A review of the potential role of cattle - slurry in the spread of bovine - tuberculosis -

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Literature review on the role of slurry in the spread of TB

To consider, through a comprehensive literature review, the role of slurry in spreading bovine TB and whether slurry from infected animals should be treated or disinfected prior to spreading.

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1 Executive summary

Bovine tuberculosis and routes of transmission

- There are several ways in which cattle can become infected with *M. bovis*. Routes of infection in cattle include the respiratory and alimentary routes, with the respiratory route considered to be predominant.
- The route of infection, infective dose and host susceptibility will determine whether infection occurs, with respiratory transmission requiring a much lower infective dose than oral transmission.
- In field cases of bovine TB and experimental models, lesion distribution and pathology show predominant involvement of the upper and lower respiratory tract and associated lymph nodes, which is supportive of infection via the respiratory route**.**
- In theory, transmission can be either direct, through close contact between infected and susceptible individuals, or indirect from exposure to viable bacteria in a contaminated environment (for example pasture, feed, housing etc).
- Indirect transmission via the respiratory route could potentially happen through the aerosol spreading of infective material including via the air-borne spreading of contaminated slurry**.**
- Droplets of contaminated water, eructation while ruminating, infected pastures or inhalation of contaminated dust particles could also be an alternative way of aerogenous infection.
- Recent studies demonstrate that *M. bovis* is capable of surviving in the environment for extended periods of time and environmental contamination has been cited to be important in the (indirect) transmission of TB from badgers to cattle and from wild deer to cattle.
- We have concentrated on potential risks posed by cattle slurry and have not discussed directly or in any detail the risks posed by badger excretions on farm or on pasture.
- The relative contribution, if any, of each of these routes has not been quantified; however, most commentators agree that direct contact is likely to be more significant than transmission via indirect routes.

Cattle slurry/manure as a source of *M. bovis* infection

Risks associated with spreading cattle manure and slurry

- Improperly managed manures could constitute a potential infection risk for livestock particularly if pathogenic organisms, such as *Salmonella*, *Clostridia*, *E. coli*, and *Mycobacteria*, are present in animal excretions.
- Solid manure is not considered to present a risk in terms of infection if it has been well composted, especially since it is less likely to generate aerosols during application to land.
- Slurry does not undergo composting during storage. As a result, slurry is extremely unlikely to reach high temperatures during storage and consequently pathogenic bacteria are more likely to survive for longer periods in stored slurry.

- The risk of infection associated with spreading of cattle slurry is likely to be much greater than spreading manure.
- Slurry containing viable *M. bovis* may theoretically contaminate pasture, soil and silage and result in respiratory/oral transmission and infection of grazing cattle (and local wildlife) for a considerable length of time after the application of slurry depending on the conditions.
- Spreading slurry can generate aerosols that potentially carry bacteria for considerable distances. Respiratory transmission to neighbouring farms via slurry aerosols, whilst probably unlikely, cannot currently be excluded.
- Studies indicate that inadequate storage of slurry is associated with an increased risk of TB transmission.

Factors influencing M. bovis exposure and infection via contaminated slurry/manure

- The number of organisms excreted by cattle into the environment will play a significant role in determining whether other animals become exposed and infected. The levels of *M. bovis* released will depend on the prevalence and severity of infection in the herd as well as lesion distribution in infected animals.
- The duration and conditions of slurry and manure storage prior to land spreading will have an effect on viability of the organism and therefore the risk of exposure and infection.
- The manner in which slurry and manure are applied on farmland may also present an additional risk. For example, exposure may occur as a result of aerosol production during spreading of slurry or if land is not harrowed following deposition of cattle manure and viable bacteria within large lumps of dung are afforded protection from adverse conditions allowing extended survival.
- The survival of the organism will play a significant role in determining the persistence of the organism in the farm environment and exposure of cattle and wildlife. The organism must survive any storage/treatment and the aerial or ground environment for long enough to contact a susceptible host and within the host, it must reach a suitable site of infection and survive to replicate.
- Animal husbandry practices, particularly grazing management, may also be important in reducing/preventing the exposure of cattle to contaminated pasture and soil.
- The risk of animals being exposed to the organism in slurry will range from high to low depending on how the variables converge on each farm. Further studies are required to determine which variables or combination of variables will result in a high risk of exposure.

Potential levels of M. bovis in cattle slurry and manure

- For cattle slurry/manure to act as a source of *M. bovis*, at least one animal in the herd must be infected and excreting bacteria in faeces, urine or milk that has been disposed of in the slurry lagoon.
- The likelihood of excretion and the number of mycobacteria excreted by an individual animal will be dependent upon infectious dose, site and level of infection, and the amount of time the animal has been infected or severity of infection.

- Current data on excretion of *M. bovis* in bovine faeces is very limited. Early studies, at a time when substantial numbers of the national herd were infected, indicate that the proportion of heavily infected cattle excreting *M. bovis* in faeces was typically 10%, but may have been as high as 80%. These early studies are not likely to be representative of the current situation in countries with established TB control programmes (including Northern Ireland).
- Excretion of *M. bovis* in faeces from experimentally infected cattle in the absence of TB lesions in the abdomen has also been reported. It was concluded that *M. bovis* in the faeces arose directly from swallowing infected mucus from the respiratory tract. This is probably more likely than the release of *M. bovis* into the gut contents from TB granulomas in the intestinal mucosa or other parts of the digestive system.
- Contamination of milk is most likely to occur when infection becomes disseminated and there is tuberculous mastitis, but the condition is now rarely observed in cows in the UK.
- TB infected cattle may be capable of excreting *M. bovis* in urine; however, there is very little evidence to confirm that this occurs. TB lesions in the kidneys, genital organs and associated lymph nodes of tuberculin test reactor cattle are exceptionally rare in the UK nowadays.
- Given the limited data available on excretion in cattle (particularly faecal excretion) it is difficult to estimate the levels of *M. bovis* that may be present in cattle manure and slurry. Detection of *M. bovis* in cattle manure/slurry is likely to be problematic due to sampling of large volumes and well-documented limitations of the methods currently available and validated for direct detection of the organism.

Environmental persistence of *M. bovis* and transmission

Environmental contamination

- Cattle manure and slurry, containing viable *M. bovis* organisms, spread on farm land constitutes a mechanism whereby the farm environment can become contaminated with the bacterium. The same is true for *M. bovis* deposited directly by infectious cattle defecating on pasture.
- Indirect transmission of *M. bovis* to cattle and wildlife via a contaminated environment may potentially occur through inhalation/ingestion of the organisms during investigation of cattle faeces deposited in the field, inhalation of potentially infectious aerosols produced during slurry spreading or inhalation/ingestion of *M. bovis* from contaminated pasture, soil and silage.
- For bovine TB to be transmitted via a contaminated environment, the organism must be capable of surviving in the environment and retaining infectivity for a sufficient amount of time before inhalation/ingestion by susceptible host.
- Although experimental investigations have produced variable results, it appears that survival of *M. bovis* is enhanced in moist, cool conditions and neutral-to-acidic substrates rich in organic matter, especially when the bacilli are protected from direct sunlight.

Potential infection via faeces

- *M. bovis* contaminated faeces may remain infective for up to six months when deposited in winter but only one to two months in the summer, depending on the temperature and the concentration of pathogens in the faeces.
- In general, cattle avoid grazing close to the faeces of other cattle, preferring to graze mature sward fertilized by the deposit, however, badgers will regularly forage cattle deposits in search of earthworms. To date, there have been no reports of *M. bovis* isolation from earthworms and the risk of TB transmission to badgers via consumption of *M. bovis* contaminated earthworms remains unknown.

Transmssion via slurry spreading

- Results from studies investigation survival of *M. bovis* in artificially infected (spiked) slurry indicate that the organism may survive for up to 6 months in stored slurry. Although the likelihood of infection may be reduced by the dilution effect (of air, uninfected soil and uninfected slurry/manure) there are risks of creating aerosols by mixing/pumping/spreading slurry.
- Investigations into the transmission of TB via contaminated pasture have produced conflicting results. Some studies have reported infection with *M. bovis* after grazing pasture contaminated both naturally and artificially.

Transmission via soil and silage

- Studies have demonstrated that *M. bovis* can remain viable in soil for about 6 months. Cattle tend to consume soil to offset mineral deficiencies and also use soil for behavioural head rubbing, during which they may create dust and potentially infectious aerosols.
- There is little information on the survival of *M. bovis* during the ensiling process. The information currently available indicates silage cannot be excluded as a risk and steps should also be taken to avoid spreading silage fields with contaminated slurry.
- Anecdotal evidence has also raised concerns about the role of silage in transmission of liver fluke, pathogenic *E. coli* etc.

Effect of disinfection and anaerobic digestion on *M. bovis* viability

Chemical disinfection

- Chemical disinfection of cattle slurry from TB reactor herds may enable rapid inactivation of *M. bovis* in cattle slurry. This may be an attractive alternative to storage especially if farms do not have adequate storage facilities for long-term storage.
- Chemical disinfection of cattle slurry contaminated with *M. bovis* presents many problems, some relating to the large volumes of slurry requiring treatment and others to the selection and evaluation of effective chemicals.

- Mycobacteria are relatively less susceptible to chemical disinfectants than many other bacterial species and this should be taken into consideration when selecting chemicals for treatment of slurry from TB reactors.
- Thick lime milk, a mixture of calcium hydroxide and water, has been shown to be effective against *M. bovis* in experimental studies. This treatment should not have a significantly adverse effect on grass composition or silage quality, although grass dry matter yield was significantly reduced when compared with grass to which untreated cattle slurry was applied.
- Other studies have investigated the inactivation of *M. bovis* by volatile chemicals - acetone, ammonium hydroxide, chloroform, ethyl alcohol, and xylene. Some of these chemicals were found to be effective against *M. bovis*, however, many of the chemicals are unsafe for use at farm level, particularly at the volumes/concentrations required for slurry disinfection

Anaerobic digestion

- Anaerobic digestion (AD) is a natural process in which bacteria break down organic matter in an oxygen-free environment to form biogas and digestate. A broad range of organic inputs can be used including manure (solid/liquid), food waste, and sewage.
- It is well documented that digestate from processing of animal manure may contain pathogenic bacteria excreted in faeces, urine and exudates. Digested residues may contain pathogenic bacteria of different species such as *Salmonella*, *Listeria*, *E. coli*, *Campylobacter*, *Mycobacteria*, *Clostridia* and *Yersinia*. Many of these bacteria are zoonotic pathogens.
- No studies investigating specifically *M. bovis* in anerobic digestion could be found upon extensive literature searches. Although the effects of anaerobic digestion on *M. bovis* have not been specifically examined, it seems likely that *M. bovis* would survive the temperatures and duration used by the majority of on-farm digestors.

Practices which should reduce the risk of TB transmission via cattle manure slurry

Storage

- Cattle slurry should be stored for a minimum of six months before spreading to land to ensure that *M. bovis* is inactivated.
- Cattle manure with low moisture levels and a high straw content should be stacked in a heap for a minimum of 30 days to permit composting (heat production and decomposition)
- Cattle manure with higher levels of moisture is not likely to undergo composting. This type of manure should be treated like slurry and stored for at least 6 months. Higher levels of moisture are more likely to occur in situations where silage rather than hay is fed.
- Following disposal of milk from reactor cattle to the slurry system, a minimum storage period of 6 months should be observed.

Treatment

- In circumstances where storage for at least 6 months is not feasible, cattle manure and slurry (and milk from reactors) should be treated by chemical disinfection.
- Experimental studies have indicated that "thick lime milk" is effective against *M. bovis*. Concentrations of 11.25 to 20 kg calcium hydroxide per m^3 are required for inactivation within 24 hours. This treatment should not have a significantly adverse effect on grass composition or silage quality.
- Other chemicals which may be useful in slurry disinfection may be found on the DEFRA-approved disinfectants list. The effect of these chemicals on grass composition and silage quality is not likely to have been investigated.

Spreading manure and slurry

- In combination with extended storage or treatment, care should be taken in how and where slurry and manure is spread. Mixing and pumping of slurry in under floor pits should be avoided while animals are present in housing to reduce/prevent inhalation of infectious aerosols.
- To minimise aerosol production during spreading, slurry should be spread in calm weather (not windy) using a downward discharge method such as band spreading or injection using attachments such as the trailing-shoe.
- Distance to neighbouring cattle when spreading should also be considered. In general, the minimum distance to nearby cattle will depend on the method of spreading. If using a method likely to produce aerosols which can travel long distances (i.e. splash plate), slurry application should be avoided when cattle are in neighbouring fields.
- Slurry should only be spread on land within the affected farm which is not accessible to other herds. The risks associated with spreading of manure on rented pasture should be considered.
- A recent local study detected a higher risk of TB associated with the use of slurry contractors. Consequently, if used, their equipment should be thoroughly cleansed and disinfected before moving off the farm to another property. However, this association may be due to some other, as yet undetermined, risk factor linked to the use of contractors.

Grazing

- If slurry is to be spread on grazing pasture, land should not be grazed for at least 2 months following spreading. Alternatively, slurry should be spread on arable land either by injection or ploughing in after spreading.

Methods -
This review was written after an extensive review of the available scientific literature. On-line resources (PubMed, Science Direct and Web of Science), were used to find appropriate peer-reviewed literature. PubMed (http://www.ncbi.nlm.nih.gov/pubmed) comprises more than 20 million citations for biomedical literature from MEDLINE, life science journals, and online books. Literature was accessed until May 2013, inclusive. The literature search had no publication date restriction and included conference proceedings and abstracts.

We purposefully selected publications that were judged most relevant for the review, with a preference for high-quality systematic reviews. Searches were conducted using combinations of the following key words: "*Mycobacterium bovis*", "bovine", "tuberculosis or TB", "transmission", "risk factors", "slurry", "faeces", "manure", "persistence", "survival", "environment", "anaerobic digestion", "ensiling", "disinfection".

The following relevant areas have been discussed:

- Bovine tuberculosis, pathogenesis and potential routes of transmission
- Risk associated with cattle slurry/manure and the potential for TB transmission
- Potential levels of *M. bovis* in cattle slurry (as a result of excretion in faeces, urine or milk disposed of in the slurry pit)
- Viability of *M. bovis* in slurry following chemical disinfection and anaerobic digestion
- Persistence of *M. bovis* in the environment and exposure of cattle and wildlife to *M. bovis* via slurry spreading, contaminated pasture, soil and silage

2 Bovine tuberculosis

2.1 Introduction

Bovine tuberculosis (bTB) is a chronic disease of animals caused by infection with the slow-growing, obligate intracellular bacterium *Mycobacterium bovis* (*M. bovis*) (OIE, 2009). In a large number of countries bovine tuberculosis is a major infectious disease among cattle and is one of the biggest challenges facing the farming industry today (Pollock & Neill, 2002; Carslake et al., 2011). As well as cattle, *M. bovis* can infect and cause TB in badgers, deer, goats, pigs, camelids (llamas and alpacas), dogs and cats and many other mammals (OIE, 2009). Whilst it is important to view bovine TB as an infectious disease which requires preventive as well as control measures, *M. bovis* infection in cattle now rarely appears to present as clinical disease. More commonly it appears as apparently healthy animals responding to an immunological test based on tuberculin, an entirely different scenario to that which existed when control programmes were first introduced (Collins, 2006). Despite the implementation of eradication programmes since the 1950s bovine TB has not been eradicated from either the UK or ROI. Indeed, there has been a sustained and largely unexplained increase over the last 20 years in parts of the UK (Gilbert et al., 2005). The problem of bovine tuberculosis in the UK is extremely complicated. Further research is required to gain a better understanding of bovine TB epidemiology. Key to understanding bovine TB epidemiology is the relationship between infection and disease (TB) and the relationship between disease and transmission (Skuce et al., 2011). In particular, there is a need to quantify the relative importance of all routes of transmission to enable the most appropriate and cost effective control measures to be implemented.

2.2 Pathogenesis and routes of transmission

2.2.1 Potential routes of transmission in cattle

There are several ways by which cattle can become infected with *M. bovis*. The most common routes of infection in cattle are the respiratory and alimentary routes, with the respiratory route considered predominant. In theory, transmission can be either direct, through close contact, or indirect from exposure to viable bacteria in a contaminated environment (for example pasture, feed, housing etc). The relative contribution, if any, of each of these routes has not been quantified (Skuce et al., 2011). Less common routes of transmission have been recorded, including cutaneous, congenital, and genital. Cutaneous infection requires contamination of other primary lesions with tubercle bacilli (Neill et al., 1994). Transmission of *M. bovis* via the umbilical vessels, due to uterine infection of the dam has been reported (O'Reilly & Daborn, 1995). Calves are believed to be congenitally-infected if they present with lesions in the liver and portal system only. However, few cows in the UK present with uterine bovine TB. No confirmed isolations of *M. bovis* were reported from uterine tissue submitted to VLA Weybridge (1986–1994). This route is probably insignificant in bovine TB epidemiology in the UK and ROI and no specific control measures are indicated currently (Phillips et al., 2003). Genital transmission can occur if the reproductive organs are infected, or if the preputial orifice is contaminated but this too is extremely rare (Francis, 1972; Neill et al., 1994). Iatrogenic transmission via the use of surgical instruments such as teat siphons, urinary catheters and hypodermic needles has also been reported (Ritchie, 1959).

2.2.2 Tuberculous lesions in cattle

In cattle as well as in other animal hosts, the route of infection with *M. bovis* can be deduced by the pattern of lesions observed in slaughtered animals. Animals with lesions restricted to the thoracic cavity are presumed to have been infected by the inhalation of aerosols, while those with lesions only in mesenteric lymph nodes are thought to have acquired the infection by ingestion (Pollock & Neill, 2002). In field cases, lesion distribution and pathology show predominant involvement of the upper and lower respiratory tract and associated lymph nodes which is supportive of infection via the respiratory route. In a study of lesions in 179 visibly-lesioned TB reactors slaughtered in England, Wales and Scotland in 1982, lesions were distributed as follows: in 52 cattle, lesions were confined to the head only; in 73, the respiratory tract only; in 14, the head and lungs, in 6 intestine only; in 14 head and intestine; in 8, lung and intestine; in 12 lesions were present in combinations of head, lung, intestine and liver. Overall, 139/179 (77.6%) lesions were observed in the head only, respiratory tract only and head/lungs only (Pritchard, 1998).

Crews (1991) examined detailed gross post-mortem findings from 1,398 lesioned bovine TB reactors from a veterinary district in New Zealand. A total of 1,808 lesions were detected. Overall, 64.7% of cases had lesions associated with the respiratory system and 16.2% of cases had lesions associated with the abdominal tract. Within the respiratory system, the mediastinal lymph nodes were most commonly affected with 41.6% of total lesions detected, followed by the bronchial lymph nodes (13.2%) and the lungs (2.6%). Similarly, a more detailed necropsy procedure was employed by Corner et al. (1994) to optimise post-mortem examinations and determine the distribution of lesions in tuberculous cattle. Results from the study indicated the majority of lesions detected were associated with the respiratory tract, in particular, the lungs (9.8%), mediastinal (28.2%) and bronchial (18%) lymph nodes. In another study in the USA, detailed post-mortem examinations were carried out on 30 cattle with bovine TB. A total of 24 tissue samples from each animal were examined for gross lesions and processed for bacteriological culture. Using a combination of macroscopic examination, histology, and bacteriology, evidence of tuberculosis was detected in lymph nodes of the thoracic, head and other sites in 86.7%, 26.7% and 20% of infected cattle, respectively (Whipple, 1996).

Experimental models of bovine tuberculosis, involving nasal, tracheal and aerosol infection and in-contact infection, support the effectiveness of the aerosol route for bovine infection. Cassidy et al. (1999) conducted experiments comprising infected "donor" cattle and non-infected "in-contact cattle." At the end of the experiment, the majority of donor calves were found to have extensive tuberculous lesions in the upper respiratory tract (URT), or lungs or both. Furthermore, six of the in-contact cattle were found to have tuberculous lesions within the lungs, URT associated and bronchiomediastinal lymph nodes. In a study by Buddle (1994) inoculation of 18 month-old cattle with 5×10^5 cfu (high dose) induced extensive lung lesions, as well as tuberculous lesions in the lymph nodes of the head, neck, thoracic and abdominal cavities. Following inoculation with 500 cfu (low dose), induced small lesions (<1cm diameter) that were localised to lungs and pulmonary lymph nodes, similar to the natural disease observed in cattle.

Studies of calves experimentally-infected with *M. bovis* also indicated that, following a lag period after inoculation, *M. bovis* can be isolated consistently from nasal mucus before shedding becomes intermittent (Neill et al., 1991). The quantity and frequency of shedding appeared inversely related to the infecting dose. Evidence of shedding was also

demonstrated in studies of field cases of tuberculosis, where up to 20% of tuberculous cattle had *M. bovis* in nasal mucus (McIlroy et al., 1986; Neill et al., 1988). The authors cautioned that failure to detect *M. bovis* in individual nasal secretions should not necessarily lead to the assumption that such cattle are not shedding, as sequential sampling would possibly facilitate better assessment than from mucus taken at one time-point. In addition, the culture methods used to detect *M. bovis*, whilst recognised currently as the "gold-standard" are relatively insensitive. Further evidence of the potential for shedding from tuberculous cattle was demonstrated in a more recent study, in which over 50% of heads from skin test-positive cattle, when critically examined, yielded *M. bovis* from nasal mucus or respiratory tissues such as nasal mucosa, turbinates and tonsil (Cassidy et al. 1999). These studies demonstrate that infected cattle have the potential to excrete *M. bovis* in nasal secretions, indicating a potential route of transmission in cattle-to-cattle and cattle-to-badger spread.

2.2.3 Aerosol transmission

Aerosol transmission would appear to be the most probable method of infection, typically through close contact between infectious and susceptible animals. The development of tuberculosis lesions which invade the airways is thought to be required to facilitate active excretion, aerosol spread of *M. bovis* and transmission. Respiratory excretion and inhalation of *M. bovis* is considered to be the main route through which cattle-to-cattle transmission occurs in bovines (Neill et al., 1994). From the evidence, it appears that inhalation of very small numbers of mycobacteria can initiate lesions in cattle, possibly equivalent in number to the quantity of organisms delivered (Converse et al., 1998; Dean et al., 1995; DEFRA SE3024).

A generally accepted concept is that infection with *M. bovis* can be established in cattle inhaling tubercle bacilli or possibly a single bacillus in an aerosol droplet (Neill et al., 1991). This droplet nucleus lodges within the respiratory tract, possibly on the alveolar surface of the lung (Langmuir, 1961; Pritchard, 1988). This is not to suggest that every bacillus that enters the alveoli is capable of causing infection, but instead that most natural infections derive from a single bacillus (Phillips et al., 2003). The establishment of infection probably depends upon the scenario of a phenotypically hardy and virulent bacillus being ingested by a relatively weak alveolar macrophage with poor microbicidal activity. It is suggested that this combination might occur only once in many bacillus/macrophage interactions (Dannenberg, 1991). Assuming infection of cattle with *M. bovis* can be caused by one organism, there can be a significant latent period following infection before excretion (approximately 87 days) (Neill et al., 1991). In the initial stages of infection, there may be regular excretion of the tubercle bacillus in nasal mucus before excretion becomes intermittent, although the quantities of *M. bovis* may vary significantly between animals.

In establishing infection, the size and consistency of aerosolized droplets appear to be of crucial importance. Fine aerosol suspensions of low viscosity appear most effective for suitably delivering their mycobacterial content (O'Reilly & Daborn, 1995). It has been calculated that only inhaled droplets of very small size are likely to reach the alveoli and avoid the muco-cilliary escalator of the host respiratory system. Estimates have been made that such a droplet could contain between 1-3 mycobacterial bacilli (Dannenberg, 1989; Wiegeshaus *et al.,* 1989; Dannenberg, 1991). It has been reported that only a very small fraction of such droplets contain viable tubercle bacilli up to 1 h after release. Viability and

virulence of such mycobacteria are vitally important but there is little precise information on the impact of environmental stress on virulence of transmitted *M. bovis*.

Indirect transmission via the respiratory route could potentially happen through the aerosol spreading of infective material particularly through air-borne spreading of contaminated slurry (Phillips et al., 2003). Slurry may become contaminated with *M. bovis* following excretion of the organism in the faeces or through the disposal of milk from TB reactors into the slurry system. In addition, droplets of contaminated water, eructation while ruminating infected pastures or inhaling contaminated dust particles could potentially be an alternative way of aerogenous infection (Skuce et al., 2011) but little or no data exist. Studies of TB pathogenesis in humans showed that dust and dried sputum were very effective in transmitting infection (Francis, 1958). Hence there is a need to consider the role of environmental contamination in transmitting and maintaining TB in cattle and wildlife. For infection to occur via a contaminated environment, the organisms must be capable of surviving in the environment for a sufficient amount of time before reaching a host. Some authors have argued that *M. bovis* survives outside the host for only a few weeks at most under natural conditions. It could, therefore, be suggested that environmental contamination plays a relatively insignificant role in the maintenance of *M. bovis* infection in cattle (Morris *et al*., 1994; Jackson et al*.,* 1995; Menzies and Neill, 2000). However, environmental contamination has been cited as potentially important in the (indirect) transmission of TB from badgers to cattle in UK studies (Wilesmith *et al.,* 1982; Clifton-Hadley et al., 1995; Krebs et al., 1997; Phillips et al., 2003) and from wild deer to cattle in the US (Kaneene et al., 2002; Fine et al., 2011).

2.2.5 Oral transmission

Indirect transmission via the alimentary route by ingestion of *M. bovis* from contaminated pastures, water or fomites is considered secondary to respiratory spread (Menzies & Neill, 2000). It is generally accepted that significantly larger numbers of *M. bovis* are required to cause infection by ingestion than via the respiratory route (Chaussé, 1913; Wells et al., 1948; Francis, 1971; O'Reilly & Daborn, 1995). Experimental infections have determined the minimum infectious dose required to establish infection via the oral route (ingestion) was up to 1,000 times that of the respiratory route. Sigurdsson, 1945 (cited by O'Reilly & Daborn, 1995) reported on findings of early research workers who demonstrated that at least 10 mg of bovine tubercle bacilli are necessary to cause alimentary infection in calves whereas 0.01 mg produced infection via inhalation. In early transmission studies, following oral challenge of cattle with *M. bovis*, many cattle developed lesions in the alimentary tract and abdomen (McFadyean, 1910), a very different lesion pattern to that observed today in UK cattle (Liebana et al 2008). Tuberculous lesions occurring solely in the mesenteric lymph nodes are not now a common finding, but do occasionally result from ingestion of a heavy bacterial load, such as from drinking infected milk. However, the consensus is that mesenteric (intestinal) lesions, if present, are more likely to be due to dissemination from other sites or swallowing of infective sputum. Transfer of organisms the other way, from the rumen to the respiratory tract, is theoretically possible due to regurgitation or eructation (Mullenax et al., 1964), although the expected accompanying intestinal lesions are not observed normally. However, in experiments, significant numbers of non-pathogenic 'indicator' bacteria were conveyed to the lungs during eructation. These findings suggest that it may be possible for the organism to be aerosolized and inhaled following ingestion and for infection derived from ingestion to present as a respiratory infection.

3 Cattle slurry/manure as a source of *M. bovis* infection

3.1 Use of cattle slurry/manure as fertiliser

In Northern Ireland, housed farm livestock produce approximately 10 million cubic metres (2,200 million gallons) of undiluted manure each year, with 88% of this being cattle manure (AFBI, 2008). Livestock manures refer to organic materials which supply organic matter to the soil, together with plant available nutrients (in relatively small concentrations compared to inorganic fertilisers). They may be either slurries or solid manures (ADAS, 2001). Slurries consist of excreta produced by livestock in a yard or building mixed with rainwater and wash water and, in some cases, milk, waste bedding and feed (ADAS, 2001). Slurries are stored in pits, tanks or lagoons and can be pumped or discharged using a variety of methods. Solid manures include farmyard manure (FYM) and comprise material from covered straw yards, excreta with a lot of straw in it, or solids from mechanical slurry separators. Solid manures can generally be stacked (ADAS, 2001).

Slurry and solid manures are widely used as fertiliser for farming, to improve the soil structure (aggregation), so that it holds more nutrients and water and becomes more fertile (NIEA, 2011). As well as improving soil structure, manure also encourages soil microbial activity, which promotes the soil's trace mineral supply, improving plant nutrition (Edmeades, 2003). Careful recycling to land allows their nutrient value to be used for the benefit of crops and soil fertility, which can result in large savings on the use of inorganic fertilisers and a reduction in the amount of animal waste for disposal (NIEA, 2011). However, improperly managed manures could constitute a potential infection risk for livestock (ADAS, 2001), particularly if pathogenic organisms, such as *Salmonella*, *Clostridia*, *E. coli*, and *Mycobacteria* are present in animal excretions (Larsen & Munch, 1981; Strauch, 1991).

3.2 Risks associated with spreading cattle manure and slurry

3.2.1 Risks of spreading cattle manure versus slurry

Solid manure is not considered to present a significant risk in terms of infection if it has been well composted, especially since it is less likely to generate aerosols during application to land (Scanlon & Quinn, 2000a). Composting is a biological process in which microorganisms convert organic materials such as manure, sludge, leaves, paper, and food wastes into a soil-like material called compost. It is the same process that decays leaves and other organic debris in nature and offers several potential benefits, including improved manure handling, enhanced soil fertility, and reduced environmental risk. During the composting process heat is produced, which drives off moisture and kills pathogens (NRAES, 1992).

Goodchild and Clifton-Hadley (2001) concluded that solid farmyard manure poses a lower risk than cattle slurry since it tends to reach a higher temperature during composting and is rarely thrown long distances by machinery. Composting of solid manure, under favourable conditions, can result in an increase of temperatures to 60-70°C (Hahesy, 1996). This was considered to be an effective way of inactivating pathogens over a three week period, thereby minimizing the risk of transmitting disease during spreading to land (Strauch, 1981). However, in a study of compost heaps on Co. Dublin farms, temperatures in excess of

60°C were recorded in only a small number of cases (Hahesy, 1996). Hahesy indicated that bacterial survival could be considerably longer in lower sections of the manure stacks, where recorded temperatures were lower. In addition, Hahesy concluded that higher moisture content along with greater compaction at increased depth would be expected to create an anaerobic environment which is unsuitable for composting. Higher moisture content is likely to occur more commonly in situations where silage rather than hay is fed (Hahesy, 1996). Thus composted manure cannot necessarily be considered safe. In Ireland, manure from *M. bovis* infected cattle or those that are suspected to be infected is not permitted to be spread on grazing land (Phillips et al., 2003).

Slurry becomes anaerobic during storage in lagoons and pits (especially at lower depths) which leads to fermentation rather than composting. In the absence of a composting stage, slurry is extremely unlikely to reach high temperatures during storage and consequently pathogenic bacteria are more likely to survive for long periods in stored slurry (Scanlon & Quinn, 2000a). Although the likelihood of infection may be reduced by the dilution effect (of air, uninfected soil and uninfected slurry/manure) there are risks of creating aerosols by spreading slurry. In summary, the risks associated with spreading of cattle slurry are likely to be much greater than the risks associated with manure.

3.2.2 Potential for TB transmission

Prompted by changes in cattle husbandry (which had led to a gradual increase in the volume of slurry produced on Irish farms) and the persistence of TB in the Irish cattle herd, Hahesy et al. (1992, 1995 & 1996) investigated the potential role of slurry/manure in the indirect transmission of TB between animals. They concluded that the disease risks from spreading slurry with less than six months storage were two-fold (Hahesy et al., 1992, 1996). Firstly, slurry containing viable *M. bovis* can contaminate grassland and result in respiratory/oral transmission and infection of grazing cattle (and local wildlife) for a considerable length of time after the application of slurry. Secondly, land-spreading slurry can generate aerosols that carry bacteria for considerable distances, leading to respiratory transmission of cattle on neighbouring farms. Phillips et al. (2003) also concluded that land spreading cattle slurry might be a potential risk on farms that had, or had recently had, infected cattle. They suggested that this risk could be minimized by prolonged storage of the slurry before spreading, or by spreading it on fields not used for cattle grazing.

Transmission of *M. bovis* in contaminated cattle slurry and manure was considered in an analysis of the risk of transmission of bTB through the disposal on farm land of cattle slurry and manure from TB breakdown herds (de la Rua-Domenech, 2007 cited by Wilsmore & Taylor, 2008). It was concluded that slurry had the potential to spread bovine TB via two routes: ingestion (via the gastrointestinal tract), and respiratory (via the lungs). For this to occur, it would require that at least one bovine in the herd was infected, infectious and shedding bacteria in faeces, urine (unlikely), or milk that was disposed in slurry. If *M. bovis* is excreted in the faeces, urine or milk of an infected bovine, the risk of cattle, other farm animals and wildlife being exposed to and infected with *M. bovis* through contact with contaminated slurry (including milk from TB reactors disposed in the slurry pit) and manure depends on a number of variables discussed by de la Rua-Domenech (2007) and listed below:

- The number of organisms excreted by cattle into the environment will play a significant role in exposure and infection. The levels of *M. bovis* released will depend on the prevalence and severity of infection in the herd as well as lesion distribution in infected animals.
- The duration and conditions of slurry and manure storage prior to land spreading will have an effect on viability of the organism and therefore the risk of exposure and infection.
- The manner in which slurry and manure are applied on farmland may also present an additional risk. For example, exposure may occur as a result of aerosol production during spreading of slurry or if land is not harrowed following deposition of cattle manure and viable bacteria within large lumps of dung are afforded protection from adverse conditions allowing extended survival.
- The survival of the organism will play a significant role in determining the persistence of the organism in the farm environment and exposure of cattle and wildlife. The organism must survive any storage/treatment and the aerial or ground environment for long enough to contact a susceptible host and within the host, it must reach a suitable site of infection and survive to replicate.
- Animal husbandry practices, particularly grazing management, may also be important in reducing/preventing the exposure of cattle to contaminated pasture and soil.
- The route of infection, infective dose and host susceptibility will also determine whether infection occurs, with respiratory transmission requiring a much lower infective dose than oral transmission.
- The risk of animals being exposed to the organism will range from high to low depending on how the variables converge on each farm. Further studies are required to determine which variables or combination of variables will result in a high risk of exposure.

3.2.3 Risk factor studies: management of cattle manure and slurry

The evidence from risk factor studies investigating the potential role of slurry management practices in the spread of bovine tuberculosis is somewhat contradictory. In a case control study on 160 farms in the Republic of Ireland, spreading of slurry on pasture without prior storage was found to present a higher probability of bovine TB occurrence in the herd than on farms producing other types of manure or storing the slurry before spreading (Griffin et al., 1993). Cattle were considered to be at risk if they were grazed on land on which slurry had been spread in the previous 2 months. It was also suggested that cattle may become infected from contaminated slurry in other ways, such as inhalation of *M. bovis* organisms during the spreading process. On the basis of the study, the authors concluded that a direct association between production of slurry and TB incidence could not be ruled out.

Christiansen et al. (1992) also reported increased risk of bovine TB following spreading of slurry stored for less than two months. Between the clearance test and six-month check test (SMC), 31/236 (13%) herds in the study population had animals graze pasture within two months of slurry being spread on it which had been stored for less than two months. In the multivariate analysis, after adjusting for all other variables in the study, herds which failed the SMC were 7.66 times (95%CI 2.72-21.59) more likely to have been exposed to slurry

stored less than two months and spread less than two months before grazing than herds which passed the test.

These studies indicate that inadequate storage of slurry prior to spread may pose a significant risk of infection for cattle and possibly wildlife. In Great Britain, a case-control study by Reilly & Courtenay (2007) identified the storage of cattle manure for 6 months or more as a risk factor for herd breakdowns occurring between 1995 and 1999. However, the authors concluded that this was a counter-intuitive finding. Defra-funded epidemiological investigations on infected farms (TB99 and CCS2005 questionnaires) identified factors significantly associated with the risk of herd TB breakdowns (DEFRA, 2004). Analysis of results from CCS2005 indicated that spreading artificial fertilizers or farmyard manure on grazing land were both "protective" and associated with decreased risk (DEFRA, 2004).

Movement of slurry tankers within- and between- farms was also highlighted as a potential risk factor in an outbreak of *M. bovis* infection at West Penrith in Cornwall 30 years ago (Richards, 1972). It was noted that slurry tankers were moved from farm to farm without being washed or cleansed and these practices may be responsible for perpetuation and spreading disease. Similarly, a recent NI case-control study reported an association between increased risk of bovine TB and the use of contractors for spreading slurry (O'Hagan et al., 2013). The study analysed results from a herd-keeper questionnaire applied in case and control farms based in County Down, N.I. during 2010-2011. The study found that most case farmers (58.1%) and control farmers (61.3%) applied slurry/manure to land grazed by cattle, however, case farms were more likely at multivariable analysis to have contractors spreading slurry on their farm (adjusted OR=2.83; 95%CI 1.24-6.49; P=0.011) compared to control farms. Also, less contractors washed their equipment before the arrival on case farms (27.1%) compared to control farms (31.8%) but this was not a significant difference. The authors concluded, with few contractors washing and disinfecting their equipment after use, the potential of *M. bovis* spreading between farms and possibly even the establishment of a wildlife reservoir appears to be plausible.

3.3 Animal waste management

In Northern Ireland TB reactor herd keepers receive an advisory letter from DARD explaining the conditions for disposal of contaminated materials including slurry, manure and milk. This is sent to all reactor herd keepers at the beginning of the breakdown, and the contents are explained by a DARD Veterinary Officer at the breakdown investigation visit. This letter states that slurry, manure or other animal waste from TB-restricted premises can only be spread on land owned or rented by the breakdown herd keeper. Disposal options and minimum timescales before cattle can access pasture after disposal are given. Herd keepers are made aware that these options may not completely remove the risk from environmental M. bovis, and the longer waste products can be stored, and the longer the land where they are spread is not grazed, the smaller the risk.

In other UK regions, DEFRA guidelines state that slurry, manure or other animal waste can only be removed from TB-restricted premises, or linked holdings, under licence. If considered necessary, restrictions on use of slurry may be applied during a TB breakdown (DEFRA, 2008). At present there are no restrictions on slurry that has moved prior to a TB breakdown. Slurry or manure can be used on land within TB-restricted premises while TB restrictions are in force. Although the guidelines suggest the risk of spreading disease to

other stock or wildlife should be considered (DEFRA, 2013), there is no information in the guidelines as to how disease may be spread to other cattle and wildlife.

3.3.1 Slurry

The literature suggests the longer slurry can be stored and the longer land where slurry is spread is left before grazing, the lower the risk of infection (Phillips et al., 2003; Hahesy, 1996). Current advice on disposal of slurry states that slurry should be stored for 6 months before spreading (DEFRA, 2013). This is supported by experimental studies that indicate slurry/manure should be stored for at least 6 months to allow for natural inactivation of *M. bovis* (Scanlon & Quinn, 2000a; Hahesy, 1996). A mixture of calcium hydroxide and water, "thick lime milk", can be used to reduce the level of *M. bovis* contamination – this may be particularly useful on farms with limited slurry storage capacity. In Germany it is mandatory to treat slurry with "thick lime milk" when certain notifiable diseases, such as TB, have been confirmed (Strauch, 1981; Hahesy, 1992). Other recommendations include the use of a downplate or direct injection to minimise the risk of aerosol production during spreading on land (Phillips et al., 2003). In addition, the slurry should not be spread when cattle are in nearby fields and steps should be taken to reduce the risk of slurry drift into adjoining fields (Phillips et al., 2003).

3.3.2 Manure

Current advice from DEFRA indicates that manure should be sprayed with an approved disinfectant, then removed and stacked for at least three weeks prior to being spread (DEFRA, 2013). However, studies by Hahesy (1996) have indicated that compost heaps may not reach temperatures required for *M. bovis* inactivation and therefore composted manure cannot necessarily be considered safe. In Ireland, manure from *M. bovis* infected cattle or those that are suspected to be infected is not permitted to be spread on grazing land (Phillips et al. 2003).

3.3.3 Milk

Milk from TB reactor cattle is not permitted in the human food chain, whether heat treated or not, and must be withheld from the bulk tank (DEFRA, 2013). Current advice indicates milk from reactor animals may be disposed of in the farm slurry system. Milk from reactors may be disposed of either by mixing it with slurry and spreading it on land or other appropriate manner (DEFRA, 2013; DEFRA, 2008). There is no indication of a required minimum storage or treatment time prior to spreading on land.

3.4 Potential levels of *M. bovis* in cattle slurry/manure

3.4.1 Likelihood of *M. bovis* excretion in TB infected cattle

For cattle slurry/manure to act as a source of *M. bovis*, at least one animal in the herd must be infected and excreting bacteria in faeces, urine or milk that has been disposed of in the slurry lagoon (de la Rua-Domenech, 2007). It has been assumed that all infected animals

excrete, sporadically, at some stage post-infection (Francis, 1946). However, the original citation is quite old, and as a result, it is not clear how the evidence relates to current field cases in regions operating comprehensive cattle-based controls (Goodchild & Clifton-Hadley, 2001). The likelihood of excretion and the number of mycobacteria excreted by an individual animal will be dependent upon infectious dose, site and level of infection, and the amount of time the animal has been infected or severity of infection (Phillips et al., 2003). Excretion of *M. bovis* in faeces, urine or milk is more likely to occur in cases of generalised or advanced tuberculosis, normally characterized by disseminated infection and lesions in organs such as the liver, kidneys and udder, or in the meninges and serous cavities (Neill et al., 2005). Dissemination is considered to arise from primary lesions, possibly in the lung or alimentary tract. Generalized tuberculosis is now observed infrequently in developed countries with active control or eradication programmes, and consequently, excretion of the organism in faeces, urine and milk is now regarded as a relatively insignificant feature of the disease (Hardie & Watson, 1992; Morris et al., 1994; Menzies & Neill, 2000). However, it is recognized that *M. bovis* can be isolated from nasal mucus (Neill et al., 1991; Cassidy et al., 1999) and this is likely to contribute to spread of infection. The low incidence of generalised or advanced disease is attributed to the fact that the current statutory bovine TB surveillance programme removes infected animals before the disease becomes disseminated. In areas with annual testing regimes in place (such as in NI), the majority of infected cattle are removed before reaching advanced disease and as a result the risk of excretion should be reduced.

3.4.1.1 Faeces

Current information from meat inspection and post mortem examination indicates that few infected cattle exhibit lesions in the intestine or mesenteric lymph nodes (Liebana et al., 2008). Tuberculous lesions occurring solely in the mesenteric lymph nodes are not now a common finding in cattle, but do occasionally result from ingestion of a heavy bacterial load, such as calves drinking infected milk. However, it has also been suggested that they may result from dissemination from primary lesions in other sites. In cases of bovine TB, lesions are found most frequently in lymph nodes of the thoracic cavity, usually the bronchial and/or mediastinal lymph nodes, and these lymph tissues are thought to be the first affected. Confirmation of infection in TB reactors is generally based on histological examination and/or bacteriological culture of affected respiratory tissues. Faecal deposits are rarely examined for the presence of *M. bovis*, and as a result, empirical data on shedding of *M. bovis* in faeces is limited.

A few studies have investigated the excretion of *M. bovis* in naturally infected cattle. Williams & Hoy (1927) reported viable tubercle bacilli could be demonstrated in the faeces of six apparently healthy cows. In a subsequent study, 24% of faecal samples from cows were found to be positive for *M. bovis* following inoculation of guinea pigs (cited by Williams & Hoy, 1930). The authors concluded that the chief source of infection of the faeces originated from the lungs, supported by the fact that at the post mortem examinations only one cow showed any naked eye evidence of tuberculous infection of the mucous membrane of the intestine. Furthermore, cows were observed to swallow coughed sputum and *M. bovis* was demonstrated repeatedly in sputum. According to Makkaievskaya (cited by de la Rua-Domenech, 1997), 93% of cattle with clinical TB and 43% of reactors without clinical signs shed *M. bovis* bacilli in their faeces.

Studies by Reuss (1955) and Schellner (1959) indicate that the proportion of heavily infected cattle excreting *M. bovis* in faeces is typically 10%. Reuss also cited three earlier studies showing that 5-80% of all tuberculin reactor cattle could excrete *M. bovis* in their faeces without exhibiting any clinical signs of TB (de la Rua-Domenech, 2007). In a more recent investigation in the Republic of Ireland, 40% of infected cattle were reported to excrete the organism in faeces (Christiansen et al., 1992). Taken together, results from these studies indicate that there may be some variation in faecal excretion in infected cattle, with the proportion of animals excreting likely to be greatly reduced for cattle in the early stages of infection. Early studies based on animals with advanced disease may not be representative of the current situation in countries with established TB control programs. The proportion of advanced bovine TB cases excreting the organism in the faeces may still be correct, however, there are now many fewer cases with advanced bovine TB.

Excretion of *M. bovis* in faeces from experimentally infected cattle in the absence of TB lesions in the abdomen has also been reported. In one study, intranasally infected donor calves were placed in-contact with uninfected calves, resulting in confirmed infection in seven out of nine "in-contact" animals. *M. bovis* was recovered from a single faeces sample from one "donor" calf and one "in-contact" calf. Samples of small intestine from three of the "in-contact" calves were positive for *M. bovis* by culture (Cassidy et al., 1998). In another study, Neill et al. (1988) reported excretion of *M. bovis* in the faeces of 9/10 cattle following intranasal inoculation with high doses of *M. bovis* (10^6 or 10^4 cfu). Excretion in faeces was not a regular occurrence despite frequent sampling. However, it is important to bear in mind that faecal sampling is complicated by inherent difficulties in isolating the organism from faeces using conventional culture. It was suggested that due to the low frequency and irregular excretion of *M. bovis* in the faeces, even in heavily infected animals, faecal excretion may be a less important mode of transmission than direct respiratory spread. The number of faeces samples positive for *M. bovis* was greatest in those animals having the longest periods of regular occurrence of the organism in nasal mucus. Based on this observation, and the absence of intestinal lesions in any animal in this study, the authors concluded it was probable that *M. bovis* in the faeces arose directly from swallowing infected mucus from the respiratory tract (Neill et al., 1998). This is probably more likely than the release of *M. bovis* into the gut contents from TB granulomas in the intestinal mucosa or other parts of the digestive system. Francis (1947) remarked that TB bacilli coughed up from the lungs into the pharynx are usually swallowed and many of them are passed out in the dung with or without concurrent intestinal lesions.

3.4.1.2 Milk

Where there is infection in the herd, either detected or undetected, routes that could lead to contamination of milk with *M. bovis* include via faeces and from the environment but the main risk is from direct contamination of the milk in the udder. Contamination of milk can occur before the animal tests positive on the skin test or before clinical signs of infection are apparent (ACMSF, 2010). A study conducted in Brazil by Zarden et al. (2013) collected milk samples from 8 SICCT negative animals for examination by culture and PCR and found that 5 milk samples were positive for *M. bovis* – one by culture and 4 by PCR. In other studies, Zumarraga et al. (2012) reported positive PCR results for milk samples from bulk tank from TB-suspected herds and also certified TB-free herds and Figueiredo et al. (2010) reported the identification of specific *M. bovis* DNA in 12% of milk samples from skin test negative cattle.

Contamination of milk is most likely to occur when infection becomes disseminated and there is tuberculous mastitis. Although *M. bovis* will not multiply in milk or will do so very slowly (Lake et al. 2002), the large number of bacteria excreted by a single cow with tuberculous mastitis is generally sufficient to render milk pooled from 100 milking cows infectious to humans (Wilson, 1942 - cited in Pritchard, 1988). When the condition remains undiagnosed it has serious consequences since one mastitic cow can infect a large number of calves fed on the milk withheld from the bulk tank (de la Rua-Domenech, 2007). Excretion of up to 10^3 colony-forming units of *M. bovis* per ml has been reported in subclinically infected cows (Zanini et al., 1998). In 1934, before the adoption of milk pasteurisation and compulsory tuberculin skin testing of cattle, it was reported that more than 40% of dairy cows in Great Britain were infected with *M. bovis* and 0.5% suffered from TB of the udder. During this period, bovine TB was widespread in humans and approximately 2,500 people died annually from the disease (de la Rua-Domenech, 2006). Since the introduction of milk pasteurisation in the UK in the early 1960s, bovine TB in humans has declined rapidly (Torgerson, 2009). Between 1993 and 2003, 315 human cases of bovine TB were confirmed (Javala et al., 2007). Among the people affected, only 14 had been born in the UK after 1960, whereas most had been born either before 1960 (265 cases) or outside the UK (36 cases).

Despite the resurgence of bovine TB in the cattle population since the late 1980s, the percentage of cows infected is much lower than it was in the 1930s and tuberculous mastitis nowadays is rarely seen in cows in the UK. This is believed to be due to the fact that the current statutory bovine TB surveillance programme removes infected animals before the disease becomes disseminated to the udder (ACMSF, 2010). Information from GB reactor cattle indicates that tuberculous lesions in the udder and associated lymph nodes are now uncommon in cows (Goodchild & Clifton-Hadley, 2001). Although rarely seen these days, cows diagnosed with tuberculous mastitis should always be assumed to excrete *M. bovis* in their milk.

As reported by Doran et al. (2009) cases of mastitis can still occur resulting in widespread effects on humans and cattle. This case describes an outbreak of TB affecting cattle and people on a dairy farm in Ireland following consumption of raw milk from a seven year old cow with tuberculous mastitis. Twenty-five of 28 calves born between autumn 2004 and spring 2005 were subsequently identified as TB reactors and 5 of 6 family members were positive on the Mantoux test. During 2005, milk from this cow had been mainly used to feed calves and was added occasionally to the bulk tank. The family collected milk from the bulk tanks and consumed it without pasteurisation. The cow had been negative to the SICCT on seven occasions since 2003, but was positive to an antibody based ELISA in July 2005.

3.4.1.3 Urine

In theory, TB infected cattle may be capable of excreting *M. bovis* in urine; however, there is very little evidence to confirm that this occurs. Shedding of *M. bovis* in the urine of infected cattle is the result of renal or genital TB. These forms of bovine TB have been described in both naturally and experimentally infected cattle, but they tend to occur as part of generalised TB following haematogenous spread of the bacterium in the late stages of the disease (Francis, 1947; Jubb et al., 1993). TB lesions in the kidneys, genital organs and associated lymph nodes of tuberculin test reactor cattle are exceptionally rare in the UK

nowadays. In a study of lesion distribution in GB reactor cattle between 1986 and 1994, only 1.9% of submissions were associated with kidney (and other sites) (Goodchild & Clifton-Hadley, 2001).

3.4.2 Assessing the levels of *M. bovis* in cattle slurry/manure

We were unable to locate any published data relating to investigation of levels of *M. bovis* in cattle slurry/manure. Given the limited data available on excretion in cattle it is difficult to estimate the levels of *M. bovis* that may be present in slurry/manure. Further research is required to determine the potential mycobacterial load in these matrices, particularly on farms with cattle herds at high risk of TB infection and those with high numbers of reactors. Investigation of the mycobacterial load will be complicated by the need to sample large volumes of slurry/manure, particularly if low numbers of bacteria are likely to be present. Furthermore, the inherent limitations of available methods for direct detection of *M. bovis* (bacterial culture, polymerase chain reaction (PCR) and IMS based methods) are likely to present additional challenges, as discussed below.

3.4.2.1 Culture

Isolation of *M. bovis* from infected tissues by culture is regarded currently as the "gold standard" for definitive diagnosis of bovine TB in cattle. In other studies bacteriological culture has been applied in detection of *M. bovis* in faeces from badgers and ferrets. Bacteriological culture has also been used to assess *M. bovis* viability in sterilised cattle slurry (Scanlon & Quinn, 2000a). Bacteriological culture could potentially be applied to slurry to provide a quantitative assessment of mycobacterial numbers; however, sampling of slurry may prove difficult due to the volumes concerned and the presence of competing microorganisms. There is also potential for *M. bovis* to become dormant during long term storage in slurry and the organism may require a period of natural or induced resuscitation before growth in culture media. The sensitivity of culture is dependent upon a number of factors including the type of samples examined, processing of samples prior to culture, the type of culture media used and the length of incubation time. Members of the *Mycobacterium tuberculosis* complex are extremely slow growing and require long incubation periods to maximise recovery, particularly on primary isolation. Due to the difficulties experienced with primary isolation of *M. bovis* from clinical specimens, a range of processing procedures (homogenisation, decontamination and concentration), and the use of culture media to inhibit competing organisms, are employed to facilitate the recovery of mycobacteria (Murray et al., 2007; Corner et al., 2012).

A variety of solid media is available for recovery and enumeration of Mycobacterial species, including Lowenstein-Jensen, Stonebrink's, Herrolds egg yolk, and Middlebrook. Recovery of *M. bovis* can be enhanced by using more than one culture medium (Corner & Nicolacopoulos, 1988; Corner et al., 2012). Colonies of *M. bovis* appeared earlier on agar-based media (Middlebrook) than on egg-based medium (Lowenstein-Jensen, Stonebrink's, Herrolds). However, more colonies grew on the egg-based medium and a higher proportion of samples were positive following culture on this medium (Corner et al., 2012). Results from this study support an earlier recommendation by Krasnow & Wayne (1969), that two different types of media should be used: agar-based media for more rapid detection of positive samples and egg-based media for greater sensitivity. As initial concentration of

bacilli in samples decrease, the time to detection increases. In samples with low numbers present, additional replicates and extended incubation will increase probability of detecting positive samples (Corner, 2012).

Since *M. bovis* is a slow growing organism, faster growing non-mycobacterial species can outgrow *M. bovis*, making it difficult to isolate the organism from samples. To prevent overgrowth, chemical decontamination is usually carried out to reduce or eliminate other contaminating bacterial species using hexadecylpyridinium chloride (HPC), oxalic acid or sodium hydroxide. Although these agents are effective in controlling contamination from competing organisms they are also toxic to *M. bovis* to varying degrees (Corner & Trajstman, 1988; Corner et al., 1995). Adverse effects of decontaminants lead to increased time to detection, a decrease in the number of positive samples, and a decrease in the number of colonies present. Experiments have demonstrated that toxicity generally increases with increasing concentration and that HPC is generally less toxic than oxalic acid or sodium hydroxide. Due to the toxic effects of decontamination, isolation using bacteriological culture may prove problematic. The numbers of bacteria may be too few to isolate, especially if chemical decontamination is required to prevent overgrowth of competing microorganisms likely present in faeces/slurry samples.

In sample matrices such as cattle slurry, *M. bovis* may become dormant in response to lack of oxygen or other unfavourable conditions such as nutrient depletion or low temperatures. Bacteria of the TB complex have been shown to actively modulate their gene expression and metabolism in response to sensing their environment. Bacilli in this physiological state do not readily grow on artificial media and may require resuscitation to restore culturability. There is considerable circumstantial evidence to suggest that persisting organisms, such as *M. tuberculosis*, may be capable of existing in physiological states that are characterized by impaired culturability (i.e. colony-forming ability) (Shleeva et al., 2003; Young et al., 2005a). Addition of resuscitation promotion factors (Rpfs) from *M. luteus*, *M. tuberculosis*, and possibly even *M. bovis* to dormant cultures may potentially resuscitate dormant cultures and stimulate the growth of viable cells. Resuscitation promotion factors, which promote the resuscitation and growth of dormant, non-growing cells, were initially discovered in *Micrococcus luteus* (Mukamolova et al., 1998; Kell & Young, 2000). In picomolar concentrations, *M. luteus* Rpf was found to increase the viable cell count of dormant cultures at least 100-fold. It was also shown to stimulate the growth of several other high GC Gram-positive organisms, including *M. avium, M. bovis* (BCG), *M. kansasii*, *M. smegmatis* and *M. tuberculosis* (Mukamolova et al., 1998). *M. tuberculosis* also possesses five *rpf* homologues, *rpf A-E* (Mukamolova *et al*., 2002), and expression of some of these rpfs factors has been observed during human infection. A study by Kana et al. (2008) demonstrated a key *in vitro* phenotype associated with progressive *rpf*-like gene loss in *M. tuberculosis* is the inability to resuscitate spontaneously from a 'non-culturable' state.

3.4.2.2 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is a method of detecting small amounts of DNA from various samples by DNA amplification. It derives its name from one of its key components, a DNA polymerase used to amplify a piece of DNA by *in vitro* enzymatic replication. As PCR progresses, the DNA generated is itself used as template for replication in the next cycle. This sets in motion a chain reaction in which the DNA is exponentially amplified. With PCR it is possible to amplify a single or few copies of DNA target across several orders of magnitude, generating millions or more copies of the DNA target to an extent where it can

be quantified, visualized, manipulated etc. (Old & Primrose, 1994). PCR has been a revolutionary molecular biology technique and an important enabling technology for the analysis of amplified DNA. In direct detection mode, it has been particularly effective in "viral load testing" in diagnostic virology. However, due to increased complexity, its application to direct detection of important bacterial pathogens, and TB in particular, has lagged substantially behind the virology field. Indeed, the DEFRA bovine TB Diagnostics Programme Advisory Group (DPAG) concluded recently that PCR was not currently "fit-for-purpose" for bovine TB detection, at least in environmental samples (DEFRA, 2010).

No information relating to the development of PCR for detection of TB in cattle faeces or slurry could be found in the literature, however, PCR assays for the detection of TB in other matrices (lymph nodes/tissues, soil, human and badger faeces) have been described. A number of PCR methods have been developed for detection of TB in tissue and lymph nodes from cattle (Skuce et al., 2003; Mishra et al., 2005; Taylor et al., 2007). In most of these studies, conventional culture was shown to be superior to PCR in detection of infected animals. Oberhelmen (2010) reported the use of an IS6110 "nested" PCR for detection of *M. bovis* in human faecal samples. In samples from adults with active pulmonary TB the sensitivity of PCR was reported as 86%, whereas in children excreting lower amounts, the sensitivity was only 38%.

Young et al. (2005b) reported the use of a PCR assay targeting MPB64/70 for the detection of *M. bovis* and *M. bovis* BCG in spiked soil. This work was carried out in an attempt to assess the suitability of PCR for detection of *M. bovis* in the environment with particular reference to identification of positive badger setts for targeted culling. Reported limits of detection for the MPB64/70 based PCR were 10^1-10^2 cells per g of spiked soil for PCR (DNA detection) and 10^4 cells per g of spiked soil for RT-PCR (detects mRNA which indicates presence of viable cells). In a study by the same group, Sweeney et al (2007) deployed a PCR assay based on the RD4 flanking region to detect the presence of *M. bovis* at badger setts. This assay was specific for *M. bovis* and identified all setts sampled although there was greater variation in cell numbers found at setts compared to latrines. In interpreting the results of these studies, it must be considered that detection of *M. bovis* specific DNA sequences may not necessarily indicate the presence of viable infectious organisms. In a further study by this group Pontiroli et al (2011) spiked bovine slurry with various concentrations of *M. bovis* BCG and attempted to detect the inoculum by a range of in-house and commercial PCR template preparations. Whilst the BCG inoculum was detected in some spiked slurry samples, some of the non-inoculated control samples were also PCR positive.

The potential advantage of using PCR assays is that a result can be obtained in days rather than the weeks required for culture, and consequently, this has the potential to reduce the time and costs associated with detection and identification. However, it is worth noting that PCR can be hampered by certain issues. Firstly, in the case of *M. bovis*, extraction of DNA may be problematic due to the nature of the mycobacterial cell wall; secondly, the presence of certain components (PCR inhibitors) in environmental samples can prevent or hinder DNA amplification; and lastly, PCR has a poor negative predictive value. At present there is very little evidence to suggest that PCR offers any advantages over culture and a review into bovine TB concluded that 'the PCR technique is not yet able to perform as well as conventional bacterial culture in the detection of *M. bovis* in terms of sensitivity, specificity or reliability' (Wilsmore & Taylor, 2008; DEFRA, 2010). Extensive validation and replication work is required to further develop available/new PCR assays to allow an assessment of

their potential value as a useful tool for direct detection of TB in clinical and environmental samples, including cattle faeces/slurry.

3.4.2.3 Immuno-magnetic separation-based methods (IMS)

Immuno-magnetic separation (IMS) is one of several "target-enrichment" methods in which specific antibodies are linked to magnetic beads and used to attempt to recover and concentrate bacterial organisms, or their DNA, from various clinical and environmental samples. Once recovered by IMS, the bacteria can then potentially be identified using a range of methods including bacteriological culture and / or PCR (Olsvik, 1994). At present, there are no reports in the literature of the use of IMS-based methods for detection of TB in faeces or slurry, however, a small number of studies have outlined the development of novel IMS based methods for direct detection of *M. bovis* from spiked tissue and naturally infected tissues (Garbaccio & Cataldi, 2010; Stewart et al, 2012; Stewart, 2013). There may be potential to improve detection of *M. bovis* from clinical specimens using IMS based methods. By using IMS to capture the organism, the need for chemical decontamination, which can be detrimental to *M. bovis* cells (Corner & Trajstman, 1988; Corner et al., 1995), is circumvented, and therefore enhanced detection of bacteria by bacteriological culture or other methods may be possible. However, further validation and replication studies of IMS-based tests, particularly IMS-PCR, using a range of sample matrices, including faeces/slurry is required.

In 2010, Garbaccio & Cataldi described an IMS-based method for detection of *M. bovis* using magnetic beads incubated with goat anti-rabbit IgG followed anti-*M. tuberculosis* H37Rv lysate polyclonal rabbit serum. In this work, immuno-magnetic capture followed by PCR (IMS-PCR) based on the IS6110 element showed a detection threshold corresponding to 10 CFU in *M. bovis*-spiked PBS. When the method was applied to infected bovine fresh tissues, the minimum value of detection was 1000 CFU in 100% of the trials (5 replicates), indicating reduced sensitivity in clinical samples. This reduction in sensitivity is likely due to difficulties in extracting mycobacterial cells from lesioned tissues in comparison to *M. bovis*-spiked PBS.

Stewart et al (2012) outlined the development of an immunomagnetic separation (IMS) method to isolate *Mycobacterium bovis* cells from lymph node tissues. In this study, gamma-irradiated whole *M. bovis* cells and ethanol-extracted surface antigens of such cells were used to produce polyclonal and monoclonal antibodies, and peptide ligands by phage display biopanning, against *M. bovis*. The various antibodies and peptide ligands obtained were assessed for cross-reactivity and non-specific binding using a range of Mycobacterial species before being used to coat immunomagnetic beads, singly or in combination, and evaluated for IMS. IMS-based methods were applied to various *M. bovis*-spiked lymph node matrices, achieving detection sensitivities (50% limits of detection of 3.2 and 57.7 CFU/ml of lymph node tissue homogenate for IMS-PCR and IMS-culture, respectively). However, it should be noted that spiked tissue is probably not a good simulation of intracellular bacteria and that it may be much more difficult to recover and detect the organisms from lesioned tissues.

In a subsequent study, Stewart et al (2013) applied the previously developed IMS based methods (IMS-MGIT(culture) and IMS-PCR) in a survey of 280 bovine lymph nodes (206 visibly lesioned, VL; 74 non-visibly lesioned, NVL) collected at slaughter as part of the Northern Ireland bovine TB control programme. Overall, 174 (62.1%) lymph node samples

tested positive for *M. bovis* by culture, 162 (57.8%) by IMS-PCR (targeting IS6110), and 191 (68.2%) by IMS-MGIT culture. There was imperfect agreement between the three methods. Depending on the IMS-based method, only between 64-74% of VL reactor tissues were IMS positive; culture would routinely detect >98% of these. It appears that no known negative tissues were blinded into the study and no data were presented on the reproducibility of the results. Twelve (6.9%) of the 174 culture positive lymph node samples were not detected by either of the IMS-based methods. However, an additional 79 lymph node samples (27 (13.1%) VL and 52 (70.3%) NVL) were detected positive by the IMS-based methods (IMS-MGIT and IMS-PCR) but not by culture. In the case of the NVL samples, the difference between results of culture and IMS-based methods was particularly marked: 54 (73%) of 74 NVL samples were positive by IMS-PCR or IMS-MGIT culture, whereas only 2 (2.7%) of 74 NVL samples cultured positive.

4 Environmental persistence and *M. bovis* transmission

Cattle manure and slurry containing viable *M. bovis* organisms and spread on farm land constitutes a mechanism whereby the farm environment can become contaminated with the bacterium. The same is true for *M. bovis* deposited directly by infectious cattle defecating on pasture. In theory, indirect transmission of *M. bovis* to cattle and wildlife via a contaminated environment may potentially occur through inhalation/ingestion of the organisms during investigation of cattle faeces deposited in the field, inhalation of infectious aerosols produced during slurry spreading or inhalation/ingestion of *M. bovis* during grazing on pasture and silage. The route of infection, infective dose and host susceptibility will determine whether infection occurs, with respiratory transmission requiring a much lower infective dose than oral transmission. For *M. bovis* to be transmitted via a contaminated environment, the organism must be capable of surviving in the environment and retaining infectivity for a sufficient amount of time before reaching a susceptible host. The ability of the organism to survive outside its hosts for prolonged periods of time has been demonstrated following both natural and artificial contamination of various environmental sites, including cattle faeces, stored slurry, pasture and soil. The survival of *M. bovis* in these environments is discussed in the following sections.

Mycobacterial species, including *M. bovis*, are well known for their ability to survive dehydration, fluctuations in temperature, moderate pH changes and the effect of sunlight (Duffield & Young, 1985). The ability of mycobacteria to survive for long periods in suboptimal conditions is attributed to the slow growth and impermeable cell wall (Scanlon & Quinn, 2000a). The survival of *M. bovis* in the environment is influenced by the presence of organic matter, temperature, moisture, the desiccating effect and ultraviolet (UV) radiation of sunlight, pH, activity of other bacteria, fungi and protozoa and the nature of the contaminated substrate (Wray, 1975). However, it is not clear how these variables interact with each other. Adequate availability of nutrients in the form of organic matter was the most critical factor according to Wray (1975). If nutrients are scarce, organisms become more susceptible to the adverse effects of other factors. Sunlight indirectly affects survival by causing desiccation, whereas high levels of moisture and relative humidity enhance survival (Wray, 1975; Tanner & Michel, 1999). In summary, survival of *M. bovis* is enhanced in moist, cool conditions and neutral-to-acidic substrates rich in organic matter, especially when the bacilli are protected from direct sunlight.

4.1 Potential infection via cattle faeces

Following deposition in faeces at pasture, *M. bovis* survival depends on the amount of sunlight and the thickness of the deposit. Typically, the faeces may remain infective for up to six months when deposited in winter but only one to two months in the summer (Mitserlich & Marth, 1984), depending on the temperature and the concentration of pathogens in the faeces. After this time most of the deposit will have been broken down by arthropods and micro-organisms (Phillips, 2003).

Maddock (1933) carried out an experiment with large doses of *M. bovis* mixed with soil or faeces stored in open jars buried in soil. Infectious material was recovered from faeces exposed to the elements for 178 days, but in particularly hot and dry weather this was reduced to 152 days, and there was a decrease in virulence after 61 days (Maddock, 1933). On the basis of these findings, Maddock concluded that faeces was only safe after approximately seven months storage, and that the degree of infectivity was related to the retention of organic matter in the faeces. Other experiments have demonstrated that *M. bovis* can survive in faeces for 2 months in summer and 5 months in a wet winter (Rudolfs and Ragotski, 1950). Williams & Hoy (1930) reported survival of *M. bovis* in faeces for 5 months in summer, 4 months in autumn and less than 2 months in winter. In another study, *M. bovis* survival was 32 days in dry faeces and 54 days in moist faeces. *M. bovis* could not be recovered from the manure of infected cattle after twenty years storage (Deutrich and Pioch, 1991). In badger faeces spiked with TB bacilli and exposed to the open air, infectivity for guinea-pigs was retained for at least 5 months from November, 4 months from September, 2 months in May and less than 2 months in summer (Benham, 1991).

In a review Phillips et al. (2003) remarked that cattle avoid grazing close to the faeces of other cattle, preferring to graze mature sward fertilized by the deposit. Therefore, it seems unlikely that there is much/any acquisition of *M. bovis* infection directly from faeces deposited by grazing cattle. However, a recent proximity study demonstrated that direct contacts (interactions within 1·4 m) between badgers and cattle at pasture were very rare (four out of >500,000 recorded animal-to-animal contacts) despite ample opportunity for interactions to occur. Indirect interactions (visits to badger latrines by badgers and cattle) were more frequent than direct contacts: 400 visits by badgers and 1,700 visits by cattle were recorded (Drewe et al., 2013).

Badgers in GB will regularly forage cattle deposits in search of food (e.g. earthworms) (Skuce et al., 2011). Earthworms feed mostly on decomposing plant mass, ingesting numerous microorganisms and carrying them both deep into the soil layer and to its surface (Aira et al., 2008). Most of the ingested microorganisms pass through the digestive tract and are excreted in earthworm faeces (Holter, 1979); however, some bacterial species can propagate in the digestive tract (Schonholzer et al., 1999). The role of earthworms as vectors of mycobacterial infection in cattle and goat farms has been identified for *M. avium* and *M. paratuberculosis* (Fischer at el., 2003). To date, there have been no reports of *M. bovis* isolation from earthworms and the risk of TB transmission to badgers via consumption of *M. bovis* contaminated earthworms remains unquantified. In summary, further work is required to assess the potential role of earthworms in TB transmission to badgers.

4.2 Transmission via slurry spreading

4.2.1 M. bovis survival in cattle slurry

Several observational studies have demonstrated the ability of *M. bovis* bacilli to survive for prolonged periods under favourable conditions in naturally- and artificially-contaminated cattle slurry. The reported survival of the organism in cattle slurry varies depending on the experimental design and conditions, which makes comparisons between studies difficult. Factors likely to play a significant role in the survival of *M. bovis* in slurry include: initial numbers of the organism, dilution effect of slurry, temperature, organic content, dry matter content (moisture), and presence of competing organisms (de la Rua Domenech, 2007; Strauch, 1991). The high water content and amount of organic material in cattle slurry offer a favourable environment for the survival of many pathogenic bacteria (Scanlon & Quinn, 2000a). Cattle slurry has a pH value close to neutral and, unlike solid manure, does not undergo composting (Scanlon & Quinn, 2000a), therefore prolonged survival of many infectious agents, including *M. bovis*, is possible in stored slurry (Strauch, 1981; Scanlon & Quinn, 2000a).

Williams & Hoy (1930) examined the viability of *M. bovis* in liquid manure. Briefly, two and a half gallons of liquid manure were taken from a dairy farm and to each cubic centimetre was added 5,000 tubercle bacilli derived from tissue from tuberculous cattle. The mixture was stored underground in a jar before inoculation into test animals (guinea pigs). There was a gradual decrease in virulence of the mixture, which was evident from the requirement for increasing quantities of inoculum as testing proceeded. Despite the decrease in virulence between week 0 and 22, viable and virulent mycobacteria were still present in the liquid manure after 4 months.

In a study by Scanlon and Quinn (2000a), *M. bovis* was added to sterilised slurry to yield a concentration of 6.5 x 10^3 cfu per ml and the suspension dispensed in 10 ml volumes in screw cap bottles stored in the dark at ambient temperature. Over 31 weeks, at weekly intervals, a sample was taken from a separate bottle and cultured for growth of *M. bovis*. The numbers of viable mycobacteria in the slurry declined over time: from the initial 6.5 x 10^3 to 2.8 x 10^3 after 27 days, to 1.7 x 10^3 after 42 days, to 2 cfu after 164 and 171 days and zero after 178 days. The authors concluded that storage conditions and absence of other viable microorganisms may have influenced the survival pattern of *M. bovis* in this study. Furthermore, the authors noted that the initial concentration of organisms in a sample usually determines the duration of survival in defined conditions and that the high number of *M. bovis* in the study may not be representative of the numbers present in slurry from TB infected cattle. Doukoupil (1964) cited by de la Rua-Domenech (2007) also reported a survival time of 176 days (approx. 6 months) in liquid cattle manure stored at 5°C.

Taken together, the results of these studies indicate that storage for at least six months may be necessary before all *M. bovis* organisms in contaminated slurry are naturally inactivated. Storage temperature will play a significant role in determining survival, which was 17 months at 40-45°C (Vera, 1988) but only 30 days at 54°C (Hahesy et al., 1995). Results from these studies indicate that survival times may be extended at lower temperatures. *M. bovis* in slurry stored in lagoons and tanks is likely to be subjected to quite low temperatures, particularly in Northern Ireland, and therefore the organism may be capable of surviving for longer periods in this environment.

4.2.2 *Aerosol production during slurry spreading*

Production of aerosols potentially containing *M. bovis* during mixing and pumping of slurry, particularly in slurry tanks under slatted floors, may pose an infection risk to humans and cattle, if these infectious particles are inhaled (Scanlon & Quinn, 2000a). Creation of aerosols by spreading of slurry on land is also a recognised risk, including for contiguous farms (Skuce, 2011). In addition to the direct risk to humans and cattle, spreading potentially infected slurry on the land may increase the likelihood of establishing a local wildlife reservoir of *M. bovis* infection, with consequent danger of spill-back transmission to cattle (Phillips et al., 2003).

The methods used for spreading and prevailing weather conditions can greatly influence dispersal of aerosols as demonstrated by Hahesy et al. (1995). In this study, dispersal and recovery of a marker bacterium (*Serratia rubidaea*), added to cattle slurry and used as a proxy for *M. bovis*, was investigated using five slurry spreading methods under field conditions. The maximum distances marker bacteria were recovered downwind following dispersal by shallow injection, band spreading, low splash plate, high splash plate and raingun spreading methods were 50m, 50m, 200m, 300m, and 800m, respectively. An association between windspeed and both the rain gun and high splash plate methods was also observed.

4.3 Transmission via contaminated pasture

Investigations into the transmission of TB via contaminated pasture have produced some conflicting results. Some studies have reported infection with *M. bovis* after grazing pasture contaminated both naturally and artificially. Maddock (1933) examined the infectivity of pasture following irrigation of grazing plots with suspensions of tuberculous organs from cattle. Maddock demonstrated that following repeated infection of grazing plots, it was possible to induce tuberculosis (via ingestion) in guinea-pigs grazed in the open and in those fed cut grass from infected plots. In a similar experiment conducted in cattle, tuberculosis was confirmed in 3/3 cattle grazed on infected pasture and 2/3 fed cut grass from infected plots (Maddock, 1934). Post mortem results indicated that infection probably occurred by the alimentary route (ingestion).

Schellner (1959) experimentally irrigated pasture plots with 10^2-10^{12} *M. bovis* per ml of water and after periods of 7, 14, and 21 days, allowed heifers to graze. Only 2 of 14 animals which grazed a plot irrigated 7 days previously became infected. All other animals remained healthy. Previously, Schellner (1956) found that after one week of resting pasture following grazing by heavily infected cows there was approximately a 6% chance of a non-infected cow acquiring the infection each day, but after two weeks rest this had declined to 2% per day. The most likely sites for the infection to reside were, in declining order, the bronchial, intestinal, mediastinal and pulmonary lymph nodes. On the basis of these findings, Schellner concluded that infection may have occurred by both the oro-pharangeal and respiratory route, possibly by eructation or aerosol inhalation during grazing. These findings support the theory that contaminated pasture may present a risk, however, the experiments are quite old and mesenteric involvement and generalized bovine TB is allegedly rare nowadays.

Experiments carried out in the Barabinska lowland region of South Central Russia in the late 1960s demonstrated the possibility of *M. bovis* transmission through naturally- and artificially-contaminated grass (Kislenko, 1972). Virulent *M. bovis* was detected in pasture grazed seven months earlier by naturally-infected cattle. Faeces from three orally-infected young bulls were then spread on a field over a three-month period. Again, virulent bacilli were demonstrated in the following three months by means of experiments in guinea pigs. Thereafter, one naïve calf grazed on the same field for 55 days was found to develop tuberculin reactivity and tuberculous lesions in bronchial, mediastinal and mesenteric lymph nodes, from which *M. bovis* could be isolated. The distribution of lesions suggests that infection may have occurred by both the oral and respiratory route.

Other studies indicate the potential risk of bovine TB transmission via contaminated pasture, even following heavy contamination, is extremely low. In one study, Maddock (1936) produced a heavy infection of pastures by allowing artificially-infected calves to graze the plots. Briefly, calves were dosed with infected whey until such time as they excreted *M. bovis* in their faeces. The excreting calves were grazed for three weeks following which 2 uninfected calves were introduced to graze for 3 weeks on one of three plots at intervals following removal of the original calves. No signs of bovine TB infection were evident in any of these calves *post mortem*. In a further experiment, a cow with TB mastitis and excreting *M. bovis* in her faeces was grazed for 9.5 weeks. Naïve calves were introduced to contaminated plots at monthly intervals. Following examination, no infection was demonstrated in the grazing calves. The information available on survival and transmission of *M. bovis* at pasture is somewhat contradictory, and therefore the possibility of infection via contaminated pasture cannot currently be excluded and may justify further investigation.

4.4 Transmission via contaminated soil and silage

4.4.1 Soil

Most studies have found that the organism remains viable in soil for about 6 months (Maddock, 1933; Saxer & Vanarburg, 1951), with one study reporting shorter survival periods but also encountering difficulties in culturing *M. bovis* (Duffield & Young, 1985). There appear to have been no attempts to measure the effects of temperature and humidity of soil on the maintenance of a viable population of *M. bovis*, even though these factors are likely to be of major significance (Phillips et al. 2003). Soil can be ingested by cattle, comprising ~5-10% of the fresh-weight intake and 10-15% of dry weight intake of grazing cattle (Skuce et al., 2011). Cattle tend to consume soil to offset mineral deficiencies and for behavioural head rubbing, during which they may create dust and potentially infectious aerosols. Relatively more soil would be ingested when pasture sward is short and soil may also contaminate silage. Providing cattle with mineral supplements in the field may reduce the attractiveness of soil (Phillips, 2003).

4.4.2 Silage

There is little information on the survival of *M. bovis* during the ensiling process. Reuss (1955) reported samples of faeces containing *M. bovis* were not infectious to guinea-pigs after being ensiled with grass for ten weeks in a mini-silo. Similarly, a study based on lab-

scale ensiling failed to recover *M. bovis* from ensiled material at 6 or 12 weeks, which the authors attributed to bacterial recovery processes (DEFRA SE3022). Studies indicate the oxygen concentration in grass silage is reduced to zero within a day of ensiling (Phillips et al. 2003), which might kill *M. bovis* or induce it to enter a state of dormancy (Hutter & Dick, 1999; Cunningham & Spreadbury, 1998). This may explain the lack of infectivity in the experiment conducted with guinea-pigs by Reuss (1955) and the inability to recover viable organisms in the DEFRA study (DEFRA SE3022). The optimum pH for *M. bovis* is 5.8-6.9 and it will survive for 20 days at pH 4-5 in yoghurt (Mitserlich & Marth, 1984). The pH of silage has been shown to decline to approximately pH 4 (Phillips, 2003), therefore the pH during the ensiling process is unlikely to have a significant effect on viability. Furthermore, the temperature during ensiling and storage of grass increases to approximately 30°C (Williams, 1997), which is close to the mammalian body temperatures at which *M. bovis* can grow (37°C). It is therefore unlikely to inactivate the organism. The information currently available indicates silage cannot be excluded as a risk and the precautionary principle would suggest that steps should be taken to avoid spreading silage fields with contaminated slurry.

5 Effect of disinfection and anaerobic digestion on the viability of *M. bovis*

5.1 Chemical disinfection of slurry

5.1.1 General considerations

Chemical disinfection of cattle slurry from TB reactor herds may enable rapid inactivation of *M. bovis* in cattle slurry. This may be an attractive alternative to storage, especially if farms do not have adequate long-term slurry storage facilities. Chemical disinfection of cattle slurry contaminated with *M. bovis* presents many problems, some relating to the large volumes of slurry requiring treatment and others to the selection and evaluation of effective chemicals. A fundamental requirement in selection of a chemical for treatment of slurry contaminated with *M. bovis* is retention of mycobactericidal activity in the presence of high concentrations of organic matter (Scanlon & Quinn, 2000b). Mycobacteria are less susceptible to chemical disinfectants than many other bacterial species (Russell, 1999). The most likely mechanism for increased resistance compared to other bacterial species is the hydrophobic nature of the cell wall due to the presence of high levels of lipid (Russell, 1996). Chemical compounds with known activity against mycobacteria include alcohol, aldehydes, halogens, phenolic compounds and sterilizing agents (Russell, 2006). Other disinfectants with mycobacterial activity may be found on the DEFRA approved list of disinfectants. In evaluating chemicals for *M. bovis* microbiocidal activity, consideration should be given to inactivation or elimination of residual disinfectant activity at the end of the treatment period (Scanlon & Quinn, 2000b). Chemically treated slurry may require a storage period to allow chemical inactivation prior to spreading on land. Alternatively, residual disinfectant may be inactivated by neutralisation, dilution or physical methods (Strauch, 1981).

5.1.2 Experimental studies

If properly applied, a mixture of calcium hydroxide and milk, known as “thick lime milk”, should inactivate *M. bovis* (Skuce et al, 2011). Current advice suggests concentrations of

11.25 to 20 kg calcium hydroxide per m^3 are required for inactivation within 24 hours. Use of calcium hydroxide at these concentrations is supported experimentally. In a study by Hahesy et al., (1995), *M. bovis* added to cattle slurry was treated by the addition of both calcium hydroxide powder and a mixture of calcium hydroxide and water ("thick lime milk") at two concentrations (equivalent to 11.25 and 20kg calcium chloride per m^3). Inactivation of the mycobacteria occurred within 24 hours with "thick lime milk" treatment while calcium hydroxide powder required up to 48 hours for inactivation. In each case, the slurry pH increased to a value above 12.0, but this was more effectively maintained in slurry treated with "thick lime milk". The study also examined the effect of the application of cattle slurry treated with calcium hydroxide powder and thick lime milk on dry grass matter yield, grass composition and silage quality. Neither treatment had a serious adverse effect on grass composition or silage quality; however, grass dry matter yield was significantly reduced when compared with grass to which untreated cattle slurry was applied (Hahesy et al., 1995).

Scanlon and Quinn (2000b) examined inactivation of *M. bovis* in cattle slurry by 5 volatile chemicals with mycobactericidal activity – acetone, ammonium hydroxide, chloroform, ethyl alcohol, and xylene. *M. bovis* suspended in sterilised cattle slurry was treated with different concentrations of the five volatile chemicals, the reaction mixture was lyophilised to inactivate chemicals and samples of slurry inoculated onto Lowenstein-Jensen medium to determine survival or inactivation of *M. bovis*. Acetone at a concentration of 22% inactivated *M. bovis* in less than 24 h. Ammonium hydroxide at a concentration of 1% was mycobactericidal after 36 h. Chloroform at a concentration of 0.5%, ethyl alcohol at a concentration of 17.5% and xylene at a concentration of 3% inactivated the mycobacteria within 48 h. The authors concluded that some of the chemicals may be potentially useful for slurry treatment but some were excluded on the basis of health and safety concerns. The authors concluded that the use of low concentrations of chloroform for treatment of slurry poses a minimal risk to persons using the chemical and is unlikely to cause pollution of the environment following land application. However, in our view, many of the chemicals tested in the study would be dangerous, particularly at the volumes/concentrations required for slurry disinfection. For example, neat chloroform is a hazardous substance and substantial volumes would be required to treat slurry at a concentration of 0.5%. The use of xylene may also pose a significant risk due to the toxic effects of the chemical which includes eye and respiratory irritation, central nervous system changes and damage to the liver and kidneys (Fay et al. 1998).

Another study assessed the ability of disinfectants used in hospitals for disinfecting non-critical and semi-critical patient care items, to inactivate mycobacteria (Rutala et al., 1991). A modified Association of Official Analytical Chemists' (AOAC) Tuberculocidal Activity Test, using Middlebrook 7H9 broth as the primary subculture medium and neutralization by dilution, was used to assess the ability of 14 hospital disinfectants to inactivate 10^6 cfu *M. tuberculosis* and 10^5 cfu *M. bovis* at 20°C using 10- or 20-minute exposure. All products tested were prepared at the manufacturers' recommended dilution. Chlorine dioxide, 0.80% hydrogen peroxide plus 0.06% peroxyacetic acid, and an iodophor achieved complete inactivation of both *M. tuberculosis* and *M. bovis*. One quaternary ammonium compound with a tuberculocidal label claim, a quaternary ammonium compound without a tuberculocidal label claim, chlorine (approximately 100 ppm) and 0.13% glutaraldehyde/0.44% phenol/0.08% phenate were not effective against both *M. tuberculosis* and *M. bovis*. Another quaternary ammonium compound with a tuberculocidal label claim was tested against only *M. bovis* and found ineffective. These results indicate

that compounds produced commercially, and sold as tuberculocidal, should be thoroughly tested before widespread use. Glutaraldehydes (2% alkaline and 2% acid), a phenolic and chlorine (approximately 1,000 ppm) demonstrated complete inactivation of *M. tuberculosis* and good inactivation of *M. bovis*.

5.2 Anaerobic digestion

5.2.1 The process of anaerobic digestion

Anaerobic digestion (AD) is a natural process in which bacteria break down organic matter in an oxygen-free environment to form biogas and digestate. A broad range of organic inputs can be used including manure (solid/liquid), food waste, and sewage, although the composition is determined by the industry, whether it is agriculture, industrial, wastewater treatment, or others (NIEA, 2010; DEFRA, 2011). Digestion, or decomposition, occurs in three stages. The first stage consists of hydrolysis and acidogenesis, where enzyme-secreting bacteria convert polymers into monomers like glucose and amino acids and then these monomers are transformed into higher volatile fatty acids. The second stage is acetogenesis, in which bacteria (acetogens) convert these fatty acids into hydrogen (H_2), CO_2, and acetic acid. The final stage is methanogenesis, where bacteria (methanogens) use H_2, CO_2, and acetate to produce biogas, which is around 55-70% methane (CH_4) and 30-45% CO_2 (ABDA, 2013). Anaerobic digesters can be designed for either mesophilic or thermophilic operation – at approximately 35°C or 55°C, respectively. The operating temperatures are carefully regulated during the digestion process to keep the mesophilic or thermophilic bacteria alive (ABDA, 2013). The process of anaerobic digestion produces biogas. The resulting biogas is combustible and can be used for heating and electricity generation, or can be upgraded to renewable natural gas and used to power vehicles or supplement the natural gas supply. Another product of anaerobic digestion is digestate which can be used as fertiliser (DEFRA, 2011).

5.2.2 Potential risks associated with products of anaerobic digestion

There is a potential health risk with digested residues from anerobic digestion, which is partly dictated by the substrate that is treated in the plant (Sahlstrom, 2003). It is well known that digestate from processing of animal manure may contain pathogenic bacteria excreted in faeces, urine and exudates. Digested residues may contain pathogenic bacteria of different species such as *Salmonella*, *Listeria*, *E. coli*, *Campylobacter*, *Mycobacteria*, *Clostridia* and *Yersinia*. Many of these bacteria are zoonotic pathogens and may cause infections in both animals and humans. Furthermore, several of the bacteria are persistent and may even multiply in the anaerobic digestion environment. Mycobacterial species may become dormant or produce survival structures in response to the anaerobic conditions and other suboptimal conditions (Dick et al., 1998; Boon & Dick, 2001; Boon & Dick, 2002). It is important to consider that these forms/structures may be much more resistant to treatments such as anaerobic digestion and may require longer treatment time/further processing for complete destruction.

There are approximately 50 anaerobic digesters nearing the end of the planning process in Northern Ireland. Most of these are on-farm anaerobic digestors which take in a mix of slurry, silage and milk by-products and will operate at temperatures of 25-35°C for 15-30

days, although some may operate at temperatures up to 45°C (DARD, 2011). The majority of on-farm digestors will use slurry from their own farms but some will source slurry from multiple farms. Currently, there are no restrictions on sourcing slurry from other farms or where the digestate can be spread as fertiliser. A likely scenario may involve a number of farms supplying slurry to a single anaerobic digester and then collecting digestate to use as fertiliser. Since there are no restrictions on slurry that has moved prior to a TB breakdown there is potential for slurry contaminated with *M. bovis* to supply anaerobic digestors.

5.2.3 Factors affecting pathogen viability in AD

The principal factors causing pathogen decay or loss of viability during anaerobic digestion include: temperature, retention period, reactor configuration, microbial competition, (Smith et al., 2005). Initial inactivation of pathogens is also dependent on the initial numbers of pathogens in the organic material (Strauch, 1991). The pH of the substrate will also have an effect on bacterial survival during anaerobic digestion (Farrah & Bitton, 1983).

Temperature has been highlighted as the most important factor concerning survival of pathogenic bacteria during anaerobic digestion (Dumontet et al., 1999). Bacterial inactivation due to temperature is related to time, with digestion at higher temperatures requiring less time for bacterial inactivation. The time required for a 90% reduction in viable counts of a microbial population or a decrease by one logarithmic unit (log10) is called the decimation reduction time (T_{90}) (Sahlstrom, 2003). This means bacteria are likely to die much faster at thermophilic temperatures (50-55°C) than at mesophilic temperatures (30-38°C) (Olsen & Larsen, 1987). Therefore operation at higher temperatures (thermophilic) may help to sterilize the digestate. In thermophilic digestion, the energy input is higher and the increased temperature increases gas yields (DEFRA, 2011).

Digester configuration, whether batch-wise or continuous, may have an effect on pathogen survival. In batch-wise systems, all the substrate is replaced at the same time but approximately 10% of the fresh substrate contains inoculated, digested material (Wellinger, 2000). A continuous system fills and removes material continuously; slurry that has been processed longest will generally be removed first as digestate. However, newly-added slurry may pass straight through the system. As a result, it is difficult to determine the amount of time that slurry has been processed in a continuous system (retention time), and whether any pathogens present have received adequate treatment time. The batch-wise method is more easily controlled in terms of temperature and time (Sahlstrom, 2003); however, for economic reasons the majority of digesters are continuous systems.

5.2.4 Survival of bacterial species during AD

No studies investigating specifically *M. bovis* in anerobic digestion could be found upon extensive literature searches. Although the effects of anaerobic digestion on *M. bovis* have not been specifically examined, it is likely that *M. bovis* will survive at the temperatures and duration used by the majority of on-farm digestors. An understanding of the survival of *M. bovis* during anaerobic digestion is required to allow an assessment of the potential risk of TB transmission via spreading digestate derived from contaminated slurry.

The survival of other pathogenic bacteria during anaerobic digestion has been investigated in a number of studies; mostly in laboratory based small-scale digestions. Olsen et al. (1985) examined the effect of anaerobic digestion on *Mycobacterium avium* subspecies *paratuberculosis* (Map), an organism closely related to *M. bovis*. In mesophilic digestion Map could be isolated at 7, 14, 21 days but not 28 days, however, in thermophilic digestion Map could not be recovered after 3 hours. In a laboratory-scale study, continuous mesophilic digestion with a maximum of 1-2 days between additions and removal could not ensure elimination of viable Map cells. In contrast, elimination of viable Map could be achieved by batch-wise, mesophilic digestion for one month or batch-wise thermophilic digestion for three hours (Olsen et al., 1985).

Similarly, experimental investigations have demonstrated that *Escherichia coli* and Salmonella spp. are not damaged by mesophilic temperatures, whereas rapid inactivation occurs by thermophilic digestion (Smith et al., 2005). Efficient mixing and organic matter stabilisation were highlighted as the main factors controlling the rate of inactivation under mesophilic conditions rather than the direct effect of temperature on pathogenic organisms. Mesophilic digestion was developed primarily as a stabilisation process and was not designed as a method of disinfecting sludge (Smith et al., 2005).

In another study, reduction of vegetative bacteria (*Salmonella*, *Streptococci* and *Staphylococci*) and spore-forming bacteria (*Clostridium perfringens* and *Bacillus cereus*) subjected to anaerobic digestion was investigated (Olsen & Larsen, 1987). At small-scale digestion at 35°C, reduction times were 2.4 days for *Salmonella typhimurium*, 2 days for *Salmonella dublin* and *Streptococcus faecalis*, and 0.9 days for *Staphyloccus aureus*. At 53°C, reduction times were 0.7 h for *Salmonella typhimurium*, 0.6 h for *Salmonella Dublin*, 1 h for *Streptococcus faecalis*, and 0.5 h for *Staphyloccus aureus*. Spores of *Clostridium perfringens* and *Bacillus cereus* were not inactivated at 35°C or 53°C. It terms of survival profile, *Mycobacterium bovis* is likely to be more resistant than vegetative bacteria such as Salmonella and less resistant than the spore-forming Clostridia and Bacillus species.

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