected by animal age and duration of infection (Chambers *et al*. 2009). IFNγ involves stimulating lymphocytes in whole blood and then detecting IFNγ by sandwich ELISA (Dalley *et al*. 2008). This method has a sensitivity ranging from 57 – 85% in badgers with lower sensitivity in cubs than adults and a specificity ranging from 93 – 98% (Dalley *et al.* 2008; Drewe *et al*. 2010; Chambers *et al.* 2009). Stat-Pak is a lateral flow serum antibody test with a sensitivity that varies from 50 – 78% (Chambers *et al.* 2009) in badgers, with sensitivity reduced in the earlier stages of disease compared to advanced infection, and specificity ranging from 93 – 97% (Chambers *et al.* 2009; Drewe *et al.* 2010). As no individual test is sufficiently sensitive or specific enough to use alone for diagnosis the combined application and interpretation of IFNγ and Stat-Pak has been recommended (Drewe *et al.* 2010). Diagnosis by immunoassay

and culture of clinical samples also requires that badgers are trapped, which is costly, logistically challenging and likely to result in limited sample sizes.

A non-invasive assay for sampling badger populations may offer an opportunity to gather information on the spatio-temporal distribution of *M. bovis* in badger populations over a larger area, more easily and cost-effectively than by trapping. A non-invasive 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 faecal shedding of *M. bovis* amongst badgers at the social group level using an existing qPCR assay developed in our group which quantifies *M. bovis* genome copy number (Travis *et al*. 2011; Pontiroli *et al*. 2011). We aimed to (i) determine the correspondence between immunoassay test results and faecal qPCR testing for *M. bovis* infection in live-trapped badgers and to (ii) establish an optimal faecal sampling regime to maximise detection of shedding in badger populations.

2.3 Materials and Methods

2.31 Sampling and Trapping

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

To determine the relative performance of qPCR we compared results to those from immunoassay testing and culture from clinical samples taken during routine trapping and sampling of the twelve targeted badger social groups. Sputum samples were collected by aspiration of both the oesophagus and the trachea using catheters. Collected samples are then flushed into physiological saline. Sputum and faeces were cultured on solid media (Gallagher & Horwill 1977) 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 two consecutive days. Badgers were trapped using baited cage traps placed around the main setts 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 twelve social groups were tested by BrockTB Stat-Pak®, IFNγ and culture of clinical samples. To establish the relative sensitivity and specificity of faecal qPCR compared to immunoassays at an individual animal level we collected faecal 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 clinical samples were positive. All work was approved by the University of Warwick and the Food and Environment Research Agency Ethical Review Committee and carried out under license granted by the Home Office under the 1986 Animals (Scientific Procedures) Act.

2.32 DNA Extraction and qPCR

Total community DNA was extracted from 0.1 g (+/- 0.003 g) of faeces 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 which 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 machine (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. PCR reactions were set up using 900 nM of each primer RD4F $^{5'}$TGTGAATTCATACAAGCCGTAGTCG$^{3'}$, (RD4R $^{5'}$CCCGTAGCGTTACTGAGAAATTGC$^{3'}$), 250 nM of Taqman probe (6FAM-AGCGCAACACTCTTGGAGTGGCCTAC—TMR), 1 mg ml^{-1} bovine serum albumen (BSA), 12.5 µl of Environmental Mastermix 2.0 (ABI), 10 µl of template and made up to 25 µl with molecular grade water (Sigma Aldrich). PCR

cycling conditions were 50 °C for 2 min followed by 95 °C for 10 min then 40 cycles of 95 °C for 15 sec and 58 °C for 1 min. Samples exhibiting amplification in one or more technical replicates were taken on to full quantification using three technical replicate per sample under the same conditions. If one or more of the technical replicates of the quantification assay exhibited amplification 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 (Pontiroli *et al*. 2011) was used to detect the possibility of false negative results due to inhibition. Each extracted sample was screened as a singlet, if ΔCT was greater than 2.5 then the sample was rescreened in doublet. If the average ΔCT was greater than 2.5 then the sample was re-extracted from frozen faecal aliquots, and 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 APHA Weybridge.

2.34 Statistical Analysis

All data analysis was performed using the statistical program R. Binomial and gaussian generalised linear models (GLM) were used to determine differences in faecal sample positivity (as a binary variable) and *M. bovis* genome equivalents shed between social groups and seasons. All GLMs were carried out with Old Oak as the baseline social group as it had the lowest prevalence of positive faecal 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) were used to determine differences in sample numbers and proportions of

trapped animals that were positive amongst social groups. Spearman's ranks were calculated to determine whether there was correspondence in the rank order of social groups based on prevalence estimated by live-testing and faecal qPCR.

Comparative sensitivity, specificity, positive predictive values (PPV) and negative predictive values (NPV) were calculated using equations 1-4 where, a = both tests positive (true positive), b = query test positive, reference test negative (comparative false positive), c = query test negative, reference test positive (comparative false negative) and d = both tests negative (true negative). As immunoassays are of limited sensitivity, false positives and false negatives are comparative (Table 2.1). Immunoassays will have failed to detect a proportion of positive individuals and therefore qPCR may appear to lack sensitivity of specificity as a result of this.

Table 2.1 Comparative a, b, c and d used in sensitivity and specificity calculations.

		Immunoassay	
		+	-
qPCR	+	a	b
	-	c	d

The confidence intervals for these values were calculated using the Wilson Score interval. When calculated for individuals a positive result was defined as a positive on any diagnostic test or culture from clinical samples per 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.

$$\text{Equation 1: Sensitivty} = \frac{a}{a+c}$$

$$\text{Equation 2: Specificity} = \frac{d}{b+d}$$

$$\text{Equation 3: PPV} = \frac{a}{a+b}$$

$$\text{Equation 4: NPV} = \frac{d}{c+d}$$

False positive rates were calculated using sixty-eight known negative faecal samples obtained from captive badgers at APHA which were routinely tested for TB using IFNγ, and forty-nine water samples. Negative samples were double blinded and randomly introduced into the experiment at both laboratories. As this qPCR assay is highly specific for *M. bovis* on a technical level (King *et al*. 2015) all false positives are expected to be from contamination introduced in the laboratory and therefore negative controls are introduced at every stage of DNA extraction and quantification.

2.4 Results

2.41 Correspondence between immunoassays and faecal qPCR on trapped badgers

Routine badger trapping at Woodchester Park took place prior to the collection of faeces from latrines. Trapped badgers were tested for *M. bovis* infection with immunoassays (IFNγ and Stat-Pak), and by culture of clinical samples and qPCR on faecal samples. In total there were one hundred and twenty trapping events with 48% found to be positive by any test. Stat-Pak identified a greater number of positives than either IFNγ or qPCR, which both identified a similar number of positives (Figure 2.1). No culture positive results were obtained from faeces or sputum samples of trapped badgers. The correlation between tests was low with StatPak and IFNγ ($r=0.27$, $p<0.05$), Stat Pak and qPCR ($r=0.11$, $p>0.05$) and INFγ and qPCR ($r=0.20$, $p<0.05$).

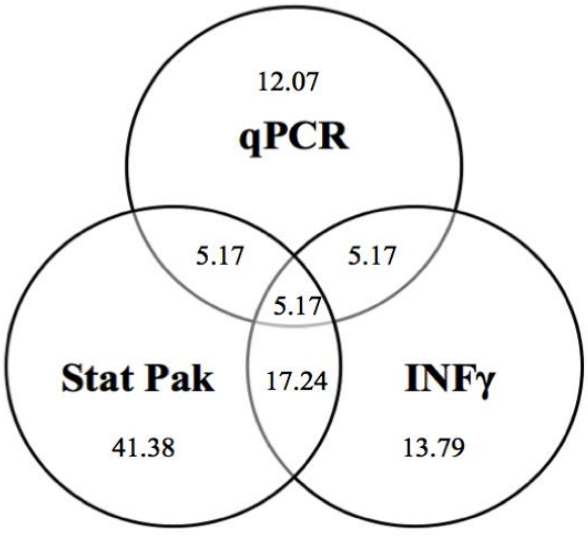

Figure 2.1 Percentage agreement between positive test results (Total trap events = 120 with 58 positive trap events, where at least one test gave a positive result).

As there is no gold standard for diagnosing infection in badgers, sensitivity, specificity, PPV and NPV calculations were carried out using Stat-Pak and IFNγ as the gold standard separately and with both tests combined. The sensitivity of qPCR compared to Stat-Pak and IFNγ both separately and combined was low, ranging from 14– 25% whereas the sensitivity of Stat-Pak and IFNγ compared to one another was higher at 32% and 59% respectively (Table 2.1). The lower sensitivity of qPCR was expected, as it is a measure of shedding, rather than infection and because infected badgers may shed *M. bovis* intermittently or not at all.

The relative specificity of qPCR was high, ranging from 91 – 93% compared with Stat-Pak and IFNγ, which have specificities of 86 and 67% respectively when compared against one another. The PPV for qPCR ranged from 43 – 64% and from 33 – 59% compared to Stat-Pak and IFNγ respectively. The high relative specificity of qPCR is due to the definitive detection of the DNA target which is unique to *M. bovis*.

The NPV ranged from 54 – 83% for qPCR and from 67 – 86% for Stat-Pak and IFNγ when compared to one another. The NPV was lower overall for qPCR than immunoassays as the former is detecting shedding, rather than infected animals, resulting in some positive animals being missed.

Table 2.1 Sensitivity, specificity, PPV and NPV of *M. bovis* diagnosis in individual trapped badgers using Stat – Pak and IFNγ both individually and combined as the gold standard against qPCR and each other (n=120).

	Sensitivity	Specificity	PPV	NPV
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)
IFNγ	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 IFNγ	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 (gamma IFNγ)	32.50 (23.89 – 41.47)	85.71 (77.76 – 91.14)	59.09 (49.53 – 68.01)	66.67 (57.21 – 74.96)
IFNγ 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)

2.42 Historically positive trapping events

As the badger population in Woodchester Park has been extensively studied for over twenty years, trapping information is available for several years prior to this study. Of the 120 trapping events discussed above, on 57% of occasions the captured animal was historically positive on at least one test. Of these historically positive captures, 29% were positive by Stat-Pak only, 25% by IFNγ only, 43% by Stat-Pak and IFNγ and 3% by culture of clinical samples, IFNγ and Stat-Pak (Figure 2.2). Contemporary (2012) trapping identified that in 53% (32 trap events) of cases the animal had at least one historical positive test result; 24% were positive by Stat-Pak only, 14% by IFNγ only, 59% by Stat-Pak and IFNγ and 3% were positive by culture and Stat-Pak and IFNγ, but none were positive by faecal culture alone (Figure 2.3).

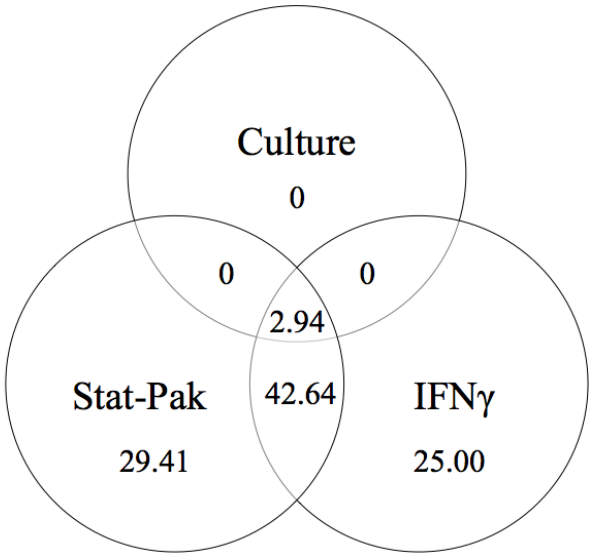

Figure 2.2 The agreement between diagnostic tests for all historically positive animals. Expressed as a percentage of trappings found positive by those measures (n=68).

The majority (93%) of historically Stat-Pak positive cases were also positive on Stat-Pak during contemporary testing. Also, 65% of historically IFNγ positive cases were also positive on IFNγ during contemporary testing (Figure 2.3). No captured animals that were historically culture positive were positive by contemporary culture however, they were all positive by faecal qPCR, indicating that they were still shedding *M. bovis*. Forty-five percent of trapped badgers that were historically positive by both Stat-Pak and IFNγ were also positive on these tests during contemporary testing. Past diagnostic tests results were pooled for all capture events for each badger, and hence IFNγ and Stat-Pak may have been positive at different capture events rather than simultaneously. Of the sixteen trap events that were positive by qPCR, 12 (75%) were historically positive on at least one of the live tests. Both of the historically culture positive animals were positive by qPCR.

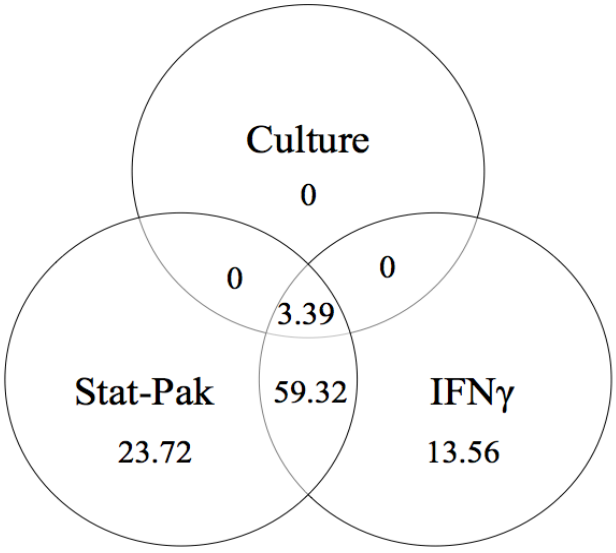

Figure 2.3 The agreement between diagnostic methods for historic positive animals, which were also contemporarily positive.

2.43 Contemporary seasonal trapping

Badgers were trapped seasonally throughout 2012 which coincided with the collection of faecal samples from latrines. Trapped badgers were routinely tested using Stat-Pak, IFNγ and culture of clinical samples. The number of badgers trapped was highly variable amongst social groups (5 – 18 animals per group) and seasons (Tables 2.2 and 2.3). The number of badgers caught per sampling day was also highly variable between seasons with between 2 and 28 badgers being trapped on a given day (Table 2.2). A greater number 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 was no difference in the number of badgers caught per social group throughout the year ($F_{(11,36)} = 1,272$, $p>0.05$) or in the percentage of positive badgers trapped per season ($F(3,44)= 0.8523$, $p>0.05$). However, there was significant variation in the percentage of positive animals (as estimated by immunoassay) per social groups across the whole year ($F(11,36) = 3.635$, $p<0.001$), with Honeywell ($t=2.563$, $p<0.05$), Nettle ($t=2.357$, $p<0.05$) and Septic Tank ($t=2.457$, $p<0.05$) having a greater percentage of test positive badgers than the other groups.

In this study badger sex was not related to the likelihood of yielding a positive test result (Female: odds 1, Male: odds: 0.86 (95% CI: 0.43 – 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 IFNγ test result.

Table 2.2 Summary of the number of badgers trapped by season (n=128).

	Spring	Summer	Autumn	Winter
Number badgers trapped	14	84	18	12
Av. Badgers trapped per social group	1.17	7.00	1.50	1.00
Median badgers trapped per sett (min, max)	0 (0,8)	6 (3,14)	1 (0,5)	0.5 (0,4)
Mean badgers trapped per trapping day	7.00	10.50	4.50	4.00
Median badgers trapped per trapping day (min, max)	7.0 (2,12)	10.5 (3,28)	4.5 (3,6)	5 (2,5)

Table 2.3 Summary of latrine faecal sample numbers by season.

Social group	Spring	Summer	Autumn	Winter	Total
Beech	50	38	58	19	**175**
Colliers Wood	40	36	50	8	**134**
Honeywell	50	37	41	8	**136**
Kennel	36	19	18	3	**76**
Nettle	49	22	32	1	**104**
Old Oak	40	35	45	13	**133**
Septic Tank	16	35	54	20	**125**
Top	46	35	70	20	**171**
West	50	37	62	13	**162**
Woodrush	38	33	63	9	**143**
Wych Elm	41	31	35	5	**112**
Yew	50	33	62	20	**165**
Total	**506**	**391**	**600**	**139**	**1636**

2.44 Seasonal and social group differences in latrine faecal sampling

The total number of faecal samples collected varied between social groups from 76 – 175 samples across the year (Table 2.3). On average more samples were collected per sampling day in spring than in other seasons with a mean of 51 per day compared to the other seasons, which ranged from 23 – 38 per day (Table 2.4). On each sampling day the aim was to collect ten fresh faecal samples, and spring and summer samples number averaged 9 and 8 samples per day respectively, with 6 samples collected per day in winter (Table 2.4).

Table 2.4 Summary statistics for latrine faecal sampling by season

	Spring	Summer	Autumn	Winter
Total samples	506	391	600	139
Mean samples per social group	42.17	32.58	50	11.58
Median Samples per group (min, max)	43.5 (16, 50)	35 (19, 38)	52 (18, 70)	11 (1, 20)
Mean samples per sampling day	50.60	32.58	37.50	23.17
Median samples per sampling day	54 (20, 65)	23 (16,64)	41 (12,57)	34 (22,50)
Mean samples per social group per sampling day (adjusted for number of sampling days visited)	8.43	8.15	7.14	5.79

The odds of finding a positive faecal sample were equal across all seasons except for summer when there was a significantly higher probability (Table 2.5). There was a significant difference in the number of *M. bovis* genome equivalents shed over the year with significantly greater numbers of cells detected in summer and autumn than in winter and spring, even though there were no more positive samples in autumn than in winter or spring (Table 2.5).

Table 2.5 The odds ratio of finding an *M. bovis* positive sample by qPCR by season, with winter as the baseline season.

Season	Odds ratio (CI)	P value
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	-

There was no correlation between the number of badgers trapped and the number of faecal 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 faeces per social group by season ($r= 0.22$, $p>0.05$).

2.45 Faecal qPCR replication at two centres

A total of 1090 (67% of all samples collected) were subjected to DNA extraction at both the University of Warwick and APHA Weybridge. Of these, 13% (140) were found to be positive, of which 32% (45 samples) were only positive at Warwick, 29% (41 samples) were only positive at APHA and 39% (54 samples) were positive at both centres. There was no statistical difference between whether a sample was positive at Warwick or APHA (McNemar: χ^2 (1,1090) = 0.165, $p>0.05$). There was a significant difference in *M. bovis* genome equivalents in samples that were positive at both centres, with fewer genome equivalents found in those analysed at Warwick (β = -2.53, $P<0.01$). Although 61% of samples were identified as positive at only one centre, the rankings of the social groups (in terms of the proportions of positive samples) were strongly correlated (Spearman's rho = 0.750, $p<0.05$).

2.46 The correspondence between live-testing with immunoassays and culture compared with faecal qPCR testing from latrines

During 2012, ten of the twelve social groups targeted for the present study were positive by Stat-Pak and IFNγ, whereas qPCR of faeces obtained from latrines identified all twelve to be positive (Table 2.6). The number of social groups positive in each season was consistently higher using qPCR of faeces from latrines compared with immunoassay results from live-captured animals for both longitudinal and intensive sampling regimes (Table 2.6). Colliers Wood and Wych Elm social groups were negative on contemporary immunoassays of live animals but were found to be positive in three and four seasons respectively by qPCR (Table 2.6). The largest discrepancy between the two diagnostic

approaches was in spring where live-trapping diagnostics identified 58% fewer social groups as positive compared to qPCR of faeces from latrines. The smallest difference was in winter with 8% fewer social groups identified by immunoassays and culture of clinical samples than by qPCR of faeces from latrines.

Each social group was ranked according to the proportion of test positives using results from live-trapping diagnostics and qPCR of faeces from latrines. The proportions of test positive samples per group as estimated by qPCR on faecal samples collected from latrines in June and from live-trapping diagnostics from summer were highly correlated (Spearman's rho = 0.87, $p<0.001$). Live-trapping diagnostic results from the whole year correlated strongly with qPCR results from faeces collected in June (Spearman's rho = and 0.71, $p<0.05$), with those for the whole years faecal sampling (Spearman's rho = 0.70, $p<0.05$) and with results from all longitudinal faecal sampling (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 (Table 2.7). The cumulative genome equivalents for the year was strongly correlated with trapping results for the year (rho = 0.63, $p<0.05$).

Table 2.6 Summary of trapping and faecal qPCR positivity for each group (trapping n = 128, faeces n= 1636).

	Spring			Summer			Autumn			Winter		
	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	−	−	−	+	+	7.69
Honeywell	−	+	10.20	+	+	33.33	+	+	4.88	+	−	−
Septic Tank	−	−	−	+	+	5.71	+	+	29.63	−	+	15.00
Top	−	+	26.09	+	+	20.00	−	−	−	−	−	−
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	+	−	−
Colliers Wood	−	+	2.56	−	+	5.71	−	+	12.24	−	−	−
Yew	−	+	2.04	+	+	15.63	+	+	3.28	+	+	5.26
Kennel	−	−	−	+	+	21.05	−	−	−	−	−	−
Old Oak	−	−	−	+	+	8.57	+	+	2.22	+	−	−
Percentage Positive	**16.67**	**75.00**	**9.64**	**83.33**	**100.00**	**17.25**	**50.00**	**75.00**	**11.50**	**41.67**	**50.00**	**13.26**

Table 2.7 Spearman's rho and p value for rankings of social groups by faecal samples seasonally and across a year, compared with equivalent trapping for five years previous to the current study and one year post study.

Year	Interval	Spearman's rho (p value)
2007	All year	0.357 (0.254)
	Spring	-0.194 (0.545)
	Summer	0.094 (0.772)
	Autumn	-0.062 (0.849)
	Winter	-0.296 (0.350)
2008	All year	0.505 (0.094)
	Spring	0.179 (0.577)
	Summer	-0.945 (0.769)
	Autumn	0.375 (0.230)
	Winter	0.403 (0.194)
2009	All year	0.338 (0.282)
	Spring	-0.438 (0.155)
	Summer	-0.043 (0.894)
	Autumn	**0.649 (0.022)***
	Winter	**0.718 (0.009)***
2010	All year	0.270 (0.397)
	Spring	-0.205 (0.522)
	Summer	-0.162 (0.613)
	Autumn	0.432 (0.161)
	Winter	0.269 (0.398)
2011	All year	0.147 (0.648)
	Spring	0.319 (0.311)
	Summer	-0.153 (0.6353)
	Autumn	0.060 (0.853)
	Winter	0.065 (0.842)
2013	All year	0.401 (0.196)
	Spring	0.163 (0.611)
	Summer	-0.118 (0.714)
	Autumn	0.398 (0.201)
	Winter	-0.229 (0.474)

2.47 Sensitivity and specificity of qPCR and immunoassays

Sensitivity, specificity, PPV and NPV were calculated at the social group level for seasonal testing of faeces from latrines by qPCR compared to the results of live diagnostic tests from a whole year's trapping results. Group-level sensitivity of qPCR was seasonally variable but consistently high with the highest noted in summer at 100% sensitivity compared with immunoassay results, and the lowest

at 50% in winter (Table 2.8). Group–level specificity was also highly variable, ranging from 0 -100% compared to immunoassays. The lower bound of this range is due to no social groups being found negative by both live-tests and qPCR. The social groups targeted in this study were all chosen as they were historically positive on live testing so 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 - 67%. This wide range may be due to inability of the test to detect the presence of infection in the absence of shedding, hence if a large number of social groups contain animals that are infected but not shedding *M. bovis* in faeces then they will not be detected by qPCR (Table 2.8).

Table 2.8 The group level sensitivity and specificity of seasonal qPCR results compared with all year trapping data using Stat-Pak, IFNγ and both Stat Pak and IFNγ as the gold standards.

Season	Measure	Stat -Pak	IFNγ	Stat -Pak and IFNγ
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)

2.48 Comparison of intensive and longitudinal faecal sampling

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

2.49 The level of faecal sampling required to detect positive social groups

Random resampling of faecal samples determined the sampling intensity required to identify positive groups with varying proportions of positive samples with 95% certainty across a year. The number of samples required varied between 5 for the group with the most positive samples (Nettle), and up to 50 for the group with the least (Yew) (Figure 2.4). In the present study up to 20 faecal samples were collected over two days but more intensive sampling would have been required to collect more. Our results indicate that the number of samples required varies with season, with the fewest samples 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 in a single sampling period by qPCR. To gain a more accurate assessment of the shedding status of a group would require sampling across a whole year.

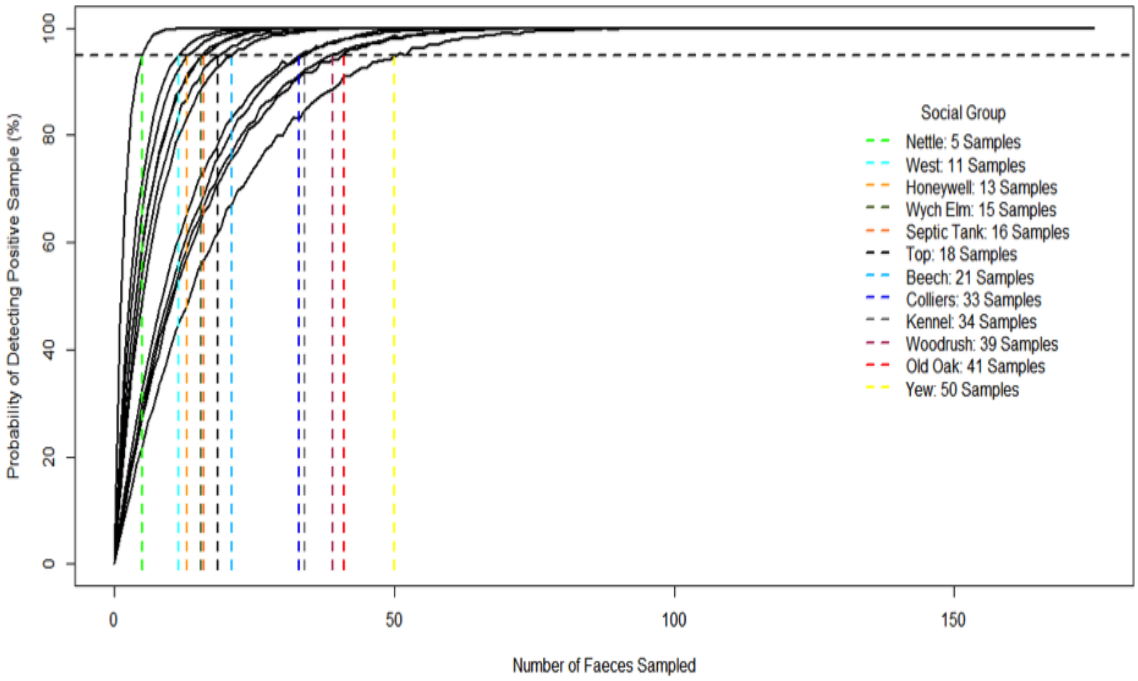

Figure 2.4 The number of faecal samples required to detect a positive social group in one year (Travis et al. 2014).

2.410 False positive rate of faecal qPCR

Of the sixty eight negative faecal samples tested two were positive (2.9%) and one of the forty-six water samples (2.1%) were positive, giving a false positive rate of 2.6%. As the number of samples analysed increases so does the probability of obtaining false positives (Table 2.9). When 5 samples are analysed, the fewest required in this study to detect a positive social group, the probability of obtaining a false positive is low at 13%, this increases to 34% when 50 samples are analyses, the largest number required in this study to detect a positive social group.

Table 2.9 The probability of obtaining a given number of false positives when analysing a set number of faecal samples.

Number of false positives	Probability of obtaining a false positive when analysing a set number of samples		
	5	**20**	**50**
0	0.86	0.56	0.23
1	0.13	0.33	0.34
2	0.01	0.09	0.25
3	2.30×10^{-4}	0.02	0.12
4	3.43×10^{-6}	2.14×10^{-3}	0.04
5	2.05×10^{-8}	2.05×10^{-4}	0.01
6	-	1.53×10^{-5}	2.59×10^{-3}
7	-	9.12×10^{-7}	4.86×10^{-4}
8	-	4.43×10^{-8}	7.80×10^{-5}
9	-	1.76×10^{-9}	1.09×10^{-5}
10	-	5.93×10^{-11}	1.33×10^{-6}
15	-	1.15×10^{-19}	6.93×10^{-12}
20	-	1.77×10^{-31}	3.45×10^{-18}

2.5 Discussion

The results presented here identify that qPCR of faecal samples from latrines is likely to be as sensitive or more so than live-testing to detect *M. bovis* in badger populations. This method therefore provides an alternative or compliment to immunoassays and culture of clinical samples, which are currently the only measures of *M. bovis* infection in badger populations that do not involve *post mortem* examinations and are themselves limited in performance.

When trapped badgers were tested by immunoassay, culture of clinical samples and qPCR of voided faeces there was low correspondence between test results within individuals as noted in other studies (Drewe *et al*. 2010). As Stat Pak positivity is associated with advanced disease and shedding, a greater positive correlation was expected between Stat Pak and qPCR on individual animals than between IFNγ and qPCR however, in this study the reverse was true. This may indicate that faecal shedding may not be restricted to the later stages of disease as is currently thought, and may also occur during early infection when cell mediated responses such as those detected by IFNγ occur. Culture of clinical samples did not produce any positives whereas qPCR identified 28% of all immunoassay positives on an individual badger level. However, in the field where qPCR was conducted on faeces from latrines, the ranking of social group shedding status inferred by qPCR strongly correlated with prevalence estimates based on immunoassays, thus demonstrating the correspondence between approaches. As qPCR quantifies the *M. bovis* genome equivalents it provides a relative measure of the level of shedding between social groups. Studies in other

hosts have found that those shedding the highest levels of pathogen are responsible for a high proportion of transmission events (Matthews and Low *et al.* 2006; Matthews and McKendrick *et al.* 2006). Whilst 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 compared with qPCR on faeces from latrines per season and across the year. QPCR also identified greater differences in the proportions of positive results between social groups than immunoassay testing. Notably, the prevalence of infection in West and Old Oak social groups was similar as estimated by immunoassay tests, but the two groups differed widely with regard to the results of faecal qPCR, with West being highly positive and Old Oak having the fewest positive faecal samples.

The higher odds of identifying a positive faecal sample from a latrine in summer suggests this this would be the optimal time to sample badger populations. This approach maximised the number of positive samples with the lowest possible sampling intensity. As the number of faecal samples collected did not differ between season, the greater odds of detecting positive samples in summer is due to an increase in positive samples rather than a greater abundance of faecal samples during this period. The optimal faecal sampling regime would involve collecting ten fresh samples per day on two non-consecutive days in early summer, which would detect the top 83% of shedding social groups, including

those excreting the most *M. bovis* genome equivalents into the environment. This sampling regime provided qPCR test data indicating relatively high sensitivity and specificity compared with live testing and also had greater odds of finding positives compared with all other seasons. Some social groups required more than 20 samples to be collected over a year to detect faecal shedding. If the motivation for sampling is to identify groups with the highest proportion of positive samples, then sampling in early summer only may be adequate. However, in order to identify all positive social groups (as determined by qPCR) in this study two separate sampling sessions would be required, once in yearly summer and once in late summer, and would be the most cost effective method of 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 because a wider window of sampling is needed to obtain faecal samples from a large proportion of animals resident in each social group.

Whilst the false positive rate of this qPCR assay is low, when large numbers of samples are tested the probability of obtaining false positives increases. When 20 samples are tested the probability of not obtaining any false positives is 56%. As false positives arise from laboratory contamination, it is possible to reduce the probability of obtaining a false positive when testing large numbers of samples by introducing known negative samples during the DNA extraction stage. If known negative samples are found to be positive by qPCR, all positive samples extracted concurrently with the known negative should be re-extracted and re-tested to confirm their positivity. Alternatively, all social groups found to be

positive could be re-extracted and re-tested to confirm they are true positives and reduce the rate of false positives.

In addition to being equally or more sensitive than live-trapping diagnostics, qPCR on latrine samples benefits from being non-invasive and less logistically challenging than live trapping and testing. Our study has identified the potential value of the qPCR of faecal 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 where the cost of the latter may be prohibitive for monitoring disease risks over relatively large areas. For example, qPCR of latrine faeces could be applied at the edges of the current TB endemic areas in the UK or throughout high-risk areas, in order to provide spatial information on relative levels of environmental contamination which may help monitor spread and target management. Although our study focuses on badgers, the same approach to the non-invasive sampling has the potential to be applied to other pathogens or in other wildlife and disease systems, particularly for elusive host species or where capture and live testing is challenging.

3. The variability and seasonality of the environmental reservoir of *M. bovis* shed by wild European badgers

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Expanded from: *Scientific Reports* **5**, Article number: 12318 (2015) (Appendix B)

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

3.1 Abstract

The incidence of *Mycobacterium bovis*, the causative agent of bovine tuberculosis, has been increasing in UK cattle herds resulting in substantial economic losses. The European badger (*Meles meles*) is implicated as a wildlife reservoir of infection. One likely route of transmission to cattle is through exposure to infected badger urine and faeces. The relative importance of the environment in transmission remains unknown, in part due to the lack of information on the distribution and magnitude of environmental reservoirs. Here we identify potential infection hotspots in the badger population and quantify the heterogeneity in bacterial load; with infected badgers shedding between 1×10^3 – 4×10^5 *M. bovis* cells g^{-1} of faeces, creating a substantial and seasonally variable environmental reservoir. Our findings highlight the potential importance of monitoring environmental reservoirs of *M. bovis* which may constitute a component of disease spread that is currently overlooked and yet may be responsible for a proportion of transmission amongst badgers and onwards to cattle.

3.1 Introduction

The incidence of *Mycobacterium bovis* in cattle herds in Great Britain (GB) has increased from 0.01% in 1979 (Garnett *et al.* 2003) to 4.8% in 2012 (Defra, 2014). Control and compensation has cost the taxpayer £500 million over the past decade and this is predicted to increase to £1 billion over the next 10 years if further geographical spread is observed (Defra, 2014); making bovine tuberculosis one of the most economically important animal health problems in the UK farming industry (Reynolds 2006).

The European badger is a wildlife reservoir involved in the transmission of *M. bovis* to cattle in the UK and RoI (Donnelly et al. 2003; Aznar et al. 2011). Once infected, badgers may intermittently shed *M. bovis* cells in sputum, faeces and urine (Clifton Hadley *et al.* 1993). One likely route of transmission to cattle is through exposure to infected badger urine and faeces. Although the location and extent of environmental *M. bovis* has not been previously quantified, indirect contact with infected faeces and urine may be an important exposure pathway (Tolhurst *et al.* 2009). *M. bovis* genomic DNA can survive outside the host for up to 21 months (Young *et al.* 2005) and cells have been shown to be viable by culture from mice fed soil in which *M. bovis* had been persisting for months (Ghodbane *et al.* 2014). The survival of shed *M. bovis* cells is likely to vary in space and time in relation to local environmental conditions and the distribution of infectious badgers. Understanding patterns in environmental contamination (defined as the presence of *M. bovis* genome equivalents in the environment) could aid in the design of more effective interventions, currently based on culling and vaccination strategies.

The availability of a method to quantify relative levels of environmental contamination with *M. bovis* could open up possibilities for monitoring spatial and temporal variation in risk and may help direct the implementation of disease control interventions. Currently the only means of measuring levels of infection in badger populations is through trapping and testing with BrockTB Stat Pak® (Stat Pak) (Chambers *et al.* 2008), Interferon gamma (IFNγ) (Dalley *et al.* 2008) and culture of clinical samples (Delahay et al. 2000). A qPCR method for non-invasive environmental monitoring of shedding was developed in our group (Travis *et al.* 2011; Pontiroli *et al.* 2011). This qPCR assay quantifies faecal shedding, a measure that correlates strongly with the level of infection within a social group as measured by immunoassay. The only other non-invasive method for monitoring shedding in badger populations is culture of faecal material, which is particularly insensitive (Figure 3.1). Using this optimised qPCR assay we are able to report on the spatio-temporal reservoir of *M. bovis* from badger faecal shedding in a natural population over the course of a year. Badgers defecate in latrines within or at the edges of their territories (Roper *et al.* 1993) and hence they can be used to identify a defined population of animals.

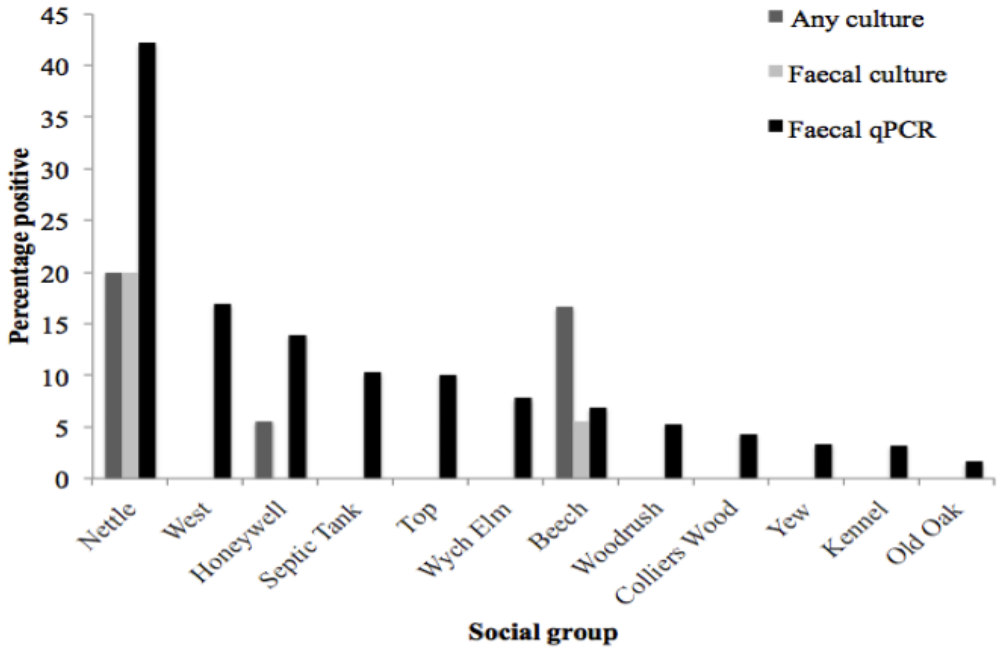

Figure 3.1 Percentage positive badgers per social group determined by any culture positive (tracheal or faecal) or faecal culture compared with positives by faecal qPCR. Data aggregated across the entire year.

3.2 Materials and Methods

3.21 Sampling and Trapping

Fresh faecal samples were obtained from latrines associated with 12 badger social groups (Table 3.1) in Woodchester Park Gloucestershire through 2012 and 2013. Two intensive sampling periods of two weeks each were undertaken during the period of peak badger latrine activity in the spring and autumn of 2012 where up to 10 unique faecal samples were obtained from latrines associated with each social group on alternate days. Faecal samples were taken from latrines in closest proximity to the main sett of each social group. A second sampling regime was undertaken over a year long period where up to 10 unique fresh, faecal samples were taken from latrines associated with each social group per day over two non-consecutive days in each season, starting two days after trapping operations took place in that location. For the purpose of this study March – May was classified as spring, June – August as summer, September – November as autumn and December – February as winter.

Each of the 12 badger social groups in the study was trapped four times throughout the year, once per season, with variable numbers of animals trapped between groups and seasons. Badgers were trapped using baited cage traps placed around the main setts of each social group and identified using a unique tattoo applied at the first capture of that animal. Trapped badgers from each of the 12 social groups were tested by BrockTB Stat-Pak®, IFNγ and culture of clinical samples. All experimental protocols were approved by the University of Warwick and the Food and Environment Research Agency Ethical Review

Committee and carried out in accordance with the approved guidelines and under the license granted by the Home Office under the 1986 Animal (Scientific Procedures) Act.

3.32 DNA Extraction and qPCR

Total community DNA was extracted from 0.1 g (+/- 0.003 g) of faeces 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 which 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 machine (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. PCR reactions were set up using 900 nM of each primer (RD4F $^{5'}$TGTGAATTCATACAAGCCGTAGTCG$^{3'}$, RD4R $^{5'}$CCCGTAGCGTTACTGAGAAATTGC$^{3'}$), 250 nM of Taqman probe (6FAM-AGCGCAACACTCTTGGAGTGGCCTAC—TMR), 1 mg ml^{-1} bovine serum albumen (BSA), 12.5 µl of Environmental Mastermix 2.0 (ABI), 10 µl of template and made up to 25 µl with molecular grade water (Sigma Aldrich). PCR cycling conditions were 50 °C for 2 min followed by 95 °C for 10 min then 40 cycles of 95 °C for 15 sec 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 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. An

inhibition control assay previously described (Pontiroli *et al.* 2011) was used to detect the possibility of false negative results due to inhibition. Where significant inhibition was detected DNA was re-extracted from frozen aliquots and qPCR assays were repeated. The number of *M. bovis* genome equivalents was quantified independently by qPCR at The University of Warwick and APHA Weybridge.

3.33 Data Analysis

All data analysis was performed using the language R. Logistic regression with social group (Old Oak) as the baseline was used to determine whether the number of positive samples varied amongst social groups throughout the year. Negative binomial GLMs were performed to determine differences in *M. bovis* cells numbers shed between groups and between seasons. For spring two sampling days per social group were chosen to represent cross sectional sampling. Variability within groups was determined by calculating the median, upper and lower quartiles and range for each social group. Two sided Kolmogorov-Smirnov tests were used to determine in the distribution in the distribution in the number of *M. bovis* genome equivalents shed per gram of faeces in positive samples between seasons and social groups. The map of *M. bovis* shedding was made using packages ggmap (Kahle & Wickham 2013) and ggplot2 (Wickham 2009).

3.4 Results

3.41 Infection levels within social groups

During the study, 53.6% of trapped badgers were *M. bovis* positive by Stat-Pak, IFNγ or culture. By qPCR faecal samples from every social group examined were found to be positive (Figure 3.2), with the odds of detecting a positive sample varying considerably between groups (Table 3.1). The odds of detecting positive sample from a social group was significantly higher for seven of the groups compared to the other five social groups.

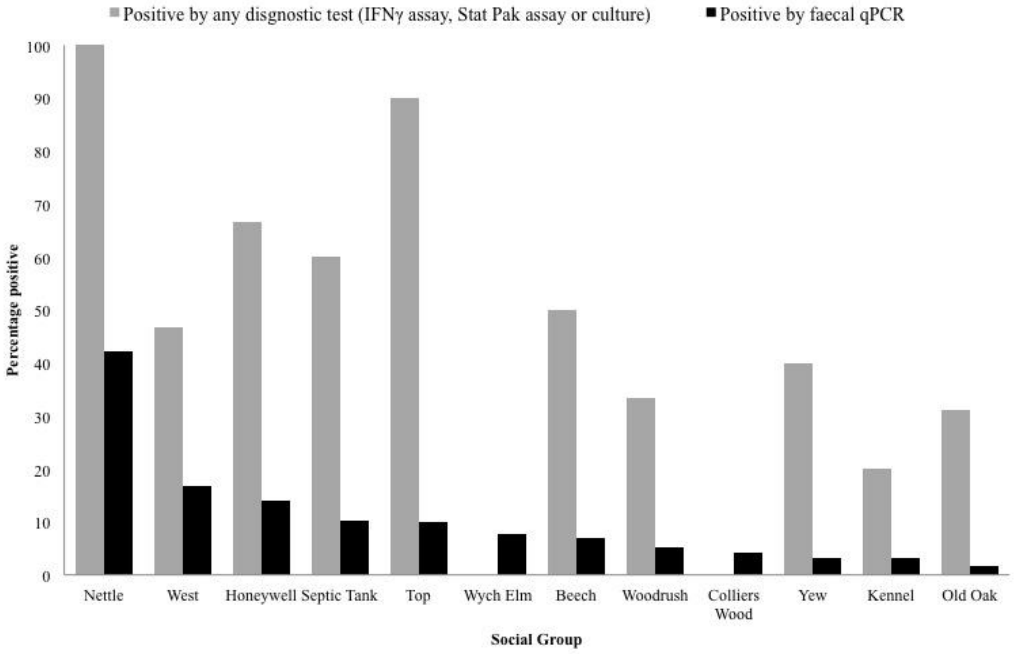

Figure 3.2 The percentage of badgers positive by any diagnostic test compared to the percentage of positive faecal samples per social group by qPCR (badgers n=128, faeces n= 1636).

Table 3.1 The relative odds ratio of obtaining a positive sample per social group across the year.

Social group	Odds of a positive	Confidence Interval	P Value
Old Oak	1	-	-
Yew	1.86	0.56 – 6.18	0.31
Kennel	2.27	0.59 – 8.73	0.23
Colliers Wood	2.32	0.70 – 7.73	0.17
Woodrush	2.42	0.74 – 7.92	0.14
Beech	3.25	1.06 – 9.95	0.04
Top	4.03	1.33 – 12.15	0.013
Wych Elm	4.61	1.47 – 14.43	8.00×10^{-3}
Honeywell	5.24	1.73 – 15.84	3.37×10^{-3}
Septic Tank	6.51	2.17 – 19.56	8.43×10^{-4}
West	7.03	2.40 – 20.57	3.67×10^{-4}
Nettle	22.73	7.81 – 66.19	1.01×10^{-8}

The percentage of infected faecal samples varied considerably (Table 3.2), as did the number of *M. bovis* genome equivalents per faecal sample which ranged from 1×10^3 to 1.48×10^6 per gram of faeces.

Table 3.3 Summary of *M. bovis* genome equivalents counts by social group from faecal field sampling and immunoassay testing results on trapped badgers (trapping n= 128, faeces n = 1636).

Social group	Percentage positive faeces by qPCR	Cumulative genome equivalents in faeces	Ranking by cumulative genome equivalents	Percentage positive IFNγ	Percentage positive Stat Pak	Percentage positive IFNγ or Stat Pak
Nettle	42.2	1.08×10^6	2	60	100	100
West	16.9	1.48×10^6	1	33.3	20	40
Honeywell	13.9	4.08×10^5	6	50	50	66.7
Septic Tank	10.3	4.57×10^5	5	40	20	60
Top	10.1	9.00×10^5	3	20	90	90
Wych Elm	7.8	4.19×10^4	10	0	0	0
Beech	6.9	4.98×10^5	4	41.2	29.4	44.4
Woodrush	5.3	1.45×10^5	8	0	33.3	33.3
Colliers Wood	4.3	8.83×10^4	9	0	0	0
Yew	3.3	2.25×10^4	12	0	40	40
Kennel	3.2	2.76×10^4	11	0	20	20
Old Oak	1.6	2.56×10^5	7	0	31.3	31.3

Significant variability in genome equivalents was identified both within and between social groups (Figures 3.3 and 3.4) with social groups Nettle, Top, Septic Tank, Beech and West shedding more genome equivalents over the year than the other social groups (Table 3.2). Social groups with a high percentage of positive samples consistently shed amongst the highest cumulative numbers of *M. bovis* genome copies during the year (Table 3.2). Social group Old Oak had the lowest percentage of positive samples in the study, but shed a greater number of genome equivalents than expected, ranking 7th in the cumulative genome equivalents shed across the year (Table 3.2). This distribution is consistent with the presence of a relatively small number of animals shedding large amounts of bacteria in some groups. However, as faecal samples could not be assigned to individuals, within-individual variation in shedding cannot be discounted as being responsible for this observation. Hence the need for further research into heterogeneity in transmission risks amongst individual badgers.

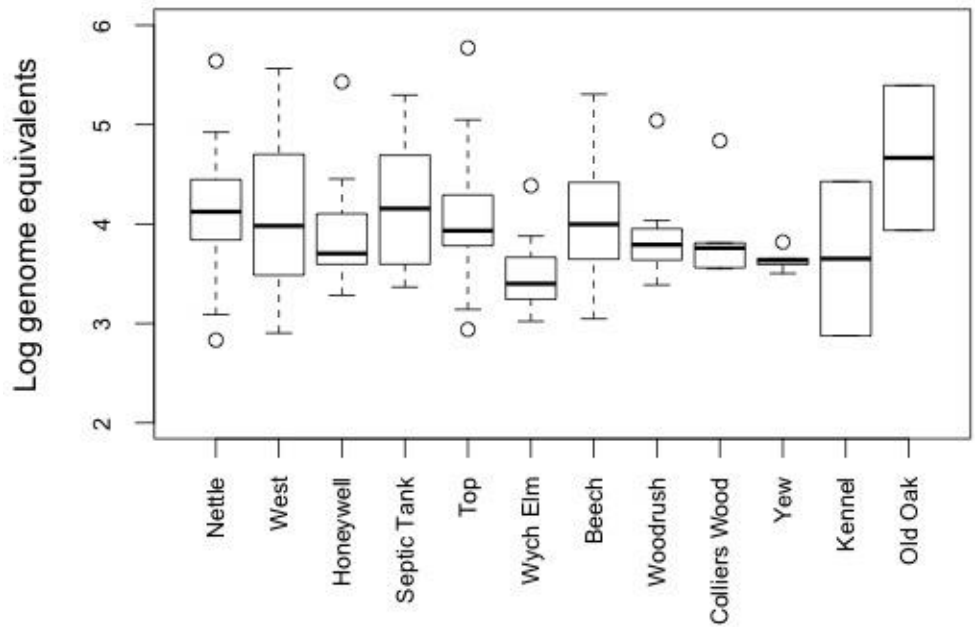

Figure 3.3 Distribution of *M. bovis* genome equivalents per positive sample by social group.

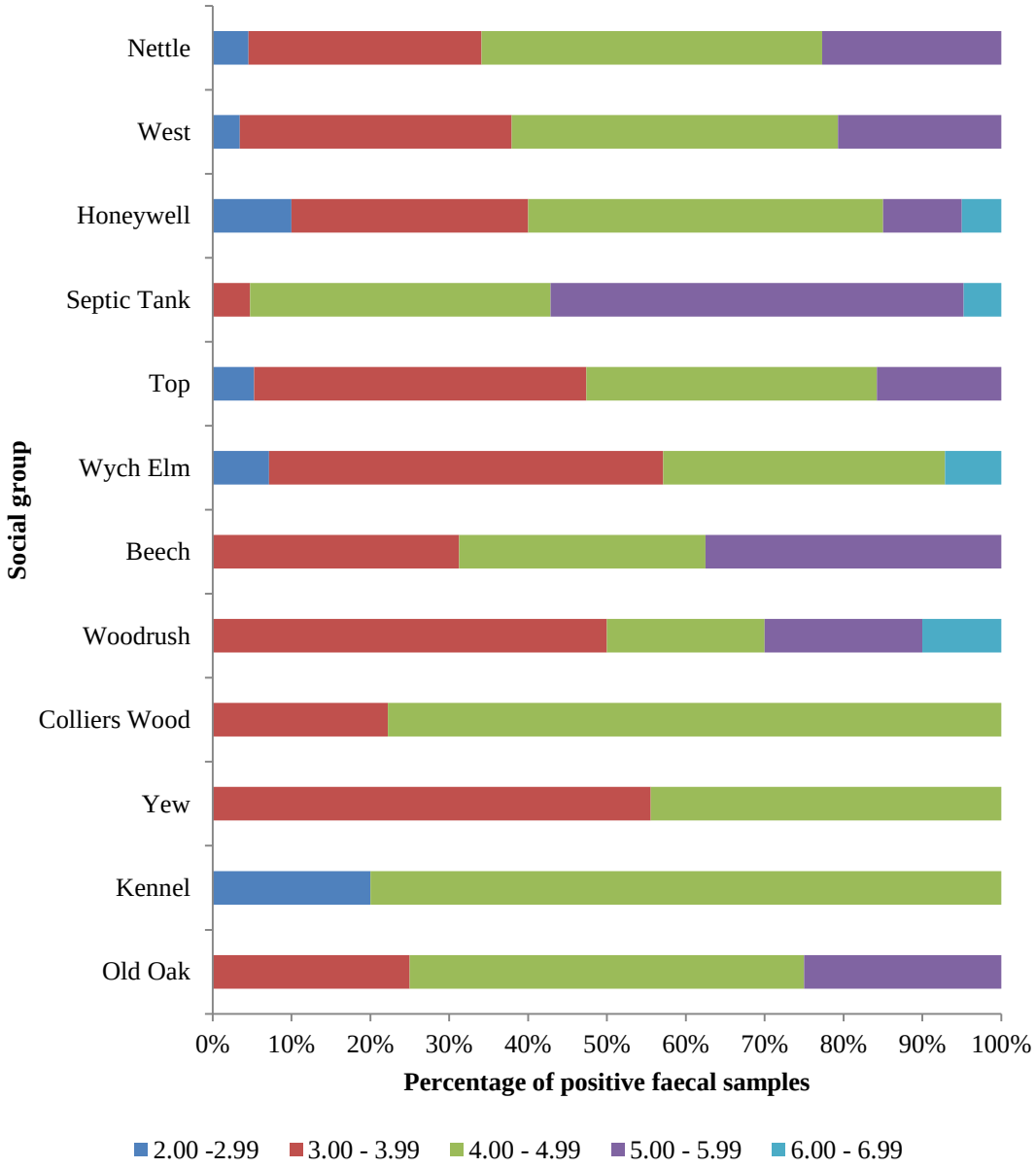

Figure 3.4 The distribution of *M. bovis* genome equivalents (log_{10} +1) per social group across the year expressed as the percentage of positive samples in each range of genome equivalents.

Across the year social groups varied in their distribution of the number of *M. bovis* genome equivalents shed per gram of faeces. Although West and Nettle shed the most cumulative cells across the year (Table 2.3) they, neither contained any samples shedding the highest number of *M. bovis* genome equivalents (Figure 3.4). Septic Tank had proportionally greater number of samples shedding large numbers of *M. bovis* genome equivalents. Whereas, other groups, such as Honeywell shed the full range, with the majority of sampling containing 3 – 4.99

log *M. bovis* genome equivalents per gram with a small number of faeces containing high (5+ log genome equivalents) and low (under log 3 genome equivalents) per gram.

3.42 Seasonal variability in *M. bovis* shedding

Overall the majority of positive faeces contained between 1 x 10^3 – 1 x 10^5 genome equivalents per gram of faeces with a small proportion of samples containing over 1 x 10^5 *M. bovis* genome copies per gram (Figure 3.5). Although autumn had a greater proportion of faecal samples containing over 5 $\log_{10}$ genome equivalents than any other season, the greater number of positive faeces in summer resulted in a significantly greater number of *M. bovis* genome equivalents were shed in summer than in any other season. Using Kolmogorov-Smirnov tests, the distribution in the number of *M. bovis* genome equivalents per gram of faeces differed in spring compared to all other seasons (summer $D= 0.36$, $p<0.01$, autumn $D= 0.57$, $p<0.01$, winter $D= 0.48$, $p<0.05$). The distribution of in the number of genome equivalents shed in summer differed from the distribution in autumn, ($D=0.39$, $p<0.01$) but not winter ($D= 0.33$, $p>0.05$) and the distributions did not differ between autumn and winter ($D= 0.25$, $p>0.05$).

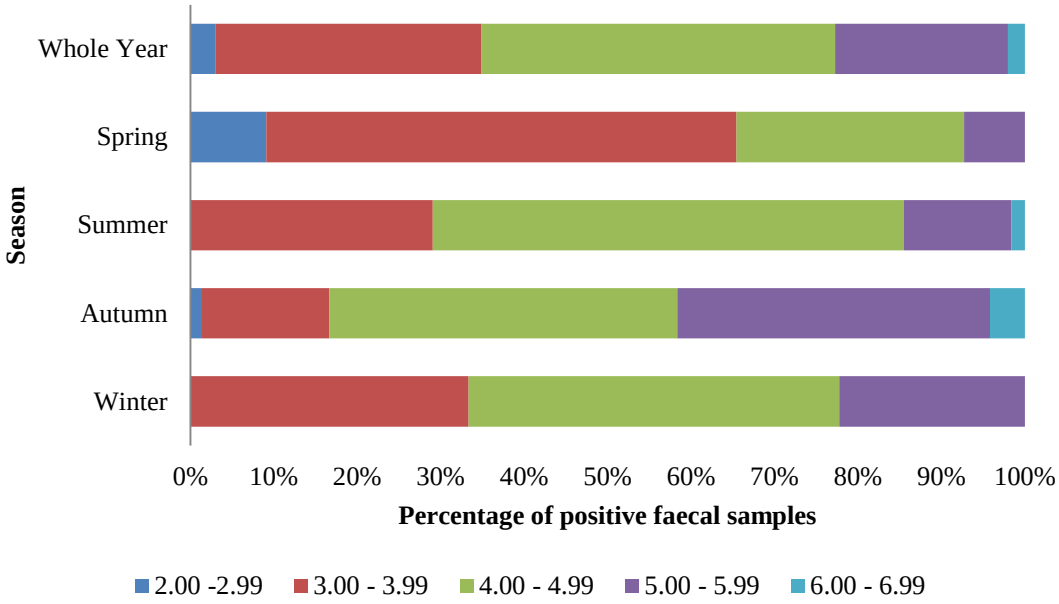

Figure 3.5 The distribution of *M. bovis* genome equivalents (log_{10} +1) per season across expressed as the percentage of positive samples in each range of genome equivalents.

3.43 Seasonal variability in shedding between social groups

There were substantial seasonal differences in the cumulative number of *M. bovis* equivalents detected per social group (Figure 3.6) with different groups identified as the largest contributors to the environmental pool of *M. bovis* throughout the year.

Although summer had the highest number of genome equivalents overall, Septic Tank shed fewer cells in summer compared to other seasons and Top and shed more cells in spring. Nettle also shed fewer *M. bovis* genome equivalents in spring compared with the rest of the year. However, five social groups (Nettle, West, Honeywell, Septic Tank, and Top) were identified as having consistently high proportions of positive faeces and relatively large quantities of *M. bovis*

bacilli shed (Table 3.2). This corresponds to immunoassay tests carried out on trapped badgers, which also identified these five groups as the most heavily infected (Table 3.2). Although there is strong correspondence between immunoassay and qPCR results there are some discrepancies, in particular Nettle and Top are 100% and 90% positive by immunoassay yet there was a large difference in the percentage of positive faecal samples with 42.2% and 10.0% respectively.

Figure 3.6 The cumulative number of *M. bovis* genome equivalents shed per social group each season. The scales for all graphs are identical.

3.5 Discussion

Detection of *M. bovis* by qPCR allows the presence of faecal shedding and hence infectious badgers to be established non-invasively and raises the possibility of identifying infectious social groups. Unlike standard diagnostic tests the qPCR approach also quantifies levels of *M. bovis* shedding, providing opportunities to assess spatio-temporal variations in the environmental distribution of this potential source of infection for cattle, badgers and other wild mammals. Environmental transmission is likely to be a complex mixture of a number of factors including the infectious load of *M. bovis* in faeces and urine and changes in these reservoirs over time, proximity to cattle pasture, the frequency and type of contact cattle have with badger excrement and the age of faecal samples. The application of qPCR to further understand the epidemiology and transmission dynamics of bovine tuberculosis may be an important component in managing the advancing frontier between endemic and non-endemic cattle infection, and to inform transmission models (e.g. Brooks-Pollock *et al* (2014)).

The heterogeneities observed in this study between social groups and the consistency with which five groups were identified as highly infected and shedding, suggesting that interventions targeted at particular high risk populations could have a larger impact than random and blanket control strategies (Lloyd-Smith *et al.* 2005). However, any perturbation of badger populations could result in increased rather than decreased transmission (Tuyttens et al. 2000; Rosie Woodroffe et al. 2005). The difference in the distribution of *M. bovis* genome equivalents shed across seasons and the

observed discrepancies in the percentage of positive faecal samples for social groups with similar prevalences of infection by immunoassay highlights the need for further work to establish the causes of these differences and determine how they may relate to changes in potential transmission risk. Whilst heterogeneity in transmission is a well-known phenomenon, this study is one of the few empirical studies which have attempted to demonstrate the extent of this variability (Matthews *et al.* 2006). Although this study does not assess the viability of *M. bovis* in faeces, previous work has identified the presence of *M. bovis* 16S rRNA in soil (Young *et al.* 2005) and badger setts and latrines (Courtenay *et al.* 2006). In addition, studies have had a culture success rate of 2.5% from badger faecal samples (Wilesmith 1986) and *M. bovis* has been cultured from cattle faeces several months after excretion (Courtenay & Wellington 2008). This indicates that at least a proportion of *M. bovis* cells shed in badger faeces can remain viable in the environment; however, further research is required to determine potential survival and transmissibility of *M. bovis* in environmental samples.

Whilst the focus in the UK and RoI is on badgers, other wildlife hosts are present (Delahay, De Leeuw, et al. 2002; Mathews et al. 2006); however, little is currently known of their contribution to environmental reservoirs and their relative importance for transmission to cattle (Delahay and De Leeuw, et al. 2002). Issues controlling *M. bovis* are not confined to the UK and RoI. Worldwide there are problems with *M. bovis* in buffalo and lions in South Africa (Renwick *et al.* 2007), brushtail possums in New Zealand (Coleman & Cooker 2001), white tailed deer in America (Miller & Sweeney 2013) and wild boar in Spain (Aranaz *et al.* 2004). This non-invasive qPCR assay can be employed to

detect shedding in other systems and samples types including milk, water and clinical tissues, is possible using this method. Whilst controlling and monitoring *M. bovis* in wildlife populations remains a challenge, non-invasive monitoring of environmental contamination may open up opportunities to identify spatio-temporal heterogeneity in disease risks and hence contribute to the development of suitable approaches fro disease control in livestock.

4. The effect of oral BCG vaccination in the field on environmental shedding of *M. bovis* in badger faeces

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H.C.K processed samples, conducted statistical analysis and produced figures and tables. D.P processed samples. D.M collected samples and was involved in conducting the trial. D.N created and maintained the database of vaccination and was involved in conducting the trial. L.C and E.G devised, obtained funding for and supervised the running of the trial. E.M.W obtained funding for the laboratory work with O.C and supervised sample processing. O.C advised on statistical methods and writing of the chapter as well as obtaining funding for the work with E.M.W.

4.1 Abstract

In the UK and RoI the European badger (*Meles meles*), a wildlife reservoir of *Mycobacterium bovis*, is implicated in the transmission of *M. bovis* to cattle. Vaccinating badgers with BCG intramuscularly has proven protective in laboratory studies, reducing the number and severity of tuberculous lesions. Intramuscular vaccination is currently licenced for use in the UK and field trials have demonstrated its use is protective and confers herd immunity. However, intramuscular vaccination requires capture, making it prohibitively expensive for a widespread control strategy. Placing vaccines in oral baits for environmental uptake may be an efficacious and more economically viable method of disease control. Although oral vaccination reduces the severity of lesions in laboratory trial, field trials had not previously taken place. Here we present the effect of oral vaccination in the field on the levels of environmental shedding of *M. bovis*, and demonstrate that while there is a trend of decreasing prevalence with BCG vaccination, overall there was no statistically significant effect of vaccination on the pool of environmental *M. bovis*.

4.2 Introduction

The incidence of *Mycobacterium bovis* in cattle herds in the UK has been increasing for over 30 years (Defra 2014a), resulting in significant economic losses (Defra 2013). Test and slaughter programmes have been sufficient to control *M. bovis* in cattle herds in the majority of developed countries, however this has not been sufficient in several countries including the UK and RoI. In the UK and RoI the European badger (*Meles meles*) is implicated as a wildlife reservoir, responsible for transmission of a proportion of *M. bovis* to cattle (Donnelly et al. 2003; Griffin et al. 2005). In the UK, badgers are estimated to be responsible for up to up to 52% of herd-level cases in some areas, with the majority of these cases due to cattle to cattle onward transmission of introduced cases from badgers, which are estimated to account for 5.7% of herd-level infections (Donnelly & Nouvellet 2013). In addition, a recent model attributed 34% of infections in cattle to the environment (defined as a combination of transmission directly from badgers and any environmental transmission) (Brooks-Pollock *et al.* 2014).

The prevalence of *M. bovis* infection in badger populations is high in many regions, with up to 38% of badgers infected (Jenkins *et al.* 2007; Murphy *et al.* 2010). The main mode of transmission between badgers and cattle is currently unknown (Krebs et al. 1997) however; direct contact between badgers and cattle is thought to be infrequent (Benham & Broom 1989, Drewe et al. 2013) and therefore unlikely to be responsible for the majority of transmission. Indirect contact with infected badger faeces, urine and sputum on pasture and in farm buildings is another potential route (Tolhurst *et al.* 2009; Hutchings & Harris

1999, Drewe et al. 2013) as once infected, badgers may intermittently shed large quantities *M. bovis* in their faeces, urine and sputum (Clifton Hadley *et al.* 1993), creating an environmental source of potentially infectious cells.

Due to the high disease incidence in cattle herds in the UK and RoI additional control interventions are required. As vaccination of cattle in the field is prohibited by European Union (EU) legislation (Council Directive 78/52/EEC), control also needs to be directed at badger populations. Control measures targeted at badger populations are based around farm husbandry, badger culling and some use of intramuscular vaccination (Defra 2014a). Badger culling trials in the UK and RoI have had mixed results, with several studies in Ireland finding a decrease in TB incidence in cattle with culling (Griffin et al. 2005; Olea-Popelka et al. 2009), whereas the Randomised badger culling trials (RBCT) in the UK found an overall increase in incidence in cattle (Donnelly *et al*. 2003) likely due to increased badger movement (Pope *et al.* 2007; Woodroffe *et al.* 2006).

As badgers are a protected species and there is substantial public opposition to culling in the UK, culling is only viable as a short to medium term control strategy (Robinson et al. 2012). Vaccinating badgers with Bacille Clamette-Guerin (BCG) is an alternative option for control. BCG vaccines are licensed for use in humans and there have been few reports of adverse effects (Robinson et al. 2012). In addition, BCG is efficacious with a single dose in many species (Robinson et al. 2012), which is essential for a vaccine that is to be used for wildlife, where administering repeated doses would be highly challenging. Furthermore, BCG has been demonstrated to be protective in other animals, such

as brushtail possums (Corner et al. 2001; Aldwell et al. 1995), wild boar (Beltrán-Beck et al. 2014; Ballesteros et al. 2009) and white tailed deer (Nol et al. 2008) as well as in mice (Vipond et al. 2008), guinea pigs (Aldwell et al. 2003) and cattle (Buddle et al. 2003). In laboratory studies brushtail possums are protected by BCG when administered by subcutaneous injection, intranasal and by intratrachael instillation, and in the field oral administration had an efficacy of 95-96% (Corner et al. 2001; Aldwell et al. 1995). In Spain, oral delivery of BCG using baits is efficacious in wild boar (Beltrán-Beck et al. 2014; Gortazar et al. 2014; Gortazar et al. 2011).

In laboratory studies, intramuscular and oral administration of BCG to badgers reduces the number and severity of lesions, the number of sites infected and the bacterial load in the lungs (Chambers et al. 2011; Corner et al. 2008; Lesellier et al. 2011; Murphy et al. 2014; Corner et al. 2010). During infection experiments BCG is administered and 12 -17 weeks later the animals are challenged with *M. bovis*, approximately 12 weeks after challenge animals are euthanized and detailed most mortems carried out and compared with animals that were challenged with *M. bovis* but did not receive BCG vaccination. When the standard human dose of 2-8 x 10^5 CFU, and a dose ten times higher is administered to badger, the animals receiving the higher dose had fewer and less widespread lesions compared to those given the lower dose, although both doses were protective compared to no vaccination (Lesellier et al. 2011). In addition, no differences in protection have been found between the vaccine strains Pasteur and Danish (Murphy et al. 2014). In no laboratory study has BCG prevented infection, this is thought to be due to the large doses (approximately 10^4 CFU)

used for challenge by the endobronchial route (Robinson et al. 2012), which is likely to be much greater than those experienced during natural exposure (Lesellier et al. 2011). When badgers were given two doses of BCG, intramuscularly and subcutaneously, only mild reactions were detected at the site of injection (Lesellier et al. 2006). This suggests that multiple vaccinations are safe in badgers, an important consideration for vaccinating wildlife as some animals may be vaccinated multiple times when vaccines are delivered in oral baits.

In field trials intramuscular vaccination has been demonstrated to be protective in a four year field study, where herd immunity was conferred to cubs (Carter et al. 2012). In setts where at least a third of adults had been vaccinated cubs were 79% less likely to become infected than cubs in setts where fewer than a third of adults were vaccinated, as measured by the culture of clinical samples and Stat-Pak assay (Carter *et al.* 2012). The duration of protection following vaccination is unknown in badgers however; brushtail possums retain protection twelve months after vaccination (Corner et al. 2001; Aldwell et al. 1995). It is not known whether re-vaccination is beneficial in badgers however, studies in red deer and wild have found increased protection with revaccination (Griffin et al. 2006) whereas in calves it has reduced protection (Buddle et al. 2003) and in wild boar increased protection (Gortazar et al. 2014). Although eradication of *M. bovis* in badgers may not be required to substantially reduce transmission from badgers to cattle, simulation modelling suggests that in England a minimum of 40-50% of the healthy badger population needs to be vaccinated annually to eradicate *M. bovis* from badger populations (Wilkinson et al. 2004). With over

30% of animals infected in some locations, and assuming there is no difference in the probability of trapping with disease status, to vaccinate 50% of the healthy population in these high prevalence areas 80% of the population would need trapped and vaccinated.

Intramuscular delivery of BCG vaccine is licenced and currently used in areas of the UK. Although it offers the potential of a less contentious and potentially efficacious method of control, it is currently expensive, with the cost estimated as £2000 – 4000 per km^2 (Chambers *et al.* 2014). In addition, the proportion of badgers that receive the vaccine and their age distribution is limited by the proportion of badgers than can be trapped, which varies from 35 – 85% of animals (Smith & Cheeseman 2007). Currently, there is no available data on the efficacy of oral vaccination in the field however, oral vaccination has the potential to reduce the cost of vaccination and increase vaccine coverage by removing the need to capture badgers by placing the vaccine in food baits in and around badger setts.

This chapter describes the effect of oral BCG vaccination in the field on faecal shedding of *M. bovis* by badgers. The trial was implemented in County Kilkenny, RoI, by collaborators at University College Dublin (UCD) between September 2009 and January 2014. The overall aim of the trial was to determine whether oral vaccination in the field reduces the level of infection in individual badgers and across the population, as determined by Stat-Pak assay and detailed post mortem examination (Aznar et al. 2011). As this was the first field trial of oral vaccination, badgers were captured and vaccine administered rather than bait

being up taken from the environment, so that badgers could be tagged and followed throughout the trial with their vaccination status known. Faecal samples were collected from latrines associated with setts involved in the trial from seventeen months after vaccination commenced and the number of *M. bovis* genome equivalents present were quantified by qPCR. The aim of the work presented here was to determine whether oral BCG vaccination reduces *M. bovis* shedding into the environment. Although indirect contact with badger excreta is a potential mode of disease transmission from badger to cattle, the effect of vaccination on environmental shedding of *M. bovis* had not previously been assessed. Faecal shedding was examined as contact with infected faeces may be a method of disease transmission and also because faecal shedding is strongly correlated with tracheal shedding (Travis et al. In preparation), a measuring of actively infectious individuals.

4.3 Materials and Methods

4.31 Vaccine trial design

The trial was carried out in Country Kilkenny, the RoI. The area is 755 km^2 and was divided into three zones with approximately equal numbers of badger setts, cattle farms, cattle and with similar land classification (Aznar *et al.* 2011). A gradient of vaccination levels was created from 0% - 100% vaccination from north to south. In the most northern zone (A) 0% of badgers were given BCG vaccine, and all captured badgers were given a placebo vaccine, in the middle zone (B) 50% of captured badgers were vaccinated and 50% were given a placebo vaccination and in the southern zone (C) 100% of captured animals were given BCG vaccine. The treatments from zones A and C were allocated at random and remained blinded until the completion of post mortem examinations and laboratory work. In zone B vaccination was randomised at the badger level so that 50% of badgers were vaccinated with BCG. This resulted in setts receiving between 0 -100% BCG vaccination depending on the number of badgers trapped and the allocation of treatment.

At the beginning of the trial zone A had a prevalence of 15%, zone B of 6% and zone C of 5% as measured by Brock TB Stat Pak, which was estimated to have a sensitivity of 50% in this study (Deirdre Ni Bhuachalla, Personal communication). Stat Pak is a lateral flow serum antibody test (Chambers et al. 2009) with a recorded range of sensitivities in badgers between 50 – 78% (Chambers et al. 2009). Starting prevalences were calculated for all setts

involved in the trial, only a subset of which had faecal samples collected from their sett related latrines for this study.

Badgers were captured each year of the trial using cage traps and stop restraints. BCG and placebo vaccines were administered orally using a syringe. BCG vaccines contained live *M. bovis* BCG Danish strain at 1 X 10^8 cells per ml in a lipid formulation and placebo vaccines contained lipid formulation alone. Vaccination took place in 6 sweeps, each lasting 21 weeks (Table 4.2).

At first capture each animal involved in the trial was given a unique identifier and the sex, weight, age, Stat Pak result and location of capture were recorded. If a badger was re-captured more than one year from its previous capture it was given the same treatment as previously administered. If the animal had received any vaccine treatment in the last year it was released without further treatment.

4.32 Faecal sample collection and storage

Faeces were collected from latrines associated with badger setts where placebo and BCG vaccination was taking place. Samples were collected in three rounds during sweeps 4, 5 and 6 by collaborators at University College Dublin. The sett ID, zone, date of collection and sample number were recorded for each faecal sample. Samples were stored at 4^{o}C before being transported to the University of Warwick where each sample was split into 10 aliquots, barcoded and stored at -20^{o}C until DNA extraction.

4.33 DNA extraction and *M. bovis* quantification

Total community DNA was extracted from 0.1 g (+/- 0.003 g) of faeces 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 which 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 machine (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. PCR reactions were set up using 900 nM of each primer RD4F $^{5'}$TGTGAATTCATACAAGCCGTAGTCG$^{3'}$, (RD4R $^{5'}$CCCGTAGCGTTACTGAGAAATTGC$^{3'}$), 250 nM of Taqman probe (6FAM-AGCGCAACACTCTTGGAGTGGCCTAC—TMR), 1 mg ml^{-1} bovine serum albumen (BSA), 12.5 µl of Environmental Mastermix 2.0 (ABI), 10 µl of template and made up to 25 µl with molecular grade water (Sigma Aldrich). PCR cycling conditions were 50 °C for 2 min followed by 95 °C for 10 min then 40 cycles of 95 °C for 15 sec and 58 °C for 1 min. Samples exhibiting amplification in one or more technical replicates were taken on to full quantification using three technical replicate per sample under the same conditions. If one or more of the technical replicates of the quantification assay exhibited amplification 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 was used to detect the possibility of false negative results due to inhibition. Each extracted sample was screened as a singlet, if ΔCT was greater than 2.5 then the sample was rescreened in doublet.

If the average ΔCT was greater than 2.5 then the sample was re-extracted from frozen faecal aliquots, and if not then the sample was considered uninhibited. As the RD4 scar is present in both *M. bovis* and *M. bovis* BCG an RD1 assay was used for each positive to confirm that *M. bovis* was present rather than *M. bovis* BCG. Each faecal sample was extracted and quantified in duplicate. When both replicate faecal samples were positive the average of the two *M. bovis* genome equivalent counts was taken. If only one replicate extract was positive then the sample was deemed positive.

4.34 Statistical analysis of the level of *M. bovis* shedding

All analyses were carried out at the sett level by dividing setts into vaccination zones (A, B and C) and by splitting all setts into two groups, vaccinated and unvaccinated, based on whether the sett had received BCG vaccination or placebo vaccination. Setts that received placebo or no treatment only were deemed 'unvaccinated' and those that received at least one BCG vaccine before faeces were collected from the sett were deemed 'vaccinated.' This analysis approach was adopted because the original trial design aimed to vaccinate all badgers captured in zone C with BCG and half in zone B, however as faecal collection commenced before the trial was completed several setts in zones B and C had not yet received treatment and therefore some setts would have been misclassified. As vaccination was randomised at the badger level a proportion of setts in zone B had received both placebo and BCG vaccination. If BCG vaccination had been delivered to a social group before faeces were collected it was deemed to have been vaccinated, even if it had also received placebo vaccinations. Setts which had not received either placebo or BCG vaccine before

faecal samples were collected were categorised as unvaccinated for this analysis. For analysis by zone setts were kept in their allocated zone regardless of whether they had received any treatment before faecal samples were collected.

For analysis of the effect of time since vaccination on the infection status of a sett, those setts that had not received either BCG or placebo were allocated the median date of placebo vaccination in surrounding setts in the given sweep and for all analyses were placed in the unvaccinated group with setts that received placebo vaccines. The number of days since vaccination (BCG or placebo) was calculated per sett by subtracting the date of vaccination from the date of faecal collection, the minimum, maximum and median number of days since vaccination was then recorded. The vaccine coverage up until the point where faecal samples were collected was calculated for each sett by assuming that a single dose of vaccine delivered before faecal samples were collected gives a maximum of 365 days coverage, given that during the study badgers were re-vaccinated each year when recaptured. The total coverage for a sett was calculated for all administered vaccines including those delivered after faecal samples had been collected.

All statistical analysis was carried out using R (version 3.0.2) using packages MASS, AER and pscl. Metadata on the badgers captured from each sett was collated including: Stat-Pak positivity, weight, sex and age of animals at each sett. These were tested as possible explanatory variables in each analysis of the changes in prevalence with vaccine treatment. The correlations between metadata was calculated using Pearson's correlation coefficient r; and correlated

variables (r >0.3) were not included concurrently in the same analysis. The $\log_{10}$ (x+1) of the number of *M. bovis* genome equivalents per gram of faeces was used in all analyses to normalise the distribution of crude count data. Data on setts containing positive faeces were analysed using generalized linear models (GLMs) by three methods: as a binary outcome (*M. bovis* positive/negative sett) using logistic models, as the proportion of faecal samples positive per sett using Poisson distributions and as the mean number of $\log_{10}$ *M. bovis* genome equivalents per sett using negative binomial models to account for the significant over dispersion ($Z=7.88$, $p= 1.68 \times 10^{-15}$) in the data. Chi-squared goodness of fit analysis demonstrated that negative binomial distributions were appropriate for analysis of the effect of both treatment zone ($p= 0.99$) and vaccination treatment ($p= 0.99$).

The relationship between the infection status (0 /1) of a sett by faeceal qPCR, the proportion of positive faecal samples per sett and the mean *M. bovis* genome equivalents per sett and explanatory variables were determined using univariate and multivariate generalized linear models (GLMs). The explanatory variables considered were: treatment zone, vaccination treatment, vaccination sweep, sett ID, the number of faecal samples tested, the number of animals vaccinated, the proportion of females, the proportion of cubs, the minimum number of days since vaccination, the starting infection status of setts (measured by Stat Pak) and the mean animal weight per sett. To convert the output of logistic regression to odds ratios the exponential of the β values were taken. The 95% confidence intervals for the odds ratios were calculated by taking $e^{\beta-1.96*SE,}$ where SE is the standard error of β. For logistic models with treatment zone as an explanatory

variable zone A (0% BCG vaccine) was used as the baseline category to which the vaccine zones were compared, where vaccine treatment was an explanatory variable unvaccinated was used as the baseline. Two sided Kolmogorov-Smirnov tests were used to determine whether the distribution in the number of *M. bovis* genome equivalents varied between zones, sweeps and treatment zones or vaccination treatment.

4.4 Results

4.41 *M. bovis* infection levels in badgers by live-testing

Each captured badger was tested for *M. bovis* infection using Stat Pak. Any animal producing a positive Stat Pak result was deemed infected, and any sett with at least one positive animal was regarded as a positive sett. The prevalence of infected setts was highest in zone A (0% vaccination), both prior to faecal sample collection and by the end of the trial (Table 4.1). The numbers of badgers captured in this zone was consistently higher than in zones B or C (Table 4.1). Although zones B (50% BCG) and C (100% BCG) had the same proportion of infected setts at the start of the trial, by the end of vaccination zone B had a greater prevalence of infected setts than zone C, despite fewer badgers having been captured and tested.

Table 4.1 The number of *M. bovis* infected setts prior to the start of faecal collection and by the end of the time the trial was completed as measured by Stat Pak.

Zone	Total setts sampled	Total badgers tested		Sett level prevalence	
		Prior faecal collection	By trial completion	Prior faecal collection	By trial completion
A	68	195	422	25.0	47.1
B	58	96	202	12.1	29.3
C	58	120	309	12.1	22.4

4.42 The number of faecal samples collected

In total 359 faecal samples were collected from 213 setts. As analysis required controlling for a range of metadata, any faecal samples that were collected from

a sett where badgers had not been trapped were excluded from further analysis. After exclusion, 324 samples remained across 184 setts (Tables 4.2 and 4.3).

Table 4.2 Summary of faecal sample numbers over the whole trial, broken down by zone and by vaccination status.

	A	B	C	Vaccinated	Unvaccinated
Number of Samples	110	98	116	174	150
Number of Setts	68	58	58	81	103
Mean samples per sett	1.64	1.61	2.07	2.15	1.46
Median samples per sett (min, max)	1 (1,5)	1 (1,4)	2 (1,6)	2(1,6)	1(1,5)

The number of faecal samples collected per sett ranged from 1 – 6 (Table 4.2), with an overall average of 1.8 samples per sett. The distribution of samples between sweeps was uneven, with more samples collected in sweep 5 than either sweeps 4 or 6 (Table 4.3).

Table 4.3 Summary of the dates and number of samples collected per sweep.

Sweep	Sampling Dates	Faecal samples collected	Number of setts sampled (faeces)	Average samples per sett	Median samples per sett
1	1st September 2009 - 28th February 2010	**0**	-	-	-
2	1st March 2010 - 31st July 2010	**0**	-	-	-
3	1st September 2010 – 7th February 2011	**0**	-	-	-
4	8th February 2011 - 30th of June 2011	61	50	1.22	1 (1,2)
5	1st September 2011 - 31st January 2012	164	76	2.16	2 (1,6)
6	30th of January 2012 - 22nd of June 2012	99	58	1.71	1 (1,4)

4.43 BCG vaccine coverage

A sett was defined as vaccinated if at least one animal had been administered BCG vaccine before faecal samples were collected. Any sett that had received only placebo vaccination was deemed unvaccinated. The aim of vaccinating 50% of setts in zone B and 100% of setts in zone C was not met by the end of the study, with only 86% of setts (50 setts) in zone C receiving BCG vaccination (Table 4.4). As a result, all further analyses were carried out by breaking down setts into treatment zones (A, B and C) and also dividing setts into those that received BCG vaccine (vaccinated setts) and those that received placebo or no vaccine treatment (unvaccinated setts) (Table 4.5). Of the vaccinated setts, 5% (4 setts) were already known to contain at least one animal Stat-Pak positive.

Table 4.4 The percentage of setts vaccinated with BCG, by zone.

Zone	Total setts	Vaccination level - aim	Actual percentage of setts vaccinated by end of trial
A	68	0	0
B	58	50	53
C	58	100	86

Table 4.5 The overall number of setts vaccinated with BCG.

Vaccination status	Number of setts	Percentage of setts
Vaccinated	81	44
Unvaccinated	103	56

The unvaccinated setts in zone B were found mainly to the centre of the zone, with the majority of vaccinated setts on the borders of zones A and C. The unvaccinated setts in zone C were clustered towards the south west of the area (Figure 4.1).

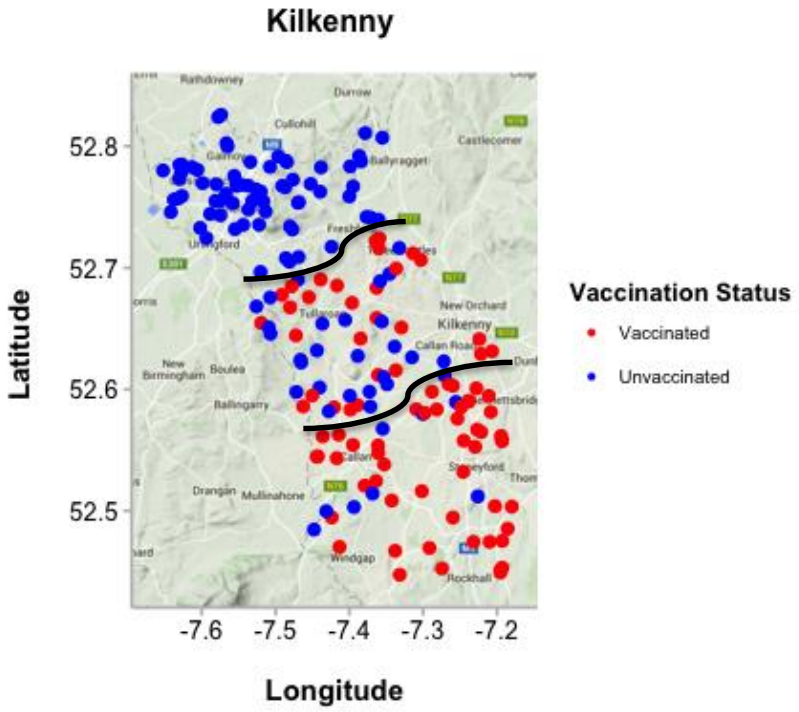

Figure 4.1 The distribution of BCG vaccinated and unvaccinated setts. Dividing lines represent approximate location of zones. Zones A-C run from North – South.

4.44 Summary of faecal sample positivity

Here prevalences have not been corrected, as the faecal samples are from latrines the badger of origin cannot be determined and multiple faeces from the same badger may have been analysed. In addition, the sensitivity of qPCR in this population is unknown and therefore prevalances should be taken as relative measures. Overall 24.5% of setts were positive across the study. however, the prevalence of *M. bovis* positive setts varied between sweeps within vaccination zones (Figure 4.2) and by vaccination status (Figure 4.3), with no clear pattern of changes over time in the proportion of positive setts. The unadjusted overall prevalence of positive setts varied between zones (Figure 4.3), with 14.8% of setts in zone B positive compared to 31.3% in zone A and 26.8% in zone C.

However, the unadjusted prevalence of positive setts varied only slightly between BCG vaccinated and unvaccinated setts, which had prevalences of 25.9% and 26.2% respectively.

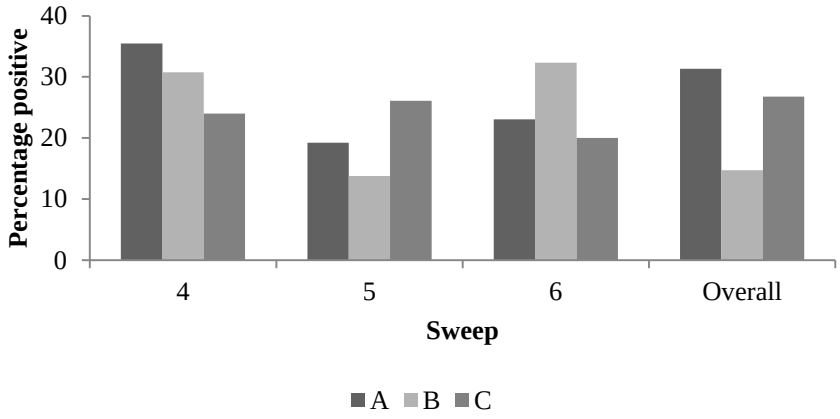

Figure 4.2 The percentage of positive setts per zone, by sweep.

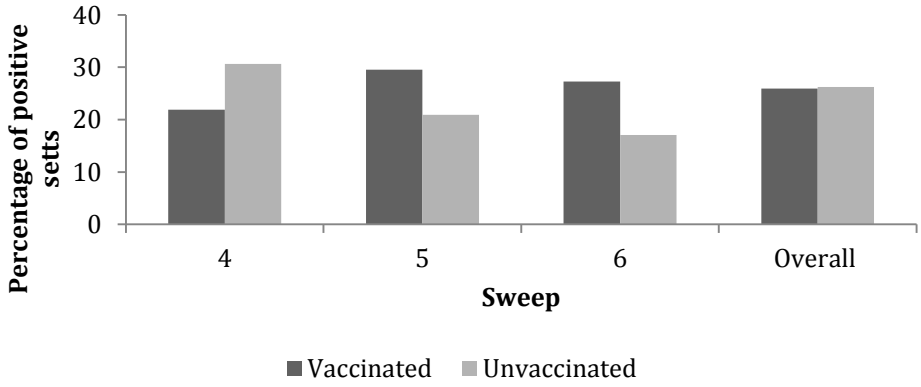

Figure 4.3 The percentage of positive setts per sweep by vaccination status.

M. bovis positive setts were distributed evenly throughout each zone, with the exception of a cluster of positive setts in the north-west of zone A (Figure 4.4). There was no clustering of infection on the borders of vaccination zones, which may have been indicative of animals from setts outside the trial area or from neighbouring zones, defecating in latrines of setts involved in the trial.

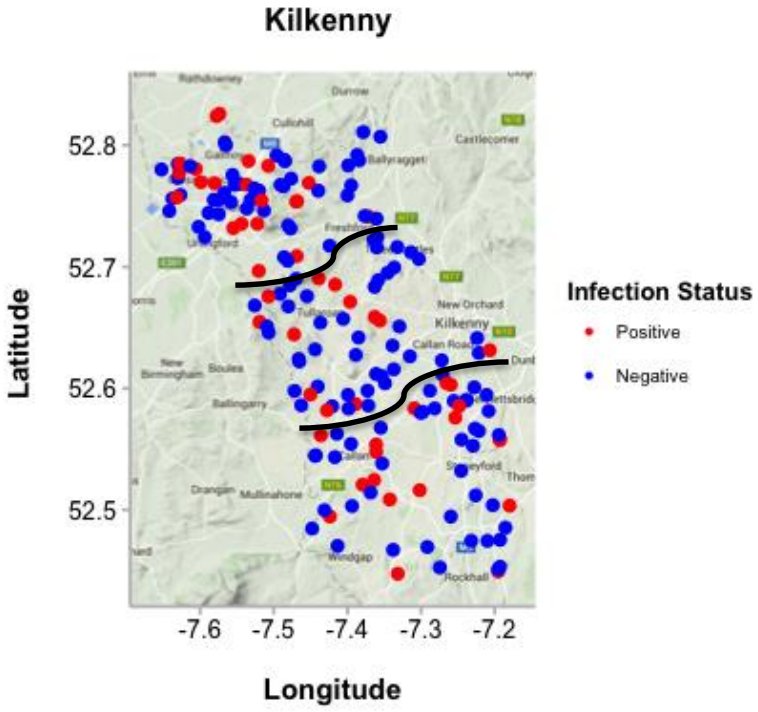

Figure 4.4 The distribution of *M. bovis* positive and negative setts after vaccination across the whole study area. Dividing lines represent approximate location of zones. Zones A-C run from North – South.

4.45 Summary of the proportion of positive faecal samples per sett

The proportion of faeces per sett that were positive for *M. bovis* ranged between 0 – 1 with the majority of setts being negative (Figure 4.5). Of the setts that were positive, the majority also had an equal or greater number of negative samples (Figure 4.5). The large number of setts where all or none of the faecal samples were positive is likely due to the large proportion of setts (41.2%) where only one faecal sample was collected over the course of the trial.

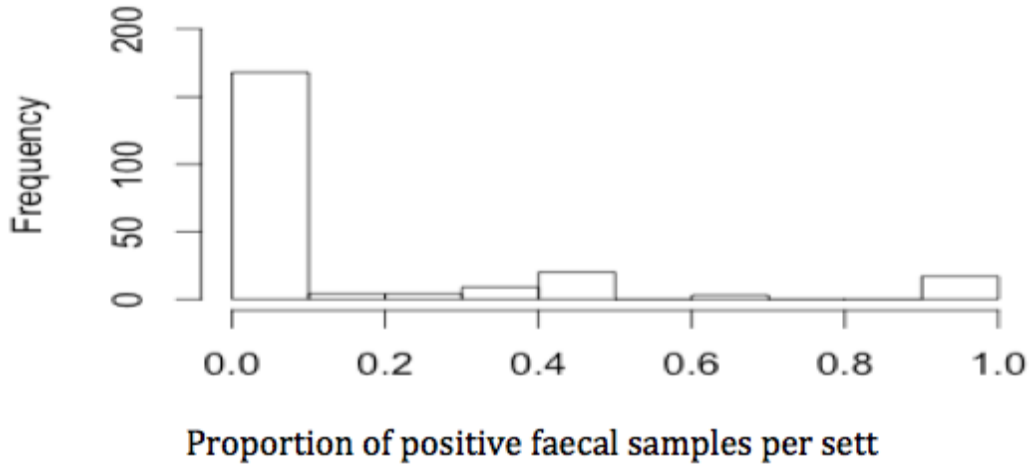

Figure 4.5 The proportion of faecal samples per sett that were positive for *M. bovis*, across the whole trial.

4.46 Summary of the average number *M. bovis* genome equivalents shed per sett

The mean number of *M. bovis* genome equivalents per positive sample per sett was calculated and used for all further analyses to normalise for the differences in sampling intensity between social groups. The average number of *M. bovis* genome equivalents per positive sett varied between 2.9 – 7.36 $\log_{10}$ per gram of faeces, with an average of 3.93 (Table 4.6). However, there was one outlier in zone C, a BCG vaccinated sett where a single sample contained 7.36 $\log_{10}$ genome equivalents per gram of faeces.

Table 4.6 Summary of *M. bovis* shedding in positive setts in log_{10} genome equivalents per gram of faeces, per sett.

	A	B	C	Vaccinated	Unvaccinated	Overall
Mean overall	0.94	0.91	1.06	0.94	0.94	0.98
Mean per positive sett	3.78	3.91	4.15	4.09	3.79	3.93
Minimum overall	0	0	0	0	0	0
Minimum per positive sett	2.90	3.13	3.03	3.03	2.90	2.9
Maximum	5.27	5.74	7.36	7.36	5.27	7.36

The distribution of the number of *M. bovis* genome equivalents in positive setts was right skewed, with the majority of setts containing between 10^3 – 10^4 genome equivalents and a small number of setts containing samples with high loads, up to 10^8 (Figure 4.6). When negative setts were excluded the mean number of genome equivalents shed did not vary between BCG vaccinated and unvaccinated setts ($t=1.67$, $p = 0.11$) or between the mean number of genome equivalents shed in positive samples between zones ($F_{53,2}= 3.17$, $p=0.39$). There was also no difference in the distribution of the number of *M. bovis* genome equivalents shed between vaccination zones (A:B – D=0.25, p=0.61, A:C – D=0.31, p = 0.28, B:C – D = 0.18, p = 0.96) or between BCG and placebo treatments (D= 0.29, p = 0.18).

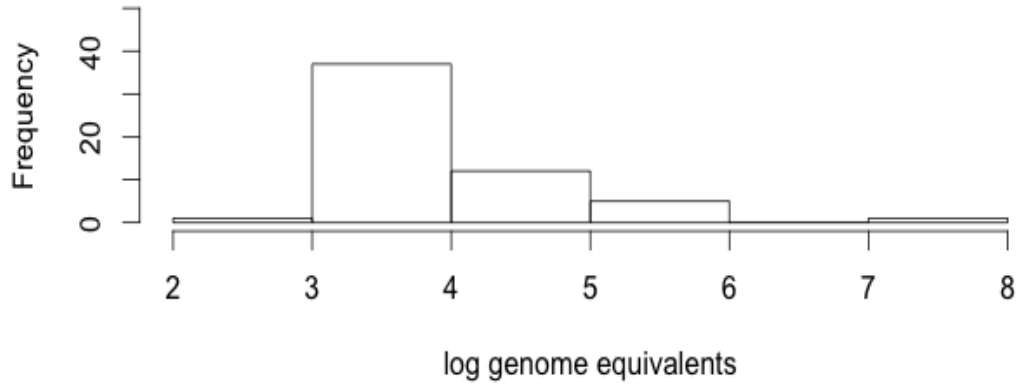

Figure 4.6 The distribution of the average number of *M. bovis* genome equivalents per positive sett.

4.47 The correlation between explanatory variables

From the metadata collected during badger capture, a range of potential explanatory variables were selected (Table 4.7). The correlation between explanatory variables was calculated and those that were significantly correlated were excluded from further analyses.

Table 4.7 Explanatory variables considered for analysis and their identifier in the correlation matrix.

Explanatory variable	Correlation matrix identifier
Number of faecal samples per sett	X1
Starting infection status per sett (Stat Pak)	X2
Total animals trapped	X3
Vaccination status	X4
Number of badgers vaccinated per sett	X5
Minimum time since vaccination on date faecal sample collection (days)	X6
Maximum time since vaccination on date faecal sample collection (days)	X7
Median time since vaccination on date faecal sample collection (days)	X8
Vaccine coverage of sett to faecal sampling date	X9
Total vaccine coverage of sett for the whole trial	X10
Proportion of males per sett	X11
Proportion of females per sett	X12
Proportion of cubs per sett	X13
Proportion of adults per sett	X14
Proportion of juveniles per sett	X15
proportion of old badgers per sett	X16
Mean badger weight	X17
Range in badger weight per sett	X18

Several variables were significantly correlated with one another (Figure 4.7) leaving: the number of faecal samples collected, the time since last vaccination, number of animals vaccinated, proportion of females per sett, proportion of cubs per sett, starting infection status per sett (Stat Pak) and the mean badger weight to be considered as explanatory variables in multivariate models. In addition, treatment zone /vaccination status, sweep and sett ID were included in all models. Correlated variables were swapped with each other in multivariate models and no difference was made to the outcome.

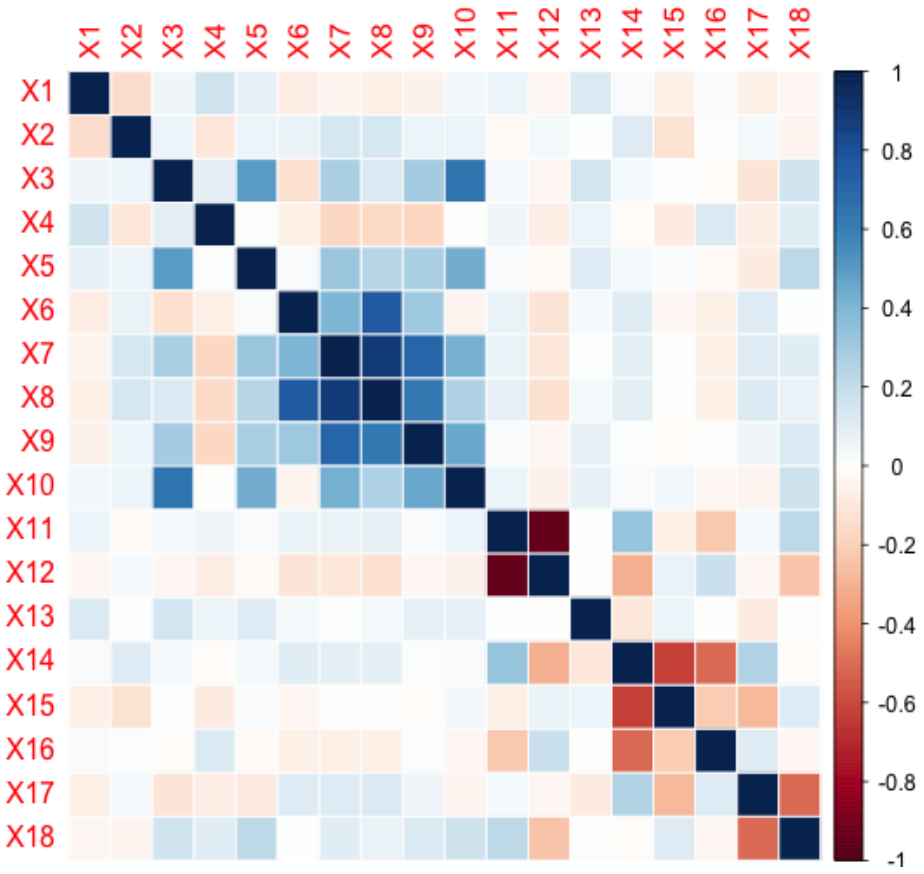

Figure 4.7 Correlation matrix of potential explanatory variables, coloured by the strength of correlation.

4.48 Univariate generalised linear models

Each explanatory variable was regressed separately against the binary infection status of setts, the proportion of positive faeces per sett and the average number of genome equivalents per sett (Table 4.8) using logistic, poisson and negative binomial models respectively. From these analyses the number of faecal samples collected per sett and the proportion of females (low, moderate, high) significantly affected the infection status of setts. These were the only variables that significantly accounted for variation in binary infection status, no variables significantly affected the proportion of positive faeces per sett or the mean number of genome equivalents per sett (Table 4.8).

Table 4.8 β and p values for each potential explanatory variable regressed against sett infection status, proportion of positive faeces per sett and the average number of *M. bovis* genome equivalents per sett.

	β (SE) p value		
	Infection status (0/1)	**Proportion faeces positive per sett**	**Average *M. bovis* genome equivalent copy number**
Zone	A: 1 B: -0.61 (0.37) 0.10 C: -0.21 (0.37) 0.56	A: 1 B: -0.57 (0.43) 0.17 C: -0.49 (0.43) 0.25	A:1 B: -0.38 (0.43) 0.37 C: -0.02 (0.44) 0.97
Vaccination status (BCG/Unvaccinated)	Unvaccinated:1 BCG: 0.11 (0.31) 0.72	Unvaccinated:1 BCG: -0.22 (0.35) 0.54	Unvaccinated:1 BCG: 0.19 (0.13) 0.15
Sweep	4: 1 5: -0.09 (0.25) 0.79 6: -0.10 (0.40) 0.78	4:1 5: -0.16 (0.39) 0.68 6: -0.12 (0.45) 0.80	4:1 5: -0.07 (0.15) 0.66 6: -0.09 (0.18) 0.61
Sett	Average: 21.57 (0.999 - 1)	Range: -22.92 – 69.31 (0.33 -1)	Average: 12.1 (0.32-0.99)
Number of faecal samples	**0.49 (0.15) 0.0009***	-0.10 (0.18) 0.60	0.29 (0.17) 0.1
Number of animals vaccinated	0.026 (0.042) 0.542	-0.001 (0.05) 0.98	0.02 (0.02) 0.306
Proportion of females (continuous)	-0.88 (0.54) 0.10	-0.91 (0.61) 0.14	-0.86 (0.62) 0.16
Proportion of females (categorical)	Low: -0.12 (0.34) 0.74 Moderate:1 **High: -0.87 (0.43), 0.04***	Low: 0.23 (0.38) 0.54 Moderate:1 High: -0.44 (0.50) 0.38	Low: -0.14 (0.41) (0.74) Moderate:1 High: -0.81 (0.46) 0.08
Proportion of cubs (continuous)	1.28 (0.18) 0.86	-7.71 (14.63) 0.60	1.44 (2.99) 0.63
Minimum time since vaccination (days)	-0.0001 (0.0007) 0.89	-0.0004 (0.0008) 0.64	-0.0001 (0.0003) 0.74
Starting infection status (Stat Pak)	Uninfected: 1 1: 0.17 (0.42) 0.70	Uninfected: 1 1: 0.28 (0.45) 0.52	Uninfected: 1 1: 0.17 (0.18) 0.34
Mean animal weight	0.08 (0.09) 0.37	0.08 (0.10) 0.42	0.11 (0.04) 0.10

4.49 The effect of BCG vaccination on the number of *M. bovis* positive setts by treatment zone

Logistic GLMs of the infection status of setts against treatment zone were carried out, adjusting for all potential explanatory variables (Table 4.6). The final models were adjusted for treatment zone, the number of faecal samples collected, sett ID, the proportion of females in the sett (continuous) and the starting infection status of setts. When adjusting for these variables there were reduced odds of detecting positive setts in zones B and C (50 and 100% BCG vaccination respectively) than in zone A (0% BCG vaccine) (Table 4.9) however, these results were not statistically significant.

Table 4.9 The odds ratio of detecting a positive sample per sett by zone, accounting for the number of faecal samples collected (β = 0.53 , SE= 0.16,p = 0.0007*), the proportion of females (β = -0.91, SE= 0.57, p= 0.11), starting infection of the sett (β = 0.33 , SE= 0.45, p = 0.47) and Zone. Unadjusted AIC = 257.93, adjusted AIC = 249

Zone	Unadjusted Odds ratio (CI) p value	Adjusted Odds ratio (CI) p value
A	1	1
B	0.54 (0.26 – 1.13) 0.10	0.52 (0.24 – 1.12) 0.09
C	0.60 (0.28-1.31) 0.56	0.62 (0.28 – 1.34) 0.22

4.410 The effect of BCG vaccination on the number of *M. bovis* positive setts by sett BCG vaccination treatment

Logistic GLMs of the infection status of setts against vaccine treatment (BCG vaccinated, unvaccinated) were carried out, adjusting for all potential explanatory variables (Table 4.8). The final models, which minimised the AIC values, adjusted vaccine treatment, the number of faecal samples collected, sett ID, the proportion of females in the sett (continuous) and the starting infection status of setts. When adjusting for these variables there was no statistical

difference in the odds of a sett being positive whether the sett had been vaccinated with BCG or not (Table 4.10).

Table 4.10 Odds ratio of detecting a positive sample per sett by vaccination status, accounting for the number of faecal samples (β = 0.51, SE= 0.15 p = 0.0008*), the starting infection status of the sett (β = 0.44 , SE= 0.45, p = 0.37) and the proportion of females (β = -0.87, SE=0.56, p= 0.12), Unadjusted AIC = 258.55, adjusted AIC = 250.05

Vaccination status	Unadjusted Odds ratio (CI) p value	Adjusted Odds ratio (CI) p value
Placebo	1	1
BCG	1.12 (0.61 – 2.05) 0.71	0.93 (0.49 – 1.77) 0.84

4.411 The impact of vaccination on the proportion of faecal samples *M. bovis* positive per sett, by treatment zone

The crude data was adjusted for treatment zone, the number of faeces sampled per sett, the proportion of females and the starting infection status per sett. None of the explanatory variables significantly explained variation in the proportion of positive faeces per sett (Table 4.11). As in the logistic analysis there was no difference in the proportion of faeces positive between zones B and C (Tables 4.9 and 4.10).

Table 4.11 Negative binomial GLM output for the proportion of faeces positive per sett unadjusted and adjusted for the number of faecal samples tested per sett (β = -0.07, SE= 0.18, p = 0.75), the proportion of females (β = -0.98, SE= 0.62, p = 0.12) the starting infection status (β = 0.14, SE= 0.46, p = 0.77) and the vaccination treatment zone. SE= standard error.

Zone	Unadjusted β (SE) p value	Adjusted β (SE) p value
A	1	1
B	-0.57 (0.41) 0.17	-0.57 (0.42) 0.18
C	-0.48 (0.44) 0.25	-0.48 (0.44) 0.27

4.412 The impact of vaccination on the proportion of faecal samples *M. bovis* positive per sett, by vaccination treatment

After adjusting the crude data for vaccine treatment (BCG vaccinated, unvaccinated), the number of faeces sampled per sett, the proportion of females (continuous) and the starting infection status per sett. There was not a statistically significant reduction in the proportion of positive setts with BCG vaccination (Table 4.12).

Table 4.12 Poisson GLM output for the proportion of faeces positive per sett unadjusted and adjusted for the number of faecal samples tested per sett (β = -0.06, SE= 0.18, p = 0.73), the proportion of females (β = -0.95, SE= 0.61, p = 0.12) the starting infection status (β = 0.25, SE= 0.46, p = 0.59) and the vaccination treatment. SE= standard error.

Zone	Unadjusted β (SE) p value	Adjusted β (SE) p value
Placebo	1	1
BCG	-0.20 (0.35) 0.58	-0.21 (0.36) 0.56

4.413 The impact of vaccination on the number of *M. bovis* genome equivalents shed per sett, by treatment zone

In addition to the explanatory variables adjusted for in previous analyses, this analysis also adjusted for the mean badger weight per sett. There was a non-significant reduction in the number of *M. bovis* genome equivalents shed in zones B and C compared to A (Table 4.13). Similarly to the logistic analysis the reduction between zones B and C was similar.

Table 4.13 Negative binomial GLM of the log_{10} *M. bovis* genome equivalents shed per sett. Adjusted for the number of faecal samples collected (β= 0.05, SE- 0.18, p= 0.79), the proportion of females (β = -0.94, SE= 0.62, p = 0.13), the starting infection status (β = 0.12, SE= 0.47, p = 0.79), the mean badger weight (β = -0.07, SE= 0.10, p = 0.72). Unadjusted AIC = 197.03, Adjusted AIC =192.32.

Zone	Unadjusted β (SE) p value	Adjusted β (SE) p value
A	1	1
B	-0.38 (0.41) 0.17	-0.56 (0.42) 0.18
C	-0.10 (0.43) 0.25	-0.48 (0.44) 0.27

4.414 The impact of vaccination on the number of *M. bovis* genome equivalents shed per sett by vaccination treatment

When the crude data was adjusted for the number of faecal samples collected, the proportion of females (continuous), the starting infection status there was no difference in the number of genome equivalents shed between BCG vaccinated and unvaccinated setts (4.14).

Table 4.14 Negative binomial GLM of the log_{10} *M. bovis* genome equivalents shed per sett. Adjusted for the number of faecal samples collected (β = -0.05, SE= 0.18, p = 0.76), the proportion of females (β = -0.92, SE= 0.61, p = 0.13), the starting infection status (β = 0.24, SE= 0.46, p = 0.59. Unadjusted AIC = 805.9, adjusted AIC = 196.98.

Zone	Unadjusted β (SE) p value	Adjusted β (SE) p value
Placebo	1	1
BCG	0.21 (0.13) 0.12	-0.18 (0.36) 0.61

4.415 The effect of sampling intensity on the proportion of *M. bovis* positive setts

As a substantial number of setts were sampled over a large area, the sampling intensity for individual setts was low. In total, 14.7% of setts had samples collected in more than one sweep, 44.0% of setts had one sample collected over

the whole trial and only 21.2% of setts had 3 or more samples collected during the study (Table 4.15). When the prevalence of *M. bovis* in the population is relatively low, here estimated by Stat Pak to be between 5-15%, and with intermittent shedding, a low sampling intensity may substantially reduce the probability of detecting positive setts. The number of samples collected was an important contributor to the findings, in univariate models the number of samples significantly altered the odds of detecting positive setts (Table 4.8) and the presence of this explanatory variable improved the model fit in all multivariate models (Tables 4.9-4.14). Zone C was the only area where the modal number of samples was greater than one, and BCG vaccinated setts also had more than one faecal sample analysed on average (Table 4.15).

Table 4.15 Sampling intensity of sett by treatment zone and vaccination treatment.

Number of faecal samples	**Number of setts**					
	A	B	C	BCG vaccinated	Unvaccinated	Total
1	37	25	19	25	56	81
2	24	18	22	30	34	64
3	2	13	9	18	6	24
4	4	2	2	2	6	8
5	1	0	5	5	1	6
6	0	0	1	1	0	1

The proportion of *M. bovis* positive setts increased with increasing numbers of faecal samples analysed (Figure 4.8). Although 6 faecal samples per sett identified all setts as positive (Figure 4.8), there was only one sett with this number of samples in the study (Table 4.15). Fifty percent of setts which had four or five faecal samples analysed were identified as positive (Figure 4.8) however, it is possible that setts where a greater number of faecal samples were available had larger group sizes, although the correlation between the number of

badger trapped and the number of faecal samples collected was low (r=0.05) so this is unlikely.

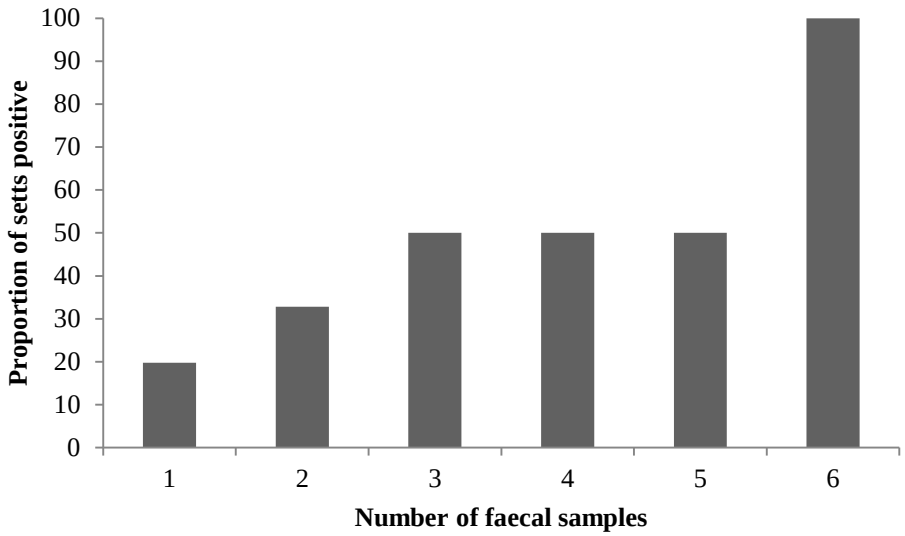

Figure 4.8 The percentage of positive setts at varying sampling intensity per sett (sample sizes: 1 = 81, 2 = 64, 3= 24, 4= 8, 5= 6, 6=1).

Calculating the prevalence of infected setts given the analysis of 1 faecal sample or more than one faecal sample, identified a substantial increase in the percentage of positive setts when more than one faeceal sample is analysed. 33% of setts where more than one sample was collected being found to be positive compared to 22% of setts that had one sample collected. However, the odds of detecting a positive sett when more than one sample is taken is not different from when one sample is analysed (Table 4.16). The percentage of positive setts is greater when more than three samples are taken compared to when 1 or two samples are taken however, and the odds of detecting a positive sett are significantly reduced when more than three samples are taken compared to one or two samples (Table 4.17), most likely due to the small number of samples in this category.

Table 4.15 The odds ratio for detecting positive setts with different sampling intensities 1 sample (baseline category) and 2+ samples.

Number of samples	Adjusted Odds ratio (CI) p value
1	1
2+	1.09 (0.58 – 2.05) 0.80

Table 4.16 The odds ratio for detecting positive setts with different sampling intensities 1-2 samples (baseline category) and 3+ samples.

Number of samples	Adjusted Odds ratio (CI) p value
1-2	1
3+	0.36 (0.17 – 0.69) 0.002*

When each number of samples is considered individually there was a trend of increased odds of detecting a positive sett with increased sampling intensity (Table 4.17). As only one sett had six samples collected over the trial, it was excluded from this analysis.

Table 4.17 The odds ratio for detecting positive setts with 1-5 samples, using one sample as the baseline category.

Number of samples	Sample size	Adjusted Odds ratio (CI) p value
1	81	1
2	64	2.53 (0.74 – 3.17) 0.26
3	24	3.61 (1.44-9.07) 0.01*
4	8	3.21 (0.81-12.69) 0.10
5	6	4.81 (0.89-26.04) 0.07

4.416 The effect of the proportion of females per sett on infection status

The odds of a sett being *M. bovis* positive was significantly reduced when the percentage of females in the sett was high (>67%) (Table 4.18) and the proportion of females per sett (continuous) was retained in all models, suggesting that the proportion of females or males in a social group influences the infection status of that group. Overall the percentage of positive setts was highest at 41%

when the proportion of females captured at a given sett was low (0-33%) compared to 27% positive with a moderate proportion of females (33< x > 67%) and 22% when the proportion of females was high (>67%).

Table 4.18 The odds ratio for detecting positive setts given different proportions of females per sett with moderate percentage of females as the baseline category.

Proportion female	Adjusted Odds ratio (CI) p value
Low (<33%)	0.88 (0.46 – 1.73) 0.74
Moderate (33< x >67%)	1
High (>67%)	0.42 (0.18-0.97) 0.04*

4.5 Discussion

The work presented here demonstrates that while there is some reduction in the level of faecal shedding with oral BCG vaccination in the field, overall vaccination did not make a significant difference to the level of environmental shedding of *M. bovis* in this study. The reductions in shedding as measured by the binary infection status of setts, the proportion of faeces positive per sett and the number of genome equivalents shed per sett do suggest that oral vaccination may demonstrate potential as a control strategy if further optimised. As differences in the prevalence of *M. bovis* in environmental faeces is strongly correlated with differences in infection at the population level, is it likely that the observed changes in the pool of environmental *M. bovis* are also reflective of a decrease in infection in the badger population. However, whether these levels of reduction are sufficient enough to reduce disease prevalence in badgers populations to a level that would result in reduced transmission to cattle is not known and would require further, more long term, trials.

Decreases in environmental shedding were only observed when examining the level of positivity by vaccination zone (A/B/C), rather than vaccine treatment (BCG vaccinated/unvaccinated). Although the aim of the study was to vaccinate half of badgers in zone B with BCG and all in zone C, this was not achieved before the start of faecal collection and more than 53% of setts were BCG vaccinated in zone B and fewer than 86% BCG vaccinated in zone C. The lack of difference in shedding observed between the BCG vaccinated and unvaccinated setts suggests that the context in which treatment is received in terms of neighbouring setts is important in determining the success of this vaccination

strategy, and is an important consideration for any implementation of this method.

This study was limited in its ability to detect differences between vaccination levels and vaccine treatments as a result of the number of samples collected per sett. There were increased odds of detecting a positive sett with increased sampling intensity. The odds of detecting a positive sett was higher when 3 samples were analysed per sett however, the lack of a significance increase in detection when 4 or 5 samples were analysed is likely due to the small number of setts in these categories. Faecal sampling was concurrent with badger capture and vaccination, this caused logistical difficulties including the time required to repeatedly sample over a large area, resulting in only one or two faecal samples being collected for the majority of setts in this study. Populations in the RoI are at lower density than in the UK with 2-3 badgers per sett, and so fewer faecal samples will be available. Once infected, *M. bovis* shedding in faeces is intermittent therefore, sampling repeatedly over a set period of time would have increased the probability of detecting positive setts. The lack of repeated sampling in this study will have reduced the number of positive setts that were identified and will have limited the power of this study.

In addition to the power of this study being limited by the number of faecal samples collected per sett, power was also limited by the number of setts in this study. As this was the first field trial of oral vaccination, the expected reduction in faecal shedding was unknown. A field trial of intramuscular vaccination found a 54% reduction in the risk of a badger testing positive after vaccination (Carter

et al. 2012). To detect the same reduction in faecal shedding in this study with 80% power and 95% confidence 153 setts were needed. There were 184 setts in this study, which suggests that if there was a reduction in shedding with vaccination the reduction was less than 54%. As the size of the reduction in shedding decreases, the number of setts needed to detect the difference increases for example at 80% power and 95% confidence 250 setts would be required to detect a 40% reduction, 465 for a 30% and 1094 for a 20% reduction in shedding. This highlights the fact that this study lacked the power to detect smaller changes in faecal shedding and emphasises the need to sample a large number of setts. However, during field trials increasing the number of setts is not always possible due limitations of the area selected and the logistical and financial challenges of vaccinating and sampling large numbers of setts.

Despite few samples being collected per sett this study does highlight the ability of this faecal qPCR method to monitor intervention strategies and detect changes in populations over a large area and over time. The initial power analysis of the whole trial suggested that sample size would not be a limiting factor in detecting changes in infection in badgers with vaccination (Aznar et al. 2013) however, it does appear to limit the power of this study due to differences in detecting infection by post mortem examination compared to detection of environmental shedding.

There were no statistically significant differences observed between vaccinating 50 or 100% of the badger population. Differences may have been observed between vaccination levels if more accurate data on pre-existing infection had

been available. Although the setts known to be infected prior to the start of the trial were clustered in zone A rather than zone C, it is likely that more setts were positive prior to the start of the trial than were identified. The number of positive setts detected was limited by the number of animals trapped at each sett and the sensitivity of Stat-Pak, which has been estimated to be 50% for this trial (Deirdre Ni Bhuachalla, personal communication).

There was a relationship between an increasing proportion of females trapped at a sett and a reduction in the proportion of infected setts. This may be due to females moving less frequently than males in this population (Byrne *et al.* 2014) and therefore experiencing fewer opportunities for transmission and aggressive behaviours. However, the results here are in contrast to other studies which found that when the proportion of females present in the group was high the risk of the sett becoming an incident case increased (Vicente et al. 2007). Males are more likely to move to a social group with a higher proportion of females (Rogers et al. 1998) and males may be more likely to be infected than females (Gallagher & Nelson 1979). Alternatively, a higher proportion of females in a group increases breeding opportunity and may result in increased aggressive behaviour between males and increased opportunity for transmission via bite wounding (Vicente et al. 2007). However, this previous work was carried out on high-density populations in the UK, where the dynamic may be different from the low density populations in the RoI.

It is unclear from the results presented here whether oral vaccination produces a large enough reduction in disease to make this is a viable and cost effective

control strategy. During this trial oral vaccines were administered to captured animals however, in the field the vaccine would be delivered in a food bait. Further studies are required to establish whether this would alter levels of uptake in wild populations. Should an oral vaccination strategy be implemented several factors will need to be considered, including the cost of the vaccine, the number of baits that will need to be deployed and the possibility of uptake by non-target species (Chambers et al. 2014). The BCG component of the vaccine is currently the most expensive; as oral vaccination will most likely require a larger dose than intramuscular vaccination (Chambers et al. 2014) the cost may be increased and if a large number of baits are required may make this method prohibitively expensive. Uptake by non target species would reduce the amount of vaccine available for badgers, potentially reducing the efficacy of the program and, if eaten by cattle they will become sensitised to the tuberculin skin test (Delahay *et al.* 2003). Although it may be possible to place baits within setts, where they cannot be accessed by cattle, to overcome this problem. In addition, the efficiency of vaccination is likely to differ between high and low density populations and therefore field trials would also need to take place in the UK before this method could be considered.

This trial has further highlighted the value of non-invasive monitoring of badger populations and demonstrated the applicability of this qPCR assay to low-density populations and large areas. However, this study did not detect statistically significant differences in the level of *M. bovis* shedding in faeces with vaccination due to the limited power of this study, as a result of both the number of setts sampled and the intensity of sampling at individual setts. As it is not

always possible to increase the number of setts sampled, future work should examine the intensity of sampling required in a low-density population to detect differences in shedding between areas over time. Overall, a substantial amount of future work is required to determine the efficiency and cost-effectiveness of this as a control method and establish whether this method has the potential to reduce transmission of *M. bovis* from badgers to cattle.

5. Characterisation and comparison of the gut and faecal microbiomes of European Badgers with a preliminary assessment of the effects of *Mycobacterium bovis* infection and BCG vaccination on these communities

Hayley C. King, Deirdre Ni Bhuachalla, Leigh Corner, Eamonn Gormley, Elizabeth M. Wellington

H.C.K processed samples, undertook data processing and analysis, prepared figures and tables and with E.M.W obtained funding for pyrosequencing. D.N conducted post mortem examinations, collected samples, created and provided a database of post mortem results. L.C with E.G devised, obtained funding for, and oversaw the running of the trial. L.C also conducted post mortem examinations and sample collection. E.M.W was involved in project design, obtained funding for pyrosequencing with H.C.K and oversaw all sample processing and analysis.

5.1 Abstract

The faecal microbiome is commonly used as a proxy for the communities present in the mammalian gastrointestinal tract (GIT) however, few studies have compared the similarity of faecal and gut communities, and those that have find large differences. Here we present the relationship between the faecal and gut (ileum) communities in European badgers (*Meles meles*) and conclude that faecal communities from different animals are more similar to each other than faecal and gut communities taken from the same animal. Comparison of the core gut and faecal communities did not increase the similarities of the communities. Although no difference in the gut communities was detected with the presence of tuberculous lesions in the lungs, faecal communities did differ based on the presence or absence of lesions, and formed separate groupings. These groups varied predominantly on changes in abundance of the Clostridiales**,** which accounted for 56% of variable taxa. The results presented here suggest that examining faecal communities allows changes indicative of altered health to be detected in hosts and that these changes may not be representative of changes in gut communities.

5.2 Introduction

The majority of microorganisms that colonise mammals are found in the gastrointestinal tract (GIT), where there are between $10^{13} – 10^{14}$ microorganisms present (Gootenberg & Turnbaugh 2011). The GIT microbiota has been implicated as an important component of defence against pathogens and influences the immune response of the host (Huse et al. 2008). Changes in the GIT microbiota have been detected with obesity (Turnbaugh et al. 2006; Turnbaugh et al. 2009) and irritable bowel syndrome (IBS) (Jeffery *et al*. 2012) as well as conditions not directly related to GIT health such as asthma (Huse et al. 2008).

As directly studying the gut microbiome requires invasive biopsies or post mortem examinations, many studies aimed at examining the gut microbiome have used the faecal microbiome as a proxy (e.g. Arumugam et al. 2013; Jakobsson et al. 2010; Turnbaugh et al. 2010; Yatsunenko et al. 2012; Muegge et al. 2011; Looft et al. 2012). Communities adhered to the surface of the GIT are likely to fulfil different roles from those present in the lumen (Eckburg et al. 2005), and therefore will have a different community composition. Although few studies have examined the similarity between the gut and faecal microbiomes, those that have found that the faecal microbiota are significantly different from the gut microbiota (Zoetendal et al. 2002; Eckburg et al. 2005; McKenna et al. 2008) and contain distinct taxa from gut communities (Eckburg et al. 2005). The faecal microbiota is extremely diverse, with studies often finding that there is not

a core set of abundant groups found in all individuals studied (Turnbaugh et al. 2010).

Badgers are susceptible to infection by *Mycobacterium bovis*, the causative agent of tuberculosis (Palmer et al. 2012). As badgers are implicated in the transmission of *M. bovis* to cattle (Donnelly et al. 2003; Griffin et al. 2005) they are the target of disease control interventions in the United Kingdom (UK) and Republic of Ireland (RoI) through culling and vaccination with BCG. Once infected, badgers may intermittently shed *M. bovis* in their faeces (Clifton-Hadley *et al.* 1993). As tuberculosis in badgers is primarily a respiratory disease (Gallagher & Nelson 1979) and lesions in the GIT are rare; the most likely source of faecal *M. bovis* is through swallowing infected sputum that has been expelled from the lungs (Corner et al. 2011). The presence of lesions in the lungs of infected animals is highly variable; with some individuals displaying no visible lesions and others several, although frequently only one visible lesion is present (Corner et al. 2011). Badgers may survive for several years with the disease however, the wider health costs and changes to the gut microbiota with infection are unknown.

As part of an orally administered BCG vaccine field trial, badgers were culled and detailed post mortem examinations carried out to determine the *M. bovis* infection status of the animals (Aznar et al. 2011). These samples provide a unique opportunity to study the correspondence between the GIT and faecal microbiomes of badgers and to confirm whether faecal shedding is a result of swallowed sputum or colonisation of the gut. In addition, characterising

bacterial communities from badgers that vary in *M. bovis* infection and shedding and vaccination status would allow for a fuller understanding of the health costs imposed by infection, and would highlight changes in microbial community structure, composition, and diversity. This study aimed to i) determine whether *M. bovis* shedding in faeces is indicative of changes in gut microbial community ii) determine changes in the gut and faecal microbiomes with *M. bovis* lesions and oral BCG vaccination.

5.3 Materials and Methods

5.31 Sample Collection

Post mortem samples were collected by collaborators at University College Dublin (UCD) from 273 badgers that had been culled as part of an oral BCG vaccine trial in Kilkenny, the Republic of Ireland (Aznar et al. 2011). The area was divided into three zones, in the first, all captured badgers were given a placebo vaccine, in the second zone 50% of captured badgers were orally administered BCG vaccine and 50% were given a placebo vaccination, and all badgers captured in the final zone were given a BCG vaccine (Aznar et al. 2011). At the end of the trial, badgers within the trial area were culled and faeces from within the rectum and a section of the ileum were removed, and stored at -20^{o}C until DNA extraction. During post mortem animals were visually determined as having tuberculous lesions, suspected lesions or no visible lesions. Samples from the lungs were taken for culture to determine the presence of *M. bovis* and other non-tuberculous mycobacteria and were confirmed as *M. bovis* by spoligotyping.

5.32 DNA extraction and sample selection

Total community DNA was extracted from the faeces of 257 badgers and the ileum of 18 badgers using 0.1 g (+/- 0.003 g) of faeces and 0.25g (+/- 0.003g) of ilium to account for badger flesh, using the Fast DNA spin kit for soil (MP Biomedicals) following the manufacturer's instructions and stored at -20^{o}C before use.

M. bovis was detected and quantified in gut and faecal extracts using a qPCR assay which targets the RD4 deletion region unique to the *M. bovis* genome (Pontiroli et al. 2011; Travis et al. 2011). An initial qPCR screen of each sample was performed using an ABI 7500 Fast qPCR machine (ABI) with two technical replicates of each sample. Positive controls (8.5×10^2 genome equivalents) and negative controls were also present in duplicate for each run. PCR reactions were set up using 900nM of each primer (RD4F $^{5'}$TGTGAATTCATACAAGCCGTAGTCG$^{3'}$, RD4R $^{5'}$CCCGTAGCGTTACTGAGAAATTGC$^{3'}$), 250nM of Taqman probe (6FAM-AGCGCAACACTCTTGGAGTGGCCTAC—TMR), 1mg ml^{-1} bovine serum albumen (BSA), 12.5µl of Environmental Mastermix 2.0 (ABI), 10 µl of template and made up to 25µl with molecular grade water (Sigma Aldrich). PCR cycling conditions were 50°C for 2 min followed by 95°C for 10 min then 40 cycles of 95°C for 15 sec 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, using a full set of standards (8.5×10^{-1} - 8.5×10^5). If one or more of the technical replicates in the quantification assay exhibited amplification 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.

5.33 Amplification and purification of fragments for pyrosequencing

All PCR reactions were set up in a dedicated PCR clean lab free of amplicons. 27F and 534R primers were used to target the V1-V3 regions of the 16S rRNA

gene. Primers followed a fusion design, with each forward containing a unique Roche MID adaptor and 27F. Each reverse primer contained the 534R sequence.

Each sample was amplified by PCR in two 50µl reactions, using a final concentration per reaction of 200µM DNTP's, 0.5µM of each primer, 3% DMSO, 0.4mg/µl BSA, 1 unit of Phusion High Fidelity Taq (Thermo Scientific), 10µl of Phusion buffer and 5µl of 1:10 diluted template and made up to 50µls with nuclease free water. Faecal samples were amplified on a cycle with an initial denaturation at 95^{o}C for 3 minutes followed by twenty-five cycles of 94^{o}C for 40 seconds, annealing at 60^{o}C for 30 seconds and elongation at 72^{o}C for 45 seconds followed by a final extension of 10 minutes at 72^{o}C. Gut samples were amplified on the same cycle except with an annealing temperature of 57^{o}C for thirty cycles.

Bands of 500bp were size selected and removed after amplicons were run on a 2% agarose gel at 120V for 60 minutes. To increase the final yield of DNA, the two PCR products per sample were pooled prior to gel DNA extraction using the Qiagen QIAquick Gel Extraction Kit following the manufactures protocol. The concentration of DNA per sample was quantified using a Qubit and a BR ds DNA kit to ensure sufficient quantities of DNA for pyrosequencing which required 100ng of DNA per 20µl.

5.34 454-Pyrosequencing of Amplicons

Data generation and analysis were carried out by the Centre of Genomic Research, which is based at the University of Liverpool where amplicons were

sequenced using unidirectional pyrosequencing on the Roche 454 platform with XLR70/titanium chemistry.

5.35 Data Processing

Data were processed and analysed using MacQIIME. Sequences were demultiplexed and filtered, no ambiguous bases were allowed, any sequence with a quality score below 30 was removed and sequences with a maximum of two primer mismatches accepted. Sequences were clustered at over 97% similarity to form operational taxonomic units (OTUs). The most abundant sequence per OTU was selected as the representative sequence of that OTU and taxonomy assigned to this sequence using UCLUST consensus taxonomy classifier and aligned with pyNAST. The alignment was filtered for gaps and chimeras detected and removed with chimera slayer to produce a final OTU table for analysis.

5.36 Statistical Analysis

Statistical analysis was carried out using R (v.3.0.2) and using standard scripts in QIIME. Two-sided student's T tests were used to determine whether there was a difference in mean diversity between sample types and samples of different infection status. Analysis of similarity (ANOSIM) was used to determine whether there were significant differences between sample groupings and was run for 999 permutations. G-tests with Bonferroni corrected p values were used to determine whether relative abundances of OTUs varied between sample types. Both weighted and unweighted UniFrac distances matrices were used to determine differences in community structure based on abundance of taxa and the presence/absence of taxa respectively (Lozupone et al. 2007). Principle

coordinate analysis was implemented to determine similarities and differences between sample communities using dissimilarity matrices.

The core microbiome was computed separately for faecal and gut communities at three levels (50, 70 and 90%) and was defined as those taxa present in at least 50% (or 70 or 90%) of samples. When OTU abundances and presence/absence of taxa were being compared at the level of sample type (e.g. gut/faeces) comparison took place using the complete set of sequences in all samples. However, as one gut sample had a considerably higher number of sequences this was excluded from non-rarefied analysis, or analyses were carried out with and without outliers (defined at samples 1.5 times greater than the interquartile range). When samples were examined individually (e.g. alpha diversity, PCoA analysis) the number of sequences per sample could bias the results therefore, for these analysis rarefied OTU tables were used, where all samples had the same number of sequences.

5.4 Results

5.41 Quantification of *M. bovis* genome equivalents present in gut and faecal samples

Seven of the two hundred and fifty seven (2.7%) faecal samples tested were positive for *M. bovis* by qPCR. One faecal sample was screened per animal. The number of *M. bovis* genome equivalents per gram of faeces ranged from $6.0x10^2$ – 6.1 $x10^5$ with a mean of 1.0 $x10^5$ copies per gram. All screened gut samples (total = 18) were negative for *M. bovis*. The seven positive faecal samples and their corresponding gut samples, plus seven randomly selected negative faecal samples with their corresponding gut samples were selected for pyrosequencing. In addition, four randomly selected faecal and four randomly selected ileum samples were also sequenced.

5.42 Sequencing depth of gut and faecal communities

A total of 885,658 sequences were analysed with an average of 25,304.5 sequences per sample, forming 20,562 unique OTUs when clustered at 97% similarity. Rarefaction analysis curves levelled off for the majority of samples (Figure 5.1). Five samples (three gut and two faecal samples) were not as deeply sequenced as the others. These samples had fewer than average sequences per sample with the exception of one gut sample with an above average number of sequences.

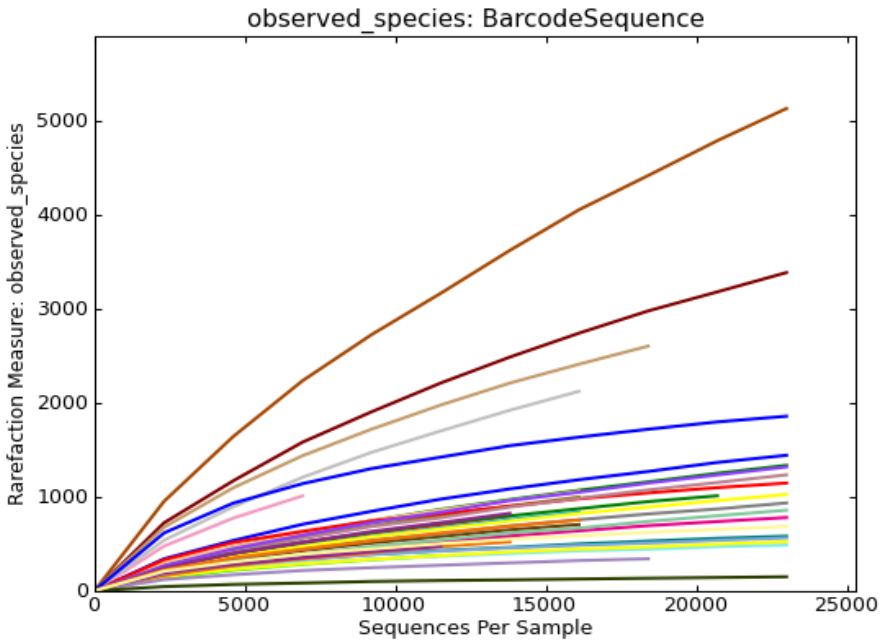

Figure 5.1 The number of sequences and species observed per sample.

The minimum number of sequences per sample was 7,633 with a median of 23,000 sequences per sample. OTU tables were rarefied to 7,633 sequences per sample to allow comparison of communities between samples. The majority of samples have begun to level off at around 7,500 sequences (Figure 5.1) suggesting that rarefying at 7,633 sequences does not result in the loss of substantial diversity for the majority of samples.

5.43 Alpha diversity of gut and faecal communities

For whole non-rarefied communities, the Simpsons inverse diversity measure ranged from 2.4 - 21.2 in faecal communities and 1.3 - 47.7 in gut communities. There was no significant difference between alpha diversity of gut and faecal samples both including (t= 0.8025, p= 0.4321) and excluding outliers (t=0.7506, p= 0.4587). There was no difference in Simpson's inverse diversity in pairs of gut and faecal samples (t= 0.912, p=0.375), or between *M. bovis* shedding status

by qPCR (t=0.7559, 0.4601), by vaccination status (F 2,32: 0.892, p = 0.42) or by whether lesions were present in the animal (F 2, 32: 1.568, p=0.224).

For rarefied communities the Simpsons inverse diversity measure ranged from 1.05 – 1.74 in faecal communities and 1.02 – 4.44 in gut communities. There was no significant difference between alpha diversity in gut and faecal samples (t= 1.10, p= 0.28119). There was no difference in Simpson's diversity between pairs of gut and faecal samples (t= 0.99, p=0.342), or between *M. bovis* shedding status by qPCR (t=0.794, 0.457), by vaccination status ($F_{2,32}$: 0.549, p = 0.583) or by whether lesions were present in the animal ($F_{2, 32}$: 0.296, p=0.746).

5.44 Composition of the gut and faecal communities

For the whole communities, large variations were observed in the taxa present and relative abundance of these taxa between individual samples and sample types. When samples were pooled into gut and faecal communities they were dominated by a small number of phyla; the Firmicutes (57.70% of total community in gut samples and 84.30% of faecal communities), Proteobacteria (gut communities: 25.91%, faecal communities: 10.98%) and Actinobacteria (gut communities: 10.61% and faecal communities: 3.10%). Although the majority of samples were dominated by the Firmicutes, two gut communities were comprised mainly of Actinobacteria (54.52% and 55.56% of total community, compared to under 19% in all other samples) and two were heavily populated by proteobacteria (75.18 and 88.19% of total community compared to under 45% in all other communities).

At the genus level few samples were dominated by any particular taxa however, the exceptions were a single gut sample where 65.68% of the community were Streptococcus and another gut sample where 87.83% of the taxa were Enterococcus. At all levels of taxa, gut communities were consistently dominated by a smaller number of taxa compared to faecal communities (Figure 5.2).

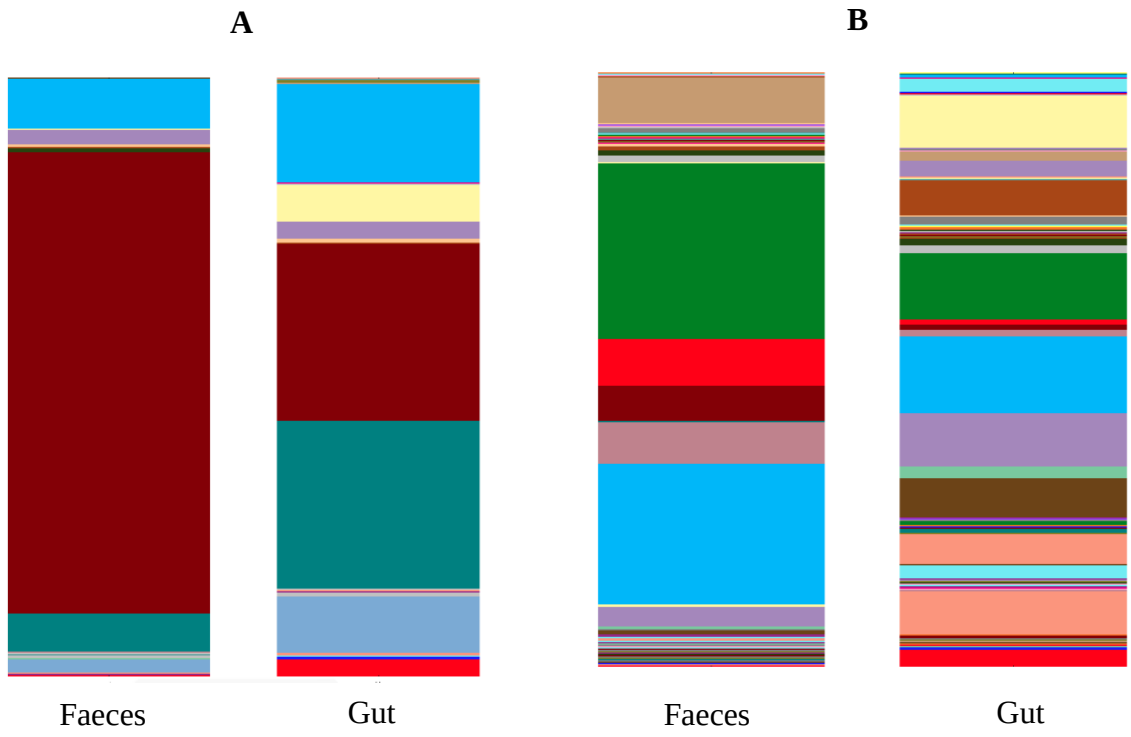

Figure 5.2 The relative abundance of classes (A) and genera (B) present in gut and faecal communities (taxa legend in appendix C).

The Mycobacteria were at equal abundance in both sample types, comprising 0.3% of the total communities. Both gut and faecal communities had a large proportion of taxa that were members of the Clostridiales (gut communities: 29.59%, faecal communities 77.12%). A number of families were present at relatively high abundance that were at very low abundance or absent in faecal communities for example Nocardiopaceae (gut communities: 7.2%, faecal communities <0.1%), Bacillaceae (gut communities: 5.30%, faecal communities 0.30%), Halomonadaceae (gut communities: 9.07%, faecal communities: 0.10%) and the Enterococaceae (gut communities: 6.6%, faecal communities: 0.80%). Only the Enterobacteriales were present at much higher abundances in faecal communities (7.90%) than in gut communities (1.5%).

5.45 Comparison of paired gut and faecal communities from the same animal

For rarefied communities, paired gut and faecal samples from the same animal do not cluster together in PCoA analysis (Figures 5.3 and 5.4). Samples cluster by sample type, rather than the animal of origin with faecal samples clustering together to form a separate group from gut samples (Figures 5.3 and 5.4). Weighted analysis (Figure 5.4) produced greater separation between gut and faecal communities than unweighted (Figure 5.3) analysis, suggesting differences in communities are caused to a greater extent by differences in taxa abundance rather than the presence or absence of taxa. However, there is also separation of gut and faecal communities, with some differences also due to the presence and absence of taxa.

When examining abundances in rarefied communities ANOSIM analysis identified that samples within the gut and faeces groups are more similar than would be expected by chance and form two separate groupings (R= 0.2797, p=0.001). When examining presence/absence of OTUs ANOSIM determined that gut and faecal communities differed significantly on the OTUs present and therefore were separate groups (R= 0.2157, p= 0.001). When examining the whole un-rarefied community both the abundance of OTUs (R= 0.2807, p= 0.001) and the presence/absence of OTUs (R= 0.2445, p= 0.001) varied significantly between gut and faecal samples, identifying them as separate communities.

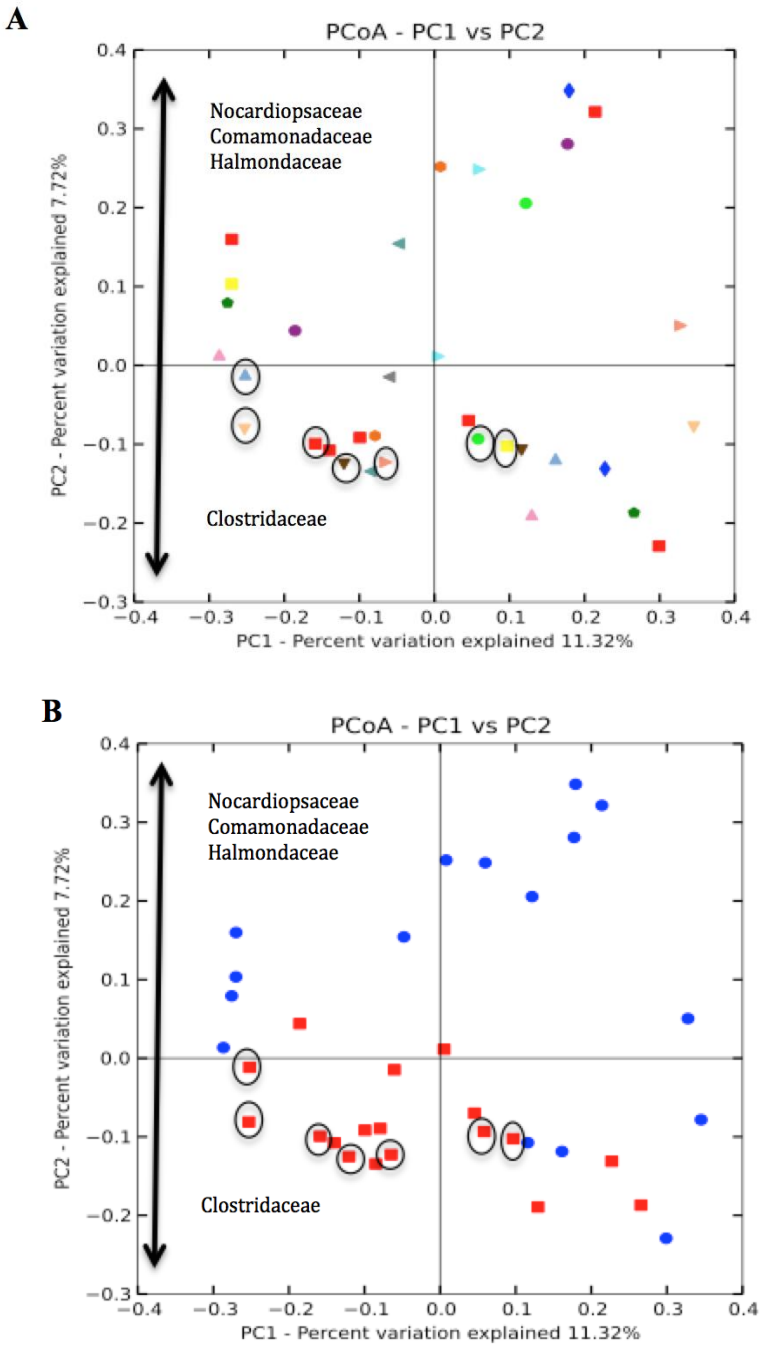

Figure 5.3 Unweighted PCoA of faecal and gut communities coloured by animal of origin (A) and sample type (gut (blue) or faeces (red)) (B). Circled samples were *M. bovis* positive.

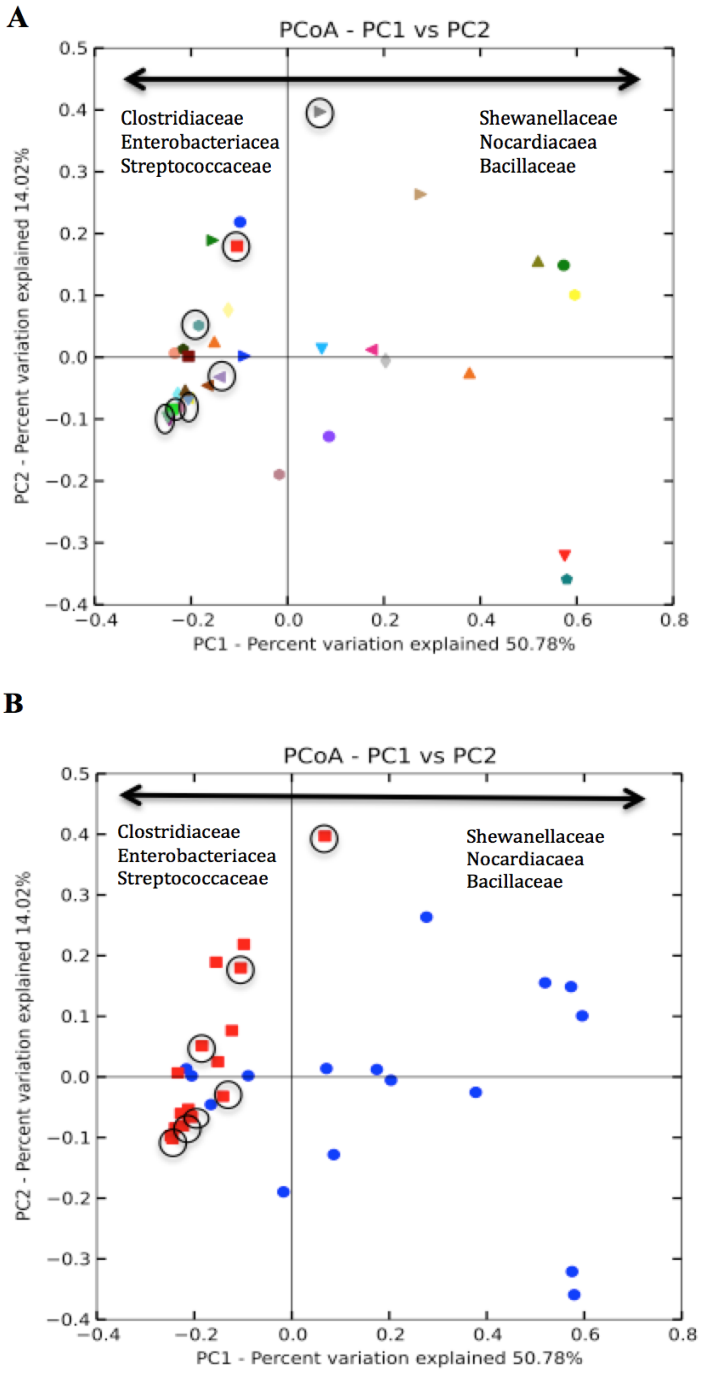

Figure 5.4 Weighted PCoA of faecal and gut communities coloured by animal of origin (A) and sample type (gut (blue) or faeces (red)) (B). Circled samples were *M. bovis* positive.

5.46 OTU similarity of complete and core communities from gut and faecal samples from non-rarefied OTU tables

Of the total OTUs identified in un-rarefied communities 17.67% (3,633 OTUs) were present in both gut and faecal microbiomes (Table 5.1). Of these, there was a significant difference in the abundance of 80 of them between faecal and gut microbiomes (Table 5.1). Of the variable OTUs 31.25% (25/80) were more abundant in faeces and 68.75% (55/80) were more abundant in gut samples. Those taxa with the greatest differences in abundances between samples types were the Enterococcus, Clostridium and Streptococcus genera and the families Nocardiopsaceae and Comamonadaceae which all had an average difference of over 1000 sequences in the mean sequences per sample between gut and faecal communities. All of these taxa contain species that are common gut commensals and all were present at significantly higher abundances in gut communities with the exception of members of the Clostridium genera, which were at greater abundance in faecal communities.

Table 5.1 The number of total and variable OTUs in complete and core gut and faecal communities using all sequences.

	Total OTUs	**Present in both**	**Absent in One**		**Proportion of variable OTUs**
			Gut	**Faeces**	
Complete	20 562	3633	6277	10 652	0.02 (80)
50	394	94	223	77	0.41 (39)
70	136	48	65	23	0.60 (29)
90	39	14	22	3	0.86 (12)

10, 652 OTUs were present in gut samples which were not present in faecal samples. These OTUs were present at low abundance, with the mean number of sequences per OTU in gut samples ranging from 14.706 sequences per sample to 0.059 per sample with a mean of 0.174 per sample (Table 5.2). 6, 277 OTUs

were present in faeces which were not identified in any gut samples. These OTUs were at low abundance, ranging from a mean of 29.167- 0.056 sequences per sample with an average of 0.123 sequences per faecal sample (Table 5.2).

Table 5.2 The number of sequences for complete and core gut and faecal community

	Complete	Gut			Faeces			
		Complete	**Core 50**	**Core 70**	**Core 90**	**Complete**	**Core 50**	**Core 70**	**Core 90**
Number OTUs	20, 562	14 285	172	71	17	9910	317	113	36
Mean sequences	1.24	2.07	143.07	312.62	820.41	2.15	48.21	159.80	431.65
Median sequences	0.06	0.06	11.71	38.82	590.47	0.06	2.55	12.17	57.19
Maximum sequences	3978.61	2598.16	2598.12	2598.12	2598.12	3978.61	3978.61	3978.61	3978.61
Minimum sequences	0	0.06	0.71	4.35	16.64	0.06	0.67	1.89	5.22

The core microbiome was defined at three levels, as those taxa present in 50, 70 or 90% of samples and was computed separately for gut and faecal communities for both rarefied and un-rarefied OUT tables. For un-rarefied communities the core microbiome at all three levels resulted in a large reduction in the number of OTUs present in both gut and faecal communities with over 99% lost in the core 90% microbiome (Table 5.1) and in all cases fewer than half of the OTUs present in the core microbiome were present in both gut and faecal communities. In addition, between 41 – 86% of taxa present in both core and faecal communities differed significantly in abundance between the two communities (Table 5.1), suggesting taxa that dominate faecal communities are at low abundance or absent from gut communities and vice versa. The few OTUs retained in core communities demonstrates the variability of gut and faecal communities, where only a small proportion of OTUs are shared between samples of the same type.

5.47 Clustering of non- rarefied gut and faecal core communities

PCoA analyses of the core gut and faecal communities at all three levels (50, 70 and 90%) increased the separation of gut and faecal communities clusters, with clustering by sample type greatest with the core 90% (Figures 5.5 and 5.6). The separation between gut and faecal samples was greatest for the weighted analysis, meaning that although differences in both the abundance and presence/absence of OTUs cause separation between sample types, differences in abundance are responsible for a larger proportion of differences between communities.

PCoA analysis of the core communities was undertaken using complete non-rarefied samples and with rarefied samples. Although this was a comparison of the clustering of faecal and gut communities overall rather than at the sample-level, the analysis itself is computed at the sample level and a single sample may alter clustering patterns. However, as patterns did not differ between rarefied and non-rarefied communities, only un-rarefied results are reported here for conciseness.

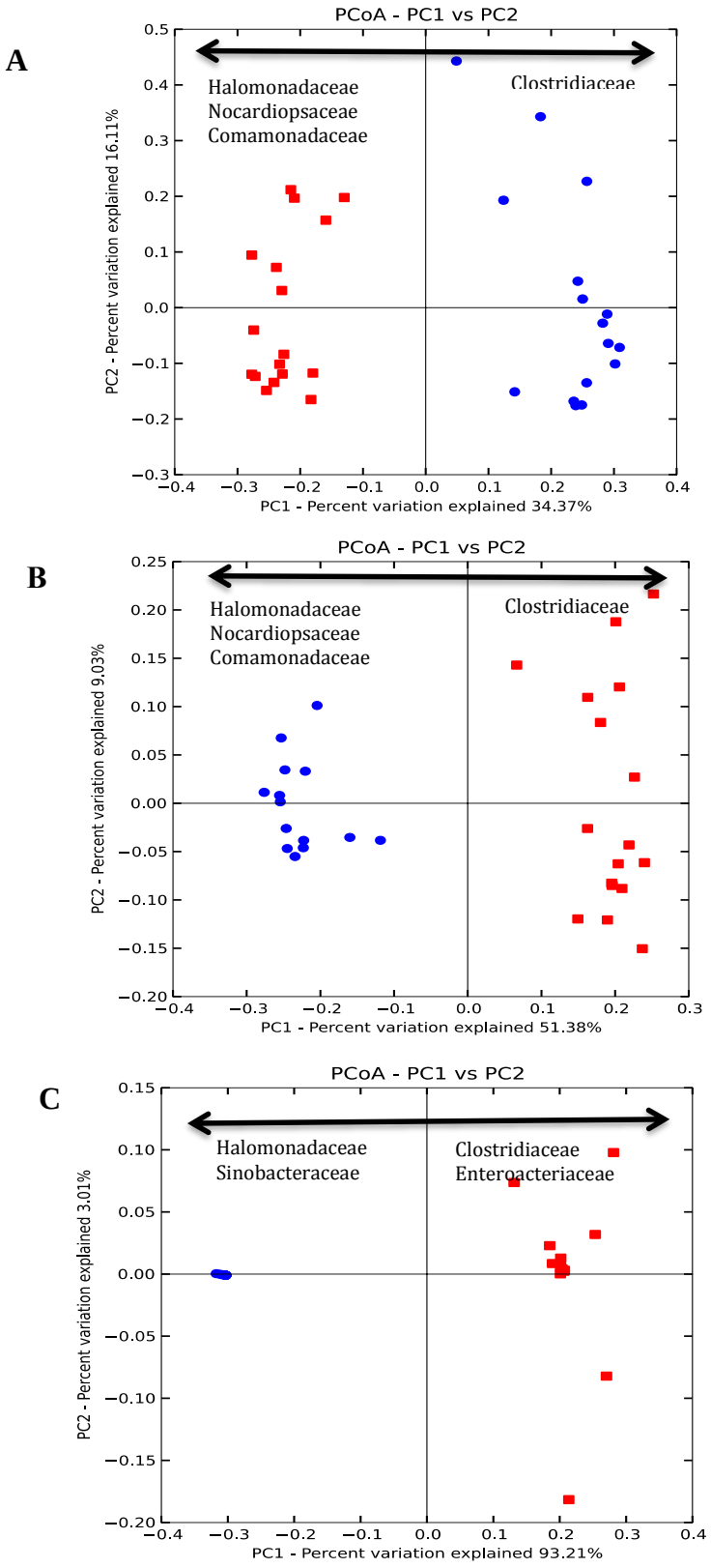

Figure 5.5 PCoA clustering of unweighted rarefied gut and faecal communities for the core 50% (A), 70% (B) and 90% (C) of taxa. Left = gut samples, right = faecal samples. Overlap between communities has reduced the number of samples visible.

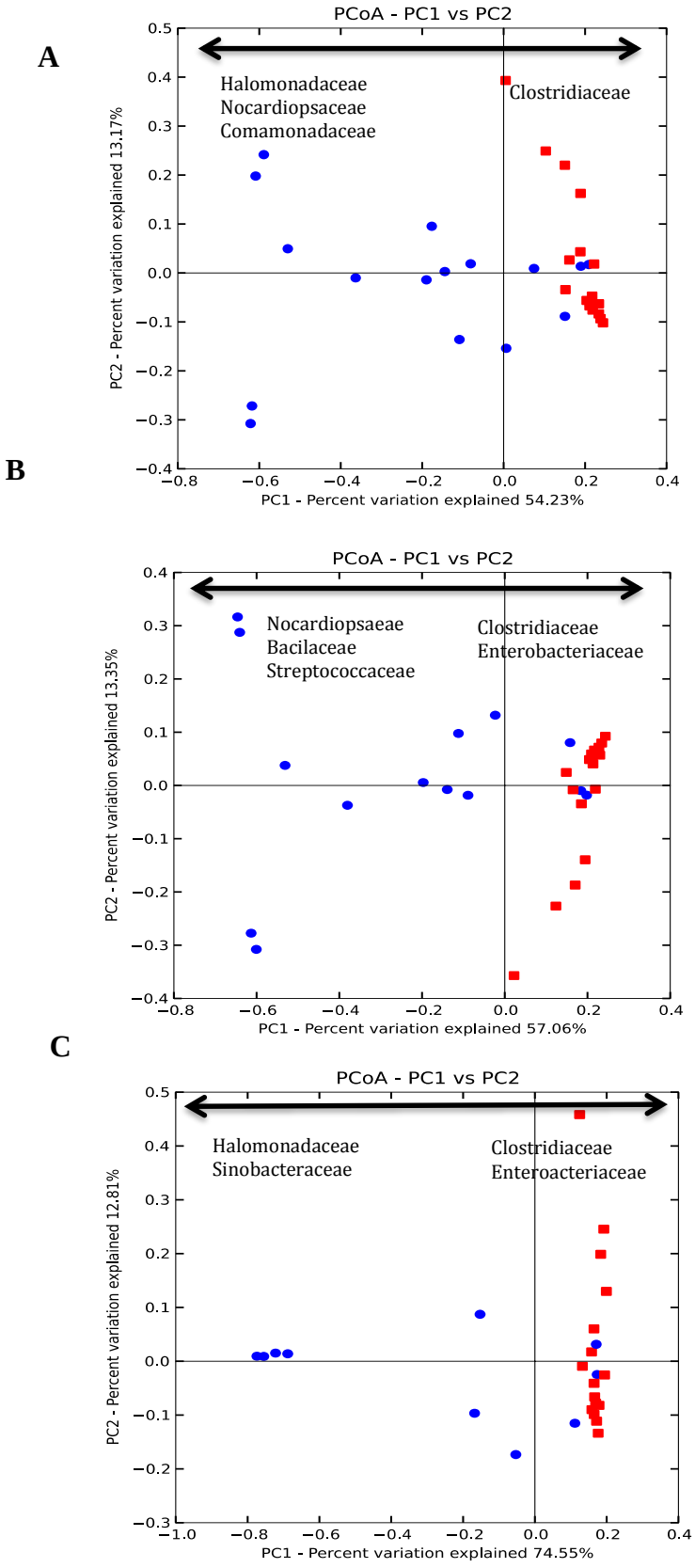

Figure 5.6 PCoA clustering of weighted gut and faecal communities for the core 50% (A), 70% (B) and 90% (C). Left = gut samples, right = faecal samples. Overlap between communities has reduced the number of samples visible.

5.48 The infection status of animals in this study

As relatively few animals were involved in this preliminary study, the number of animals in each category of infection and colonisation was low (Table 5.3). As culture work had not been completed by collaborators at UCD at the time of this analysis, a number of animals were classified as culture pending. All animals had a BCG vaccination status, had been tested by qPCR for faecal shedding of *M. bovis* and had been classed as having lesions, suspect lesions or no visible lesions in the lungs at post mortem. All further analyses were carried out at the sample level, using rarefied communities.

Table 5.3 The number of animals in each category of analysis.

Category	Number of animals
BCG vaccinated	4
***M. bovis* faecal shedding**	7
***M. bovis* culture positive**	3
Non-tuberculous mycobacteria culture positive	3
Culture negative	7
Culture pending	8
Lesions	3
Suspect lesions	6
No visible lesions	12

5.49 Clustering of faecal communities in animals shedding *M. bovis* in faeces

Weighted and unweighted PCoA analysis did not identify separation between faecal samples shedding or not shedding *M. bovis*, demonstrating there is no change in community composition with faecal shedding. ANOSIM analysis of shedding and non-shedding faeces found shedding and non-shedding groups are not separate in terms of taxa abundances ($R = -0.0205$, $p = 0.509$) and in terms of the taxa present ($R = 0.0554$, $p = 0.252$).

5.410 The effect of BCG vaccination on the gut and faecal microbiomes

Weighted and unweighted PCoA analysis for both faecal and gut communities separately did not identify clustering by BCG vaccination status, with no separation of communities from animals that had received oral BCG vaccination from those that had not received vaccination. ANOSIM found that communities from different vaccine treatments did not form separate grouping in gut samples when considering the abundance ($R = -0.0850$, $p = 0.769$) or presence/absence ($R = -0.1023$, $p = 0.868$) of taxa. The same was observed for faecal communities when examined by abundance of taxa ($R = -0.0316$, $p = 0.484$) and the presence/absence of taxa ($R = -0.004$, $p = 0.431$).

5.411 The effect of lung lesions on the gut and faecal microbiomes

There was no clustering by lesion status (present, suspect, no visible lesions) using weighted and unweighted PCoA for either gut or faecal communities. ANOSIM found that these groups were not separate groups in gut in terms of relative abundance ($R = 0.1389$, $p = 0.152$) or presence/absence ($R = 0.0789$, $p = 0.217$) of taxa. However, lesion groups did form separate groupings in faecal communities in terms of the presence/absence of taxa ($R = 0.3751$, $p = 0.013$) but not in terms of the relative abundance of taxa ($R = 0.0446$, $p = 0.332$). Despite these differences there was no visual separation in unweighted PCoA analysis which may be due to the small sample numbers of animals with lesions and suspect lesions.

5.412 Variation in specific taxa in the faecal microbiome with lesion status

46 OTUs varied between animals presenting lung lesions, those with suspect lesions and those without lesions. Most notably the mean number of sequences per sample of members of the Clostridium genus were significantly higher in animals with suspect lesions (mean = 3847) compared with animals not presenting lesions (mean = 3) and those with lesions (mean = 1). Members of the family Clostridiaceae, genera SMB53 and 02d06 were overrepresented in in animals with lesions (means = 6053 and 2270 respectively) compared to those without lesions (means = 4277 and 1360) and those with suspect lesions (mean = 2492 and 288). Members of the Streptococcus genus were present at significantly higher abundances in animals without lesions (mean = 1036) compared to those with lesions (mean = 65) and with suspect lesions (14).

Particular members of the family Enterobacteriaceae were at low abundance in animals with lesions but absent in those without lesions and with suspected lesions. Other members of the Streptococcus genus were present at low abundance but absent in animals without lesions and those with lesions. Twenty-six of the forty-six variable taxa are members of the order Clostridiales, seven were in the Enterobacteriales and four in the Lactobacillales. Three members of the Rhizobiales were present and all were overrepresented in animals that had no visible lesions.

Although these groups all varied significantly with lesion status, it was the differences in the presence/absence of OTUs that resulted in the separation of faecal communities with lesions, suspect lesions and no visible lesions. Of the

5633 OTUs identified 94.8% of them were absent in at least one of the lesion groupings. All OTUs that were absent in at least one group were at very low abundance in the other groups, with all having fewer than 200 sequences per sample on average and include all groups previously identified as differing in abundance between these groupings.

5.413 The effect of infection status on gut and faecal communities

On the basis of the results of culture from the lungs communities were divided into those samples positive for *M. bovis*, positive for non-tuberculous mycobacteria, negative samples and those with culture results pending. Due to time limitations the analyses were carried out before all culture results had been obtained. All further analyses were carried out without samples where culture results were pending.

Weighted and unweighted PCoA analysis did not show separation between these groupings in either gut or faecal communities. Gut communities do not form separate groups based on this classification when in terms of the relative abundance of OTUs ($R= 0.028$, $p=0.417$) or the presence/absence of OTUs ($R= -0.2493$, $p=0.988$). Faecal communities do not form separate groups based on this classification when in terms of the relative abundance of OTUs ($R= 0.0497$, $p=0.324$) or the presence/absence of OTUs ($R= 0.0234$, $p=0.390$).

5.414 The effect of *M. bovis* infection on gut and faecal communities

Samples were classified as being from an animal that was positive or negative for *M. bovis* by culture. By this classification gut communities did not differ in terms

of the relative abundance of taxa (R= -0.1455, p=0.832) or in terms of the presence/absence of taxa (R= -0.1667, p=0.906). Faecal communities also did not differ in terms of the relative abundance of taxa (R= 0.2853, p=0.101) or in terms of the presence/absence of taxa (R= 0.2312, p=0.156). Neither weighted nor unweighted PCoA analysis showed separation by *M. bovis* infection status in either gut or faecal communities.

5.415 The effect of mycobacterial colonisation on gut and faecal communities

Animals were divided into those with lungs colonised by any mycobacteria and those without colonisation. Clustering was not observed by these groupings in either weighted or unweighted analysis for both gut and faecal communities. The relative abundance of OTUs in these groups did not differ significantly in gut (R= -0.040, p=0.525) or faecal (R= -0.1360, p=0.945) communities. The presence and absence of OTUs in these groups also did not differ significantly in gut (R= -0.1227, p=0.889) or faecal (R= -1.067, p=0.807) communities.

5.5 Discussion

The results presented here demonstrate that complete gut and faecal communities are not comparable and that faecal communities from different animals are more similar to each other than paired gut and faecal communities from the same animal. PCoA analyses highlight the separate clustering of gut and faecal samples both in terms of the absence and relative abundance of taxa, and the lack of clustering of paired communities. Examining the core microbiome at various levels (50, 70, 90%) compared with the whole and core gut communities (50, 70, 90%) increases the tightness of clustering by sample type and reduces the correspondence between the gut and faecal microbiomes.

Constructing the core microbiome at 50% results in 98% of OTUs and the vast majority of variation being removed from the complete communities, losing information without improving the similarity of gut and faecal communities. As in human studies (Turnbaugh *et al*. 2010), the core faecal microbiomes contained few of the OTUs present in the complete faecal microbiome. The small number of shared OTUs within sample types emphasises the diversity of these communities, where a small proportion of OTUs are present in at least 50% of samples of the same type. The ileum is known to be less diverse in mammals than faeces, with the terminal ileum containing between 10^7 – 10^8 bacteria (Balzola *et al*. 2010). Whilst this study focuses on the ileum, communities in other parts of the gut do vary and sections of the large intestine are likely to be more similar to faeces than sections of the small intestine, such as the ileum. It has been suggested that the faecal microbiome is composed of non adherent luminal populations and shed mucosal microbes (Eckburg *et al.* 2005) and the

presence of a small proportion of shared OTUs between gut and faecal communities support this suggestion.

Neither the gut nor faecal microbiomes were significantly altered by oral vaccination with BCG, *M. bovis* shedding in faeces or by *M. bovis* and other non-tuberculous bacteria in the lungs. If *M. bovis* were colonising the gut, large shifts in community structure would be observed as well as overrepresentation of mycobacteria in gut communities where *M. bovis* was detected in faeces. This was not observed, which supports the hypothesis that *M. bovis* in faeces originates from swallowing infected septum. Although the presence of lung lesions did not alter the gut microbiome it did alter the faecal communities with animals with lesions, suspect lesions and without visible lesions forming separate groupings. The majority of differences between these groups were caused by differences in the presence or absence of OTUs however, all OTUs that were absent in at least one group were at very low relative abundance. Several OTUs differed significantly in abundance between these groups but the variable OTUs were dominated by four taxa: the Clostridiales, Enterobacteriales, Lactobacillales. These three groups accounted for 89% of variable OTUs, of these the Clostridiales accounted for 56% of variation in faecal communities due to lesion status. The Rhizobiales are predominantly environmental organisms and in all cases were overrepresented in animals without lesions, this could potentially identify differences in behaviour and feeding in infected animals compared to healthy animals.

Considering that *M. bovis* and other non-tuberculous mycobacterial infections did not change gut or faecal communities, the changes observed with lesions may be indicative of changes with diminished health rather than a particular infection. It is rare for badgers to develop tuberculous lesions in their GIT (Corner et al. 2011) therefore, the changes in community with the presence of lesions in the lungs is most likely not due to colonisation of the gut by *M. bovis*. The number of animals in this study was small and included few animals exhibiting lesions or suspect lesions, which may have contributed to identifying community changes with lesions status. Further work on a larger number of animals exhibiting lesions is required to determine which taxa within these dominant groups are varying with lesion status. Methods with greater resolution, such as Illumina MiSeq sequencing to the genus level combined with oligotyping would identify lower level variable taxa and begin determining why they vary in the faeces of animals with lesions.

As taking samples of gut is invasive and often not possible, faeces are frequently used as a non-invasive proxy. However, care needs to be taken when assuming faecal communities are representative of gut communities or when assuming changes in the composition of the faecal microbitoa are indicative of changes in the gut microbiota. Similarly to other studies the work presented here displays the ability of faecal communities to identify changes in the health of the host that are not necessarily related to disease or disruption of the GIT. However, these results highlight that there is often low correspondence between the faecal and gut communities from the same animal and that whilst examining faeces can

reveal changes in animal health, these same changes may not necessarily be reflective of changes in gut communities.

6. General Discussion

6.1 Benefits of non-invasive monitoring by qPCR

The work presented in this thesis demonstrates the ability to use faeces as a tool for diagnosing and monitoring *M. bovis* and health changes in badger populations. It identifies that there are no significant changes in gut communities with *M. bovis* infection and faecal shedding; supporting the idea that faecal *M. bovis* is from swallowing infected sputum. The same qPCR assay was used to diagnose *M. bovis*, establish the extent and variability of the environmental pool in faeces and monitor the effect of vaccination in the field, demonstrating the versatility and applicability of this test as an epidemiological tool.

An advantage of this assay is that it is quantitative; giving a measure of disease burden that is strongly correlated with the prevalence of infection in the population as measured by immunoassay. Furthermore, this assay quantifies shedding, a measure of actively infectious individuals, and therefore identifies groups that are more likely to be responsible for onward transmission. Other work in the group has found that high levels of faecal shedding is correlated with the severity of disease as described at post mortem (Travis et al. 2015), suggesting that the highly shedding groups identified here may contain members that have the largest disease burden and are therefore potential targets for control. As this method is non-invasive it allows a much larger number of animals to be sampled and removes biases and ethical considerations involved in capture. Using non-invasive field sampling less field training is required than for trapping, with field staff only needing to locate setts and follow badger tracks to latrines, allowing this method to be used intensively and over a large scale. However, to accurately determine which sett a particular latrine belongs to, bait

marking would be required which would increase the labour required to carry out this test.

6.2 Limitations of non-invasive monitoring by qPCR

Although this assay has been demonstrated to be a valuable epidemiological tool, there are currently potential limitations to its use and areas that require further development including: the sampling intensity required, its use in low density and low prevalence populations, the costs involved, and the requirement of a contaminant level three facility.

Identifying correlations with trapping and variability in shedding at Woodchester Park involved collecting a large number of faecal samples consistently over a year, with a mean of 136 faecal samples per group. The intensity of sampling required potentially limits the use of this method as a tool for monitoring on a large scale. However, the ability to detect changes during an intervention strategy in Kilkenny, an area over one-hundred times larger than Woodchetser Park, with an average of 1.7 samples per sett is promising for application of this method on a large scale. Although the number of samples collected limited the differences that could be detected with BCG vaccination, it suggests that sampling may not need to be as intensive as the regime undertaken at Woodchester Park. However, the population density in Kilkenny is much lower than at Woodchester Park, and collecting fewer samples is likely to be more representative in a low-density population than of a high density population, where a greater sampling intensity is likely to be required.

The number of faecal samples required to detect a positive social group will vary with the prevalence of disease in that population. This work has established that in a high-density population, a high prevalence social group can need as few as 4 samples to determine it is positive however, in lower prevalence groups as many as 50 may be required. Analysing a large number of samples will reduce the specificity of this assay by increasing the probability of obtaining false positives. However, as false positives are introduced by contamination in the laboratory it is possible to maintain the sensitivity of the test by introducing known negatives at the DNA extraction stage and checking each batch of DNA extractions for contamination. In low density populations as few as one sample was required to detect a social group as positive however, it is highly likely that more setts would have been identified as positive should more samples have been collected. As the Kilkenny and Woodchester Park populations both have high prevalences of disease, a greater number of samples would be required to diagnose a group as positive and estimate the prevalence of *M. bovis* in a low desnity population. Furthermore, the intermittent nature of shedding means that if few samples are collected, it is possible that social groups will need to be visited on multiple occasions to detect those groups that are positive. Depending on the aims of the study the sampling intensity could be reduced by only re-visiting negative social groups, but estimates of prevalence may require multiple re-sampling events.

One of the benefits of non-invasive sampling is that highly trained field workers are not required however, containment level three facilities and appropriately trained laboratory stuff are required to carry out DNA extractions. This may limit where faecal samples can be analysed and constrain the speed at which samples

can be processed. Using the DNA extraction method utilised in this work between 24 - 48 faecal samples can be processed in a day by an experienced operator. New, high throughput DNA extraction methods are in development in our group to increase the number of samples processed daily to 192 per operator. Alternatively, or in conjunction with high throughput extraction methods, mixing separate samples together before processing may allow a greater number of samples to be processed however, with the heterogeneity of *M. bovis* distribution in faeces and the small amounts (0.1g) used per extraction the probability of detecting positives is likely to reduce considerably.

Although not necessarily a limitation, the best performance and utilisation of this test is at the population rather than individual level. Due to the intermittent nature of shedding, immunoassays are more appropriate for individuals as qPCR is likely to produce a substantial number of false negatives. Nonetheless, this qPCR assay could be utilised as replacement or an addition to culturing from sputum or faeces as it is more sensitive than culture at detecting shedding. If faecal samples could be trace to individuals that deposited them, then this qPCR assay could also be used for detailed behavioural studies into movement and latrine use. Such studies would improve understanding of the effects of this disease on animal behaviour and further knowledge of spread, as behavioural studies are currently based on collaring and monitoring (Garnett et al. 2005; Cheeseman & Mallinson 1981) which are limited by small sample number.

Environmental badger faeces have been previously used to identify individuals (Wilson et al. 2003; Frantz et al. 2005; Frantz et al. 2003; Carpenter et al. 2003)

however, as genotyping errors are common when using DNA from faece (Wilson et al. 2003; Note 2002; Piggott 2004; Broquet et al. 2006) several PCR reactions are required per faecal sample which limits it applicability on a large scale (Wilson et al. 2003). Furthermore, work as part of this PhD project identified that microsatellite typing of badger faeces was not sufficiently reliable or reproducible to use for large scale monitoring (data not shown). The lack of reliability or reproducibility may be the result of using DNA extraction methods optimised to extract *M. bovis*, which may damage mammalian DNA or may not be optimal to extract sufficient quantities of mammalian DNA.

6.3 Questions raised by the variability in patterns of shedding

While this study has identified differences in the number of *M. bovis* genome equivalents shed between groups and seasons, the relationship between these differences, disease severity in individuals and its role in transmission is currently unknown. Although recent work suggests that high levels of shedding in individuals is related to advanced disease, the majority of shedding individuals do not have lesions at post mortem (Travis et al. 2015). As the number of *M. bovis* positive badgers in the study was low, further work is required to establish patterns.

Furthermore, the relative transmission potential of social groups with very high levels of shedding merits investigation as it has important implications for control. It is well recognised that for many infections particular individuals, superspreaders, are responsible for a disproportionate level of transmission (Lloyd-Smith et al. 2005; Matthews and McKendrick, et al. 2006; Lawley et al.

2009). Using qPCR is has been possible to identify social groups and setts that shed larger quantities of *M. bovis* than other groups. Although individuals were not identified in this study but the distribution of shedding in individual faecal samples in Woodchester and Ireland highlights that there are a small number of individuals shedding large quantities of *M. bovis* in faeces. This raises the question of the relative contribution to transmission of the few individuals shedding large quantities of *M. bovis* compared to the majority of shedding animals that appear to be shedding moderate amounts of *M. bovis*. The importance of shedding levels for disease transmission has already been identified for other disease systems (e.g. Matthews and Low, et al. 2006; Matthews et al. 2006; Lawley et al. 2009; Lawley et al. 2008; Pradhan et al. 2011; Capparelli et al. 2009) but is yet to be investigated for *M. bovis* transmission between badgers and from badgers to cattle.

In order to pose a risk for transmission *M. bovis* must remain in a viable state in the environment over a period of time. Although this study did not assess the viability of environmental *M. bovis*, previous culture (Cheeseman et al. 1985; Wilesmith et al. 1986; Hewson & Simpson 1987), immunomagnetic capture (Sweeney et al. 2006), RNA based (Young et al. 2005) and infection studies (Ghodbane et al. 2014; Courtenay & Wellington 2008) have all identified viable environmental *M. bovis* surviving for varying periods of time. As a greater prevalence of *M. bovis* in environmental faeces has been identified here than in previous studies (Cheeseman et al. 1985; Wilesmith et al. 1986; Hewson & Simpson 1987), it is likely that by using methods other than culture, the survival time of *M. bovis* in soil is likely to differ from previous studies. Several studies

have detected pathogens using qPCR where several or all culture attempts were negative (e.g. Slana et al. 2008; Slana et al. 2009; Kawaji et al. 2007). Given the low infective dose in cattle, which can be as few as one cell via inhalation (Menzies & Neill 2000), it seems likely that, even discounting for potential non-viability and free DNA, that badger faeces could be a significant source of infection to cattle. As it is possible for DNA to survive in the environment for long periods of time (Levy-Booth et al. 2007; Nielsen et al. 2007), not all genomic equivalents detected will correspond directly to the number of *M. bovis* cells in the lung of an animal at a given time point. The relationship between the number of genome equivalents shed and the number of viable cells present is an important one to establish for further development of this method and to further broaden its use. Several studies find fewer viable cells than qPCR genome equivalent counts (e.g. Delgado-Viscogliosi et al. 2009; Kärkkäinen et al. 2010; Haugland et al. 2005) and it is expected this would be the same in this system. Future work could examine the ratio of 16SrRNA/rDNA which is a measure of active cells present or the permeability to an intercalating dye, propidium monoazide (PMA) could be used to confirm the presence of live cells (Fittipaldi et al. 2012). These methods could be applied to fresh faeces and samples that have been left to age in natural conditions to determine changes in viable cell number over time and how this relates to genome equivalents measured by qPCR.

The level of contact cattle have with infected badger excreta will also affect the potential for transmission. The probability of transmission for a given encounter with infected badger faeces has been estimated as low (Benham & Broom 1989)

however, given the prevalence of *M. bovis* in faeces identified in this study, the building evidence that cattle investigate badger faeces (Moses 2015; Davies 2015), and the low percentage of cattle cases contracted directly from badgers (Donnelly & Nouvellet 2013), it seems plausible that this could be a significant route of transmission. Furthermore, as badgers are observed sniffing faeces in latrines (Roper 2010a), the environment may also be an overlooked transmission route between badgers. Environmental transmission has also been identified as a potential transmission route of *M. bovis* in white tailed deer.

6.4 Questions raised by environmental monitoring of oral BCG vaccination

In addition to raising questions concerning the level of sampling needed to detect changes in shedding in a population the results of this vaccine trial also raise questions about the viability of oral vaccination in the field as a control mechanism. Although there were trends of decreasing environmental *M. bovis* with increasing BCG vaccination, these failed to reach statistical significance. Whether the observed effect sizes as those observed here would be sufficient to reduce transmission to cattle to great enough extent to outweigh the cost of vaccination is not known, but it is likely that larger effects would be required to justify the expense. However, the limited effects detected may be as a result of the low intensity of sampling, with greater numbers of samples it may have been possible to detected greater differences between treatments. Oral vaccination has been successfully used to control other wildlife diseases such as rabies in Europe (Cross et al. 2007) and brucellosis in bison in Yellowstone park (Treanor et al. 2010) and oral BCG delivery in the field is efficacious in reducing *M. bovis* in brushtail possum populations in New Zealand (Corner et al. 2001) which is

promising for further development of vaccination as a control method in badger populations.

6.5 Animal heath and the gut and faecal microbiota

The differences identified between the gut and faecal communities of badgers highlight the limitations of inferring changes in gut communities from examining faecal communities as well as emphasising the ability to detect health changes in animals by examining faeces and suggesting an potential behavioural change with ill health that is not related to a particular disease. As gut samples are invasive and difficult to obtain, faeces will need to be used to examine changes in communities with altered health however, the results but must be interpreted with caution. As *M. bovis* rarely colonises the GIT of badgers infection and shedding were thought to be unlikely to alter either the gut or faecal microbiome and this was confirmed. Oral BCG vaccination also did not alter the abundance of the mycobacterial community in the gut or faeces, confirming that faecal *M. bovis* does not originate from the GIT. However, the use of the 16S rRNA pyrosequencing and the relatively small number of samples available limited the resolution achievable in this study. Information on the presence of lesion and/or *M. bovis* infection in all culled animals is expected to be available within a year, allowing a larger study targeted at animals with differing lesion status to be examined in higher resolution. Illumina Mi-Seq technology targeted at the same V1-V3 region for comparison with the current study and also targeted sequencing of more variable regions specific to variable groups identified in this study and analysed using oligotyping could be to differentiate the species

responsible for these changes and gain a better understanding of the changes occurring with reduced health.

6.6 Relevance to policy

Control of *M. bovis* in brushtail possums in New Zealand has been through intensive culling programmes (Nugent et al. 2015), however, these animals are not native to New Zealand and are seen as pests, making it more publically acceptable to cull (Waters et al. 2012). There is opposition in the UK to badger culling and although this opposition is not present to the same extent in the RoI, badgers are protected species in both counties and therefore culling cannot be a long term control strategy (Gormley & Collins 2000). Badger culling is part of the control policy in the RoI and culling is continuing is two areas of England (Defra 2014c). In England, removing enough animals has proven challenging and has only been met in one area, once during the first two years of culling. Should targeting animals remain challenging, qPCR could potentially direct culling to specific areas or setts which have a high prevalence of disease in order to maximise the number of infected animals being removed. With further optimisation, this test could also be implemented to monitor the effect of culling on infection in badger populations both in the UK and RoI. Furthermore, this tool may be used as a method of surveying badger populations in order to identify areas that may potentially become at risk of *M. bovis* transmission to cattle or to further understand the differences in risk posed by badgers in high incidence, low incidence and edge areas.

To further develop and apply this method it could be applied to vaccination programs such as the Badger Edge Vaccination Scheme (BEVS) (Defra 2014d) to monitor the effect of vaccination in these populations and to identify areas that may benefit most from vaccination. However, as the work presented here has highlighted, a large number of setts would need to be intensively sampled in order to detect changes in shedding after vaccination. Although in this study oral BCG vaccination did not significantly reduce the prevalence of *M. bovis* positive faeces, this method of control likely holds the most promise as an alternative to culling due to the cost and impracticalities of intramucular vaccination. Although some variables such as dose, coverage, bait placement, uptake by non-target wildlife and prior exposure to environmental Mycobacterium species and *M. bovis* will be difficult to control and all require further research. Suitable baits have been created for delivery to white-tailed deer (Waters et al. 2012) and application in the environment has been viable for delivery to other species, including sachets nailed to trees for brushtail possoms and in selective feeders that target wild boar piglets (Gortazar et al. 2011; Gortazar et al. 2014). In other disease systems such as rabies, oral vaccination schemes have utilised aerial delivery has been to cover large areas (Waters et al. 2012) however, due to the potential for cattle exposure to vaccine, this is unlikely to be a viable delivery system for BCG vaccines.

6.7 Future work and potential implementations

The work presented here has identified this qPCR assay as a valuable monitoring tool for the levels of infection and changes in environmental shedding over time at the population level. As discussed previously future work could apply this

technique to further monitoring intervention strategies such as culls and other vaccination programmes in addition to surveying badger populations. Furthermore, the test is not limited badger faeces and has been successfully used on goat faeces, soil, water, untreated milk and dust (Yu-Jing Hung, personal communication). A recent model identified that the majority of within-farm transmission is through the environment (Brooks-Pollock et al. 2014), although this may not necessarily be through infected badger material, and so farm surveys including pasture, slurry, feed and water troughs may provide more insight into sources of infection and new directions for control. Preliminary qPCR testing of a small number of cattle faeces has identified *M. bovis* in cattle slurry (data not shown), highlighting the need to investigate other areas of transmission and persistence. The importance of pathogen survival in farms has been noted in other disease systems for example, *E. coli* O157 has been shown to survive in milking areas and grass on farmland (Porter et al. 1997).

Further development should focus on the application of this assay to low density and low prevalence populations in addition to increasing the number of samples that can be processed. Studies similar to those carried out at Woodchester Park could take place in low prevalence populations in low incidence areas of the UK, and RoI to determine the level of sampling required for low density high prevalence populations. These studies would provide better estimates for the number of samples required to detect disease and variability in populations. To summarise, using molecular methods to monitor *M. bovis* in faeces can diagnose and monitor changes in *M. bovis* in badger populations, making it is a potentially valuable tool for epidemiological studies. Future work is required to determine

the bacterial species altering with poor health and to further develop the qPCR assay. Furthermore, success in other systems and the efficacy of intramuscular vaccination in the field are promising and suggests that with optimisation and further trials, oral BCG vaccination may a viable control method.

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Appendix A

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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

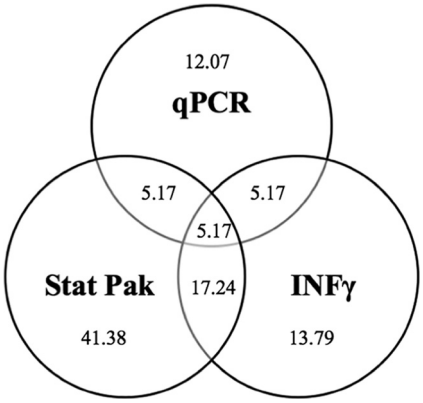

FIG 1 Percentage agreement between positive test results. There were 120 total trappings, with 60 positive trappings for which at least one test gave a positive result.

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%.

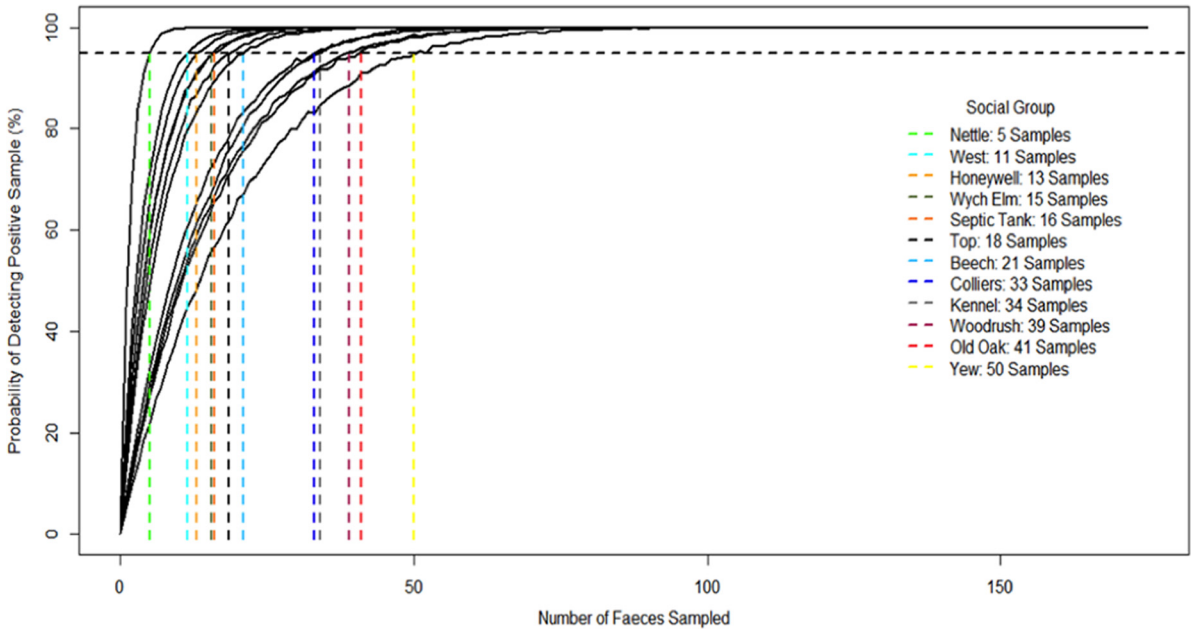

FIG 2 Numbers of fecal samples required to detect a positive social group across a year.

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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Appendix B

SCIENTIFIC REPORTS

OPEN

The variability and seasonality of the environmental reservoir of *Mycobacterium bovis* shed by wild European badgers

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The incidence of *Mycobacterium bovis*, the causative agent of bovine tuberculosis, has been increasing in UK cattle herds resulting in substantial economic losses. The European badger (*Meles meles*) is implicated as a wildlife reservoir of infection. One likely route of transmission to cattle is through exposure to infected badger urine and faeces. The relative importance of the environment in transmission remains unknown, in part due to the lack of information on the distribution and magnitude of environmental reservoirs. Here we identify potential infection hotspots in the badger population and quantify the heterogeneity in bacterial load; with infected badgers shedding between $1 \times 10^3 - 4 \times 10^5$ *M. bovis* cells g^{-1} of faeces, creating a substantial and seasonally variable environmental reservoir. Our findings highlight the potential importance of monitoring environmental reservoirs of *M. bovis* which may constitute a component of disease spread that is currently overlooked and yet may be responsible for a proportion of transmission amongst badgers and onwards to cattle.

The incidence of *Mycobacterium bovis* in cattle herds in Great Britain (GB) has increased from 0.01% in 1979[1] to 4.8% in 2012[2]. Control and compensation has cost the taxpayer £500 million over the past decade and this is predicted to increase to £1 billion over the next 10 years if further geographical spread is observed[3]; making bovine tuberculosis one of the most economically important animal health problems in the UK farming industry[4].

The European badger is a wildlife reservoir involved in the transmission of *M. bovis* to cattle in the UK and RoI[5,6]. Once infected, badgers may intermittently shed *M. bovis* cells in sputum, faeces and urine[7]. One likely route of transmission to cattle is through exposure to infected badger urine and faeces. Although the location and extent of environmental *M. bovis* has not been previously quantified, indirect contact with infected faeces and urine may be an important exposure pathway[8]. *M. bovis* genomic DNA can survive outside the host for up to 21 months[9] and cells have been shown to be viable by culture from mice fed soil in which *M. bovis* had been persisting for months[10]. The survival of shed *M. bovis* cells is likely to vary in space and time in relation to local environmental conditions and the distribution of infectious badgers. Understanding patterns in environmental contamination (defined as the presence of

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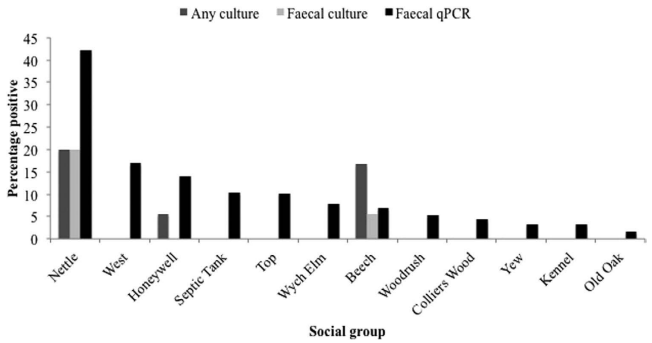

Figure 1. Percentage positive badgers per social group determined by any culture positive (tracheal or faecal) or faecal culture compared with positives by faecal qPCR. Data aggregated across the entire year.

Social group	Percentage positive faeces by qPCR	Cumulative genome equivalents in faeces	Percentage positive IFNγ	Percentage positive Stat Pak	Percentage positive IFNγ or Stat Pak
Nettle	42.2	1.08×10^6	60	100	100
West	16.9	1.48×10^6	33.3	20	40
Honeywell	13.9	4.08×10^5	50	50	66.7
Septic Tank	10.3	4.57×10^5	40	20	60
Top	10.1	9.00×10^5	20	90	90
Wych Elm	7.8	4.19×10^4	0	0	0
Beech	6.9	4.98×10^5	41.2	29.4	44.4
Woodrush	5.3	1.45×10^5	0	33.3	33.3
Colliers Wood	4.3	8.83×10^4	0	0	0
Yew	3.3	2.25×10^4	0	40	40
Kennel	3.2	2.76×10^4	0	20	20
Old Oak	1.6	2.56×10^5	0	31.3	31.3

Table 1. Summary of *M. bovis* genome equivalents counts by social group from faecal field sampling and immunoassay testing results on trapped badgers.

M. bovis genome equivalents in the environment) could aid in the design of more effective interventions, currently based on culling and vaccination strategies.

The availability of a method to quantify relative levels of environmental contamination with *M. bovis* could open up possibilities for monitoring spatial and temporal variation in risk and may help direct the implementation of disease control interventions. Currently the only means of measuring levels of infection in badger populations is through trapping and testing with BrockTB Stat Pak® (Stat Pak)[11], Interferon gamma (IFNγ)[12] and culture of clinical samples[13]. Cultivation, particularly from faecal material, has low sensitivity and is qualitative (Fig. 1). A qPCR method for non-invasive environmental monitoring of shedding was developed in our group[14,15]. This qPCR assay quantifies faecal shedding, a measure that correlates strongly with the level of infection within a social group as measured by immunoassay (Spearman's rho = 0.92, $p < 0.001$)[16]. The only other non-invasive method for monitoring infection in badger populations is culture of faecal material, which is particularly insensitive (Fig. 1). Using this optimised qPCR assay we are able to report on the spatio-temporal reservoir of *M. bovis* from badger faecal shedding in a natural population over the course of a year. Badgers defecate in latrines within or at the edges of their territories[17] and hence they can be used to identify a defined population of animals.

Methods

Sampling and Trapping. Fresh faecal samples were obtained from latrines associated with 12 badger social groups (Table 1) in Woodchester Park Gloucestershire through 2012 and 2013. Two intensive sampling periods of two weeks each were undertaken during the period of peak badger latrine activity in the spring and autumn of 2012 where up to 10 unique faecal samples were obtained from latrines associated with each social group on alternate days. Faecal samples were taken from latrines in closest proximity to

the main sett of each social group. A second sampling regime was undertaken over a year long period where up to 10 unique fresh, faecal samples were taken from latrines associated with each social group per day over two non-consecutive days in each season, starting two days after trapping operations took place in that location. For the purpose of this study March—May was classified as spring, June—August as summer, September—November as autumn and December—February as winter.

Each of the 12 badger social groups in the study was trapped four times throughout the year, once per season, with variable numbers of animals trapped between groups and seasons (Table S1). Badgers were trapped using baited cage traps placed around the main setts of each social group and identified using a unique tattoo applied at the first capture of that animal. Trapped badgers from each of the 12 social groups were tested by BrockTB Stat-Pak®, IFNγ and culture of clinical samples. All experimental protocols were approved by the University of Warwick and the Food and Environment Research Agency Ethical Review Committee and carried out in accordance with the approved guidelines and under the license granted by the Home Office under the 1986 Animal (Scientific Procedures) Act.

DNA Extraction and qPCR. Total community DNA was extracted from 0.1 g (+/−0.003 g) of faeces 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 which targets the RD4 deletion region unique to the *M. bovis* genome (Specificity data Table S2). An initial qPCR screen of each sample was performed using an ABI 7500 Fast qPCR machine (ABI) with two technical replicates of each sample. Positive controls (8.5×10^5 genome equivalents) and negative controls were also present in duplicate on each plate. PCR reactions were set up using 900 nM of each primer (RD4F $^{5'}$TGTGAATTCATACAAGCCGTAGTCG$^{3'}$, RD4R $^{5'}$CCCGTAGCGTTACTGAGAAATTGC$^{3'}$), 250 nM of Taqman probe (6FAM-AGCGCAACACTCTTGGAGTGGCCTAC—TMR), 1 mg ml^{-1} bovine serum albumen (BSA), 12.5 µl of Environmental Mastermix 2.0 (ABI), 10 µl of template and made up to 25 µl with molecular grade water (Sigma Aldrich). PCR cycling conditions were 50 °C for 2 min followed by 95 °C for 10 min then 40 cycles of 95 °C for 15 sec 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 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. An inhibition control assay previously described[14] was used to detect the possibility of false negative results due to inhibition. Where significant inhibition was detected DNA was re-extracted from frozen aliquots and qPCR assays were repeated. The number of *M. bovis* genome equivalents was quantified independently by qPCR at The University of Warwick and APHA Weybridge (Supplementary Figure 1).

Data Analysis. All data analysis was performed using the statistical program R. Logistic regression with social group (Old Oak) as the baseline was used to determine whether the number of positive samples varied amongst social groups throughout the year. Binomial generalised linear models (GLM) were performed to determine differences in *M. bovis* cells numbers shed between groups and between seasons. For spring two sampling days per social group were chosen to represent cross sectional sampling. Variability within groups was determined by calculating the median, upper and lower quartiles and range for each soil group.

The probability of detecting a false positive rate was 2%, calculated using known negative faecal samples obtained from captive badgers at APHA which were routinely tested for bTB using IFNγ. Negative samples were double blinded and randomly introduced into the experiment at both centres. The probability of detecting x false positive for a given number of samples was calculated using equation 1 where $p(x)$ = the probability of exactly x false positives, f = the false positive rate, n = the number of samples and x = the number of false positives.

$$p(x) = \frac{n!}{(n-x)!x!} \times (1-f)^{n-x} \times f^x \quad (1)$$

The number of confirmatory re-extractions (e) needed to result in the probability (p) of exactly x false positives was calculated using equation 2.

$$p(x) = \left(\frac{n!}{(n-x)!x!} \times (1-f)^{n-x} \times f^x \right) \times (1-f)^e \quad (2)$$

Results

Infection levels within social groups. During the study, 53.6% of trapped badgers were *M. bovis* positive by Stat-Pak, IFNγ or culture. By qPCR faecal samples from every social group examined were found to be positive (Fig. 2). Although the percentage of infected faecal samples varied considerably (Table 1, Table S3), the numbers of *M. bovis* genome equivalents per faecal sample also varied widely ranged from 1×10^3 to 4×10^5 per gram of faeces (Table 1).

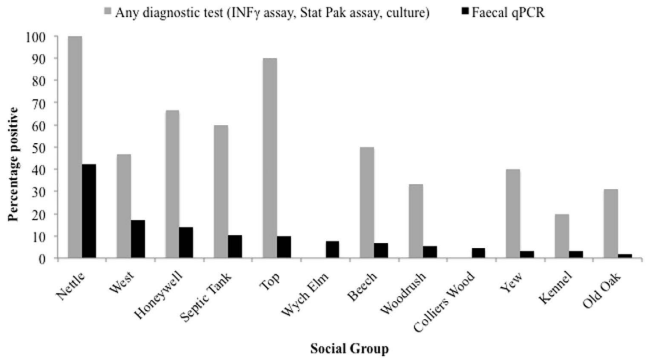

Figure 2. The percentage of badgers positive by any diagnostic tests compared to the percentage of positive faecal samples by qPCR per social group.

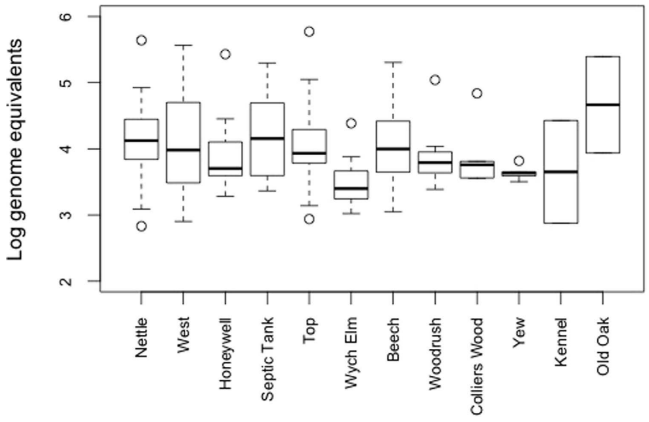

Figure 3. Distribution of *M. bovis* genome equivalents in positive samples by social group.

Significant variability in genome equivalents was identified both within and between social groups (Fig. 3) with social groups Nettle, Top, Septic Tank and West shedding more cells over the year than the other social groups (Table 1). Social groups with a high percentage of positive samples consistently shed amongst the highest cumulative numbers of *M. bovis* cells during the year (Table 1). Social group Old Oak was exceptional as it has one of the highest cumulative *M. bovis* genome equivalent values yet had the lowest percentage of positive samples in the study (Table 1). This distribution is consistent with the presence of a relatively small number of animals shedding large amounts of bacteria in some groups. However, as we could not assign faecal samples to individuals we cannot discount within-individual variation in shedding from being responsible for this observation. Hence the need for further research into heterogeneity in transmission risks amongst individual badgers.

Seasonal variability in *M. bovis* shedding. Overall a significantly greater number of *M. bovis* genome equivalents were shed in summer than in any other season. There were substantial seasonal differences in the cumulative number of *M. bovis* equivalents detected per social group (Fig. 4) with different groups identified as the largest contributors to the environmental pool of *M. bovis* throughout the year. Although summer had the highest number of genome equivalents overall, Septic Tank shed fewer cells in summer compared to other seasons and Top and shed more cells in spring. Nettle also shed fewer *M. bovis* genome equivalents in spring compared with the rest of the year. However, five social groups (Nettle, West, Honeywell, Septic Tank, and Top) were identified as having consistently high proportions of positive faeces and relatively large quantities of *M. bovis* bacilli shed (Table 1). This corresponds to immunoassay tests carried out on trapped badgers, which also identified these five groups as the most heavily infected (Table 1). Although there is strong correspondence between immunoassay and qPCR results there are some discrepancies, in particular Nettle and Top are 100% and 90% positive by immunoassay yet there was a large difference in the percentage of positive faecal samples with 42.2% and 10.0% respectively.

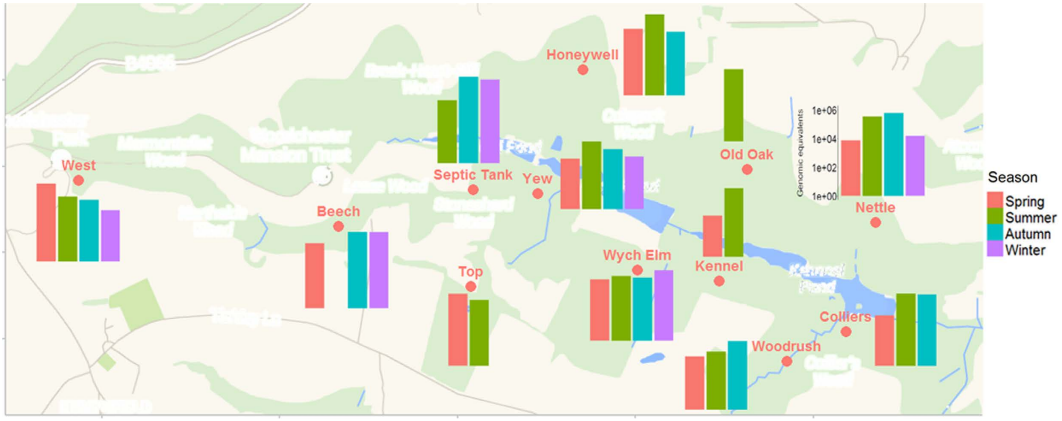

Figure 4. The cumulative *M. bovis* genome equivalents shed by each social group per season. Created in R version 3.0.2 using the packages ggplots 2[31] and ggmaps[32]. The scales for all graphs are identical.

Discussion

Detection of *M. bovis* by qPCR allows the presence of faecal shedding and hence infectious badgers to be established non invasively and raises the possibility of identifying infectious social groups. Unlike standard diagnostic tests the qPCR approach also quantifies levels of *M. bovis* shedding, providing opportunities to assess spatio-temporal variations in the environmental distribution of this potential source of infection for cattle, badgers and other wild mammals. Environmental transmission is likely to be a complex mixture of a number of factors including the infectious load of *M. bovis* in faeces and urine and changes in these reservoirs over time, proximity to cattle pasture, the frequency and type of contact cattle have with badger excrement and the age of faecal samples. The application of qPCR to further understand the epidemiology and transmission dynamics of bovine tuberculosis may be an important component in managing the advancing frontier between endemic and non-endemic cattle infection, and to inform transmission models (e.g. Brooks-Pollock *et al.* (2014)).

The heterogeneities observed in this study between social groups and the consistency with which five groups were identified as highly infected and shedding, suggesting that interventions targeted at particular high risk populations could have a larger impact than random and blanket control strategies[18]. However, we are mindful that any perturbation of badger populations could result in increased rather than decreased transmission[19,20]. The observed discrepancies in the percentage of positive faecal samples for social groups with similar prevalences of infection by immunoassay highlights the need for further work to establish the causes of these differences. Whilst heterogeneity in transmission is a well-known phenomenon, this study is one of the few empirical studies which have attempted to demonstrate the extent of this variability[21]. Although this study does not assess the viability of *M. bovis* in faeces, previous work has identified the presence of *M. bovis* 16S rRNA in soil[9] and badger setts and latrines[22]. In addition, studies have had a culture success rate of 2.5% from badger faecal samples[23] and *M. bovis* has been cultured from cattle faeces several months after excretion[24]. This indicates that at least a proportion of *M. bovis* cells shed in badger faeces can remain viable in the environment; however, further research is required to determine potential survival and transmissibility of *M. bovis* in environmental samples.

Whilst the focus in the UK and RoI is on badgers, other wildlife hosts are present[25,26]; however, little is currently known of their contribution to environmental reservoirs and their relative importance for transmission to cattle[25]. Issues controlling *M. bovis* are not confined to the UK and RoI. Worldwide there are problems with *M. bovis* in buffalo and lions in South Africa[27], possums in New Zealand[28], white tailed deer in America[29] and wild boar in Spain[30]. This non-invasive qPCR assay can be employed to detect shedding in other systems and samples types including milk, water and clinical tissues, is possible using this method. Whilst controlling and monitoring *M. bovis* in wildlife populations remains a challenge, non-invasive monitoring of environmental contamination may open up opportunities to identify spatio-temporal heterogeneity in disease risks and hence contribute to the development of suitable approaches fro disease control in livestock.

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Author Contributions

H.C.K. was involved with sample collection and processing, undertook statistical analysis, prepared tables and figures and wrote the manuscript with assistance from E.M.W. P.J. collected and processed samples and undertook statistical analysis. A.M. collected and processed samples and undertook statistical analysis. E.T. undertook statistical analysis and advised on the project. D.P. collected and processed samples. Y.H. produced figures for the manuscript. J.S. coordinated and oversaw processing of samples at A.P.H.A. Weybridge. J.C. processed samples at APHA Weybridge. R.J.D. contributed to the study design, reporting and data evaluation, organised and oversaw the fieldwork at APHA Woodchester. W.G. was involved with 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 set up and supervised all field and practical work and was responsible for biosafety.

Additional Information

Supplementary information accompanies this paper at http://www.nature.com/srep

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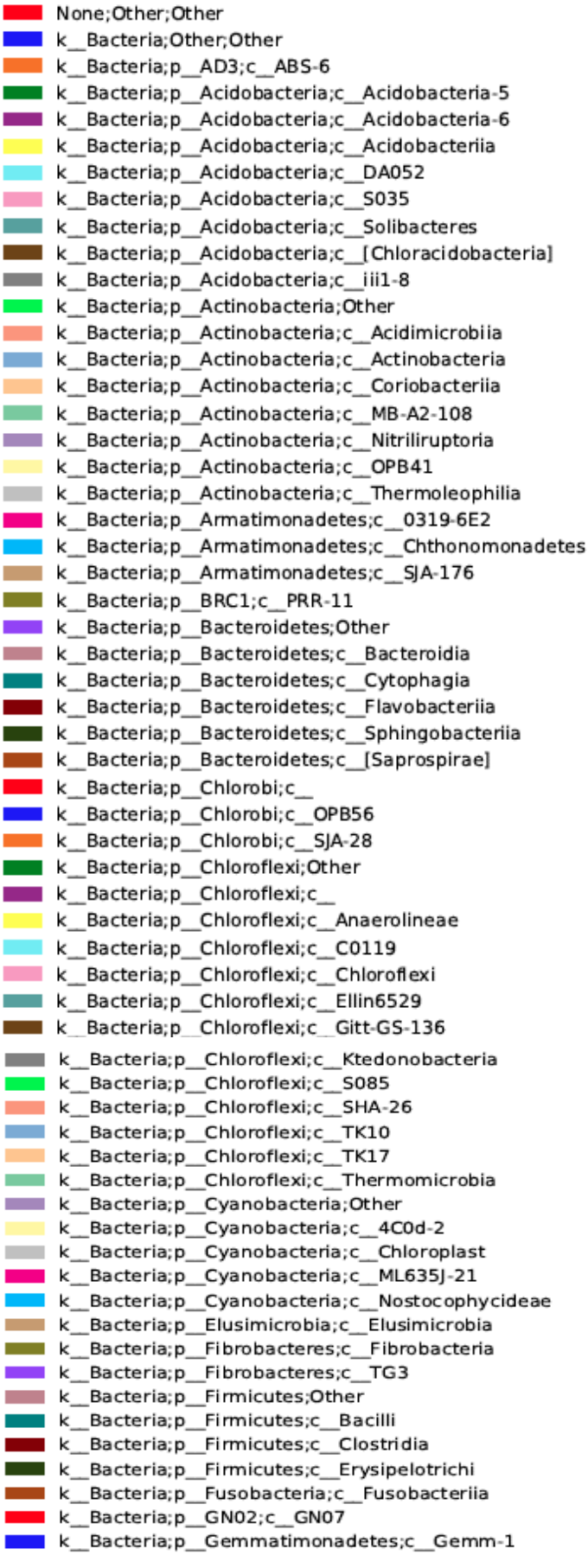
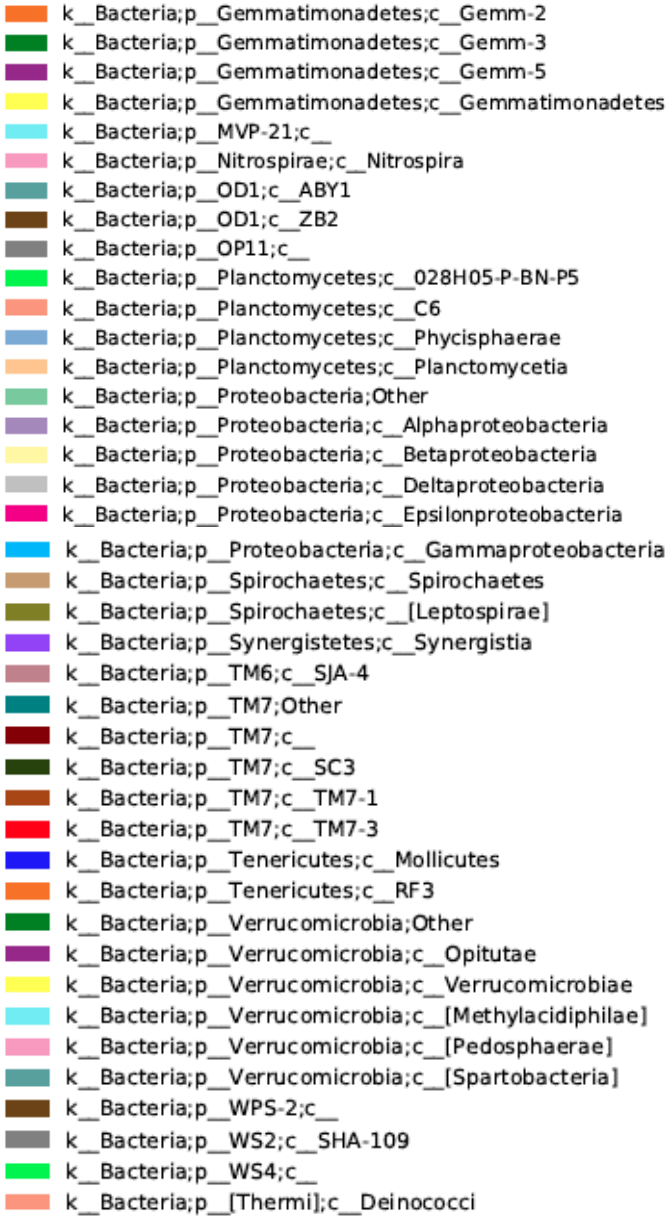
Appendix C

Key for figure 5.2. The classes present in badger gut and faecal samples.

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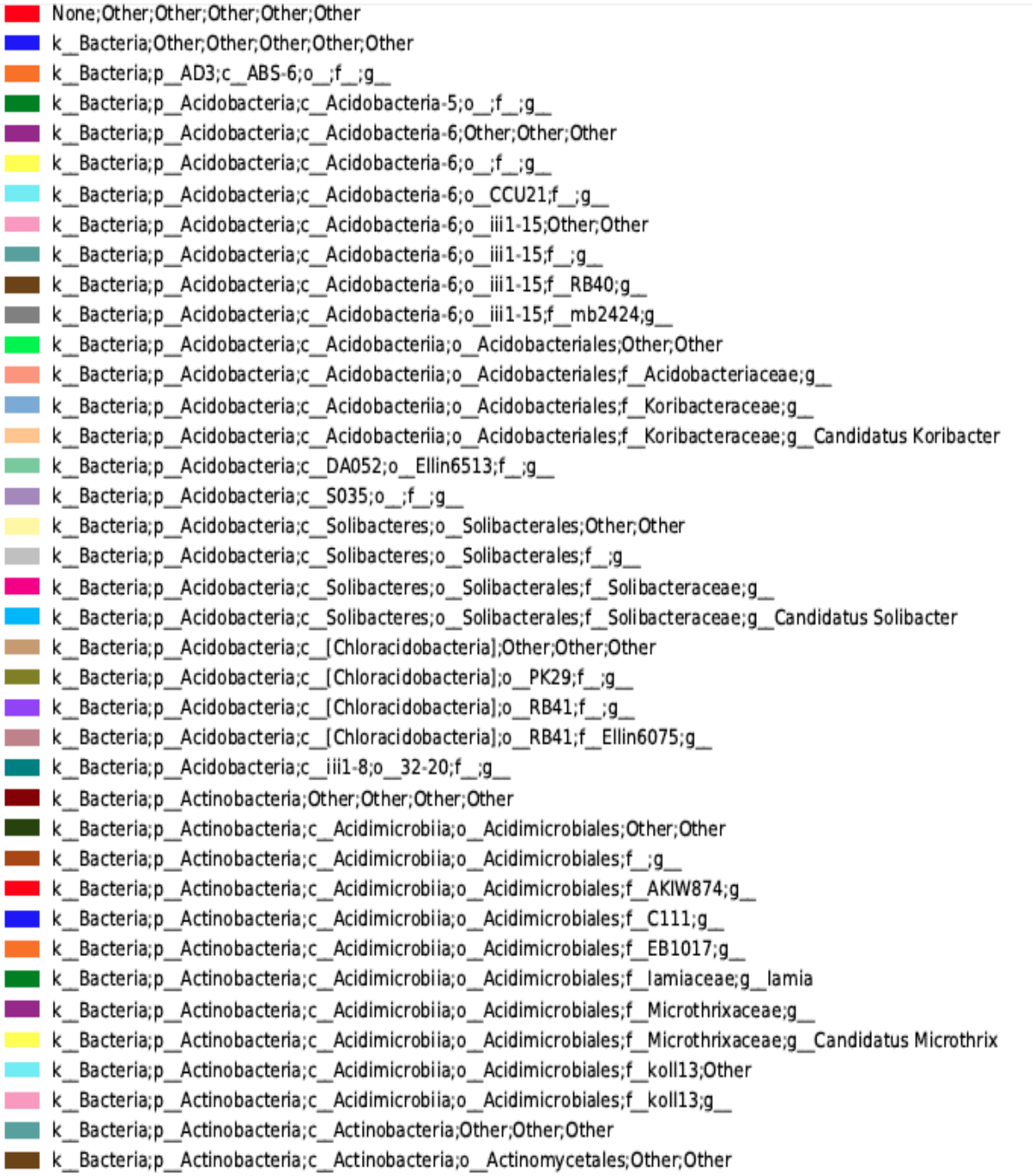
Key for figure 5.2. The genera present in badger gut and faecal samples.

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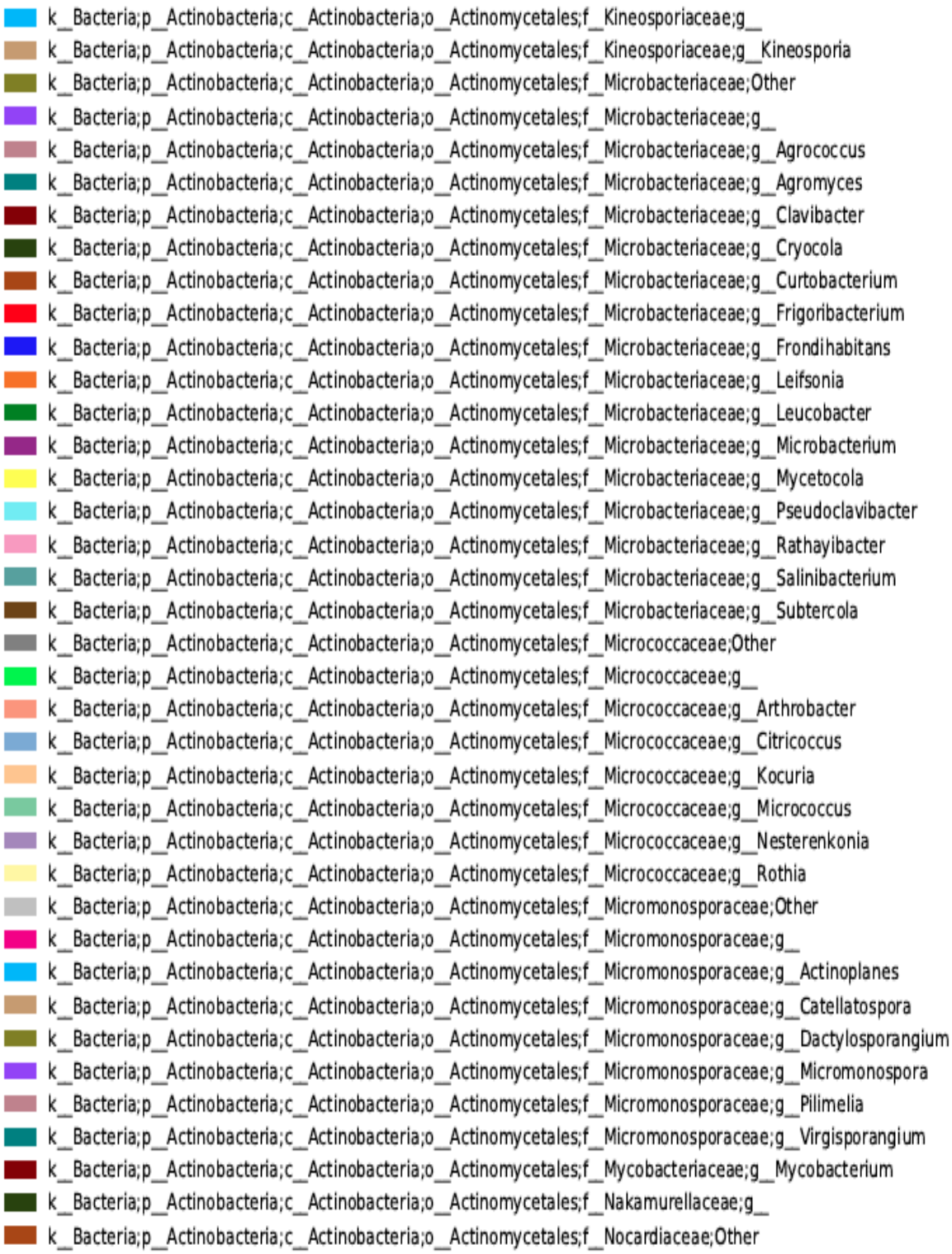
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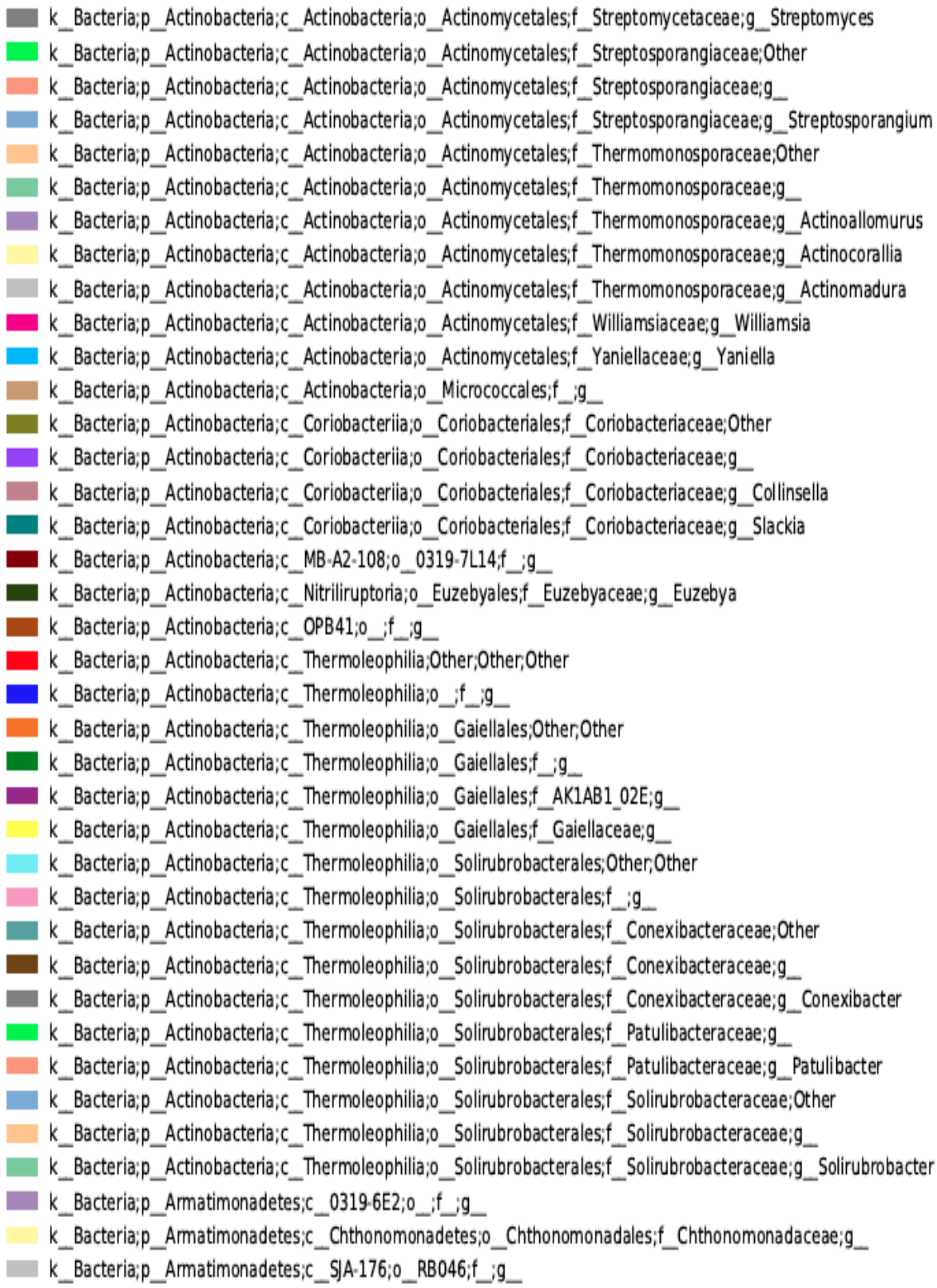
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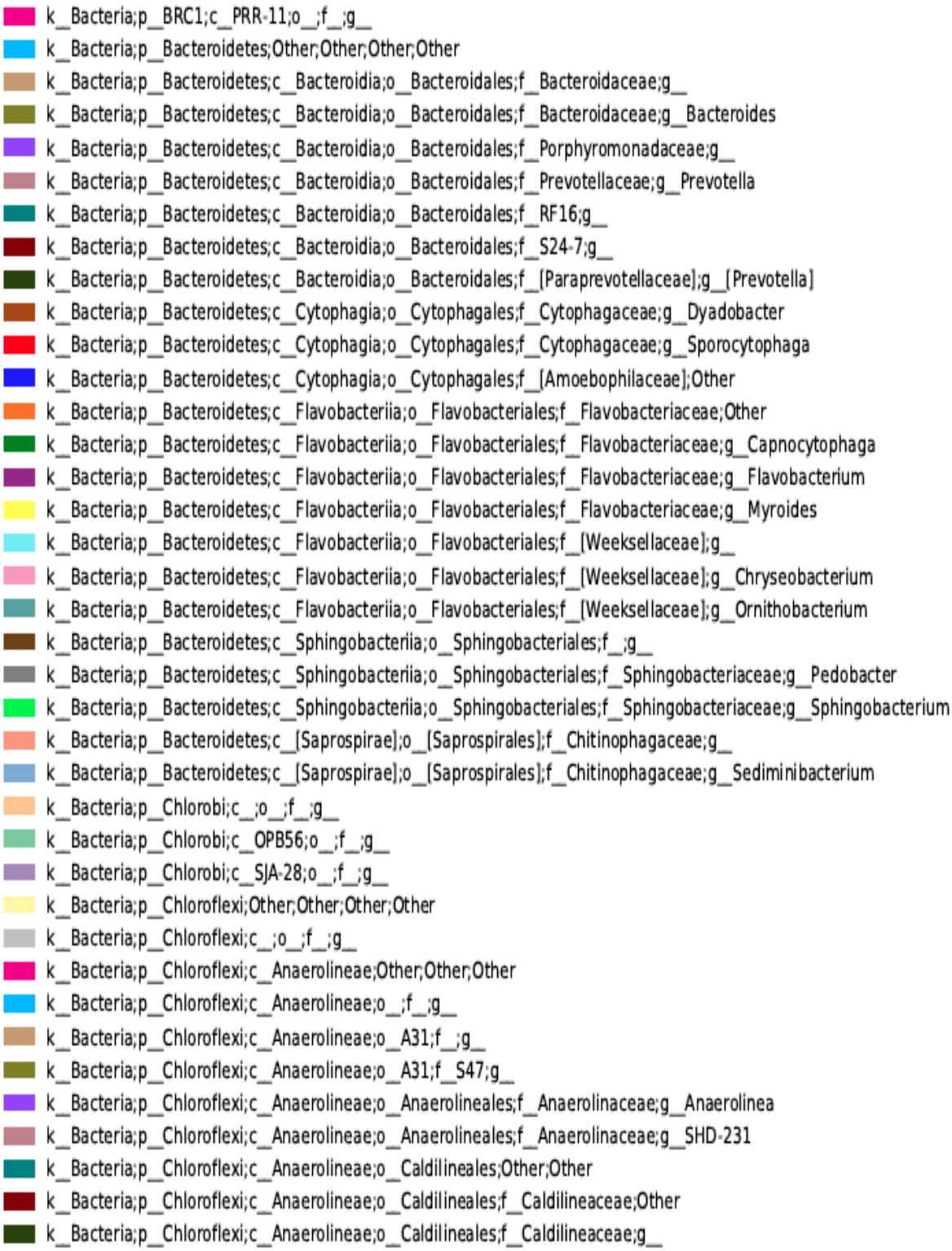
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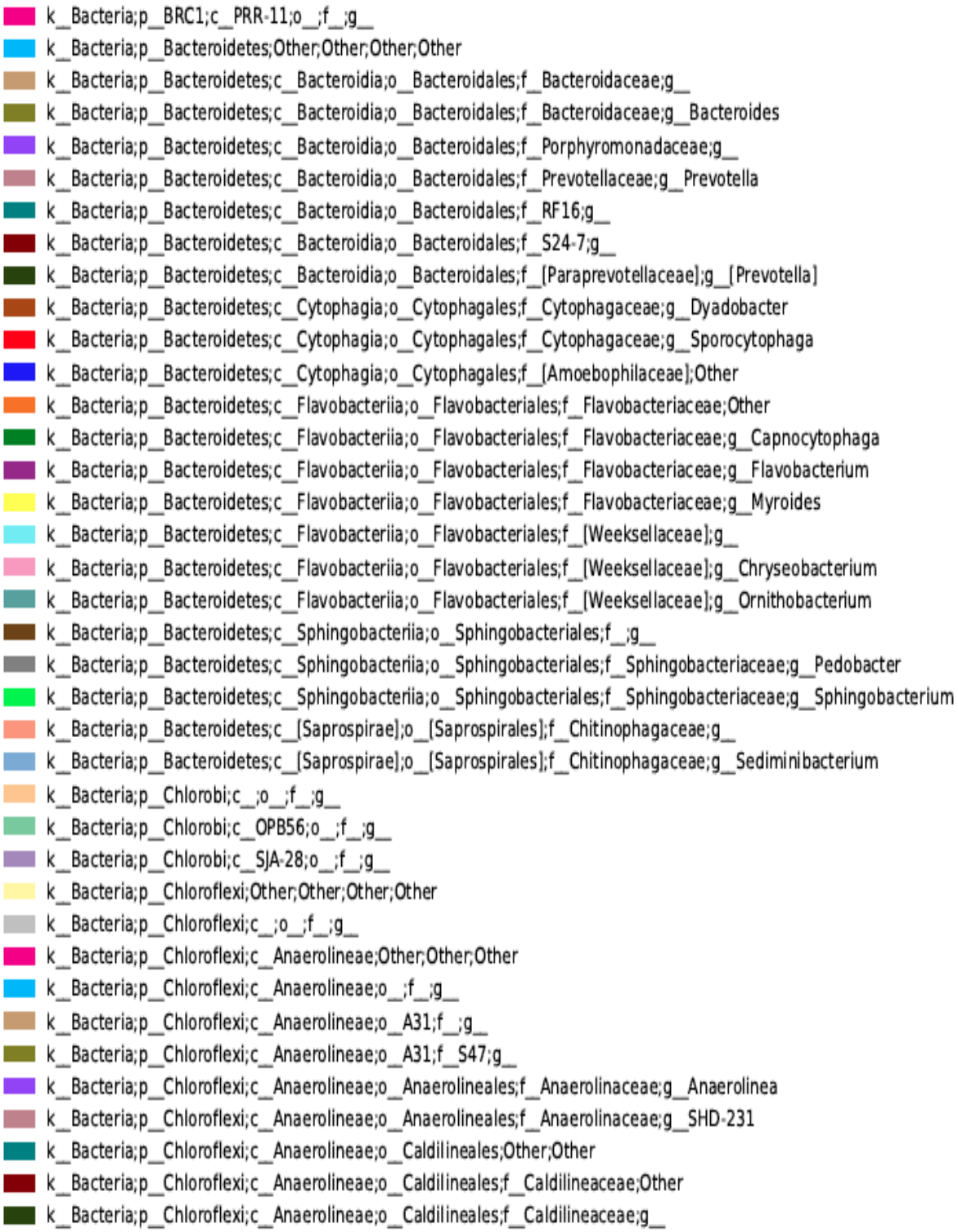
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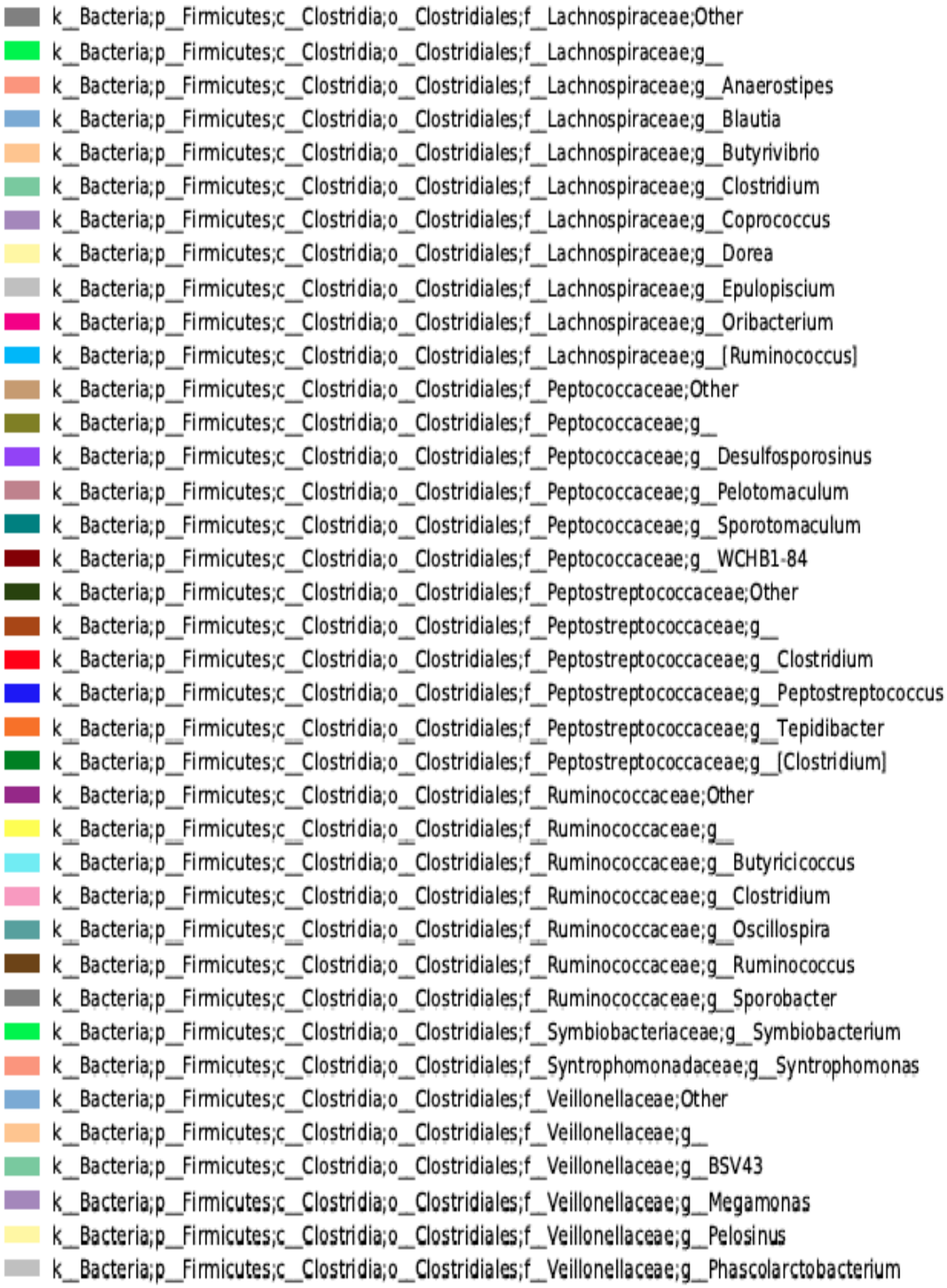
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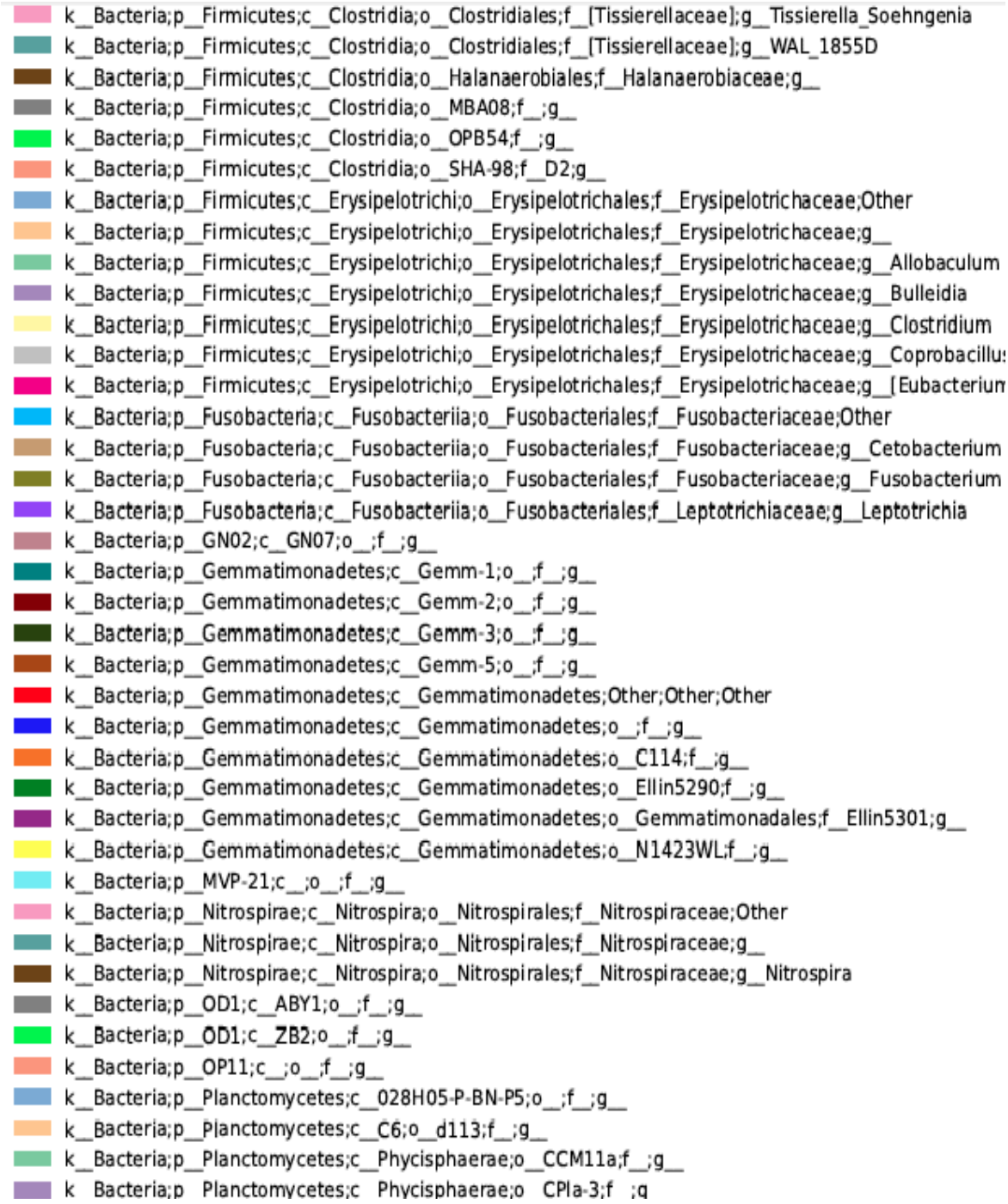
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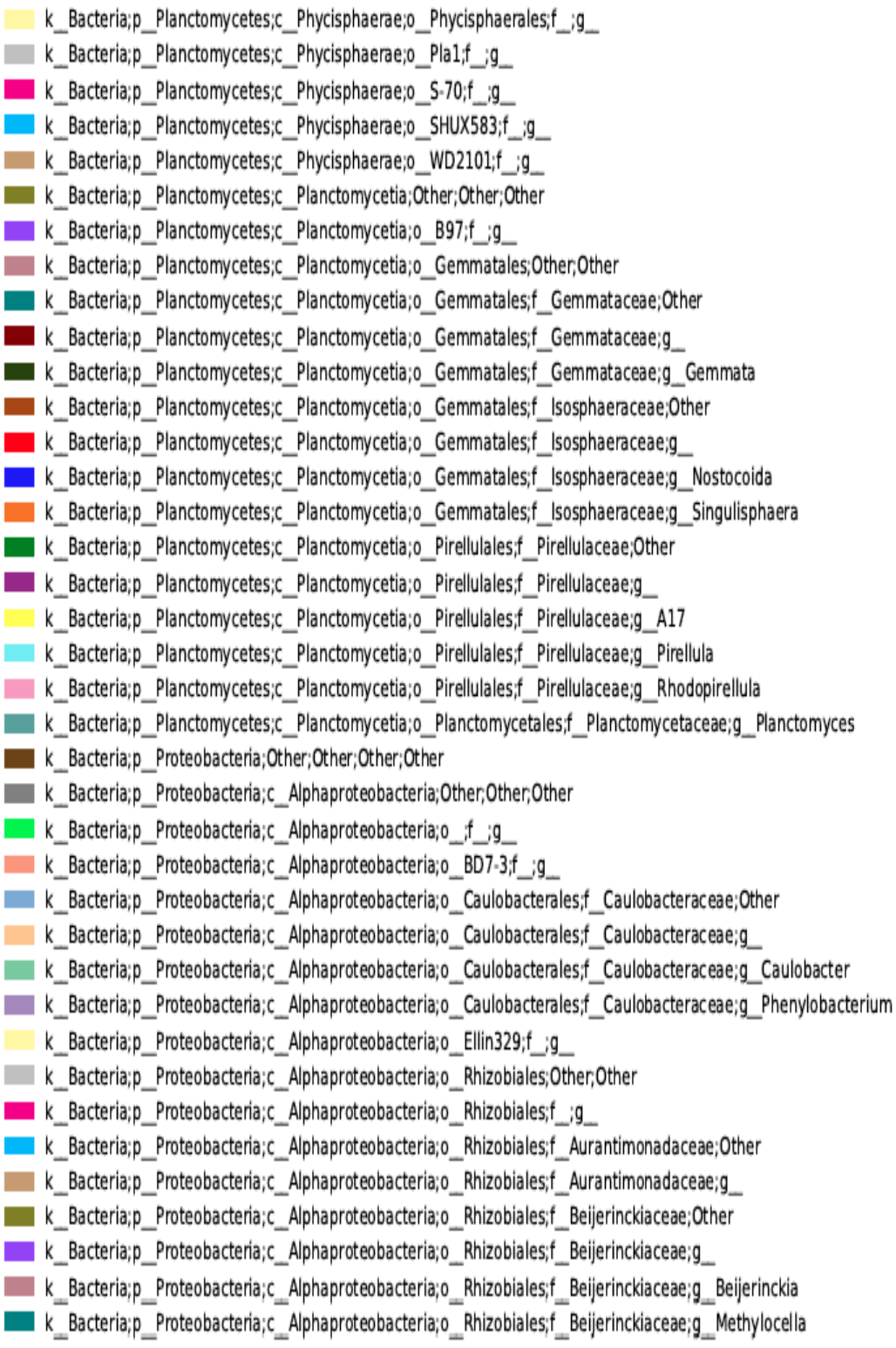
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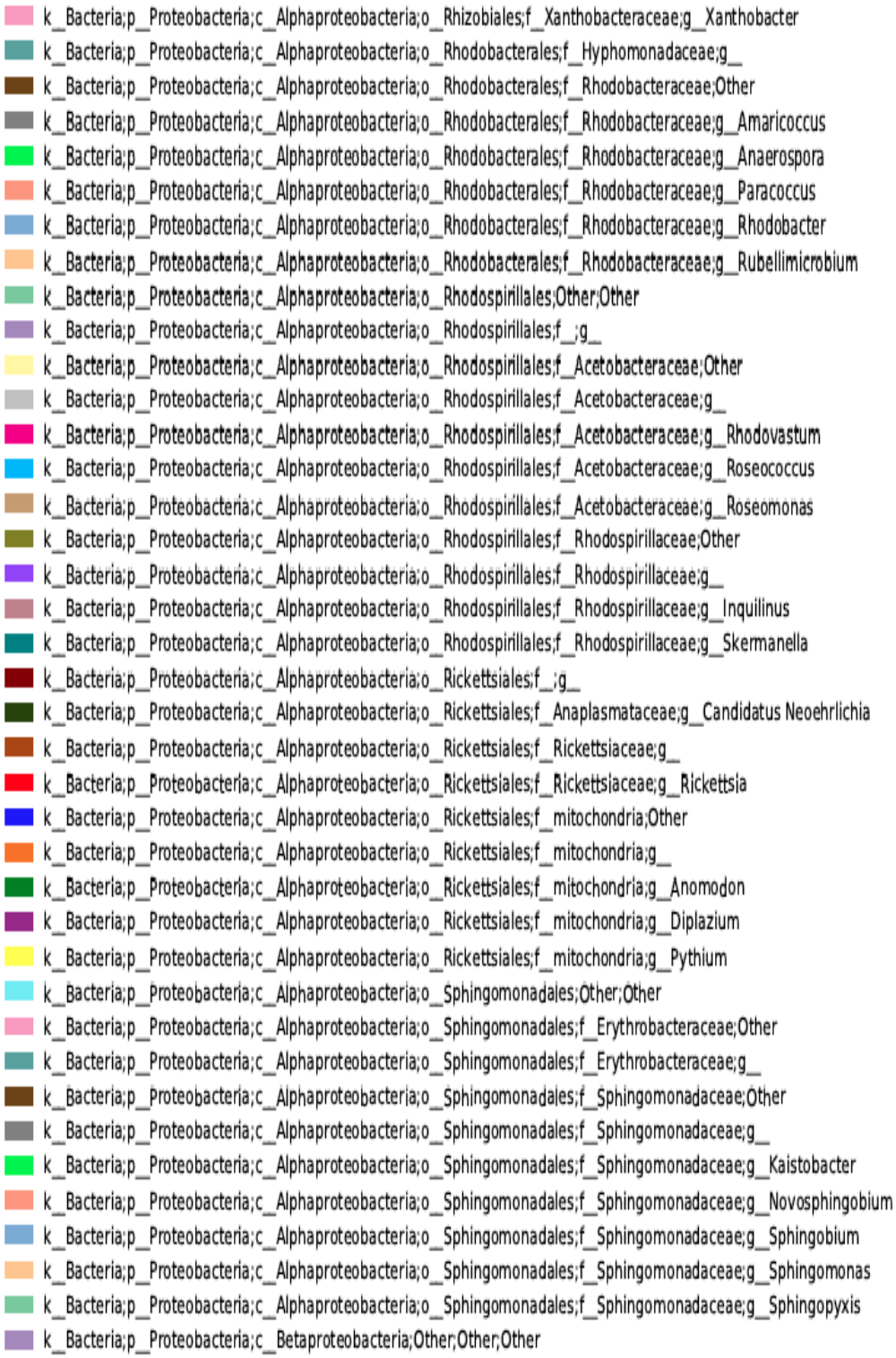
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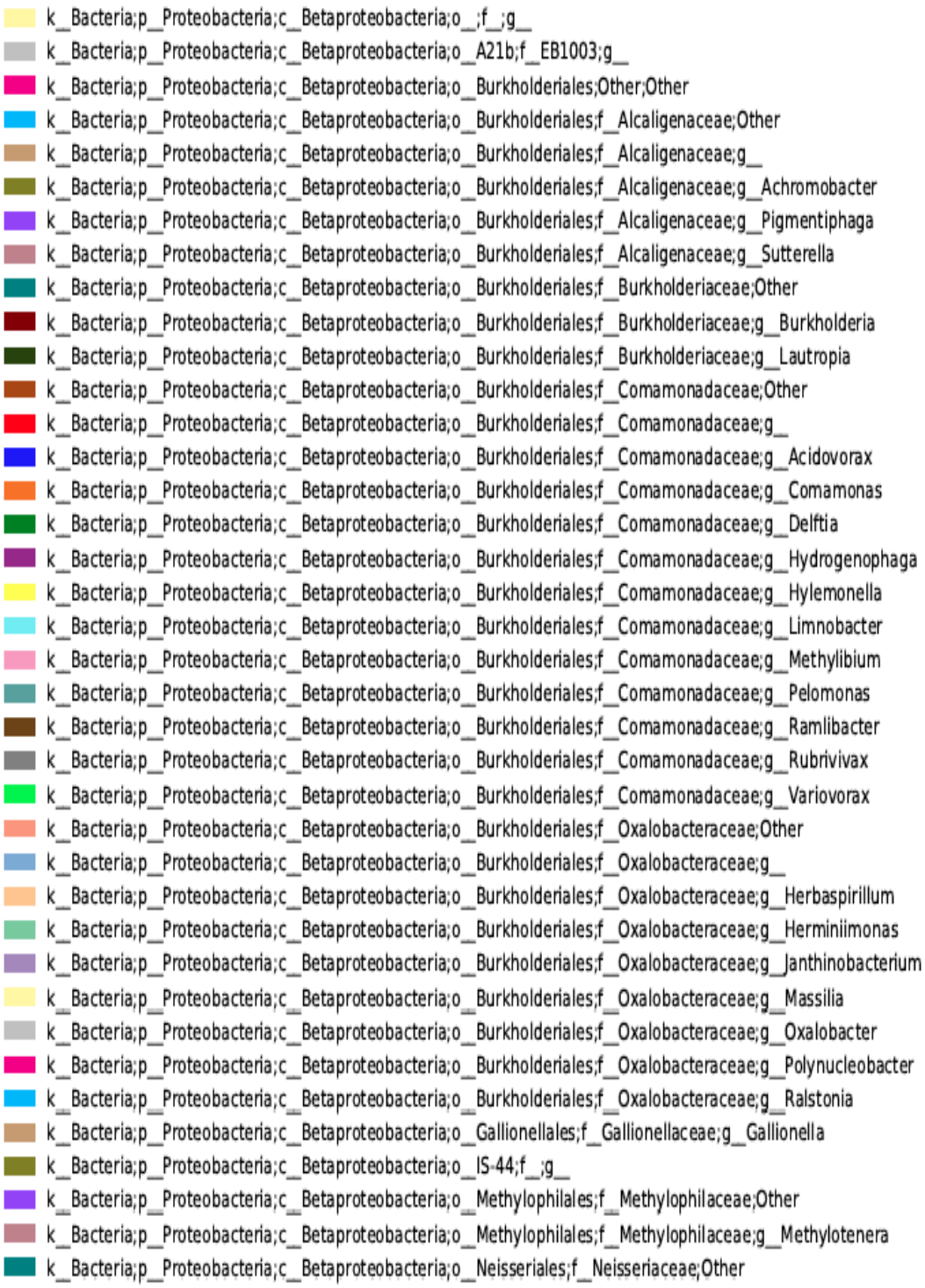
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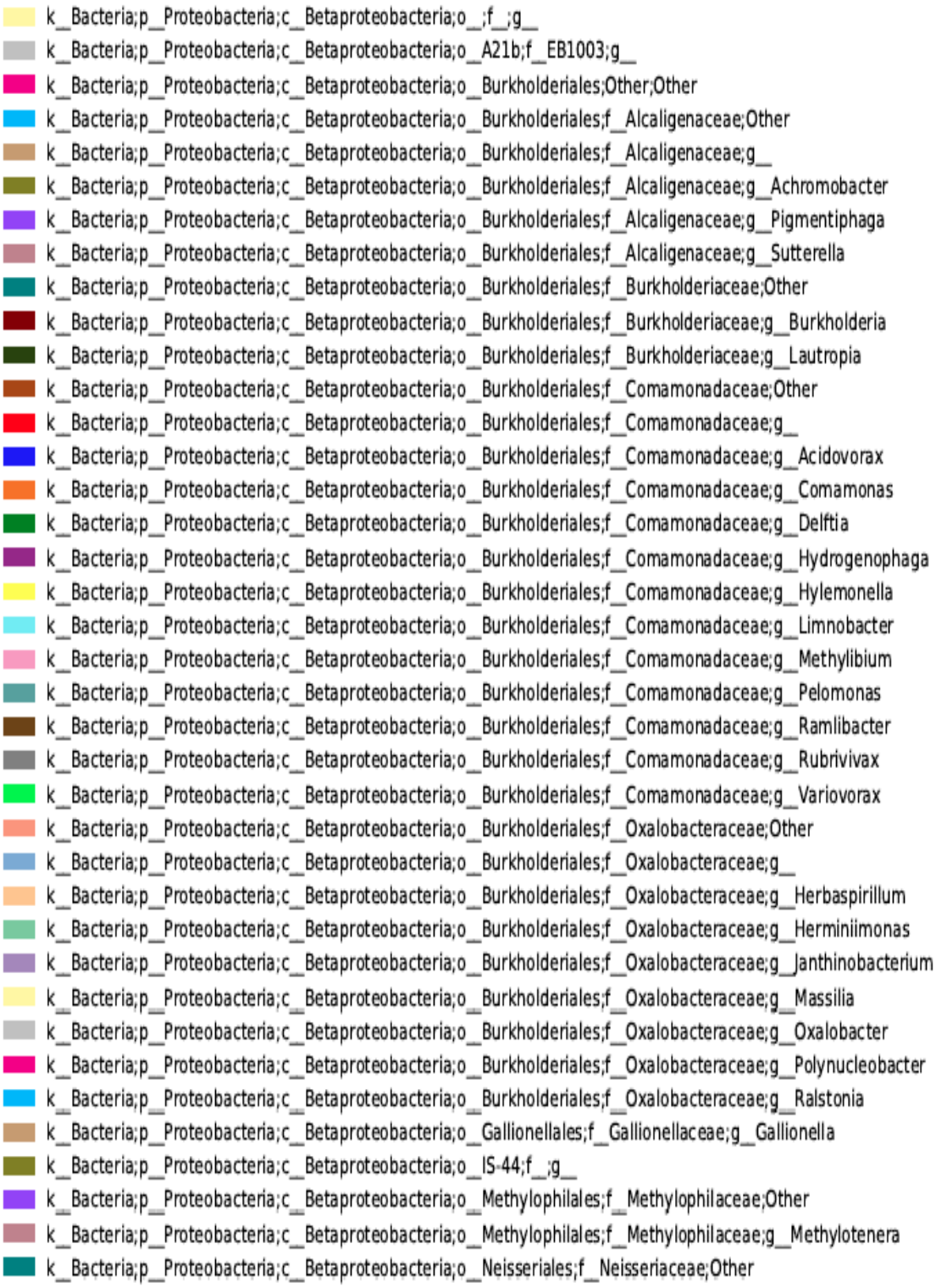
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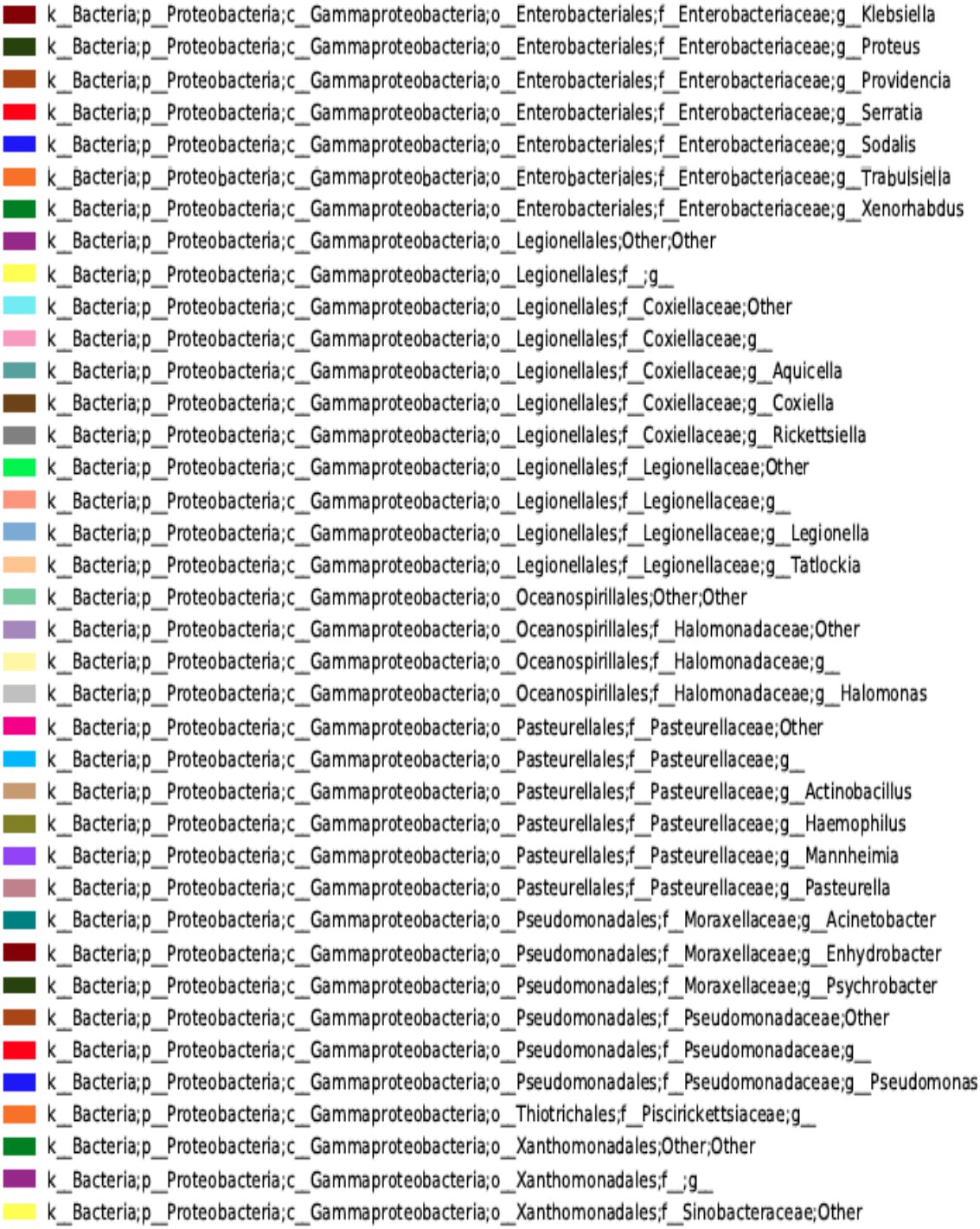
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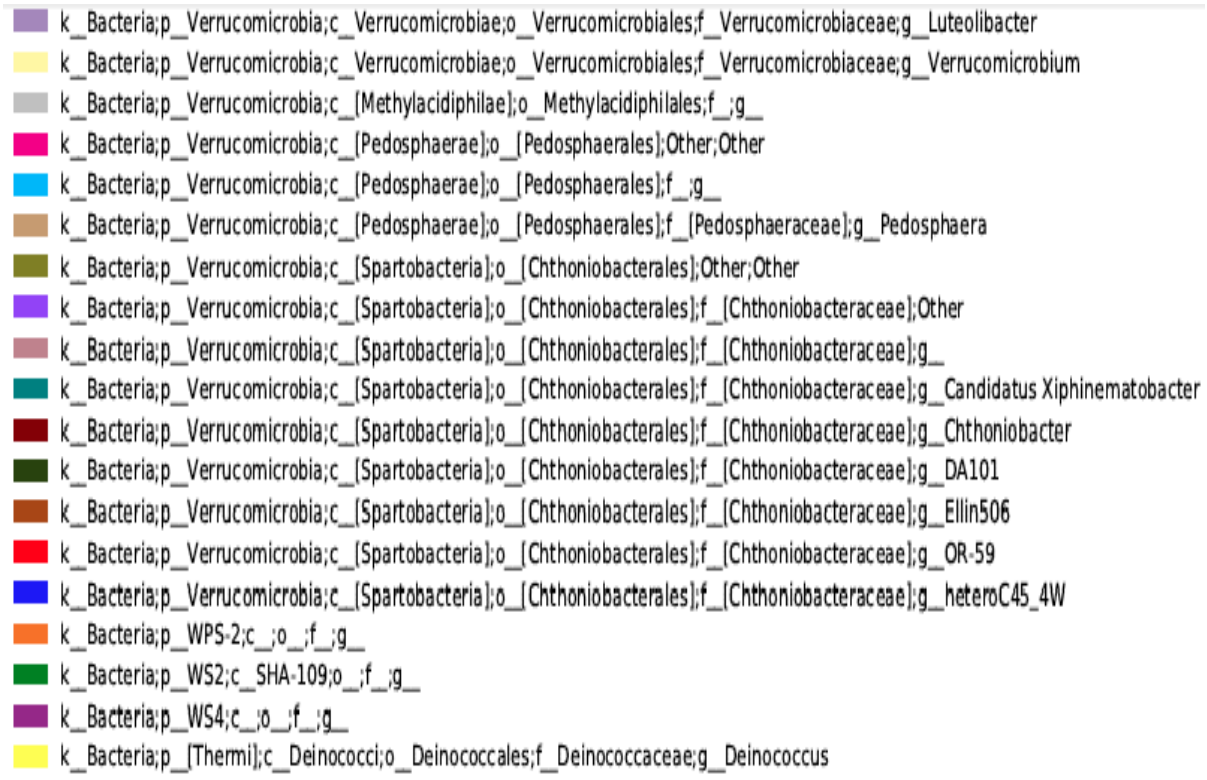
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