Investigating the Epidemiology of *Mycobacterium bovis* Infection in Humans in Ireland.

A thesis for the degree of Doctor of Medicine

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Summary:

Mycobacterium bovis is the main cause of bovine tuberculosis. It can cause zoonotic infection in humans. An average of 6 human *M. bovis* infections are notified annually in Ireland. Little is currently known about the epidemiology of this disease in humans in Ireland. The aim of this study was to (i) investigate the epidemiology of human *M. bovis* infection in Ireland through review of patients' medical records for host risk factors for infection (ii) perform molecular typing of human *M. bovis* isolates and compare them with circulating animal *M. bovis* isolates from cattle and wildlife.

Basic demographic details were collected on patients (n=66) who had *M. bovis* isolates processed at the Irish Mycobacterial Reference Laboratory (IMRL), St James's Hospital (SJH), between 2000 and 2020. A retrospective medical chart review of patients with *M. bovis* infection who had attended SJH for medical care (n=27) was carried out.

Whole genome sequencing (WGS) was performed by the IMRL on 61 human and 3 domestic pet *M. bovis* isolates recovered by, or referred to, the IMRL between 2000 and 2020. Human *M. bovis* genomic sequence data was compared with sequence data from 123 Irish animal *M. bovis* isolates collected by the School of Veterinary Medicine at University College Dublin (n=45) and the Department of Agriculture, Food and the Marine (n=78) from infected cattle and wildlife. Core

genome multilocus sequence typing (cgMLST) was used to create minimum spanning trees and to identify “clusters”. Cluster threshold was set as < 12 allelic differences between isolates. Where clusters were identified by cgMLST, further single nucleotide polymorphism (SNP) analysis was performed on each cluster using the bioinformatic pipeline MTBseq.

Sixty-two percent of patients with *M. bovis* infection were male, average age at diagnosis was 50.1 years. Where disease site was recorded (n=57), pulmonary infection occurred in 71.4%, extrapulmonary disease in 14.2%. and disseminated disease in 14.2%. Frequent comorbidities, where recorded (n=22), included HIV infection (n=3), malignancy (n=4) and autoimmune conditions (n=7). Four patients were receiving biologic immunosuppressants. The most recorded occupations were farmer (n=5) and meat factory worker (n=5).

Among the 61 human and 3 domestic pet *M. bovis* isolates sequenced by the IMRL and the 123 externally derived animal isolates, cgMLST identified 20clusters involving 37 patients, 3 domestic pets and 60 animal isolates. Seventeen of these clusters contained one or more human *M. bovis* isolates. Fourteen clusters contained both human and animal isolates. Subsequent whole genome SNP analysis performed on these clusters showed that 1 cluster involved patient isolates (n=2) that were <6 SNPs apart from each other, suggestive of close

epidemiological link. One cluster found multiple patients (n=7) had *M. bovis* isolates that were less than 6 SNPs apart from circulating animal isolates, including a domestic pet isolate, again suggestive of close epidemiological links.

This study highlights the changing epidemiology of human *M. bovis* infection. It also demonstrates that there is likely ongoing transmission of *M. bovis* between animals, between animals (including domestic pets) and humans, and potentially between humans. Prospective studies are needed in this area to further understand the epidemiological links between animal and human cases of *M. bovis* infection.

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List of Abbreviations:

AFB	Acid Fast Bacilli
AIDS	Acquired Immunodeficiency Syndrome
ATM	Amplicon Tagment Mix
APHA	Animal and Plant Health Agency
BCG	Bacille Calmette-Guerin
bTB	Bovine Tuberculosis
CDT	Elution Buffer
CFU	Colony Forming Unit
CIDR	Computerised Infectious Disease Reporting
CL2	Containment Level 2
CL3	Containment Level 3
cgMLST	Core Genome Multi-Locus Sequence Typing
CNS	Central Nervous System
COPD	Chronic Obstructive Pulmonary Disease
CT	Computed Tomography
CVRL	Central Veterinary Reference Laboratory
CXR	Chest X-Ray
DAFM	Department Of Agriculture Food and the Marine
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
DPIA	Data Protection Impact Assessment

DST	Drug Susceptibility Testing
ECDC	European Centre for Disease Prevention and Control
EDT	Proteinase K
EFSA	European Food Safety Authority
EtOH	Ethanol
EU	European Union
FSA	Food Standards Agency
FSA	Food Safety Authority of Ireland
GATK	Genome Analysis Toolkit
GC	Guanine-Cytosine
GI	Gastrointestinal
GORD	Gastro-Oesophageal Reflux Disease
HIV	Human Immunodeficiency Virus
HT1	Hybridization Buffer
HPSC	Health Protection and Surveillance Centre
HRCDC	Health Research Consent Declaration Committee
HAS	Health and Safety Authority
IDSA	Infectious Disease Society of America
IFN	Interferon
IGRA	Interferon Gamma Release Assay
IMRL	Irish Mycobacteria Reference Laboratory
JREC	Joint Research Ethics Committee

La	Lineage
LDT	Lysis Buffer
LJ	Lowenstein-Jensen
LMIC	Low- and Middle-Income Countries
LN	Library Normalisation
LNS1	Library Normalization Storage Buffer 1
LNW1	Library Normalization Wash 1
M.D.	Medical Doctorate
MDR	Multidrug Resistant
MIRU-VNTR	Mycobacterial Interspersed Repetitive Unit-Variable-Number Tandem Repeat
MPP	Meat Processing Plant
MRCI	Migrant Rights Centre Ireland
MRN	Medical Record Number
MST	Minimum Spanning Tree
MTB	Mycobacterium Tuberculosis
MTBC	Mycobacterium Tuberculosis Complex
NaOH	Sodium Hydroxide
NCBI-SRA	National Center for Biotechnology Information Sequence Read Archive
NT	Neutralize Tagment Buffer
NTM	Non-Tuberculous Mycobacteria
OTF	Officially Bovine Tuberculosis Free

PCR	Polymerase Chain Reaction
PDE	Phosphodiesterase
PHE	Public Health England
PPE	Personal Protective Equipment
RD	Regions Of Difference
REA	Restriction Endonuclease Analysis
RFLP	Restriction Fragment Length Polymorphism
RMI	Raw Milk Ireland
Rpm	Revolutions per Minute
RSB	Resuspension Buffer
SICTT	Single Intradermal Comparative Tuberculin Testing
SJH	St James's Hospital
SNP	Single Nucleotide Polymorphism
SPRI	Solid Phase Reversible Immobilisation
ST	Sequence Type
TB	Tuberculosis
TCD	Trinity College Dublin
TD	Tagment DNA Buffer
TNF	Tumour Necrosis Factor
TST	Tuberculin Skin Test
TUH	Tallaght University Hospital
UCD	University College Dublin

UK	United Kingdom
UKHSA	United Kingdom Health Security Agency
UN	United Nations
US	United States
WDT	Wash Buffer
WGS	Whole Genome Sequencing
WHO	World Health Organization
XDR	Extensively Drug-Resistant
zTB	Zoonotic Tuberculosis

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Chapter 1: Human and Animal *Mycobacterium bovis* Infection: Literature Review

1.0 Introduction:

The World Health Organization (WHO) End TB Strategy aims to eradicate tuberculosis (TB) infection by 2030 (1). Among the strategy's key activities is the diagnosis and treatment of every person with TB. However, it has been highlighted that people at risk of zoonotic TB (zTB) have largely been neglected to date. As a result, the "Roadmap for Zoonotic Tuberculosis" was published in 2017 by the WHO and identified the conducting of systematic surveys and the reporting of high quality data on zTB among 10 key areas of priority(2).

1.1 Zoonotic Tuberculosis (zTB):

Zoonosis is an infection transmissible from animals to humans. For this to happen regularly there must be a reservoir in an animal population(3). Most zoonoses occur where there is close contact between humans and a relatively abundant animal species(3).

Of the 10 million new TB cases diagnosed in humans annually worldwide, an estimated 1.4% are thought to be new cases of zTB, of whom 11 400 are estimated to die(3). The majority of zTB cases occur in Africa and Southeast Asia(4). zTB is largely under reported and the burden is likely much higher(3). Estimates are based on *Mycobacterium bovis* as it is the most common cause of zTB globally(3).

M. bovis is part of *Mycobacterium tuberculosis* complex (MTBC), which comprises 11 species with variable host tropism and virulence(5). While *M. tuberculosis* (MTB) is the leading cause of TB in humans, *M. bovis* has a broader host range primarily infecting cattle but also humans and other animals(6).

Other organisms from the MTBC that may be present in animals and can cause zTB include *M. caprae, M. canetti, M. microti, M. pinnipedii, M. mungi* and *M. orygis(3)*.

Robert Koch first isolated *M. bovis* from cattle in 1882, however, at that time, he did not believe that it was a potential hazard to human health(7). The British Royal Commission concluded in 1911 that *M. bovis* was a hazard to human health(8).

M. bovis has one of the broadest host ranges of all the MTBC pathogens which explains its persistence in the environment(9). It is considered the main causal agent of bovine TB (bTB), however *M. caprae* has been reported in many European countries as another cause of bTB(10) and *M. orygis* has recently emerged as a zoonotic threat in South East Asia(11).

Cattle are considered *M. bovis's* true host. However, it has been detected in other domestic animals such as sheep, goats, pigs, horses, dogs and cats and also in wild animals including badgers, deer, possums, wild boars, llamas, cap buffalo etc(12). The natural movement of reservoir animals increases spread of the disease to domestic animals.

M. bovis can survive in cold, dark and moist environments for many months and survives best in frozen tissue(13). The organism is resistant to a multitude of environmental conditions, however, is less resistant during warmer seasons(4).

The burden of human *M. bovis* infection is likely underestimated due to several factors. Diagnostic challenges occur as many common laboratory procedures are unable to differentiate between *M. bovis* and *M. tuberculosis(14)*. There may be

poor public health surveillance and reporting(14), lack of local awareness as well as associated cost and cultural issues(2). As such, the reporting of data is likely unreliable and highly variable across geographical regions.

There has been a resurgence of zTB even in industrialised countries(4) due to reservoir animal populations, human immigration from endemic areas and importation of infected foods or animal products(15). As a result, human *M. bovis* infection is likely to be underestimated even in high income countries.

The human burden of *M. bovis* infection cannot be reduced without improving food safety standards and the control of bovine TB within the animal reservoir(2). The "One Health" approach acknowledges the interdependence of human and animal health, as well the impact of the environment on both (2). The three core themes of One Health are: the improvement of the scientific evidence base, the reduction of disease transmission at the animal to human interface and the strengthening of multidisciplinary collaboration(13).

1.2 *Mycobacterium bovis* infection in cattle:

Disease in cattle, known as bTB is chronic and progressive and mainly presents as a respiratory disease, but signs may be absent even in advanced cases(13). Subclinical signs include weakness, dyspnoea, emaciation, enlarged lymph nodes and cough(13). Involvement of the digestive tract may cause intermittent diarrhoea or constipation. In terminal stages, acute respiratory distress may occur(13). Hardened udders may occur in TB mastitis(16). Most infections in cattle are caused by aerosol infection, with as little as a single Colony Forming Unit (CFU) of *M. bovis* capable of causing infection(17).

bTB has largely been eradicated from herds in the developed world by animal TB control and elimination programs. However, in developing countries animal TB is widely distributed and control measures are not applied or are applied sporadically(18).

bTB herd prevalence is reported at 39.65% in Mozambique, 13% in Uganda and 17% in Chad (4). In 2022, bTB herd prevalence across the EU was 0.61% compared with 0.54% in 2021(19). The number of infected herds increased from 9,384 to 9,845 during this time(19). National herd level prevalence ranged from <0.01% in Belgium and Poland to 12.2% in Northern Ireland. Herd prevalence in Ireland is estimated at 4.89%(20).

European Union (EU) legislation mandates that all Member States must report annual surveillance data for bTB (21). “Disease free status” or “OTF”, officially bTB free status, is assigned to a Member State if at least 99.8% of their bovine

herds have been disease free over 3 years and their herd incidence has been less than or equal to 0.1% during the last year(21).

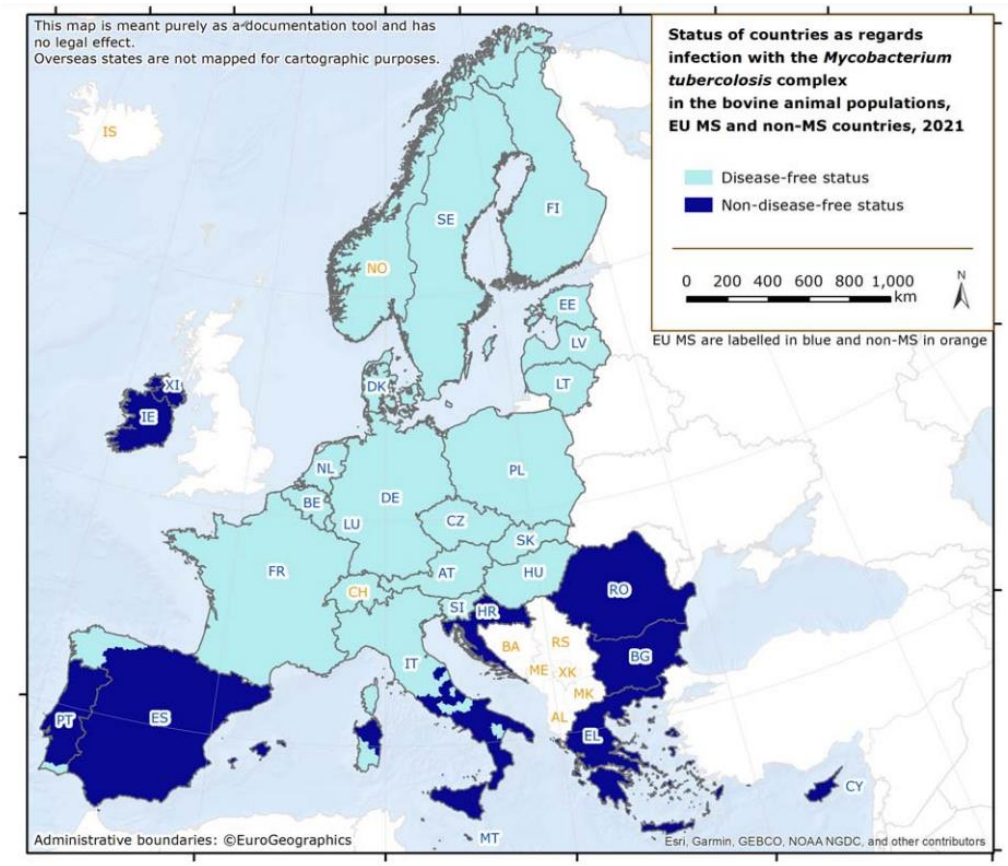

FIGURE 1.1: STATUS OF COUNTRIES FOR INFECTION WITH *MYCOBACTERIUM TUBERCULOSIS* COMPLEX IN THE BOVINE POPULATION IN 2021. THIS IMAGE IS FROM THE EUROPEAN UNION ONE HEALTH 2021 ZOONOSES REPORT. DATA WAS NOT AVAILABLE FROM THE UK AS IT NO LONGER A MEMBER STATE OF THE EUROPEAN UNION (22).

Animal TB control programmes are important for many reasons including the risk of zTB infection, the risk of economic losses due to loss of affected livestock and the benefit of bTB "free" status to international commerce. EU Member States who do not have a "free from bTB" status must have an effective eradication programme in place to be allowed to trade bovine animals and products within the EU(22). The Irish bTB Eradication Programme was introduced in 1954. It is overseen by the Department of Agriculture, Food and the Marine (DAFM) and focuses on finding *M. bovis* infection, confining it and eradicating it (16).

The Australian Brucellosis and Tuberculosis Eradication Campaign (BTEC) ran for 27 years from 1970 to 1997(23). It is a rare example of a successful bTB eradication plan, with Australia being declared bTB free in 1997. The campaign included whole herd test and slaughter programmes using single intradermal testing, introduction of a cattle tracing system and abattoir surveillance. Additionally, improved farming infrastructure and herd management proved essential to disease control (weaner and age segregation, partial destocking of high risk/older cattle)(23).

1.2.1 Herd Screening for bTB with Single Intradermal Comparative Tuberculin Testing:

All cattle in Ireland over the age of 6 weeks must undergo Single Intradermal Comparative Tuberculin Testing (SICTT) at least annually (16). This involves injecting bovine tuberculin intradermally into the neck of the bovine and examining the site for thickening (13). This "bovine tuberculin" is purified protein derivative (PPD) tuberculin prepared from a culture of *M. bovis(24)*. This is similar in nature to the human Mantoux test, however the human Mantoux involves injection of purified protein derived from *M. tuberculosis* culture(25). SICTT is imperfect and sensitivity analysis among Irish populations has ranged between 52.9% to 60.8% (26)with a specificity of 100%(27).

If a cow has been exposed to *M. bovis*, SICTT will elicit an immune response and a lump/thickening will appear at the injection site(16). If cattle have been exposed to other *Mycobacterium* species, for example *M. paratuberculosis subsp. avium* or *M. hiberniae(28)*, they may also elicit an immune response. For

this reason, cattle are also injected with protein from the avian TB bacteria(16). If skin thickening is greater at the bovine tuberculin injection site compared with the avian TB injection site, the animal is deemed a “reactor”(16).

Only cattle with a negative SICTT within the last 12 months are allowed to move on the open market(16). Where reactors are detected within a herd, they must be sent directly to an abattoir for slaughter and no cattle are allowed to enter or leave the herd until all remaining animals have passed two consecutive SICTT at 60-day intervals(16). Following an outbreak within a herd, neighbouring herds are screened on a 4-monthly basis until no further cases are detected. Inconclusive reactors, even if subsequent tests are negative, are confined to the same herd for life(16).

1.2.2 Ante-mortem and Post-mortem inspection:

Cattle are assessed visually ante-mortem and post-mortem for TB lesions by Veterinary Inspectors at abattoirs or slaughterhouses(16). All cattle slaughtered in Ireland have their head and thoracic lymph nodes examined for TB. Meat with suspected TB lesions does not go for human consumption, samples from suspect lesions are sent to the Central Veterinary Research Laboratory (CVRL) for culture and the herd from which the infected bovine came from is restricted from trade pending this result (16). Between 2005 and 2019, 20,116 herds lost their TB free status due to TB lesions detected upon slaughter(29). For context, 5078 herds were restricted in 2023 in total across the Republic of Ireland (20).

1.2.3 Other Animal Reservoir Populations:

Badger populations are managed as part of Ireland's national bTB Eradication Programme. Where badgers are implicated in a herd breakdown, i.e. the loss of a bTB free status, they may be culled to prevent further disease transmission(16). From 2018, BCG vaccination of badgers has been rolled out as an alternative to culling(16). EU and other trading blocs prohibit the use of the bacille Calmette-Guerin (BCG) vaccine in cattle as vaccination compromises the interpretation of traditional TB diagnostic tests(3).

While deer are considered reservoir animals, little is known with regards to their role in the spread of disease and they are not currently managed under the Irish bTB Eradication Programme.(30) Infection in deer ranges from minimal pathology, with deer surviving for many years post infection, to a less common generalised infection with multi organ involvement and high fatality rates(30). Wicklow has high rates of bTB and a high density of deer in that County has been implicated in the spread and persistence of disease within cattle(30).

1.3 *Mycobacterium bovis* Infection in Humans:

The most common routes of *M. bovis* transmission to humans are via airborne transmission, consumption of unpasteurised milk or milk products or via direct contact with infected animals or untreated animal products(6). Patients who are more prone to becoming infected with *M. bovis* include: the elderly with emergence of latent disease, the immunocompromised especially those with Human Immunodeficiency Virus (HIV) infection, those exposed to infected animals or animal products either via consumption or occupational exposure, and migrant populations from endemic areas(8).

Prior to milk pasteurisation schemes, *M. bovis* was a major public health issue in the early 20th century(31). It was reported that in 1936 in Sweden – one cow with TB mastitis amongst a herd supplying milk to a local community was responsible for ninety percent of children who consumed this milk subsequently testing positive by Tuberculin Skin Test (TST) - compared with 7% of those who did not drink this milk (32). It is estimated that in Ireland in the 1950s, 230 per 100,000 people developed TB from contact with infected animals or infected food products(33).

1.3.1 Presentation of *M. bovis* Infection in Humans:

The presentation of infection varies depending on mode of disease acquisition. *M. bovis* can affect all organs in the body including the respiratory tract, gastrointestinal (GI) tract, lymph nodes, genitourinary system, bones, joints, peritoneum and central nervous system (CNS)(4).

Symptoms may be indistinct including fever, night sweats and weight loss. In children cervical lymphadenopathy or lymphadenopathy of abdominal or peritoneal lymph nodes are a common presentation(32). This predominantly involves the anterior cervical chain as the oral cavity is often the primary site of inoculation through consumption of unpasteurised dairy products and is preferentially drained by the anterior cervical chains(32). The posterior cervical chain is more commonly implicated in MTB infection(32). Dental abscess secondary to *M. bovis* infection has also been documented(34).

FIGURE 1.2: SUBMANDIBULAR NECK MASS SECONDARY TO *M. BOVIS* INFECTION IN INFANT. IMAGE FROM HAN *ET AL* (32).

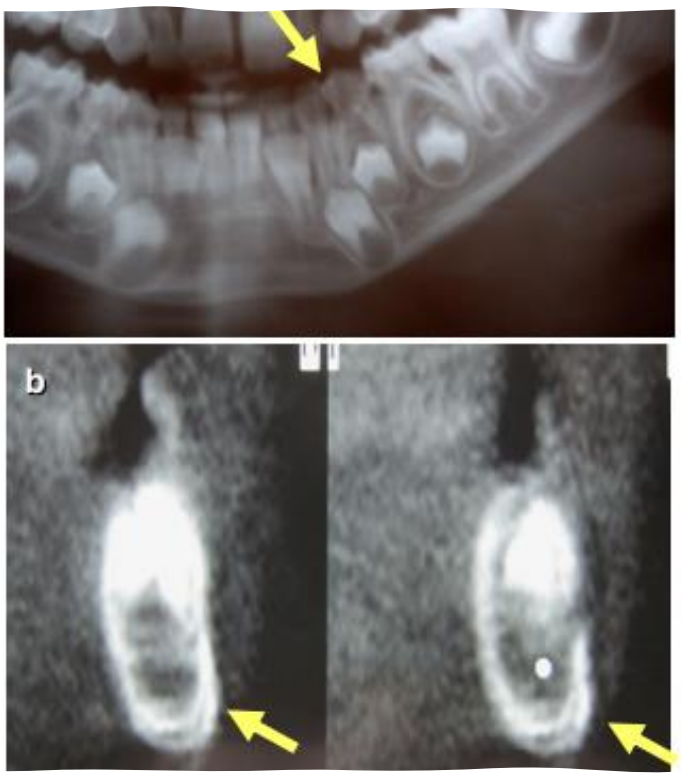

FIGURE 1.3: X-RAY AND CT IMAGING OF PRIMARY TOOTH ABSCESS AND OSTEOMYELITIS SECONDARY TO *M. BOVIS* INFECTION IN A CHILD. IMAGE FROM MARAGOU *ET AL* (34).

Scrofula derma was previously a common manifestation of TB lymphadenitis, often associated with *M. bovis* infection(35). Lupus vulgaris is a form of chronic TB skin infection, which can also be associated with *M. bovis* infection(36).

Cutaneous manifestations are common, particularly due to occupational exposure, for example direct inoculation through injury by slaughterhouse workers. Such lesions are sometimes referred to as “butcher warts”. Pulmonary spread has also been documented in a slaughterhouse worker where inoculation was felt to have occurred via the skin with haematogenous spread to the lungs(37).

FIGURE 1.4: ULCERATIVE CUTANEOUS *M. BOVIS* LESION ON DORSUM OF HAND OF A SLAUGHTERHOUSE WORKER. IMAGE FROM MERTOGLU *ET AL* (37).

Respiratory disease is a common presentation of *M. bovis* infection and may arise where transmission has occurred via inhalation of infected aerosols from an animal or human with *M. bovis* disease.

CNS infection is also reported. Clifford *et al* reported a case where a young female, resident on a farm in Mexico, presented with disseminated infection complicated by meningitis and stroke secondary to TB vasculitis(38). Indeed, in the early 20th century, *M. bovis* reportedly caused approximately 25% of cases of TB meningitis and was a concern particularly for young children(38). A retrospective case series conducted in Mexico found that CNS *M. bovis* infection was associated with worse outcomes when compared with MTB CNS infection(39).

FIGURE 1.5: COMPUTED TOMOGRAPHY (CT) SCAN OF PATIENT BRAIN WITH TB MENINGITIS AND STROKE WITH TB VASCULITIS DUE TO *M. BOVIS* INFECTION. ARROWS HIGHLIGHT TUBERCULOMAS. IMAGE FROM CLIFFORD *ET AL* (38).

The prognosis for *M. bovis* infection is likely worse than disease due to MTB *(40).* This is particularly the case within HIV co-infected patients. This may be due to delay in diagnosis, insufficient treatment due to pyrazinamide resistance, older patient age and higher frequency of miliary or CNS infection(4).

1.3.2 Transmission of *M. bovis* infection:

Transmission may occur between animals, from animals to human, from human to human, and, from human to animal in reverse zoonosis. Transmission can occur via the airborne route by inhaling infected droplets, the consumption of infected dairy products and rarely via percutaneous exposure(4).

Gastrointestinal infection due to the consumption of unpasteurised milk is a main route of transmission of infection especially in countries where there is

limited sanitary control of milk(4). Consumption of unpasteurised cheese may also be a source of infection as *M. bovis* is less affected by the pH of cheese than other pathogens(4). Transmission from consumption of meat from animals with zTB, while considered a potential risk, is less likely to occur as cooking (>60°C) will likely eliminate this risk(41).

Animals can also infect humans via the airborne route be they cattle, wildlife or domestic pets (4). There are rare reports of pet dog and cat transmission of *M. bovis* infection to their owners(42). The risk of acquiring bTB from pets is considered low but not negligible and will be discussed in Section 1.5.4.

Transmission can occur from percutaneous exposure via skin cuts and abrasions; this is a risk in those who manipulate animal carcases with bTB lesions(4).

Despite being a zoonotic disease, transmission can also occur between humans and indeed humans may act as amplifier hosts. They can act as sources for both animal and human hosts. Patients with HIV infection are more likely to develop TB and to infect others.

A UK study described 6 epidemiologically related patients with progressive TB caused by *M. bovis* between 2004 and 2006(43). All patients lived within 10 miles of each other and were linked by social networks in 2 areas (local bar and city centre nightclub). Five had pulmonary disease, two also had cervical lymphadenopathy, one had meningitis. Person to person transmission likely occurred due to prolonged and repeated contact in a confined space with poor ventilation, together with a noisy environment resulting in shouting. Except for the index patient, zoonotic links were absent, as was the consumption of unpasteurised dairy products. All patients had indistinguishable isolates using

Mycobacterial Interspersed Repetitive Unit-Variable-Number Tandem Repeat (MIRU-VNTR) and spoligotyping analysis (these molecular assays will be discussed further in section 1.3.4). Four patients had underlying immunocompromise including diabetes, alcohol misuse, HIV and use of anabolic steroids.

Reverse zoonosis of *M. tuberculosis* has been reported in zoo elephants, primates and felines which raises the worrying prospect of the potential for animals to act as a vehicle of transmission of drug resistant MTB where there is close contact between human and animal populations(3). Where poor animal management is coupled with Multidrug Resistant (MDR) TB, this could create drug resistant reservoirs of infection in animals(3).

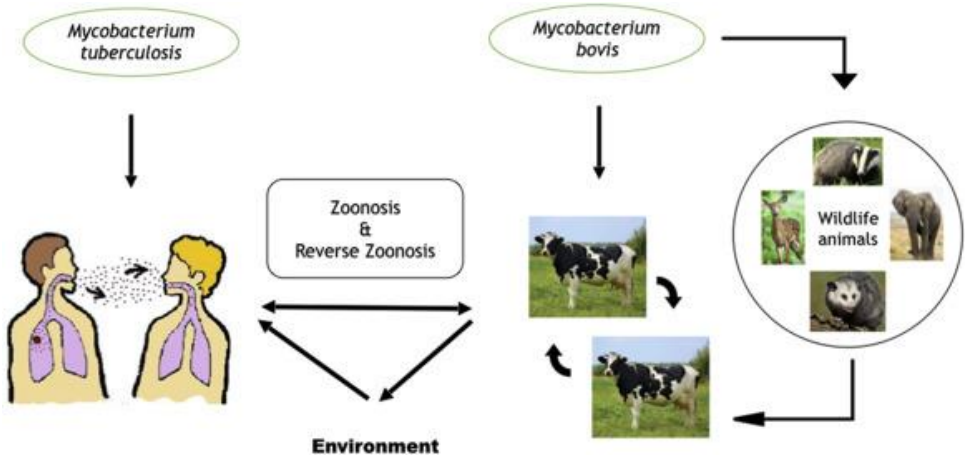

FIGURE 1.6: TRANSMISSION ROUTES *OF M. BOVIS*. *M. BOVIS* TRANSMISSION CAN OCCUR BETWEEN ANIMALS, FROM ANIMALS TO HUMANS (ZOONOSIS), FROM HUMANS TO ANIMALS (REVERSE ZOONOSIS) AND FROM HUMANS TO HUMANS. THE ENVIRONMENT CAN SERVE AS AN INTERMEDIARY HOST. IMAGE FROM PAPER REFAYA *ET AL* (44).

1.3.3 Diagnosis of *M. bovis* infection:

The clinical presentation of MTB and *M. bovis* infection in humans is largely indistinguishable clinically and radiographically; differentiation is only possible through diagnostic bacterial culture and molecular methods. It is thus likely to be underdiagnosed. Additionally, *M. bovis* infection is frequently extrapulmonary in nature which may also hinder diagnosis.

Distinguishing between the species is important for epidemiological investigation and for adequate treatment, however many low to middle income countries (LMIC), where the risk of zTB is higher, may lack the laboratory facilities to allow differentiation between members of the MTBC(4).

Mycobacteria are thick gram variable bacilli, 1 – 10 µm length and 0.2 – 0.6 µm in width (13). They are facultative intra-cellular organisms, obligate aerobes and have a thick waxy cell wall containing mycolic acid(13).

Diagnosis of TB in many parts of the world is based on sputum smear examination for acid fast bacilli (AFB) or rapid molecular assays including the Xpert ® MTB/RIF, however this does not allow for differentiation between species(4). TST and Interferon gamma release assays (IGRA) will also detect *M. bovis* but will not differentiate between MTBC species, nor will it differentiate between active or latent infection.

TST, also referred to as a Mantoux test, is a delayed hypersensitivity test which involves the injection of Purified Protein Derivative (PPD) intradermally and observing for a reaction(45). PPD is a mixture of antigens conserved in MTB, *M. bovis*, BCG and NTMs(45).

The IGRA, also known as the QuantiFERON test, quantifies a patient's interferon gamma response to specific peptides associated with pathogens of the MTBC, including *M. tuberculosis* and *M. bovis,* but not BCG vaccine strains or NTMs. There are no studies validating its use in patients with known *M. bovis* infection(46).

However, a San Diego study by Pong *et al* on children with TB found similar sensitivity of the IGRA in both *M. bovis* and MTB cases, but a slightly lower, but not a statistically significant difference in, sensitivity of the TST in *M. bovis* cases compared with MTB infections(46). Little seems to be known about the relative sensitivity and specificity of the IGRA and TST in screening for latent *M. bovis* infection.

Up until the end of the 20th century differentiation between species of the MTBC was based primarily on phenotypic characteristics and biochemical tests, even in developed countries(8). The introduction of molecular tests over the last 30 years has significantly changed the diagnosis of *M. bovis* infection.

Following culture on solid media for up to 8 weeks, *M. bovis* displays dysgonic colonies and negative biochemical test results for nitrate reduction and niacin accumulation. However, biochemical tests are typically not applicable to growth within liquid media(47). While *M. bovis* is intrinsically resistant to pyrazinamide, reliance of pyrazinamide resistance alone to differentiate between *M. bovis* and MTB is not appropriate as pyrazinamide sensitivity testing is technically difficult and may not be performed in all laboratories(48).

The GenoType MTBC line probe assay (Bruker-Hain) is a kit used to differentiate between *M. tuberculosis, M. bovis* BCG, *M. bovis, M. africanum, M. caprae*, and

M. microti(49). It uses DNA Strip technology and relies on Single Nucleotide Polymorphism (SNP) differences in the *gyrB* gene(50).

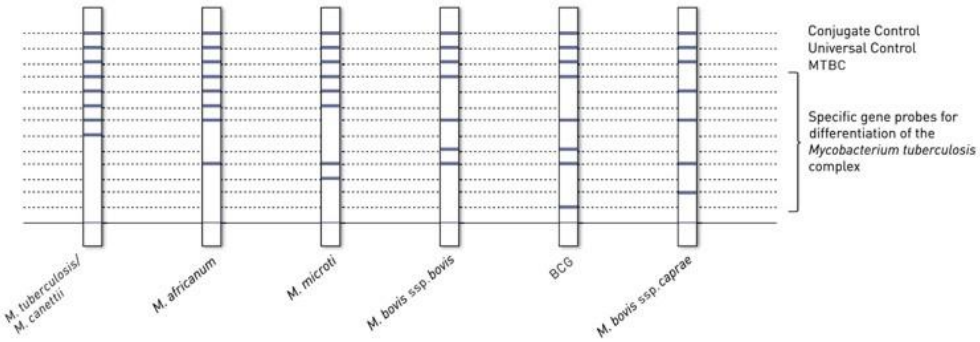

FIGURE 1.7: GENOTYPE MTBC LINE PROBE ASSAY BY BRUKER-HAIN. THIS USES DNA STRIP TECHNOLOGY TO DIFFERENTIATE BETWEEN MTBC ORGANISMS (49).

1.3.4 Genomics and Molecular Analysis:

The genomic sequence of *M. bovis* AF2122/97 was published in 2003(51). The average *M. bovis* genome is 4.3Mb, comprising approximately 4200 genes(5). It has around 65% Guanine-Cytosine (GC) content(5). Like other MTBC genomes, *M. bovis* has a significant number of repetitive elements which may complicate genomic analysis (5)

MTBC genomes are highly similar, >99.95%. These pathogens have evolved solely though SNPS, indels (the incorporation or deletion of one or more nucleotides), deletions of up to 26Kb, insertion sequences and duplications(5). Large deletions, also called regions of difference (RD), have traditionally formed the basis of molecular diagnosis and differentiation of MTBC species(5). There are 14 such RDs ranging from 2 to 12.7Kb in size in *M*. *bovis*. *M. bovis* can be differentiated from other members of the MTBC by deleted regions RD9 and RD4 (5).

Polymerase chain reaction (PCR) based tools including spoligotyping and MIRU-VNTR are useful tools for investigating *M. bovis* transmission(52). Spoligotyping relies on the analysis of DNA polymorphisms of the RD region at one particular chromosomal locus unique to MTBC bacteria (53).

Variable Number Tandem Repeat (VNTR) is a locus of tandem repeats within a nucleotide sequence. Twenty-four loci MIRU-VNTR is used as it has a high discriminatory power and can define potential clusters of related strains. However, it cannot differentiate between strains that are closely related in an outbreak.(54)

Spoligotyping and MIRU-VNTR can be used to analyse local clustering on larger scales. However, the discriminatory power within cluster events is limited especially due to high similarity of strains particularly within certain MTBC lineages. WGS is often preferred due to its high discriminatory ability.

WGS is not only a useful tool for identifying pathogens and for evaluating antimicrobial resistance mechanisms and the genes involved, but also to further understand the epidemiology of infection and its spread. It is increasingly being used in outbreak response and infectious disease surveillance, particularly in MTB. Furthermore, it is also being applied in developed countries in bTB control programmes(5). Unfortunately, there is no standardisation of WGS analysis pipelines and no common nomenclature to facilitate data exchange across laboratories and geographical regions(55).

Phylogenetic trees can be constructed from the alignment of SNPs(5). Mapping sequenced nucleotide reads to a reference genome is preferred in order to detect high quality variants as opposed to *de novo* gene assembly(5). Common

practice is to exclude indels and repetitive DNA regions. This is unlike MIRU-VNTR and spoligotyping which rely on these rapidly evolving regions or repetitive DNA. WGS relies on slowly evolving regions within the genomes.

1.3.5 Lineage Nomenclature:

Human adapted MTBC is classified into 9 phylogenetic lineages: Lineages 1 to 9. Lineages (L) 1 -4 and L7 represent *M. tuberculosis sensu strictu*, while L5 and L6 correspond to *M. Africanum*. L8 and L9 remain poorly characterised(56).

Up until recently, genetic diversity among *M. bovis* isolates was described using four clonal complexes based on genotypic deletions and SNPs(56). These clonal complexes were named European 1 and 2 (Eu1 and Eu2) and African 1 and 3 (Af1 and Af2)(56).

In 2021, Zwyer *et al* proposed a new nomenclature for animal adapted MTBC (*M. bovis, M. caprae, M. orygis*) based on phylogenetic principles and genetic diversity patterns(56). They named *M. bovis* "La1", *M. caprae* "La2" and *M. orygis* "La3". These lineages differ greatly in their global distribution suggesting local host adaptation. The evolutionary success of La1 and its global distribution may relate to its broad host tropism(56). Lineage La1 as described by Zwyer *et al* is further subdivided into clades or sub lineages La1.1 – La1.8 differing in geographical distribution. SNPs specific to each lineage and sub lineage can be used as genotypic markers.

FIGURE 1.8: GLOBAL DISTRIBUTION OF *M. BOVIS* LINEAGES: LA1, LA2 AND LA3. IMAGE FROM ZWYER *ET AL (*55).

1.3.6 Antimicrobial Treatment:

As stated earlier*, Mycobacterium bovis* is intrinsically pyrazinamide resistant. As most patients start treatment without identification of the causative *Mycobacterium* species, there is thus a risk of inadequate treatment and the development of drug resistance in patients with undiagnosed *M. bovis* infection who do not have drug sensitivity testing performed, or indeed where *M. bovis* has been diagnosed but where DST is not possible(2).

As per the Guidelines on the Prevention and Control of Tuberculosis in Ireland, the treatment of *M. bovis* infection comprises of two months treatment with rifampicin, isoniazid and ethambutol followed by a further 7 months of isoniazid and rifampicin(57).

The Infectious Disease Society of America (IDSA) similarly recommends 2 months of rifampicin, isoniazid and ethambutol followed by a further 7 months of isoniazid and ethambutol for extrapulmonary and pulmonary *M. bovis* infection (58). This is longer than the commonly used 6-month rifampicin, isoniazid, pyrazinamide, ethambutol treatment regimen used for pulmonary MTB infection, likely due to the loss of pyrazinamide which is synergistic with

rifampicin(14). A longer duration of treatment may be required in CNS *M. bovis* infection(38).

A 2016 study of 3 published articles which reviewed drug regimens and outcomes in human *M. bovis* infection found that while there is limited evidence supporting current guidelines, treatment regimens involving rifampicin and isoniazid appeared adequate, while the benefit of the addition of ethambutol to this regimen was unclear(59).

1.3.7 Drug Resistance:

M. bovis strains bear a mutation in amino acid position 169 in the *pncA* gene which confers intrinsic resistance to pyrazinamide(60). There are rare reports of pyrazinamide susceptible *M. bovis* strains(61).

MDR TB is defined as TB isolates resistant to rifampicin and to isoniazid and it is an increasing problem globally(62). Whilst most MDR TB outbreaks are associated with MTB infection, outbreaks and sporadic cases of MDR *M. bovis* infection have also been reported(63).

Between 2008 – 2012, 28.5% of human *M. bovis* isolates received at the Irish Mycobacteria Reference Laboratory (IMRL) were resistant to both isoniazid and pyrazinamide(64) . A Mexican study characterising 3 human MDR *M. bovis* strains identified *katG* and *rpoB* mutations conferring resistance to first line TB medications(65). There was no epidemiological link between these isolates. Another Mexican study found that resistance to isoniazid, rifampicin and streptomycin in previously untreated individuals was higher among *M. bovis* strains than MTB strains (10.9% vs 3.4%) and appeared to be increasing(66).

Rather dramatically, an Extensively Drug-Resistant (XDR) *M. bovis* outbreak in a Madrid hospital in the 1990s affected 19 hospitalised patients with HIV. All patients died and the *M. bovis* isolates in question were resistant to 11 anti-TB medications(67).

In most bacteria, drug resistance is most often acquired via horizontal transfer of mobile genetic elements conferring resistance. However in MTB, resistance is usually acquired by spontaneous mutation in chromosomal genes(65). As isoniazid and rifampicin are not routinely used in veterinary practice – it is likely that the development of resistance is acquired during infection within a human host(65).

1.4 Epidemiology:

The burden of zoonotic disease is likely underestimated, due in part to two factors. The first, the absence of systematic surveillance of *M. bovis* as causative the agent of TB, particularly in low income - but high TB burden - countries, and secondly, the inability of routine laboratory techniques to differentiate between species within the MTB complex(68).

In 2016, there were an estimated 147 000 cases of zTB with 12 500 associated deaths(69). As mentioned previously, the highest burden of human disease is in the African region, followed by South-East Asian region(69).

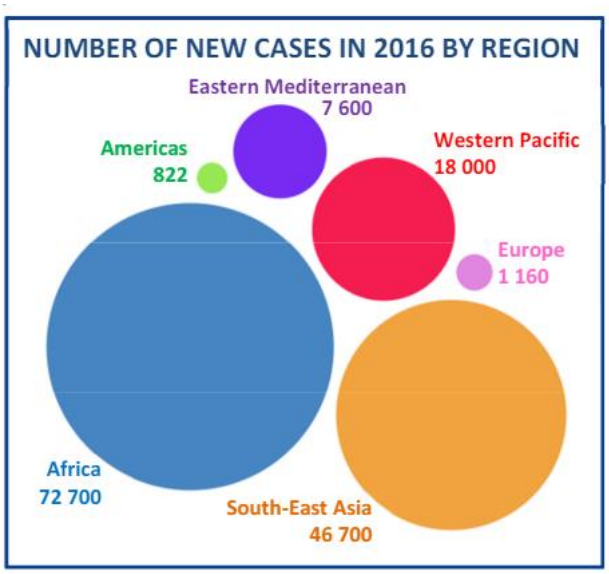

FIGURE 1.9: NUMBER OF ZOONOTIC TB CASES BY WHO REGION IN 2016. IMAGE FROM WORLD ORGANISATION FOR ANIMAL HEALTH (67)

A metanalysis by Taye *et al* reviewed 19 studies published across the world between 2009 and 2019 and the reported prevalence of zTB among MTBC cases ranged from 0.4% to 76.7%(70). A review by Muller *et al* in 2013 looked at data collected from 61 countries and estimated prevalence of zTB to be 1.4% in countries outside of Africa, and 2.8% in countries within Africa. The study also

found that the prevalence of *M. bovis* detected from extrapulmonary cases was 3 times higher (4.1%) than from pulmonary cases(71).

1.4.1 European context:

The overall TB incidence in the WHO European region is in consistent decline since 2015 and amongst the lowest in the world at 10 cases per 100,000(3). zTB in this region accounts for 0.04% of cases, most caused by *M. bovis* and *M. caprae(71)*.

The European Centre for Disease Prevention and Control (ECDC) reported that there were 130 confirmed cases of *M. bovis* or *M. caprae* in humans with 16 associated deaths in 2022 within the EU, Northern Ireland and 11 non-EU Member States(19). This corresponds to a 0.03 per 100,000 population notification rate which was a 13.2% increase when compared with 2021(19). The notification rate among non-officially bTB free (non-OTF) states was 0.04 per 100,000(19). Most cases (68.5%) were of EU origin -where the patient was native or originating from other EU Member States(19).

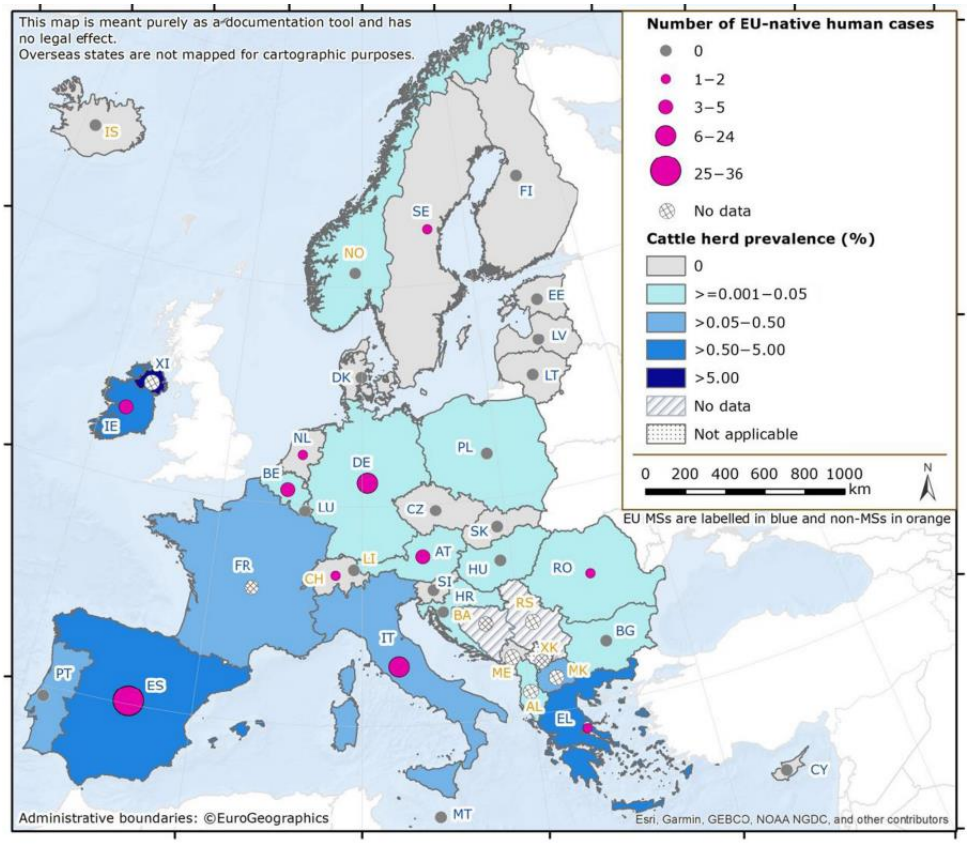

FIGURE 1.10: MAP OF THE NUMBER OF CONFIRMED TB CASES DUE TO *M. BOVIS* OR *M. CAPRAE* IN INDIVIDUALS OF EU ORIGIN AND NATIONAL HERD PREVALENCE OF TB. IMAGE FROM THE EUROPEAN UNION ONE HEALTH 2022 ZOONOSES REPORT (19)

1.4.1.1 Italy:

A study in Northern Italy, between 2011 and 2015, found 1.76% of MTBC diagnoses to be attributable to *M. bovis*, such cases were more commonly associated with extrapulmonary disease, older age and more commonly involved patients born in Italy (72). The authors concluded that the rate of *M. bovis* infection in humans was not negligible and remained a public health issue. The primary method of transmission to humans was via ingestion of unpasteurised contaminated dairy products, less commonly via contact with diseased animals or rarely by human-to-human transmission. Of note, Bologna, in Northern Italy,

where the study took place, has an *M. bovis* free status with low incidence of TB, but high prevalence of immigration from countries with high TB incidence.

The data suggested that most infections in elderly people were not due to recent transmission, but probably due to the reactivation of *M. bovis* infection acquired before the introduction of bTB eradication interventions.

1.4.1.2 Spain:

An epidemiological study in Asturias, Northern Spain between 2006 and 2014 found that 1.2% of TB infections were caused by *M. bovis* (73). The study found that all human *M. bovis* isolates were from Spaniards and that *M. bovis* infection was associated with a higher mortality rate than MTB infection. There were no cases in individuals aged under 24 years, the majority of cases were pulmonary in nature (13/19) and risk factors for *M. bovis* were identified in 6 patients. Eight of these patients were over the age of 45 years.

1.4.1.3 France:

A 2006 retrospective 5-year study in Lyon, France, found *M. bovis* infection accounted for 2% of TB disease. Seventy percent of patients with *M. bovis* infection were French born and most were aged over 50 years, suggesting infection had likely occurred prior to the introduction of widespread milk pasteurisation(74).

1.4.1.4 Netherlands:

A 1993-2007 review of TB cases in the Netherlands found 16,059 patients were registered with culture proven TB, 1.4% of whom had *M. bovis* infection(40). The majority were Dutch (59.7%) while most foreign born (23.4%) were from

Morocco. A significant difference in mean age was noted between those born in the Netherlands and immigrants (64.8 vs 38.6 years). The majority presented with extrapulmonary TB alone (58.9%), 29.4% had pulmonary TB alone, 11.7% both. Lymph node TB was more common among women, whilst pulmonary TB was more common among men. The proportion of deaths for *M. bovis* (19.9%) was higher than for MTB disease. Of note, there appeared to be two age peaks with the second/older peak likely being secondary to late endogenous reactivation - spoligotyping showed signal patterns in keeping with an old, predominantly cattle, endemic strain. Over time, the proportion of patients with *M. bovis* infection living in major cities increased from 33.3% to 41.8%, while declining elsewhere in the Netherlands. During 1993 – 1997, mainly elderly Dutch patients had disease, whilst later, between 1998-2007, disease was more evenly divided between natives and immigrants. Contrary to other studies *M. bovis* infection was more common in women compared with men.

1.4.2 New Zealand:

Combined epidemiological and laboratory investigation of human TB cases over the period 1995–2002 showed that *M. bovis* accounted for 2.7% (54/1997) of laboratory-confirmed human TB cases (75).

Seventy-two percent of patients diagnosed with *M. bovis* infection were born in New Zealand. Pulmonary infection occurred in 56. 7%, the remaining extra-pulmonary sites included: peritoneum, lymph nodes, arm/wrist (tenosynovitis), genitourinary system, hip joint, and proximal interphalangeal joint. The case-fatality rate was 15.4%. Patients were more likely to be male and over the age of 60 years.

A study by Yen *et al.* found that *M. bovis* accounted for 0.6% of culture positive TB cases in New Zealand from 2010 to 2011(76). Analysis of *M. bovis* cases in humans between 2006 and 2010 found that males accounted for 80% of cases, median age at diagnosis was 53 years and 30% of cases were aged over 70 years.

1.4.3 Australia:

In 1997, Australia was officially deemed free of bTB following an extensive 27 yearlong eradication campaign(23).

Cousins *et al*. sought to determine the epidemiology of human *M. bovis* infection in Australia between 1970 and 1994(77). During this time, 236 cases of human *M. bovis* infection were notified to public health, accounting for 1.4% of culture confirmed TB cases in Australia.

Patient information was available on 150 of these patients. Mean age at diagnosis was 54.2 years, 70.5% were male, pulmonary infection accounted for 71.6% of cases while extrapulmonary infection occurred in 25.7%. Extrapulmonary infection was more common in female patients. The majority of cases (67.5%) were from patients born in Australia, and the majority of foreign-born patients originated from Europe. No cases were diagnosed in children.

Notable risk factors included working in the meat (24.7%) or livestock (14.6%) industries, contact with others with a TB diagnosis (4.7%) and working in a laboratory where bTB samples were processed (2%).

The authors commented that infection via the aerosol route, resulting in pulmonary disease, has become a more important route of transmission than via the GI tract in countries where bTB eradication is nearing an end. They stated

that the eradication of bTB from Australian herds has reduced the risk of primary exposure, but, that ongoing human case detection was likely due to reactivation of latent disease(77).

1.4.4 Americas:

A review of studies from the Americas found that 0.3% of reported MTBC infections were attributable to *M. bovis* (71).

One United States (US) epidemiological study from 2006 to 2013 found that proportion of TB cases attributable to *M. bovis* did not change substantially (1.3-1.6%) during this time. Patients were more likely to be Hispanic (93%), many of whom were born in Mexico and the cumulative incidence of *M. bovis* infection was higher at the Mexico–US border than the national average. Patients born in the US were more often diagnosed with exclusively extrapulmonary disease compared with foreign born patients (54.6% vs 40.8%). Patients with *M. bovis* were younger than those with MTB, women had a higher frequency of exclusively extrapulmonary disease compared with men. Among 78 paediatric patients, 85.9% were diagnosed with exclusively extrapulmonary disease compared with 39.4% of adult patients. Isolates were pyrazinamide resistant in 93.6% of cases and 0.4% were resistant to isoniazid and rifampicin. Similar mortality rates were observed when comparing *M. bovis* and MTB infections.

A study in California found that while TB cases had decreased from 2003 to 2011, there was a relative increase in the proportion of *M. bovis* cases during this time (3.4% to 5.4%)(78). From 2001 and 2005, 10% of TB cases observed in San Diego were caused by *M. bovis*: almost all cases were reported in Hispanic individuals.

1.4.5 Low- and Middle-Income Countries (LMIC):

Livestock play a key part in the livelihoods of approximately 70% of the world's 1.4 billion people living in poverty(2). This close relationship leads to a potential public health risk where transmission of zoonotic disease via direct contact with an infected animal or consumption of unsafe food may occur (2).

Muller *et al*. reported that among studies from Africa, a median of 2.8% of all human MTB cases were caused by *M. bovis*. A large variation was noted in reported rates of *M. bovis* infection by country (range 0% - 37.7%) (71). Review of 15 studies from LMIC reported a prevalence of human zTB ranging from 0% to 28% with cattle and raw dairy likely representing primary exposures(53). India reported a 10.3% rate of zTB among patients with clinically suspected TB, while 8.7% had mixed *M. bovis* and MTB infection(79). India has an estimated 21.8 million infected cattle in a rapidly growing dairy sector(3). In India, *M. orygis* appears to be a major pathogen(3).

1.5 Risk factors:

1.5.1 Unpasteurised dairy product consumption:

Consumption of unpasteurised milk has long been recognised as a risk factor for human *M. bovis* infection(80). Despite efforts to reduce this risk by pasteurisation of milk, particularly in developed countries, outbreaks associated with contaminated unpasteurised milk do occur. An international systematic review estimated the prevalence of *M. bovis* in an individual cow's milk on dairy cattle farms to be 5% independent of TST status and 8% in TST positive cows(81). The half-life of *M. bovis* in raw milk at 20 °C is approximately 12 hours(82). Raw cheese may also be a source of acquisition as *M. bovis* can survive up to 393 days where there is a high bacterial burden in maturing raw mild cheddar(83).

A 2005 report in the Irish Veterinary Journal detailed an outbreak of *M. bovis* infection in both animals and humans on a farm owing to the consumption of unpasteurised milk(31). Milk from a 7-year-old cow with TB mastitis was fed to 29 calves on this farm and was occasionally added to a bulk tank from which the household members drank the milk without pasteurising. Twenty five of the 29 calves were subsequently identified as TB reactors, additionally 5 of the 6 family members were Mantoux positive. One 4-year-old boy from this family had anterior cervical lymphadenopathy. This lymph node was positive for AFB on microscopy, with features highly suspicious for TB on histology however culture failed to yield MTBC.

Disease arising from oral consumption of *M. bovis* is mainly extrapulmonary in nature, commonly presenting as visceral disease, peritoneal or cervical lymphadenopathy, also termed “scrofula”(14).

Several countries in the EU, including Ireland, permit the sale of unpasteurised dairy products under the condition that sanitary conditions are met(4).

1.5.2 Occupational Exposure:

The WHO “Roadmap for Zoonotic Tuberculosis” identifies staff at meat storage facilities, butchers and abattoir workers as being at high risk of zoonotic infection(2). A major route of transmission to such individuals is by aerosols or cutaneous contact. In such cases the minimum infective dose is much lower than acquisition via the GI tract(84). Humidity from water used to wash carcasses, the presence of residual organic material combined with the colder temperature can prolong survival of *M. bovis* within these environments and thus increase occupational exposure(85).

An Italian study sought to evaluate the environmental contamination by *M. bovis* within an abattoir where reactor cattle were slaughtered (85). Extensive testing of the environment revealed the presence of *M. bovis* on the hands of a worker and in washing water, highlighting ineffective cleaning methods within this setting. The researchers noted that use of Personal Protective Equipment (PPE) by workers was almost non-existent highlighting that the perception of biological risk within this workforce remains low.

A Portuguese study highlighted the risks of zTB within game hunters(86). With a rise in animal TB not just confined to domestic livestock but now present in wild

animals too, such as wild boar and red deer, hunters are at increased risk of exposure. On review of practices among Portuguese game hunters, compliance with hygienic and sanitary practices at the time of carcass evisceration were found to be suboptimal. Only 51% of cases observed used gloves on handling of carcases, and these were often not disposable. There was also scarce practice of disinfecting knives used on TB-like lesions on carcasses – raising a concern for potential cross contamination.

A study among dairy farm workers and their household contacts in Mexico found a high prevalence of latent TB infection among those workers exposed to infected cattle(87). Seventy-six percent were positive on TST, and while this was likely reflective of a high baseline prevalence of latent infection, increased risk was noted among those exposed within non ventilated spaces (e.g. milkers). Two workers were found to have active pulmonary infection, with one worker sharing the same *M. bovis* spoligotype as that circulating among the cattle, thereby highlighting the occupational hazard within this setting.
A Spanish hospital case of MDR *M. bovis* infection in a male patient, who had not been isolated for 10 days prior to diagnosis, prompted an occupational health review of members of staff in contact with the patient(88). This highlights another risk group for occupational exposure to *M. bovis* infection.

1.5.3 Immunosuppression and *Mycobacterium bovis* Infection:

Multiple case reports have been published describing either reactivation or new acquisition of *M. bovis* infection among immunocompromised patients.

1.5.3.1 HIV:

Immunosuppression due to HIV infection is a known risk factor for *M. bovis* infection(89). Cobo *et al* describe a hospital outbreak of MDR *M.* bovis in a Spanish hospital between 1993 – 1995, where an *M. bovis* strain resistant to 11 drugs infected 19 HIV positive patients over 2 Infectious Disease wards(67). CD4 count of less than 50/µl was found to be a significant risk factor for disease acquisition.

A retrospective review of 86 TB cases among HIV infected individuals at a hospital in San Diego between 2000 and 2007, identified 30 (34.9%) as being due to *M. bovis* (90). In contrast, *M. bovis* only accounted for 6.4% of adult TB cases in San Diego between 1994 and 2005. The study found that *M. bovis* infection was strongly associated with Hispanic ethnicity and disseminated disease with abdominal infection. CD4 counts did not differ substantially between patients with *M. tuberculosis* infection versus *M. bovis* infection. However, *M. bovis* was more likely to occur in patients with advanced immunosuppression, with no cases of *M. bovis* infection occurring in patients with CD4 counts >200/µl. Eighteen percent of patients with MTB infection had CD4 counts of >200/µl.

1.5.2.1 Immunomodulatory Medications:

Reactivation of MTB infection in patients receiving Tumor Necrosis Factor (TNF) alpha inhibitors has long been documented. In 2008, Larsen *et al* reported the first case of *M. bovis* reactivation following treatment with TNF alpha inhibitors in Denmark(91). This patient was started on infliximab for management of her rheumatoid arthritis. TST and chest X-Ray (CXR) were negative on commencement of treatment, QuantiFERON test was indeterminate. Eleven months later, the

patient presented with fever, decreased appetite, nausea and new ascites. TST again was negative, however ascitic fluid was positive on culture for *M. bovis*. Repeat QuantiFERON was positive. On further review, the patient gave a history of working at a dairy during her childhood with probable exposure to unpasteurized milk from infected cattle. The authors remarked that indeterminate QuantiFERON tests should be interpreted with caution in immunocompromised patients and should be repeated at a minimum.

Rodríguez-Orozco *et al* describe the case of a Mexican patient on treatment for rheumatoid arthritis with methotrexate who developed peritoneal *M. bovis* infection following the commencement of infliximab therapy of his autoimmune condition. TST and CXR were negative for TB at treatment commencement, and he did not receive isoniazid prophylaxis (92). The authors highlighted that the high prevalence of *M. bovis* infection within the Mexican population should be considered when starting an immunosuppressive agent.

Viera *et al* describe *M. bovis* infection in a Moroccan patient receiving Apremilast, a phosphodiesterase 4 (PDE-4) inhibitor, for treatment of Bechet's syndrome(93). Following 4 weeks of treatment, a painless cervical node appeared which was culture positive for *M. bovis*. The patient had a positive QuantiFERON study upon initiation of treatment. The authors thus advocated the potential benefit of TB prophylaxis in at-risk patients prior to commencing Apremilast. It was hypothesized that PDE-4 Inhibitors decrease cyclic adenosine monophosphate levels, reducing the production of proinflammatory mediators such as TNF alpha and Interferon (IFN) gamma. TNF alpha and IFN gamma both effect granuloma maintenance and inflammation mediated tissue damage, thus, PDE-4 inhibitors

may disrupt granuloma microbe-containing capacity, leading to disseminated infection.

Winger *et al* describe *M. bovis* enterocolitis in a 16-year-old female patient from Mexico on mycophenolate mofetil and hydroxychloroquine for systemic lupus erythematous with a history of Mexican soft cheese consumption(94). The young patient presented with fever and bloody diarrhoea. TST showed an induration of 34mm. Colonic biopsies confirmed a diagnosis of *M. bovis* infection and symptoms resolved with anti-tuberculosis therapy. An early differential diagnosis for the authors in this case, prior to culture results, was Crohn's colitis, management of which would have included steroids which may have disseminated her TB infection. Recognition of epidemiological and exposure history were key to this patient's successful outcome.

A case report by Chen *et al* (95) from California describes an 8-year-old girl with a history of renal transplant 4 years prior to presentation with cough, chest pain and fevers. She had been receiving tacrolimus, sirolimus and prednisolone immunosuppression. CT thorax had findings suspicious for TB infection and TST had 22.5 cm induration. Of note, TST performed 1 year following transplant was negative. Diagnosis of *M. bovis* infection was confirmed by positive sputum culture. The patient was successfully treated, however the authors remarked on difficulties encountered titrating tacrolimus levels due to its interaction with rifampicin. Whilst some practitioners may be reluctant to combine the two medications, loss of anti TB medicines rifampicin and pyrazinamide (secondary to inherent resistance) may compromise efficacy of TB treatment. This patient's risk factor for *M. bovis* infection was travel, one year prior to her transplant, to a family

farm in Mexico where she had consumed unpasteurized cheese. The authors recommended that all solid organ transplant patients should be advised to avoid consuming unpasteurized dairy products.

Sotoudeh *et al* reported disseminated *M. bovis* infection in a patient whilst receiving immunosuppression post liver transplant. The patient reported exposure to unpasteurised dairy whilst living on a farm in Mexico as a child(96).

Transmission from person to person, particularly among immunocompromised individuals has also been reported. Bourgoin *et al* detail the case of a son and his mother developing *M. bovis* pulmonary infection within 2 years of each other (97). The son, a butcher at a slaughterhouse, presented initially with respiratory symptoms with sputum culture positive for *M. bovis* but AFB negative on microscopy. He was treated for 6 months with rifampicin, isoniazid, pyrazinamide and ethambutol. His mother, a patient in her 70s on long term steroids and methotrexate for rheumatoid arthritis, subsequently presented with respiratory symptoms. She had a positive TST result and sputum cultured *M. bovis*. The two patient strains were compared using MIRU-VNTR and spoligotyping which showed identical DNA profiles. There was mention of previous family cattle breeding activity, however the authors hypothesized that the son became infected with *M. bovis* by occupational zoonotic exposure, subsequently transmitting infection to his immunocompromised mother with disease reactivation 2 years later.

A Netherlands case series highlighted the occurrence of pulmonary *M. bovis* infection in 2 patients following treatment with anti-TNF medications for 1 year despite negative TST and normal CXR prior to initiating treatment(40). One patient also had suspected cerebral tuberculomas. Neither received TB

prophylaxis. The authors highlighted that reactivation of latent TB usually occurs within the first 3 -4 months of anti TNF alpha therapy whilst these patients' developed infection after a year of treatment, raising the question of whether their TB was newly acquired or the result of reactivation and also whether bacteriologic virulence of *M. bovis* affects the pace of potential disease activation. Their cases also raise the question of should patients have a QuantiFERON test rather than TST and should immunosuppressed patients be warned about travel to endemic regions.

1.5.4 Domestic Pets and *Mycobacterium bovis*:

Companion animals, including cats and dogs may be susceptible to mycobacterial infection. In the UK, *M. bovis* isolates from companion and non-bovine farmed animals represented <1 % of culture positive isolates. Of note, however, active surveillance is not in place for these animals, unlike for cattle, and so infections within these animals may be under estimated(98).

In cats, TB is primarily caused by *Mycobacterium bovis*. There have even been reports of *M. bovis* infections in cats potentially associated with a commercial raw food diet (99). In the early 20th century, prior to routine milk pasteurisation, TB in cats primarily presented in the form of GI tract infection. However more recently, clinical presentation may be with persistent abscesses or skin lesions refractory to antibiotics, weight loss or lymphadenopathy (100). Between 2004 and 2010, 116 cases of *M. bovis* infection in domestic cats was reported to the Animal and Plant Health Agency (APHA) in the UK(100).

A case report from Buenos Aires reported a human case of glenohumeral joint septic arthritis caused by *M. bovis(101)*. The patient's only epidemiologic risk factor was that they owned cats that were fed raw bovine lungs. *M. bovis* was cultured from the BAL fluid of one of the cats. In 2013 in England, a cluster of infection in cats resulted in the occurrence of 2 cat to human transmission events confirmed by genotyping and 2 cases of latent TB detected on screening by a combination of IGRA and TST(102).

Randas *et al* reported a case in Texas where a household cat was diagnosed with *M. bovis* infection months after the death of their owner due to pulmonary *M bovis* infection(103). The deceased man's wife eventually tested positive on TST 2 months after his death, despite initial negative TST. The case report occurred in an area where bTB is not endemic and neither cat owners nor the cats had contact with cattle. The gentleman had relocated from Mexico 15 years earlier and consumed unpasteurised Mexican cheese. WGS indicated the cat and human isolates were closely related but not identical (8 SNPs apart) indicating that direct transmission between human and cat was unlikely.

Dogs are rarely infected with *M bovis* or *M. tuberculosis* (104). Only 7 cases of *M. bovis* infection in dogs were reported to the APHA between 2004 and 2010 (100). However, an outbreak was reported in 2017 among working Wolfhounds in England (105). Of 164 hounds tested from a kennel due to suspicion of an outbreak raised by the attending veterinarian, 85 tested positive on IGRA. Test-positive hounds and clinically unwell hounds were euthanised, 14 were confirmed to have *M. bovis* infection by culture. It was hypothesised that recent movement of hounds from a high-risk area for bTB likely introduced infection to

the kennel. It was likely that these hounds may have been fed fallen stock infected with *M. bovis*. What ensued was a complex public health investigation, eleven close human contacts were screened with QuantiFERON and CXR, one of whom tested positive on QuantiFERON and was treated with prophylactic isoniazid. No human contacts had signs of active infection.

In 2009, Shrikrishna *et al* reported a case of household transmission of *M. bovis* infection between human and canine (42). The suspected index case was a woman in her 40s who presented with cough, malaise and an apical cavitary lesion on CXR. Her sputum was positive for AFB, and culture later identified *M. bovis*. Her asymptomatic 12-year-old daughter was QuantiFERON and TST positive upon screening and she was treated prophylactically. Four months later, the woman's dog developed persistent cough with tracheal mucus positive for AFB. The dog was euthanised and subsequent cultures confirmed *M. bovis* with genotype identical to its owner's isolate. The family lived in an area of high bTB prevalence, and the woman had contact with reactor cattle through occupational contact as a veterinary nurse, though she also reported contact with local badgers. Airborne transmission within the household from woman to dog and daughter was thought likely.

A cross sectional study in Michigan sought to evaluate *M bovis* infection among domestic pets on 9 farms with *M. bovis* infected cattle (106). Nineteen cats and 5 dogs were included in the study. None of the 51 specimens from these domestic pets grew *M. bovis*. There was thus no evidence to suggest that the domestic pets acquired *M. bovis* from infected cattle. However, this may be due

to the low burden of disease on the selected farms - the herds on participating farms had a low prevalence of infected cattle (1.6%)

Public Health England (PHE), now known as the UK Health Security Agency (UKHSA), has recommended that all household contacts of domestic pets with *M. bovis* infection should be assessed by a public health specialist. Children, immunocompromised patients and those with extended contact with an infected animal are prioritised for screening (107). Additionally in England and in Wales, all newly diagnosed human cases of *M. bovis* infection are explicitly asked about contact with pets with suspected or confirmed *M. bovis* disease as part of an enhanced surveillance system(108). Legislation introduced in the UK in 2006 makes notification to authorities compulsory for any animal keepers, veterinarians and meat inspectors of any suspect TB lesion detected in any mammal excluding humans(100).

1.6 Research Aims and Relevance:

The aim of this study was to investigate whether ongoing human *M. bovis* infection in Ireland is due to reactivation of latent TB infection with *M. bovis* that had been acquired years earlier, prior to widespread milk pasteurisation, or whether it is due to new infection transmitted to humans from infected cattle or other animals.

With a growing number of patients on novel immunomodulatory agents, and a recent rise in M*. bovis* infections, particularly among this cohort of patients, it is important to assess whether there is a link between the two. The research could potentially lead to a discussion on the advice given to patients on immunomodulatory agents. For instance, if infection among this cohort of patients is thought to be new, this may have relevance to advice given regarding the consumption of unpasteurised foods, proximity to farm animals and even domestic pets. It could also potentially impact on whether these patients are considered for prophylactic anti TB therapy. Indeed, a recent Irish case report detailed *M. bovis* infection in a human likely acquired from an infected, immunosuppressed domestic pet(109).

To this end, we reviewed the demographics, and medical charts where available, of all patients who had samples for *M. bovis* processed for diagnostic purposes at the IMRL between 2000 and 2020. In parallel to this, human *M. bovis* isolates and a small number of domestic pet isolates stored at the IMRL from 2000 to 2020 underwent WGS. These genomic sequences were then compared with a selection of genome sequences of Irish animal *M. bovis* isolates provided by University College Dublin (UCD) on behalf of the Department of Agriculture, Food

and the Marine (DAFM) and with a number of Irish animal genomic *M. bovis* sequences published online.

Patient meta data from the retrospective chart review was then linked to the relevant *M. bovis* genomic data to better ascertain potential transmission events and risk factors for development of zTB infection.

An analysis of the epidemiology of human *M. bovis* infection in Ireland will be described in Chapter 2. Chapter 3 will focus on comparing animal and human *M. bovis* sequences and describing any potential transmission links between the two.

Chapter 2: Human *M. bovis* Infection in Ireland.

2.1 Introduction:

2.1.1 Epidemiology of Human *M. bovis* Infection in Ireland:

In 2020, Ireland had a 0.06 per 100,000 notification rate for human *M. bovis* infection, while the notification rate was 0.14 per 100,000 in 2019 (110). This was above the EU notification rate of human *M. bovis* infection of 0.02 per 100,000 for both officially bTB free (OTF) and non-officially bTB free countries and is the highest across the EU (110). The EU notification rate for human *M. bovis* infection has ranged between 0.02 and 0.05 per 100,000 population between 2016 and 2020(110).

In 2019, the Health Protection Surveillance Centre (HPSC) reported 7 cases of human *M. bovis* infections, accounting for 3.6% of total TB cases in Ireland(111). For comparison, across Europe zTB accounts for 0.4% of TB cases, mostly caused by *M. bovis* and *M. caprae*(71). Despite predictions that cases of human *M. bovis* infections would continue to fall, there is ongoing disease detection within Ireland(112).

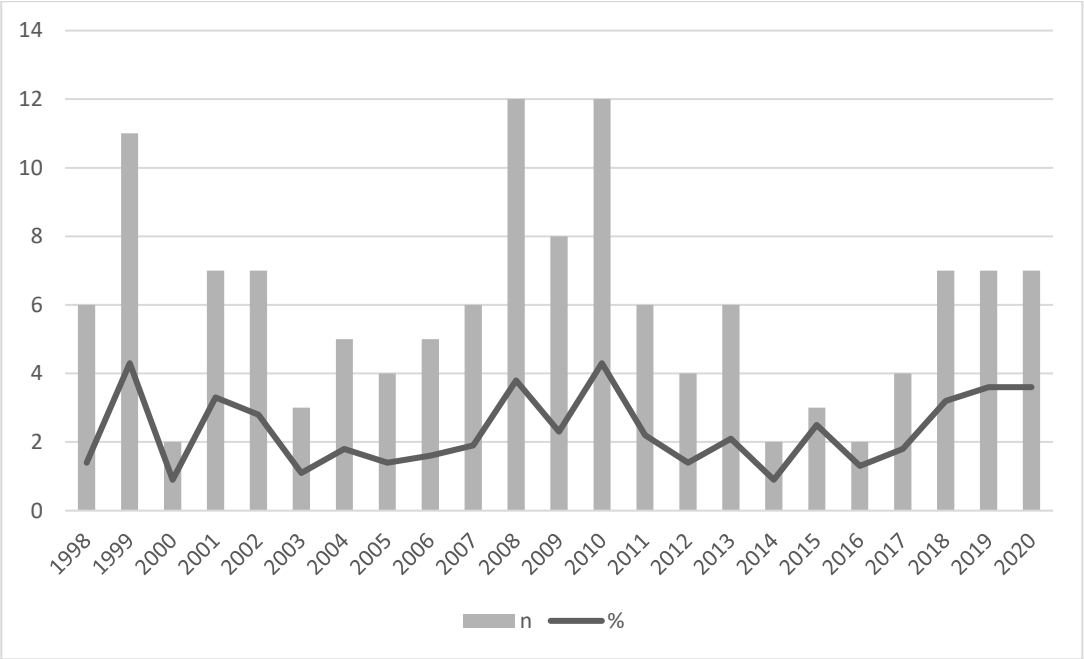

FIGURE 2.1: NUMBER OF HUMAN *M. BOVIS* CASES (N) AND PERCENTAGE OF TOTAL MTBC CASES BY YEAR AS PER ANNUAL SURVEILLANCE REPORTS PRODUCED BY THE HPSC. N= NUMBER OF CASES BY YEAR, % = PERCENTAGE OF TOTAL MTBC CASES.

Cotter *et al* examined the epidemiology of *M. bovis* infection in South-West Ireland between 1983 and 1992. They reported a mean annual incidence of human *M. bovis* infection of 0.56 per 100,000 versus 9 per 100,000 population for MTB (112). Males accounted for 63.4% of *M. bovis* cases. Pulmonary disease occurred in 43% of cases of *M. bovis* infections versus 85% in MTB infection. Average age at diagnosis was 58.4 years for *M. bovis* infection versus 48.5 years in MTB. The authors predicted that cases of human *M. bovis* infection were likely to fall further, with potential elimination of human disease in the following 10 – 15 years. They hypothesised that infections were likely secondary to reactivation of latent disease rather than newly acquired infection.

In 2008, Ojo *et al* examined the epidemiology of *M. bovis* infection in South-West Ireland(57). Between 1998 and 2006, *M. bovis* infection accounted for 3% of

human TB infections. Researchers used spoligotyping and 10 locus MIRU-VNTR to further characterise their isolates. Among their 11 isolates, they identified 8 different spoligotypes of which SB0140 accounted for 3 isolates and this was also noted to be the prevalent spoligotype among Irish cattle. The authors hypothesised that primary infection likely occurred in patients decades before diagnosis of clinically evident infection and likely represented reactivation of latent infection acquired prior to the widespread pasteurisation of milk.

Despite the extensive financial investment that has been made into the control of bTB among Irish herds - 97 million euro annually(113)-, there are few Irish studies examining the epidemiology of *M. bovis* infection among humans. As *M. bovis* infection in humans is a statutorily notifiable infectious disease, this research is of Public Health importance. Assessing the disease epidemiology among patients may help assess the effectiveness of current bTB control measures.

2.1.2 The Health Protection Surveillance Centre (HPSC):

The HPSC carries out national surveillance of communicable diseases. This organisation works closely with health service providers across Ireland to prevent and control the spread of infectious diseases in order to improve population health(114). Its six main areas of responsibility are: disease surveillance, providing expert advice, provision of training to individuals on disease control, research, advising on policy and providing information to the public(114).

TB, including disease caused by *M. bovis,* has been a notifiable disease since 1948 and so is reportable to the HPSC(115). Laboratories and Departments of Public Health across the state notify cases of TB to the HPSC via the Computerised Infectious Disease Reporting (CIDR) system. Enhanced surveillance forms (see Appendix 1) are completed for each case by local public health departments(115). The collated data is summarised into quarterly and annual surveillance reports.

Guidelines on the Infection Prevention and Control of Tuberculosis in Ireland (25) published by the HSPC in 2010 advised that there is no evidence that zTB is a major public health issue in Ireland and state that the risk of human *M. bovis* infection is negligible thanks to the stringent TB testing of animals in Ireland. They highlight occupational exposure in farmers, veterinarians, and others working with animals or animal carcasses, as well as the consumption of unpasteurised milk, extremes of age and immunosuppression as risk factors for disease. The document describes ingestion, rather than inhalation, as the principal route for acquisition of human infection. The document also advises that infection is most often due to reactivation of latent disease rather than new

infection, as between 2002 and 2006 over 75% of patients were aged over fifty years and average age at diagnosis was 62.3 years.

2.1.3 The Irish Mycobacteria Reference Laboratory:

The Irish Mycobacteria Reference Laboratory (IMRL) is located within St James's Hospital (SJH), Dublin 8, and was established in 2001(116). It provides a culture and specimen referral service to microbiology laboratories throughout Ireland. It receives about 6000 diagnostic specimens and 500 mycobacterial cultures annually(116). The laboratory provides expertise in TB and mycobacterial disease diagnosis, surveillance and detecting drug resistance. The large repository of mycobacterial isolates stored at the IMRL offers a unique opportunity for research projects nationally and internationally. The IMRL is fully accredited to ISO15189:2012 standards and has been a member of the European Reference Laboratory Network for Tuberculosis since 2009(116). Services provided include rapid diagnostic molecular tests to identify MTBC and screen for drug resistance directly on specimens, mycobacterial species identification using molecular techniques, WGS and drug susceptibility testing (DST) of MTBC and referral of non-tuberculous mycobacteria (NTM) for DST. Additionally, clinical advice on treatment and isolation can be provided by the clinical directors of the laboratory(116).

The advent of WGS has revolutionised the microbiology laboratory, particularly in the realm of TB diagnosis and the analysis of outbreaks and evolving drug resistance (117). Molecular epidemiological surveillance using WGS has been performed at the IMRL in SJH since 2020. Since then, all MTBC samples processed at the IMRL undergo WGS using the MiSeq™ Illumina short read

sequencer(118). Bioinformatic analysis is done using Ridom™ SeqSphere+ Software which uses core genome multilocus sequence typing (cgMLST) to define clusters and create minimum spanning trees (MST)(119). Further bioinformatic analysis can be performed using the automated pipeline MTBseq to determine global lineages, the presence of variants associated with antimicrobial resistance and to assess isolates' relatedness using an assessment of the Single Nucleotide Polymorphism (SNP) differences between isolates(117). Both bioinformatic tools map WGS created reads to the reference *M. tuberculosis* H37Rv strain (NC000962.3).

2.2 Methods:

2.2.1 Overview:

A retrospective review was undertaken of the healthcare records of patients who attended SJH, Dublin 8, for diagnosis, investigation, or treatment of their *M. bovis* infection between 2000 and 2020. In parallel to this, WGS was performed on DNA extracted from 64 *M. bovis* isolates. These isolates were originally recovered from patients attending SJH or other Irish hospitals (n=61), and a small number of domestic animals (n=3), that had been collected by the IMRL and stored over the last 20 years (2000 to 2020) as part of routine clinical care, diagnosis, and national reference work.

Available patient metadata, collected by reviewing patient charts, was linked to corresponding sequenced *M. bovis* isolates with the hope of allowing a better understanding of risk factors for, and epidemiology of, *M. bovis* infection in Ireland.

The HPSC kindly shared anonymised data collected on *M. bovis* cases across Ireland as part of enhanced surveillance between 2000 and 2020. This data is summarised in Section 2.3.1 of the results section below.

2.2.2 Relevant Approvals:

This project was approved by the SJH Research and Innovations Office - Project Number 6378.

Ethics Committee approval was obtained for the project from the St. James' Hospital (SJH) / Tallaght University Hospital (TUH) Joint Research Ethics Committee (JREC) on 8th June 2021 with subsequent amendments approved on the 10th of March 2022 and 16th December 2022 -Project Reference Number: 1889.

As consent was not sought from patients for inclusion in this study, a consent declaration was obtained from the Health Research Consent Declaration Committee (HRCDC). Project reference number: 21-003-AF1/AMD1. A number of transparency measures were put in place including a project specific privacy notice on the SJH website and notices were placed in the SJH TB outpatient clinics (see Appendix 2 and 3).

Applying to the HRCDC for a consent declaration involved extensive discussion with both the Trinity College Dublin (TCD) and SJH Research Data Protection Officers, completion of a Data Protection Impact Assessment (DPIA) - in addition to the detailed application forms required by the HRCDC.

The original consent declaration was made on 18th May 2021. Subsequent amendment was approved on 22nd April 2022 to include TCD as a joint data controller, in addition to SJH. Further amendment was approved on the 30th June 2023 to extend the duration of the project until April 2024 and also to include patients from 2021 and 2022. The approval made on the 30th June 2023 included

a condition that attempts should be made to consent the relevant additional patients, however due to time constraints and the lack of direct involvement in these patients' clinical care, it was not possible to consent patients from 2021 and 2022 and so their data was not included in the project.

2.2.3 Retrospective Patient Chart Review:

A list of medical record numbers (MRNs) of patients with a history of *M. bovis* infection who had their samples processed at the IMRL was generated. The electronic patient records of these patients were reviewed, along with physical patient charts where available. Sixty-six patients had samples processed for human *M. bovis* infection at the IMRL between 2000 and 2020 of which 61 were available for WGS analysis. The laboratory information system (Telepath) records were reviewed for all 66 of these patients to ascertain basic demographic details. Twenty-seven patients had attended SJH for review and thus had a physical or electronic patient record available for review.

Where available, patient age category, gender, country of birth, co-morbidities, rural or urban dwelling, time since entry to Ireland (if foreign born) was recorded. Clinical factors such as site of disease, previous history of TB diagnosis and use of immunomodulatory medications was obtained. Information on potential past exposures to *M. bovis* was also gathered, if documented in the patient chart. Such risk factors for potential exposure included contact with a human TB patient, travel (for >2 weeks) to, or residence in, a country with high TB incidence (where TB incidence is > 100 per 100 000 population(120)), consumption of unpasteurised milk products, occupational contact with animals,

physical contact with wild animals or pets and physical contact with any animal with TB.

The above-mentioned patient data and risk factors for *M. bovi*s exposure were selected for inclusion in this research project as they mirror those recorded in a recent study by Davidson *et al* (121) that investigated the epidemiology of *M. bovis* infection in the UK. By focusing on the same categories of patient data and risk factors as Davidson *et al*, results of the UK study will be directly comparable with the results of this study.

Data minimisation was among the conditions of the consent declaration issued by the HRCDC. Therefore, in order to minimise the amount of personal data collected, patients' names were not recorded. Only decade of birth was recorded as opposed to date of birth. Site of disease was recorded as either "pulmonary" or "extrapulmonary" or "both".

Patient information relevant to the project, as listed above, was recorded on password protected Excel sheets or Word files stored on a SJH computer. Data was pseudonymised during collection.

2.2.4 Whole Genome Sequencing Overview:

Whole genome sequencing (WGS) refers to DNA sequencing of an entire genome. It uses massively parallel sequencing of either short or long reads of DNA at the same time (122). Short read sequencing, as used in this project with the Illumina Miseq platform, involves fragmenting the bacterial genome into small fragments of between 50 – 300 bases in length(122). Long read sequencers, such as the MinION (Oxford Nanopore Technologies), fragment DNA into fragments of approximately 10,000 bases before sequencing(122).

The Illumina Miseq "sequences by synthesis" (Figure 2.2). DNA polymerases catalyse the incorporation of fluorescently labelled deoxyribonucleotide triphosphates (dNTPs) into a DNA template and nucleotides are identified by fluorophore excitation. This process is extended across millions of DNA fragments in a parallel manner at the same time(123).

The first step in the process is library preparation. This involves random fragmentation of the DNA sample followed by ligation of specific adaptors to each fragment's ends(123). PCR is then used to amplify the adaptor ligated fragments before loading of the library onto a "flow cell" within the Miseq(123). The adaptor ligated DNA fragments are hybridised to the flow cell and fragments are then amplified into clonal clusters through bridge amplification. The Illumina sequencer then adds fluorescent labelled nucleotides (A, C, T or G) to each of the DNA template strands, the flow cell is imaged and the emission wavelength and intensity is used to identify the incorporated base(123). This cycle is repeated for each base incorporated to create a read length.

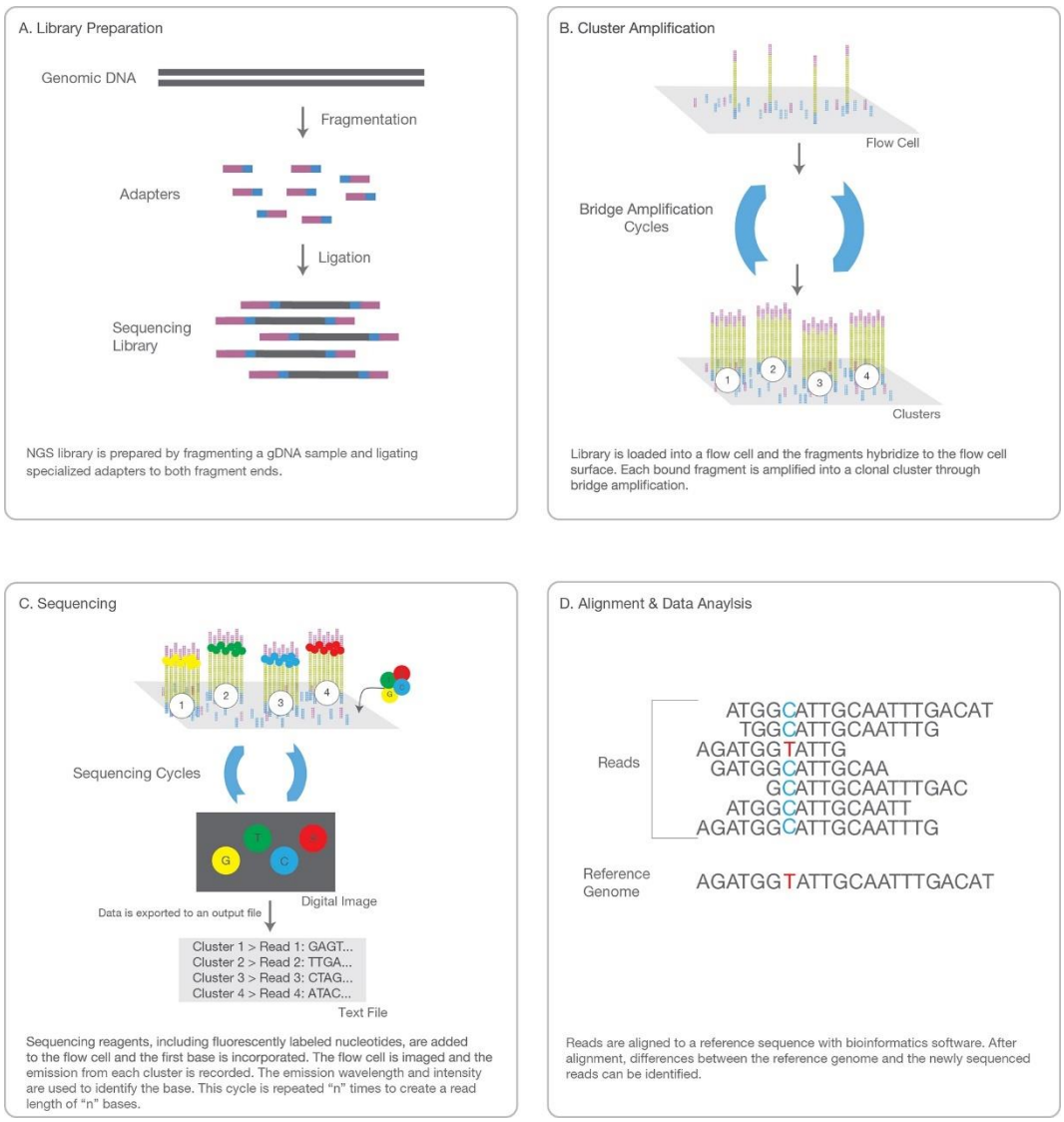

FIGURE 2.2: SEQUENCING BY SYNTHESIS - OVERVIEW OF WORKFLOW DESCRIBING LIBRARY PREPARATION, CLUSTER AMPLIFICATION, SEQUENCING, ALIGNMENT AND DATA ANALYSIS. IMAGE COURTESY OF ILLUMINA INC.

The created raw short read DNA sequences are stored as FASTQ files and are then aligned to a reference genome. Bioinformatic software tools can then be used to analyse SNPs and to identify insertion-deletion elements and perform phylogenetic analysis(123).

Studies have looked at defining SNP threshold to distinguish whether isolates belong to a "cluster" of potentially genomically related isolates or not. Many of these evaluate MTB which has an evolutionary rate of 0.3 – 0.5 SNPs per genome

per year, the estimated *M. bovis* evolutionary rate is 0.15 to 0.53. Many studies have defined a maximum of 5 SNP differences between isolates to determine a potential transmission cluster, with 10 – 12 SNPs considered a more relaxed threshold. However, thresholds have not been determined for *M. bovis*(5).

2.2.5 Laboratory preparation:

MTBC isolates, including *M. bovis* recovered from patient specimens at SJH and other Irish hospitals were archived and stored securely at -80°C in the Containment Level 3 (CL3) laboratory in the IMRL, within the SJH Central Pathology Laboratory. Mycobacterial isolates for long term storage were inoculated into sterile Middlebrook broth containing enrichment media, allowed to culture at 35°C for at least 2 weeks, before freezing at -80°C.

M. bovis isolates (n=64) retrieved from long term storage at -80°C were heat inactivated at 95°C in the CL3 by IMRL staff laboratory prior to being processed for WGS within the Containment Level 2 (CL2) laboratory.

2.2.5.1 Extraction and Purification:

Within the CL3 laboratory, 1 µl of *M. bovis* inoculum from Löwenstein–Jensen (LJ) slopes was suspended into 400µl of 0.85% saline in a screw cap tube. Sealed tubes were sonicated for 20 minutes at a frequency of ≥35kHz. Sonicated tubes were then immersed into a preheated 95°C water bath and left for 2 hours. At this stage, mycobacteria were inactivated and thereafter samples were safe to remove from the CL3 laboratory.

DNA purification and extraction was performed using adapted Fuji Quickgene protocol. This technique was performed by the IMRL scientists and observed by the M.D candidate at the CL2 stage of the process.

Twenty µl EDT (Proteinase K) and 300µl lysis buffer (LDT) were added to each sample and the contents of each tube transferred to the Lysing Matrix B tubes which contain silica beads used to lyse the mycobacteria to release DNA during bead bashing. The tubes were placed in the magNA Lyser instrument for two cycles of bead-bashing (40s each at 6m/s) with a 5-minute interval between beatings.

Samples were centrifuged at 13,600 revolutions per minute (rpm) for 10 min. Supernatant was transferred to a fresh tube carefully without transferring beads. Samples were heated to 70°C for 10 min. 240µl of 99% ethanol (EtOH) was added and vortexed for 15 seconds.

The Quick-gene machine was then set up (Figure 2.3), the front row filled with discard tubes, the back with final tubes ready to catch purified DNA and filters placed within the movable rack.

Sample contents were transferred to the filter overlying the discard tube. The machine was run by turning the handle until all fluid had passed through the filter, then 750µl of wash buffer (WDT) was added to each filter and passed through the filter until all the WDT had passed, this was repeated 2 more times.

The movable rack was moved backwards so that the filters overlaid the final catch tubes and the samples eluted with 100µl elution buffer (CDT).

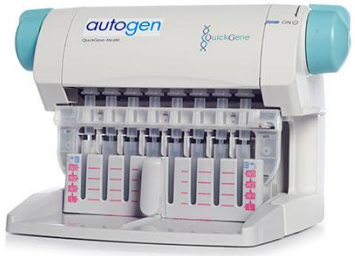

FIGURE 2.3: IMAGE OF QUICK-GENE INSTRUMENT. IMAGE FROM AUTOGEN.COM

2.2.5.2 DNA Quantification:

The Qubit™ fluorometer was used to determine DNA concentration by measuring the fluorence intensity of fluorescent dye binding to double stranded DNA(124). Two DNA standards were used to create a calibration curve. DNA quality was assessed using the Nanodrop 2000 spectrophotometer. This was performed by IMRL scientists and observed by the M.D candidate.

2.2.6 Whole genome sequencing:

2.2.6.1 DNA Library Preparation :

DNA Library Preparation was performed using the Nextera protocol but with modified reagent volumes(125). The Nextera protocol was optimised for 1ng of input DNA. This was performed by IMRL scientists and observed by M.D candidate.

2.2.6.2 Step One - Tagmentation:

Tagmentation is a process that fragments and tags DNA with adaptor sequences using the Nextera XT transposome. Tagment DNA Buffer (TD) was added to 1ng of DNA in a 96 well plate and mixed using a pipette tip. Five µl of Amplicon Tagment Mix (ATM) was added to each well and mixed. The plate was sealed and centrifuged - 280 × g at 20°C for 1 min. The plate was then run on a preprogrammed thermal cycler (preheat lid option, set to100°C, reaction volume 50 µl, 55 °C for 5 minutes and hold at 10°C).

Once the thermocycler had reached 10 °C, 5 µl of Neutralize Tagment Buffer (NT) was added to each well and pipetted 10 times. The plate was sealed, centrifuged for 1 minute and then left to incubate at room temperature for 5 minutes. This was performed by IMRL scientists and observed by the M.D candidate.

2.2.6.3 Step Two – Amplifying Libraries:

Tagmented DNA was amplified using a limited cycle PCR program which added Index 1 (i7), Index 2 (i5) adaptors, sequencing and common adaptors required for cluster generation.

Index adaptors i7 and i5 were added to each sample using TruSeq Index Plate Fixture and 15µl of Nextera PCR Master Mix added to each well before pipetting and sealing. The plate was centrifuged (280 × g at 20°C for 1 minute) before being placed on a preprogramed thermal cycler and the NXT PCR program was run. (Set to 100°C, reaction volume to 50 µl, then 72°C for 3 minutes, 95°C for 30 seconds, then: 12 cycles of: 95°C for 10 seconds, 55°C for 30 seconds, 72°C for 30

seconds, 72°C for 5 minutes, hold at 10°C). This was performed by IMRL scientists and observed by the M.D candidate.

2.2.6.4 Step Three - Library Clean up:

Amplified libraries were purified using single-sided bead purification which relies on solid phase reversible immobilisation (SPRI) of genomic DNA.

Samples were centrifuged (280 x g at 20°C for 1 minute) to collect contents at the bottom of the well. 50 µl of supernatant was transferred from each well to the corresponding well of a new midi plate. 15 µl AMPure XP beads was added to each well. Plates were sealed and put on a plate shaker at 1800rpm for 2 minutes before incubating at room temperature for 2 minutes. The plate was placed on a magnetic stand until the liquid was clear. The supernatant was discarded without disturbing the beads.

The samples were washed by adding 110 µl fresh 80% EtOH without mixing, incubating for 30 seconds and removing the supernatant without disturbing the beads. This step was repeated one more time.

Residual EtOH was discarded using a 20 µl pipette and samples left to airdry on the magnetic stand for 15 minutes. Resuspension Buffer (26.5 µl) was added to the beads, the plate was sealed and put on a plate shaker at 1800rpm for 2 minutes and then incubated at room temperature for 2 minutes. The plate was put back on the magnetic stand and left until the liquid was clear. Fifty µl of supernatant was transferred to the corresponding wells of a new 96 well PCR plate.

At this point libraries were quantified using Qubit to calculate each DNA concentration. This was performed by IMRL scientists and observed by the M.D candidate.

2.2.6.5 Step Four – Normalising Libraries:

This step normalised the quantity of each "library" to ensure equal library representation within the final pooled library. In this step, DNA was bound to and eluted from magnetic beads at approximately the same concentration for each sample.

Twenty µl of supernatant from each well of the PCR plate was transferred to the corresponding well of a new midi plate.

Library normalisation (LN) master mix was prepared in a 15 ml conical tube with Library Normalization Additives 1 (46 µl) and Library Normalization Beads 1 (8 µl) added for every sample on the run. This was pipetted 10 times and transferred to a trough. Using a multi-channel pipette 45 µl LN master mix was added to each well on the midi plate before sealing and placing on the plate shaker at 1800 rpm for 30 minutes.

The plate was placed on the magnetic stand until the liquid was clear, and, without disturbing the beads the supernatant was removed and discarded. The plates were washed by adding 45 µl Library Normalization Wash 1 (LNW1) to each well, then sealing the plate and placing back on the plate shaker for 5 minutes. The plate was placed on the magnetic stand and left until the liquid was clear before removing and discarding the supernatant. This washing step was

repeated. Thirty µl of 0.1 N Sodium Hydroxide (NaOH) was added to each well, the plate was sealed and placed on the plate shaker for a further 5 minutes.

Once resuspended the midi plate was placed on the magnetic stand until the liquid was clear, then 30 µl of supernatant was transferred from each well to the corresponding well of another midi plate which had already had each well inoculated with 30 µl of Library Normalization Storage Buffer 1 (LNS1). The plate was centrifuged at 1000 × g for 1 minute.

Samples were diluted to a starting concentration using resuspension buffer (RSB). This was performed by IMRL scientists and observed by the M.D candidate.

2.2.6.6 Step Five – Denaturing and Pooling:

0.2 N of NaOH was added per 4 nM library and then vortexed briefly before centrifuging at 280 × g for 1 minute. 990 µl prechilled HT1 Hybridization Buffer was added, inverted to mix and then pulse centrifuged. Then 10 µl of each diluted library was added to a tube to create a multiplexed library pool. A PhiX control that had been denatured and diluted was then added to the sample before sequencing. PhiX is a ready to use control library for sequencing runs. This was performed by IMRL scientists and observed by the M.D candidate.

2.2.6.7 Loading to sequencer:

Pooled libraries (600µl) were loaded onto the reagent cartridge of the WGS platform, the MiSeq. This cartridge is a single use consumable with sealed reservoirs prefilled with sequencing and clustering reagents(126). Sample

libraries were loaded on the cartridge through position 17. Libraries automatically transfer from the cartridge to a flow cell as the run begins. The MiSeq uses a flow cell which is made of a single-use glass-based substrate where the sequencing reaction is performed (Figure 2.4). The output from a sequencing run is a set of quality-score base call files (*.bcl). These bcl files are then converted into FASTQ files. This was performed by IMRL scientists and observed by the M.D candidate.

FIGURE 2.4: MISEQ FLOW CELL. IMAGE COURTESY OF ILLUMINA INC.

FIGURE 2.5: IMAGE OF FLOWCELL BEING INSERTED INTO MISEQ. IMAGE COURTESY OF ILLUMINA INC.

2.2.7 Bioinformatic analysis:

Ridom™ SeqSphere+ Software was the bioinformatics software tool used to analyse the output FastQ files. This software imports, assembles and analyses read files - creating a minimum spanning tree (MST) based on core genome Multi-locus Sequence Typing (cgMLST) schemes(119). A predefined public cgMLST scheme for the *M. tuberculosis* complex was used. Minimum spanning trees were created and cluster analysis performed.

Where clusters were identified, i.e. where isolates were <12 allelic differences apart, a SNP based pipeline MTBseq analysis was performed to further elucidate genomic relatedness. FASTQ files were also run though this pipeline to determine lineage and mutations associated with antimicrobial resistance.

IMRL scientists assisted the M.D candidate in performing bioinformatic analysis.

2.2.7.1 Core Genome Multi-locus Sequence Typing:

SNP calling is often complicated by variation between laboratories and genomic factors such as repetitive regions. Kohl *et al* developed cgMLST for use in TB surveillance activities(127). cgMLST extends the concept of MLST (which focuses on 6 – 7 housekeeping genes) to a genome wide level(128). This method combines traditional MLST and WGS and uses allelic variation based on 2891 genes present among MTBC isolates(55). SNPs are translated into an allele number system and each isolate is given a sequence type (ST) based on a combination of allele numbers.

Kohl *et al* utilised the software Ridom™ SeqSphere to perform cgMLST, which they validated for members of the MTBC including animal pathogens such as *M. bovis*. Critical thresholds for linked isolates were similar to SNP analysis with less than or equal to 5 distinct allelic differences representing potentially epidemiologically linked isolates. Allelic variation of 6– 12 between isolates is considered indeterminate for recent transmission. Where there are greater than 12 allelic differences between isolates, close transmission events are not likely (54, 123, 124). A rate of change of 0.55 alleles per MTBC genome per year was calculated based on their analysis. The resolution level retained by cgMLST was deemed sufficient to analyse outbreaks and transmission chains. The analysis of 755 accessory genes could be used to improve resolution. Twelve allelic differences or less was chosen as the cut off in this study to define clusters of potentially closely related *M. bovis* isolates.

FASTQ files were uploaded to Ridom SeqSphere Software. Quality control was performed using FASTQC, adaptor trimming using Trimmomatic, contamination check using MASH, mapping of reads to *M. tuberculosis* H37Rv NC_000962.3 using BWA(129, 130). A mean read coverage depth of a least 30 x was required as well as 95% coverage of the reference genome(130). Ridom™ SeqSphere Software creates minimum spanning trees where branch length between nodes corresponds to allelic differences between isolates(130).

2.2.7.2 MTBseq analysis:

MTBseq is an automated pipeline that uses a number of command line tools to perform read mapping, variant calling and resistance mutation detection and to determine lineage(117). *M. tuberculosis* H37Rv is used as the default genome for

analysis within the IMRL, however the reference genome can be changed by the user to any MTBC or non-MTBC bacterial reference genome(117).

The pipeline is depicted in Figure 2.6 and consists of two workflows: 7 modules for sample specific analysis and 3 modules for comparative analysis of multiple data sets(117).

The sample specific analysis consists of i) mapping of FASTQ files to the reference genome using programmes BWA and SAM Tools in the step “TBbwa”, ii) base call recalibration and realignment of reads around insertions or deletion using Genome Analysis Toolkit (GATK) in the “TBrefine” step, iii) Variant calling using SAMtools in the “TBpile” step, iv) “TBlist” applies thresholds for coverage and base quality, v) “TBvariants” annotates SNPs and insertions or deletions including resulting amino acid changes and associations with antimicrobial resistance, vi) “TBstats” creates descriptive statistics of the mapping and variant detection steps, vii) “TBstrains” performs phylogenetic classification(117).

Comparative analysis of multiple samples can be provided by “TBjoin”, “TBamend” and “TBgroup”(117). FASTA formatted sequences are generated which can be used in tree reconstruction algorithms or to create pairwise SNP distance matrices(117).

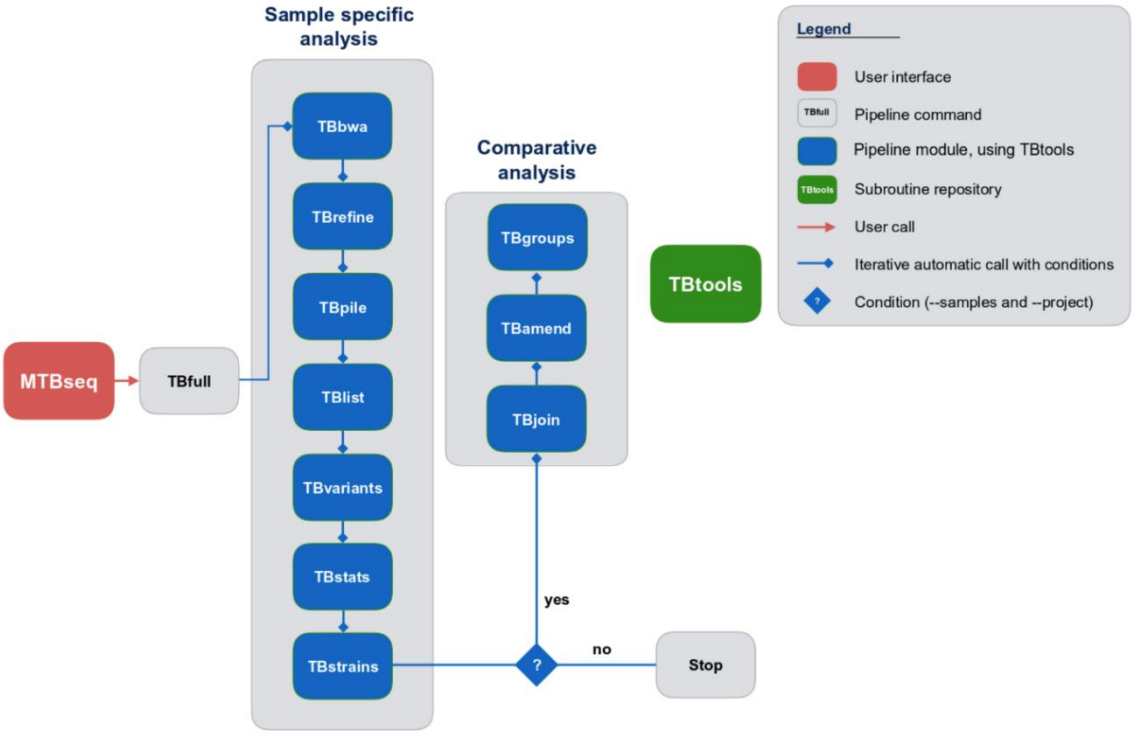

FIGURE 2.6: REPRESENTATION OF THE MTBSEQ WORKFLOW DESCRIBING PIPELINE STEPS FOR BOTH SAMPLE SPECIFIC ANALYSIS AND COMPARATIVE ANALYSIS. IMAGE FROM KOHL ET AL (110).

2.3 Results:

2.3.1 HPSC Data

Human *M. bovis* infection is a notifiable disease in accordance with public health legislation in Ireland. Between 2000 and 2020, 116 cases were notified to the HPSC. The HPSC collate data on age group, gender, disease site, occupation, country of birth, immunosuppressive risk factors and previous personal history of TB or contact with another TB case. This section will summarise data collected by the HPSC on cases of human *M. bovis* collected over this 20-year period.

Sixty (60.3%) percent of cases occurred in men and 39.6 % occurred in women. Case distribution by year of diagnosis is depicted in Figure 2.1 in Section 2.1.1 with peaks in cases between 2008 and 2010 and then again between 2018 and 2019.

The number of cases by age group at diagnosis and by country of origin, where documented, is summarised in Figure 2.7. There is a bimodal distribution of age groups with peaks between ages 20 – 39 years and then again between ages 50 – 74 years. Of note, most documented foreign-born individuals fall into the younger age categories. Most patients notified were Irish born (97/116), 16 were foreign born, there was no record of place of birth for 3 individuals (Figure 2.8).

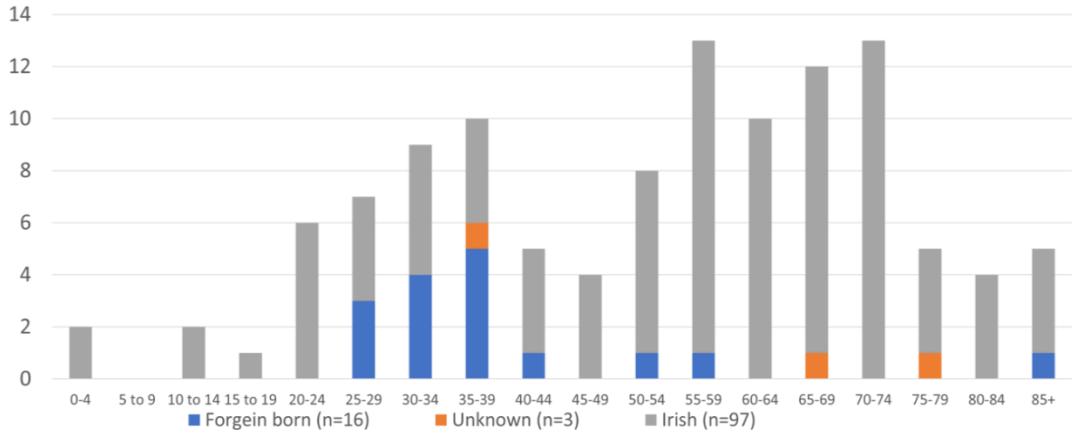

FIGURE 2.7: NUMBER OF CASES BY AGE GROUP AND COUNTRY OF BIRTH REPORTED TO THE HPSC BETWEEN 2000 – 2020. IRISH CASES ARE GREY, FOREIGN-BORN CASES ARE BLUE, CASES WHERE COUNTRY OF BIRTH IS UNKNOWN ARE REPRESENTED IN ORANGE. FIGURE GENERATED USING HPSC DERIVED DATA.

FIGURE 2.8: COUNTRY OF BIRTH FOR *M. BOVIS* CASES REPORTED TO THE HPSC BETWEEN 2000-2020: IRELAND N=97, NIGERIA N=3, N=1 FOR EACH OF: SUDAN, ERITREA, ALGERIA, ETHIOPIA, ZIMBABWE, SOUTH AFRICA, ROMANIA, BELGIUM, PORTUGAL, BRAZIL, INDIA, IRAQ. LITHUANIA FIGURE GENERATED USING HPSC DERIVED DATA.

Pulmonary disease occurred in 70.7% of cases, exclusively extrapulmonary disease occurred in 17.2% and both pulmonary and extrapulmonary disease occurred in 10.3%. Disease site was not documented in 1.7 % of cases. Number

of cases by site of extrapulmonary disease is shown in Figure 2.9. Of note, one case of *M. bovis* meningitis was reported in a 10-14-year-old child in 2017 and another case in a patient aged over 65 years in 2015. There were no significant differences in disease site between Irish born and foreign-born patients with 72.9% of Irish born cases reported to present with pulmonary disease compared with 68.8% among foreign born cases (p=0.82).

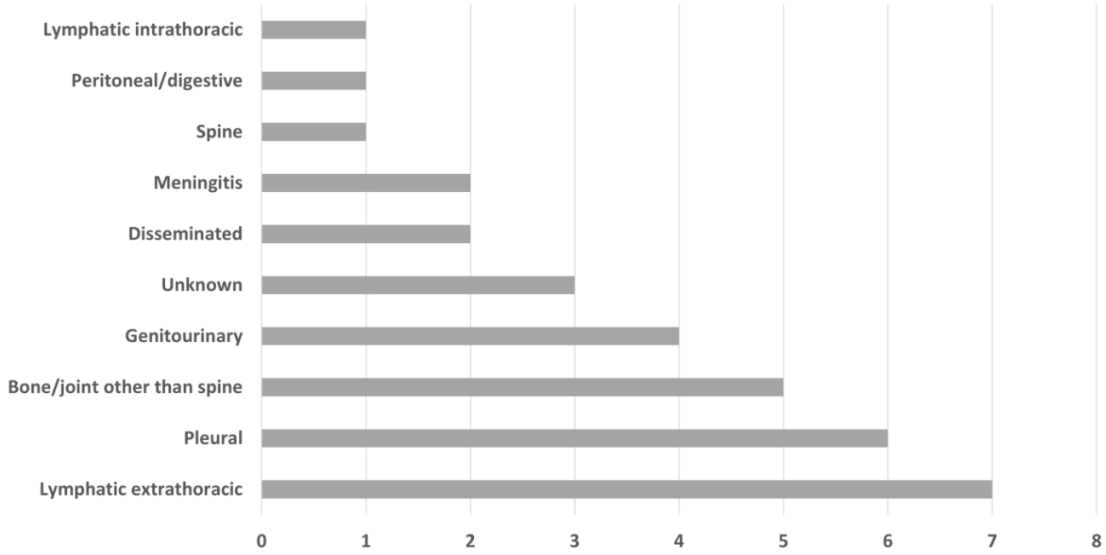

FIGURE 2.9: NUMBER OF CASES (N=32) BY EXTRAPULMONARY DISEASE SITE REPORTED TO THE HPSC BETWEEN 2000-2020. FIGURE GENERATED USING HPSC DERIVED *M. BOVIS* DATA

Relevant risk factors were recorded for some patients. For instance, HIV status was documented in 21 individuals, of whom 2 were positive. Six patients were documented to be receiving TNF alpha blockers, an additional 2 patients had other immunosuppressive conditions.

Five patients had previous contact with an individual with TB infection and 6 patients had a documented previous history of TB. History of alcohol misuse was documented in 12 patients.

Occupation was documented for 86 patients who had their *M. bovis* infection notified to the HPSC, the most common occupation being farmer (n=16) with a further 3 documented as retired farmers. Two were noted to be meat factory workers. Other frequent recorded occupations included: “retired” (n=14), “housewife/househusband” (n=9), “student” (n=8), “unemployed” (n=6).

2.3.2 Retrospective Review of Patient Records at the IMRL and SJH:

Between 2000 and 2020, IMRL had record of 72 patient isolates and 3 pet isolates that had been processed within the laboratory. Of these patient isolates, 4 were deemed contaminated samples, and 2 were duplicate samples. This left 66 patient isolates that had been processed during this time at the IMRL.

Basic demographic details including age of patient, gender and site of disease were available for most of these patient isolates through review of their Telepath records (n=66). Twenty-seven patients had attended SJH at some point in time and so had patient records available for review.

An average of 3.1 cases were recorded per year between 2000 and the end of 2020. The breakdown of number of cases per year is shown in Figure 2.10.

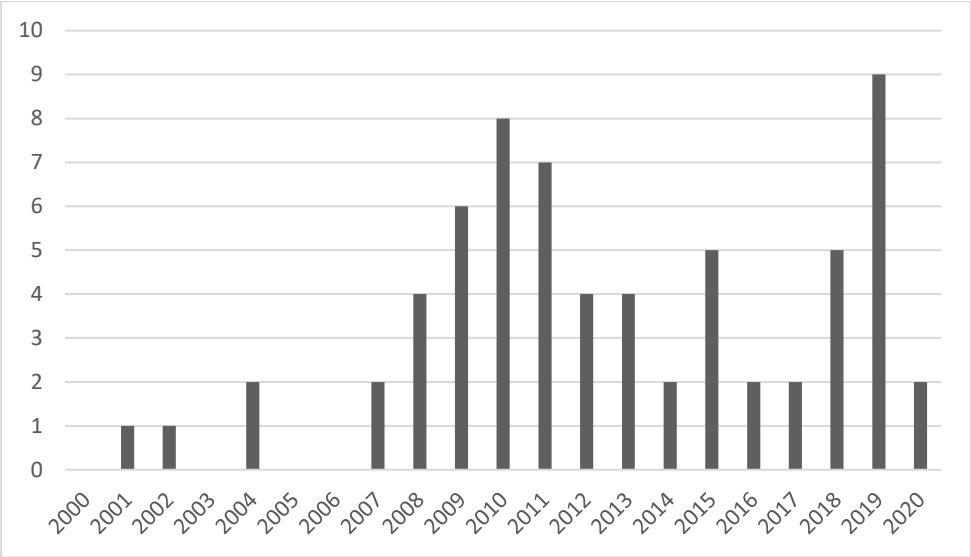

FIGURE 2.10: *M. BOVIS* CASES BY YEAR OF DIAGNOSIS BETWEEN 2000 AND 2020 BASED ON IMRL RECORDS. OF NOTE THERE WERE NO ISOLATES COLLECTED IN THE YEARS 2000, 2003, 2005 AND 2006.

2.3.2.1 Age and Gender:

Average age at diagnosis among this patient cohort was 50.1 years. Similar to HPSC collected data, male patients accounted for 62.1% of cases (41/66), while female patients accounted for 36.4% of cases (24/66) (p=0.0033). Gender was not recorded for one patient. Average age amongst male patients was 53.9 years compared with 44.4 years among women (p=0.0384 using Mann-Whitney test). A third of patients (33.3%) were aged over 60 years.

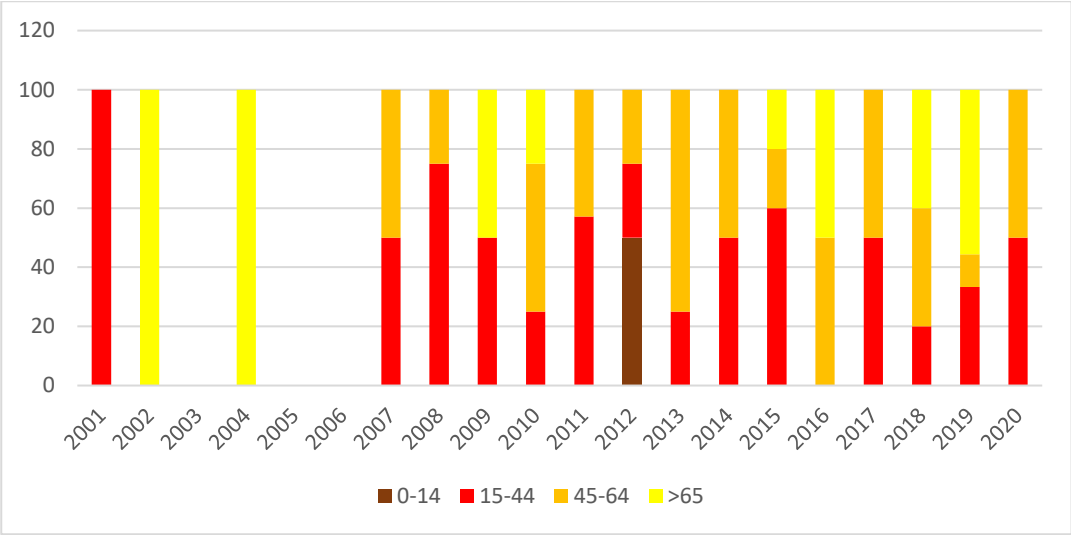

FIGURE 2.11: ANNUAL PROPORTION OF *M. BOVIS* CASES BY AGE GROUP (0-14 YEARS, 15 – 44 YEARS, 45 – 64 YEARS AND OVER 65 YEARS) BETWEEN 2000 AND 2020.

2.3.2.2 Disease Site:

Where site of disease was recorded (57/66), the majority of cases were pulmonary in nature at 71.9% (41/57), 14% (8/57) were solely extrapulmonary, while 14% (8/57) had disseminated (both pulmonary and extrapulmonary) disease on presentation. Site of disease was not recorded for 9patient samples. These percentages were comparable with HPSC data on disease site described in Section 2.3.1. Where documented, among male patients 65.7% (23/35) presented with pulmonary infection, vs 81.8 % (18/22) in women (p=0.19using "N-1" Chi-squared test). Thirty-four percent (34.2%) of men presented with extrapulmonary or disseminated infection vs 18.2%of women (p=0.1943).

2.3.2.3 Occupation:

Occupation was recorded in 19 patients. The most common documented occupations were meat factory worker (n=5), farmer (n=5) and housewife (n=3) (Table 2.1).

TABLE 2.1: OCCUPATION AMONG IMRL M. BOVIS CASES WHERE DOCUMENTED BETWEEN 2000 AND 2020

Occupation	Number of patients
Meat Factory Worker	5
Farmer	5
Housewife	3
Tradesperson	2
Hospitality	2
Unemployed	1
Security	1

2.3.2.4 Country of Origin:

Country of origin was documented in 14 cases, 5 of whom were foreign born (Algeria (n=1), Brazil (n=1), South Africa (n=2), and Portugal (n=1)), whilst 9 were documented as Irish born. The average age for those patients documented as foreign born was 35 years of age versus 53.1years of age where Irish born.

2.3.2.5 Risk Factors:

Where past medical history was recorded, comorbidities were documented in 21 out of 22 patients. The average number of comorbidities recorded per patient was 2.6(58/22). Relevant immunosuppressive conditions recorded included haematological disorder (n=3), malignancy (n=4), HIV (n=2), autoimmune conditions (n=7). One additional person whose chart was not available for review had a history of HIV infection documented on their specimen collection form. See Table 2.2 for further break down.

Four patients were receiving immunosuppression therapy with TNF alpha inhibitors for Crohn's disease (n=2) and rheumatoid arthritis (n=2). Three were receiving adalimumab and one was receiving etanercept. Two of these patients

were also receiving methotrexate with one of these also receiving hydroxychloroquine. A history of unpasteurised milk consumption was documented in 4 patients. One patient reported a history of contact with a family member diagnosed with TB infection.

TABLE 2.2: FREQUENCY OF COMORBIDITIES BY DISEASE CATEGORY AMONG PATIENTS (N=21) WITH M. BOVIS INFECTION WHERE COMORBIDITIES RECORDED IN SJH PATIENT CHART BETWEEN 2000 AND 2020.

Comorbidity by System:	Frequency (n=):
Haematological Disorder:	3
Malignancy history:	4
HIV:	2
Autoimmune:	
– Vitiligo	1
– Hypothyroidism	2
– Crohn's disease	2
– Rheumatoid Arthritis	2
Respiratory:	
– Smoker	6
– COPD	3
Cardiac:	
– Ischaemic Heart Disease	2
– Arrhythmia	3
Metabolic:	
– Hypertension	4
– Hypercholesterolemia	2
– High Body Mass Index	1
– Type 2 diabetes mellitus	2
Vascular:	
– Aortic aneurysm	1
Gastrointestinal:	
– Peptic ulcer disease/GORD	5
– Diverticular disease	1
– Colitis (unspecified)	1
Hepatobiliary:	
– Alcohol Liver disease	
– Hepatic steatosis	1
– Pancreatitis	1
	1
Bone and Joint:	
– Osteoarthritis	2
Other:	
– Alcohol excess	3
– Pregnancy	1
– Depression	1
– Fibromyalgia	1

COPD: Chronic Obstructive Pulmonary Disease.
GORD: Gastro-oesophageal Reflux Disease.

2.3.2.6 Comparison of patients born pre and post 1960:

Thirty patients (45.5%) were born pre-1960. As widespread pasteurisation of milk was introduced to Ireland in 1958, these patients were considered as born prior to the introduction of widespread dairy pasteurisation measures. Of these patients, 11 had charts available for review, however, 5 charts had very limited information documented. Four patients had no documented risk factors, 6 patients had one documented risk factors, and one patient had 3 risk factors. The most common risk factor within this cohort was occupational history (n=4), followed by significant immunosuppressive comorbidity (n=2), iatrogenic immunosuppression (n=2), previous contact with the TB case (n=1).

Thirty-six of the sixty-six patients (54.5%) reviewed in this study were born from 1960 onward. Of these patients, 16 charts were available for review. All but 3 patients had documented risk factors for *M. bovis* infection. Five patients had one documented risk factor, 6 patients had two risk factors and another 2 patients had 4 risk factors.

The most common risk factor was significant comorbid immunosuppressive condition (n=7), followed by unpasteurised dairy consumption (n=4), occupational exposure (n=4) travel history (n=4), exposure to domestic or farm animals (n=3) and two patients were receiving iatrogenic immunosuppression.

Significant comorbid immunosuppressive conditions included HIV infection, malignancy, haematological disorder, pregnancy, viral hepatitis and alcoholic liver disease.

TABLE 2.3: COMPARISON OF FREQUENCY OF RISK FACTORS FOR *M. BOVIS* INFECTION AMONG PATIENTS BORN PRE- AND POST- 1960.

Risk factor:	Frequency among those born pre-1960 (n=11)	Frequency among those born post-1960 (n =16)
Iatrogenic immunosuppression	2	2
Significant immunosuppressive comorbidities	2	7
Occupation	4	4
Unpasteurised milk consumption	0	4
Domestic animals	0	3
Travel to endemic area	0	5
Personal history of TB	0	0
Contact with patient with TB	1	0

Among those born prior to the pasteurisation of milk, 50% had pulmonary infection, 20% had extrapulmonary infection, 6.7% had disseminated infection and in 23.3% site of infection was not recorded. For those born after 1960, 72.2% had respiratory infection, 5.6% had extrapulmonary infection, 16% had disseminated infection. Site was not recorded in 5.5% (2/36). Those born prior to the widespread pasteurisation of milk were more likely to be male, 83.3% (25/30) compared with 44% (16/36) of those born post 1960 (Table 2.4).

TABLE 2.4: CHARACTERISTICS OF *M. BOVIS* INFECTION IN THOSE BORN PRE-1960 AND POST-1960.

	Born pre-1960	Born post-1960	P value
Number of patients	30	36	-
Gender (%)			
- **Male**	83.3	44.4	*0.0013**
- **Female**	16.7	52.8	*0.0026**
- **Not recorded**	0	2.7	
Disease site (%)			
- **Pulmonary**	50	72.2	*0.066*
- **Extrapulmonary**	20	5.6	*0.0768*
- **Both**	6.7	16.7	*0.2192*
- **Not recorded**	23.3	5.6	*0.0385**

*P<0.05 was considered statistically significant. P values were calculated with Medcalc software using N-1 Chi-squared test for comparing proportions. * = p value considered statistically significant*

2.3.2.7 Comparison of patients diagnosed between 2000-2010 and 2011-2020:

To assess change in epidemiology of *M. bovis* infection over our study period, patients diagnosed between 2000-2010 and 2011- 2020 were compared. Twenty-four patients had *M. bovis* samples processed at SJH between 2000 and 2010, compared to forty-two between 2011-2020.

TABLE 2.5: COMPARISON OF CHARACTERISTICS OF M. BOVIS INFECTION IN PATIENTS DIAGNOSED BETWEEN 2000-2010 AND BETWEEN 2011-2020.

	2000-2010	2011 – 2020	P value
Number of patients	24	42	-
Average age (years)	52.2	48.9	0.66
Gender (%)			
- **Male**	62.5	61.9	0.96
- **Female**	37.5	35.7	0.88
- **Not recorded**	-	2.4	-
Disease site (%)			
- **Pulmonary**	45.8	71.4	*0.0407**
- **Extrapulmonary**	16.7	9.5	0.3922
- **Both**	12.5	11.9	0.9432
- **Not recorded**	25	7.1	0.0429*

**P<0.05 was considered statistically significant. P values were calculated with Medcalc software using N-1 Chi-squared test when comparing proportions. P values were calculated using Mann Whitney test for comparison of average age.*

2.3.3 Cluster Analysis:

Analysis of the human only isolates (61) by cgMLST yielded 8 “clusters”. Cluster threshold was set at 2 of more isolates with less than 12 allelic differences between them. As mentioned previously, isolates within a cgMLST “cluster” underwent pairwise SNP distance analysis using MTBseq software. Isolates <6 SNP differences apart were considered very closely genomically related, isolates between 6 – 12 SNPs apart considered closely related and isolates >12 SNPs apart not considered closely related.

Isolates of note, which will be discussed in more detail in sections 2.3.3 and 3.3.1, have been labelled A through to Z and 1 through to 6. Isolates “S” and “P” were subsequently removed from the analysis as found to be contaminants.

The initial analysis focused on the cgMLST “clusters” where isolates were < 6 SNP differences from each other - see Section 2.3.3.1. Among human only isolates there was one such grouping which is detailed below (Figure 2.12).

This is followed by an analysis of the clusters where isolates have > 6 SNPs but less than 12 differences between them. Among human only isolates, there were 4such groupings which are detailed below – see Section 2.3.3.2.

Finally, cgMLST clusters that contained isolates that were >12 SNP distances apart were not detailed as isolates were not considered closely genomically related (n=3).

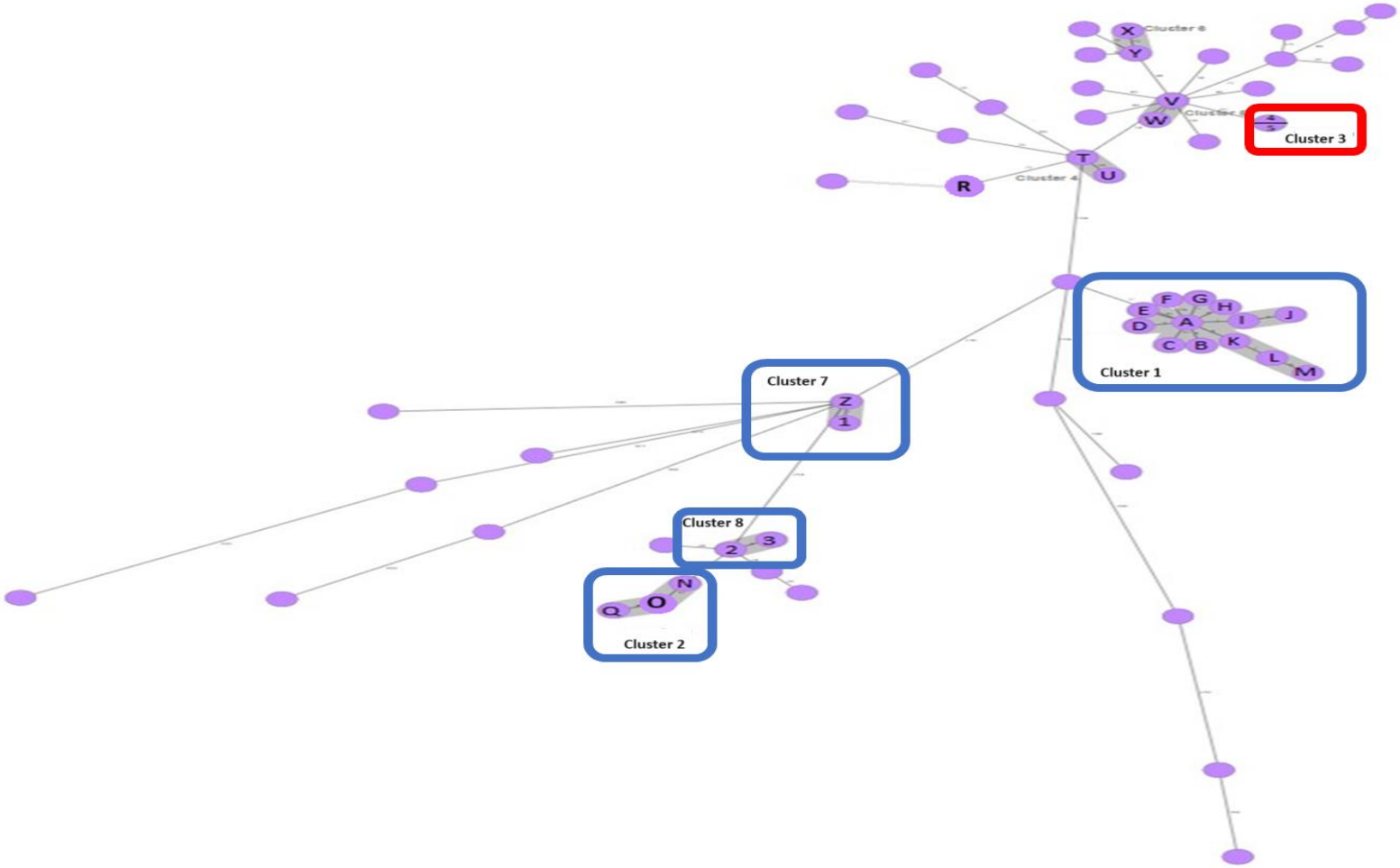

FIGURE 2.12: MINIMUM SPANNING TREE (MST) OF IMRL HUMAN *M. BOVIS* ISOLATES FROM 2000 -2020 CREATED BY CGMLST.
EIGHT CGMLST CLUSTERS WERE IDENTIFIED. RED BOX HIGHLIGHTS CGMLST CLUSTER WITH ISOLATES LESS THAN 6 SNP DIFFERENT FROM EACH OTHER. (CLUSTER 3)
BLUE BOXES HIGHLIGHT CGMLST CLUSTERS WITH ISOLATES BETWEEN 6 AND 12 SNPS DIFFERENCES BETWEEN EACH OTHER. (CLUSTERS 1, 2, 7 AND 8)

2.3.3.1 Less than 6 SNP Differences:

CLUSTER 3:

Cluster 3 comprised 2 human *M. bovis* isolates from patients ("4" and "5") diagnosed with pulmonary infection in 2013. These isolates were zero SNP differences apart. One patient was a male aged 25-29 years who was a smoker, however little else is known. The other a female aged 50-54 years on whom little else is known on history.

FIGURE 2.13: CLUSTER 3 MINIMUM SPANNING TREE (MST) OF IMRL HUMAN M. BOVIS **ISOLATES CREATED BY CGMLST.** TWO PATIENT ISOLATES ARE INCLUDED WITHIN THIS CLUSTER LABELLED "4" AND "5".

2.3.3.2 Clusters with Isolates 6 – 12 SNP differences:

CLUSTER 1:

Cluster 1 was the largest cgMLST cluster containing 13 human isolates ranging in diagnosis year from 2008 to 2020. Patient age on diagnosis ranged from early 20s to mid-80s, with average age at diagnosis of 48.5 years. There were 6 males and 7 females. Three patients were born prior to 1960, while the remainder were born after. None of the isolates were <6 SNP apart from another human isolate, however many of the isolates were closely genomically related to animal isolates as will be described in more detail in Chapter 3.

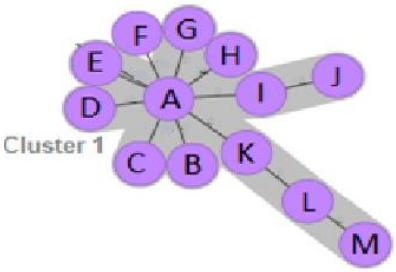

FIGURE 2.14: CLUSTER 1 MINIMUM SPANNING TREE (MST) OF IMRL HUMAN *M. BOVIS* ISOLATES CREATED BY CGMLST. THIRTEEN PATIENT ISOLATES WERE INCLUDED WITHIN THIS CLUSTER AND ARE LABELLED "A" THROUGH TO "M".

CLUSTER TWO:

Cluster 2 involved 3 human isolates, within this cluster only 2 isolates were closely related (<12 SNPs apart). Isolate "O" was 10 SNP differences apart from isolate "Q."

Patient "O" was from a female with pulmonary infection diagnosed in 2011. She was aged between 40 and 44 years with 2 comorbidities, including a potentially immunosuppressive haematological disorder, however little else is known about her risk factors for disease.

Isolate "Q" came from an Irish born housewife aged 40 – 45 years who presented with pulmonary infection in 2007. This patient reported living on a farm previously and had consumed unpasteurised dairy products. This patient had one documented comorbidity, however, she was not actively immunosuppressed.

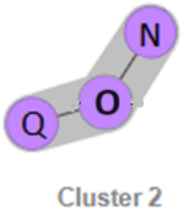

FIGURE 2.15: CLUSTER 2 MINIMUM SPANNING TREE (MST) OF IMRL HUMAN *M. BOVIS* ISOLATES CREATED BY CGMLST. THREE PATIENT ISOLATES WERE INCLUDED WITHIN THIS CLUSTER LABELLED "N", "O" AND "Q".

CLUSTER 7:

Cluster 7 involved two patient isolates which are 9 SNP differences apart. Isolate "1" came from a male aged 70 – 74 years with multiple comorbidities (n=7) without active immunosuppression who presented in 2009 with pulmonary infection. Isolate "Z" derived from a female aged 20-24 with pulmonary infection diagnosed in 2010.

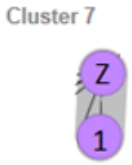

FIGURE 2.16: CLUSTER 7 MINIMUM SPANNING TREE (MST) OF IMRL HUMAN *M. BOVIS* ISOLATES CREATED BY CGMLST. TWO PATIENTS WERE INCLUDED WITHIN THIS CLUSTER LABELLED "Z" AND "1".

CLUSTER 8:

Human isolates "2" and "3" were 7 SNP differences from each other. Isolate "2" was from a male farmer aged between 55-59 years who presented in 2010 with extrapulmonary infection. Isolate "3" was from a male patient aged between 55 - 59 years who presented with pulmonary infection in 2011.

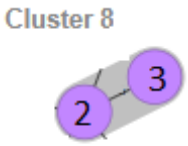

FIGURE 2.17: CLUSTER 8 MINIMUM SPANNING TREE (MST) OF IMRL HUMAN *M. BOVIS* ISOLATES CREATED BY CGMLST. TWO PATIENTS WERE INCLUDED WITHIN THIS CLUSTER LABELLED "2" AND "3".

2.3.3.3 Clusters with isolates >12 SNPs apart:

Clusters 4, 5 and 6 all contained isolates that were >12 SNP distances apart and thus were not considered closely genomically related.

2.3.4 Lineages:

Most (87.5%) of the sequenced human isolates were designated *M. bovis* lineage (La) 1.8.1, while there was one of each of La 1.2, La 1.3 and La 1.4, three of La 1.6 and two of La 1.7. One of the patients whose isolate was lineage La 1.7 was from Algeria, whilst one of the patients whose isolate was lineage La 1.6 was from South Africa.

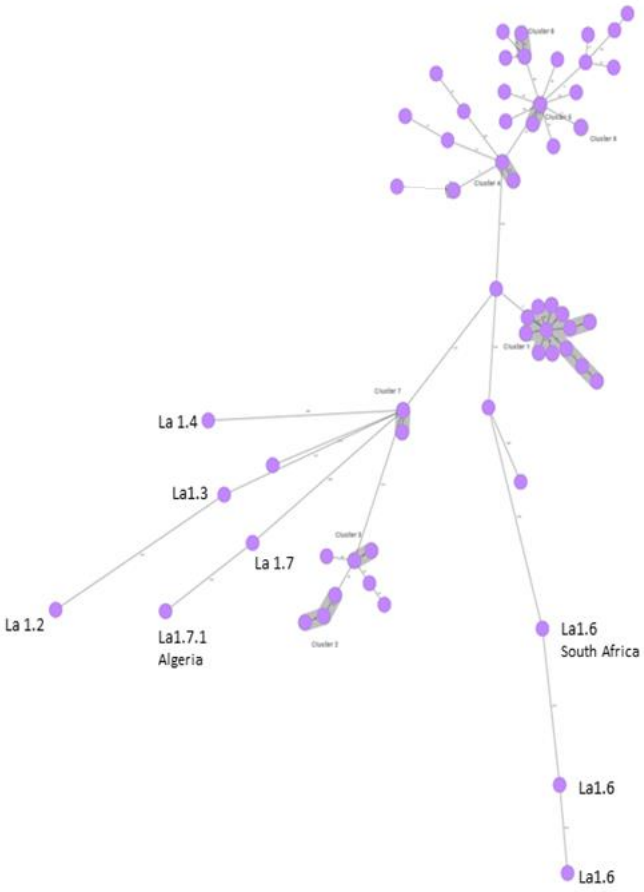

FIGURE 2.18: MINIMUM SPANNING TREE (MST) OF IMRL HUMAN *M. BOVIS* ISOLATES CREATED BY CGMLST HIGHLIGHTING NON 1.8.1 LINEAGE ISOLATES AND COUNTRY PATIENT ORIGIN WHERE DOCUMENTED.

2.4 Discussion:

2.4.1 Demographics:

The incidence of *M. bovis* infection in Ireland has decreased significantly since Cotter *et al* reported a rate of 0.56 per 100,000 in 1996(112) to 0.06 per 100,000 in 2020(110). Cotter *et al* examined the epidemiology of *M. bovis* infection in South-West Ireland between 1983 and 1992. Average age of *M. bovis* infection within this cohort was 58.4 years compared with the average age among this study's' patient cohort of 50.1 years. Males accounted for a similar proportion of patients, 63.4% in their study versus 62.1% among in this study. Pulmonary infection accounted for 43% of cases in Cotter *et al's* study versus 71.9% of presentations, where recorded, within this study's cohort.

Unfortunately, their prediction that human *M. bovis* cases may be eliminated in the coming years did not come to be. Additionally, their observation that most cases were due to reactivation of latent infection does not reflect what we have observed within our patient cohort. Rather - average age of presentation has fallen, not risen, which is not in keeping with disease activity being solely due to reactivation of latent disease. The epidemiology of human *M. bovis* infection has certainly changed since their report almost 30 years ago and even since the HPSC publication on the Guidelines of the Prevention and Control of Tuberculosis in Ireland in 2010.

When patients in the first half of this study cohort, i.e., those diagnosed between 2000 and 2010, were compared with those diagnosed in the later half, the average age of patients decreased from 52.2 years to 48.9years.

Patient numbers were lower between 2000 and 2010, likely because the IMRL had only just been established at SJH in 2001 and so may not have received many of the clinical isolates cultured in other hospitals. Illness was more likely to be respiratory in patients diagnosed from 2011 onwards than those diagnosed between 2000 and 2010.

As routine pasteurization of milk was not standard until the late 1950s, it could be assumed that many patients born prior to the 1960s may have been exposed to infection via the consumption of unpasteurised milk at a time where herd incidence of disease was high. The cohort may thus be at increased risk of latent infection becoming active as they age or develop comorbid conditions.

Although information from charts was not available for many of the patients born prior to 1960, the number of risk factors per patient for developing *M. bovis* infection did seem to increase in those born post 1960, in particular the number with immunosuppressive conditions. This highlights the more complex aetiology of disease in this younger cohort of patients, where multiple risk factors combine to increase risk of disease. Further to this, when we look at disease site within these patient cohorts, excluding those patients where site was not recorded, percentage of pulmonary infection increased from 65.2% among those born pre 1960 to 76.5% (p=0.3558). By comparison, in those born from 1960 onwards, disseminated disease increased from 8.7% of cases to 17.6% (p=0.3465) of cases and extrapulmonary cases decreased from 26.1% of cases to 5.9% of cases (p=0.0328). This is perhaps reflective of a change in route of transmission which may be predominately respiratory as

opposed to by dairy product consumption. Also, an increase in disease dissemination may be reflect the increased complexity and concomitant immunosuppression of this patient cohort.

The epidemiology of disease thus appears to be changing with patients being younger at diagnosis and more likely to present with respiratory or disseminated infection. This is at odds with the commonly held perception that *M. bovis* infection is limited to the elderly with reactivation of latent TB.

2.4.2 Comparison with other countries:

As described previously in Chapter 1, Ireland, a non-OTF country, has the highest incidence rate of human *M. bovis* infection within the EU. Like in many other epidemiology studies of *M. bovis* infection, the majority of our patients were male (62.1%), and most patients, where recorded, had pulmonary infection (71.9%)(121), although this contrasts with a study from the Netherlands(40).

Among the European studies summarised below, infection was more common in native individuals(40, 121, 131, 132), however it is of note that in a Californian study(78), infection was more common in foreign-born patients, a large majority of whom were from Mexico.

Men in our study were more likely to have extrapulmonary infection than women, they were also older upon diagnosis. Of note, the proportion of cases diagnosed in patients > 60 years old was lower in Ireland than compared with other countries.

TABLE 2.6: COMPARISON BETWEEN HUMAN M. BOVIS EPIDEMIOLOGY STUDIES.

	Irish Data IMRL + HPSC	Northern Ireland (131)	England/ NI/Wales (121)	Spain (132)	Netherlands (40)	California (78)
Study Period	2000-2020	2000-2012	2002-2014	2006-2015	1993-2007	2003-2011
N	66	22	357	75	231	176
% of MTBC	2.26^	3	-	2.43	-	-
Age (years)	50.1	60	-	66.2	-	-
>60 – 65 yrs. (%)	33.3#	50#	48.6	63	50.3#	23.9
M (%)	63.1	86	55.1	63	45.5	51.8
F (%)	36.9	14	44.9	37	54.5	48.2
Pulmonary (%)	71.9	68	58.8	59	29.4	62.5
Extrapulmonary (%)	14	32	41.2	34.6	58.9	-
Both (%)	14	-	-	6.6	11.7	37.5
Native (%)	83.6^	-	72.1	92	59.7	18.9
Foreign born (%)	13.8^	-	27.9	8	40.3	80.1

IRISH *M. BOVIS* DATA WAS DERIVED FROM DATA COLLECTED FROM IMRL BETWEEN 2000 AND 2020 EXCEPT FOR DATA HIGHLIGHTED BY (^) WHICH WAS DERIVED FROM HPSC PROVIDED DATA. IMRL *M.BOVIS* DATA ON DISEASE SITE EXCLUDES PATIENTS FOR WHOM DISEASE SITE WAS NOT AVAILABLE (N=9). . *M. BOVIS* DATA ON PATIENT GENDER EXCLUDES ONE PATIENT FOR WHOM GENDER WAS NOT RECORDED
"#" REPRESENTS PERCENTAGE OF PATIENTS OVER 60 YEARS, AS OPPOSED TO OVER 65 YEARS.
"-" REPRESENTS WHERE INFORMATION HAS NOT BEEN PRESENTED IN A STUDY.
N=NUMBER OF PATIENTS IN EACH STUDY.
M=MALE GENDER, F = FEMALE GENDER.
MTBC =MYCOBACTERIUM TUBERCULOSIS COMPLEX.

2.4.3 Comparison with *Mycobacterium tuberculosis* infection:

One-hundred and ninety-six culture positive cases of TB were reported in 2019 in Ireland, *M. tuberculosis* accounted for 177 cases (90.3%), *M. bovis* for 7 (3.6%) and *M. africanum* for one (0.5%), the remainder were not identified to species level. MTB cases in Ireland have been in decline in recent years, particularly in Irish born individuals, however an increase in cases in foreign born individuals has been noted as well in the number of drug resistant

strains. Between 2018 and 2022, TB cases were higher among foreign born individuals as compared with Irish born individuals(133).

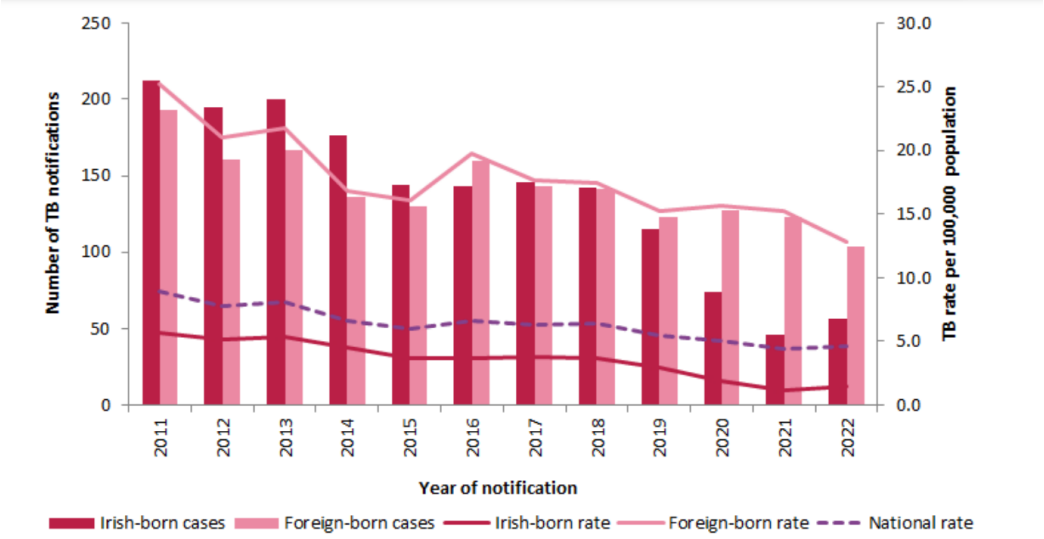

FIGURE 2.19: TB NOTIFICATIONS AND RATE BY GEOGRAPHIC ORIGIN AND YEAR. GRAPH FROM HPSC "TUBERCULOSIS IN IRELAND: PROVISIONAL TRENDS IN SURVEILLANCE DATA" (127)

As per the HPSC National Tuberculosis surveillance in Ireland 2019 Report, 59.6 % of MTBC infections occurred in males(111). Twenty- four percent of cases were aged over 65 years at diagnosis. Forty percent were Irish born while 44.2% were foreign born. Fifty-six percent were pulmonary in nature, 10.5% were both pulmonary and extrapulmonary and 27.7% were extrapulmonary.

Data from 2022 found that among foreign born individuals with TB, rates were highest in those aged between 24 – 34 years of age, whilst cases among Irish born individuals were highest in those aged 55 – 64 years(133).

When comparing our *M. bovis* data between 2000 and 2020 with the 2019 HPSC data on MTBC (which included 7 *M. bovis* isolates), there was no

statistically significant difference (p<0.05) between percentage of patients aged over 65 years or between percentage of male patients. Of note, exclusively extrapulmonary disease was more common among the MTBC diagnosed patients in 2019 than in our cohort of patients with *M. bovis* infection. Patients with *M. bovis* infection were more likely to be Irish born, whilst patients with MTBC infection in 2019 were more likely to be foreign born (Table 2.7).

TABLE 2.7: COMPARISON OF 2000-2020 M. BOVIS DATA WITH MTBC INFECTION DIAGNOSED IN 2019 DATA FROM HPSC.

	***M. bovis* Data**	**MTBC HPSC Data**	**P value**
Study Period	2000-2020	2019	-
N	66	196	-
>=65 yrs. (%)	25.8	24	0.769
M (%)	62.136.4	59.6	0.7202
F (%)		40.1	-
Pulmonary (%)	62.16	56	0.3868
Extrapulmonary (%)	12.112.113.6	27.7	0.0102*
Both (%)		10.5	0.7188
Disease site not recorded		5.2	0.0237*
Native (%)	83.6^	40	<0.0001*
Foreign born (%)	13.8^	44.2	<0.0001*
Country of birth not recorded (%)	2.6^	15.7	0.0003*

P<0.05 CONSIDERED STATISTICALLY SIGNIFICANT. CALCULATED USING N-1 CHI-1 SQUARED TEST ON MEDCALC.ORG
MTBC HPSC DATA INCLUDED PATIENTS DIAGNOSED WITH MTB, M. BOVIS AND M. AFRICANUM IN 2019.
M. BOVIS DATA WAS DERIVED FROM DATA COLLECTED FROM IMRL BETWEEN 2000 AND 2020 EXCEPT FOR DATA HIGHLIGHTED BY (^) WHICH WAS DERIVED FROM HPSC PROVIDED DATA.

2.4.4 Genomic analysis:

One of the cgMLST determined clusters included patient isolates that were zero SNPs different from each other (n=2 patients), suggesting these isolates were closely genomically related.

Within this cluster, the patients with identical genomic isolates (zero SNP differences apart) had been diagnosed with *M. bovis* infection within the same year as each other Both patients presented with pulmonary infection. This contrasts with the typical historical presentation of extrapulmonary infection secondary to unpasteurised product consumption. Human to human transmission, while rare, has been reported, and generally occurs via the respiratory route(134).

Within Cluster 2, patient isolate "O" was 10 SNPs different from the isolate from patient "Q", a patient who had previous remote exposure to unpasteurised milk whilst living on a farm. This close, but not identical, relationship with isolate from patient "Q" may suggest that patient "Q" may have experienced a reactivation of latent TB acquired earlier in life while patient "O" was newly infected with *M. bovis* infection, possibly whilst immunosuppressed. Of course, this is only a hypothesis and without further details on the patients involved and their risk factors it is not possible to ascertain exactly their source of acquisition.

Clusters 7 and 8 included patients with isolates between 6 – 12 SNP differences apart (9 and 7 SNPs respectively) suggesting close but not immediate transmission links. Noted again is that the year of diagnosis for each of these patients with closely genomically related isolates are very similar, with cases only diagnosed a year apart from each other. Three of the 4 patients involved had pulmonary TB, whilst the patient with extrapulmonary TB was noted to be a farmer.

As per the HPSC, an outbreak of TB is defined as an the occurrence of active TB disease above the expected level over a given period of time, usually 6 months, within a geographic area, facility or within a specific population(115). This definition does not extend to cover latent infection; however such outbreaks are notifiable if food borne transmission of infection is suspected as the source(133).

To assess whether a cluster represents an outbreak, the HPSC advises the consideration of indicators such as epidemiological links between cases, similar characteristics among cases and supporting WGS data. Without knowing the geographical location of the patients involved in the above described genomic “clusters”, or whether there are any epidemiological links between cases, it is difficult to ascertain whether they can be considered potential “outbreaks” of infection. However, the WGS data, their similar pulmonary presentation, and the time frame that cases were diagnosed (within a year of each other) could support the hypothesis of potential “outbreaks”.

Most human isolates belonged to lineages La 1.8.1, which is to be expected as it is the dominating lineage among cattle in Ireland. Of the 8 isolates from alternative lineages, 2 were documented to have come from the African continent. Of note, none of these 8 isolates clustered with any other isolate by cgMLST analysis. This suggests that they are not contributing largely to outbreaks or ongoing transmission in Ireland, instead it is likely that a large proportion of human *M. bovis* is acquired locally rather than from foreign countries, which is perhaps opposite to MTB infection.

Lineage 1.8.1 corresponds to previous clonal complex, Eu1. *M. Bovis* Lineage 1.8.1 is common in the UK and in former trading partners of the UK.

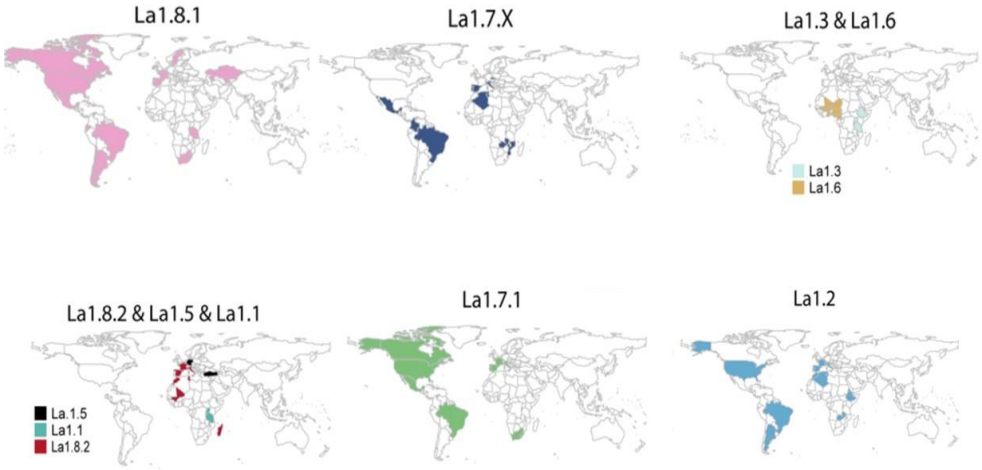

FIGURE 2.20: GEOGRAPHICAL DISTRIBUTION OF M*. BOVIS* SUB LINEAGES. IMAGES FROM ZWYER ET AL (55)

Whilst we have accepted less than 6 SNP differences for closely linked isolates as a stringent threshold and 12 SNP differences for a more relaxed threshold, this is based on previous analyses of *M. tuberculosis* transmission and not *M. bovis(5)*. *M. bovis* likely has different evolutionary pressures and so interpretation of SNP differences in *M. bovis* infection may not have the same implications(5). Also, if MTB genomes are used as references for *M. bovis* studies, as was the case in our analysis, the number of detected SNPs rise leading to errors in read mapping and variant calling(5). More studies are needed on the effect of reference genome on SNP identification.

The estimated base substitution rate for *M. bovis* is 0.15 – 0.53 per genome per year, but- little is known about whether *M. bovis'* broad host tropism influence rates of replication and substitution over time(5). Due to the slow evolving rates for MTBC over time and potential differences in substitution and replication rates between latent and active replication states, delineating routes and intervals of transmission is complicated. MTBC has a slow rate of nucleotide substitution and so even with zero or very few SNPs between isolates, potential genomic links may carry little epidemiological meaning(5). To increase the resolution for determination of transmission routes, dense unbiased genetic data and high quality epidemiological and demographic data are needed.

2.4.5 Limitations:

Our study has several limitations. For one, limited charts were available for review, and we did not have access to patient charts from other hospitals. Where charts were available to review, often clinical information was lacking, including information on relevant risk factors and country of origin. Additionally, as patients were not consented for involvement in this study, and, as we relied on a consent declaration issued by the HRCDC, the patient metadata that we were allowed to collect was clearly defined and limited in nature to ensure data minimisation. Data could not be collected on geographical location of the patients. Nor could data be collected on treatment and patient outcome or mortality rates. Additionally, comparison with the epidemiology of *M. tuberculosis* infection within Ireland through

analysis of records held at SJH would have been useful to further characterise disease trends in Ireland.

Chapter 3: Animal and Human *Mycobacterium bovis* infection in Ireland.

3.1 Introduction

3.1.1 Bovine Tuberculosis in Ireland:

The Eradication of Bovine TB (bTB) Programme was introduced in Ireland in 1954 at which point herd bTB prevalence was approximately 80% and 17% of the 4.5 million cattle in Ireland were infected with TB(16). As a result, trade in livestock products, particularly with the UK, was severely impacted. Additionally, many people, particularly children, contracted *M. bovis* infection by drinking unpasteurised milk of infected cattle or through close contact with an infected animal. Disease progression in cattle was common, and cattle were often riddled with TB lesions particularly in their lungs. Such cattle were referred to as “piners” and often had hardened udders or TB mastitis (16). This advanced clinical presentation of TB among cattle is now rare in Ireland. However, it has been projected that the current epidemiological condition of bTB in developing countries could be similar to that of Europe in the 1930s prior to the pasteurization of milk(70).

Herd rates of bTB in Ireland, whilst a vast improvement from 1954, have risen in recent years from an all-time low herd incidence of 3.27% in 2016 to 4.89% in December 2023 (20, 113) (Figure 3.1). Wicklow is known to have the highest herd prevalence of *M. bovis* infection in Ireland (30). Other areas of high herd prevalence include Monaghan, Cavan, Louth and North Meath, as well as parts of Clare, Cork, Tipperary, Wicklow, Offaly and Westmeath(135).

Ireland has the highest levels of bTB in the EU(136). To trade cattle and their products within the EU, Member States which are not bTB free must have an

effective bTB eradication programme in place(136). Deterioration in TB disease trends in Ireland over the last number of years has led to funding penalties imposed by the EU(33).

The increase in bTB cases is multifactorial, with larger herd sizes playing a contributory role(137). Ireland now has a cattle population of 6.5 million and over 100,000 herds(138). In 2018, the Department of Agriculture Food and the Marine (DAFM) agreed on a proposal to eradicate bTB by 2030(139).

Since 1954, 2.5 million reactor cattle have been culled and an estimated 5.5 billion euro (in today's monetary terms) has been spent in an effort to eradicate bTB in Ireland(140). In 2020 alone, 97 million euros was spent on bTB eradication programmes(140). There is thus a strong financial incentive to eradicate TB disease among cattle by 2030.

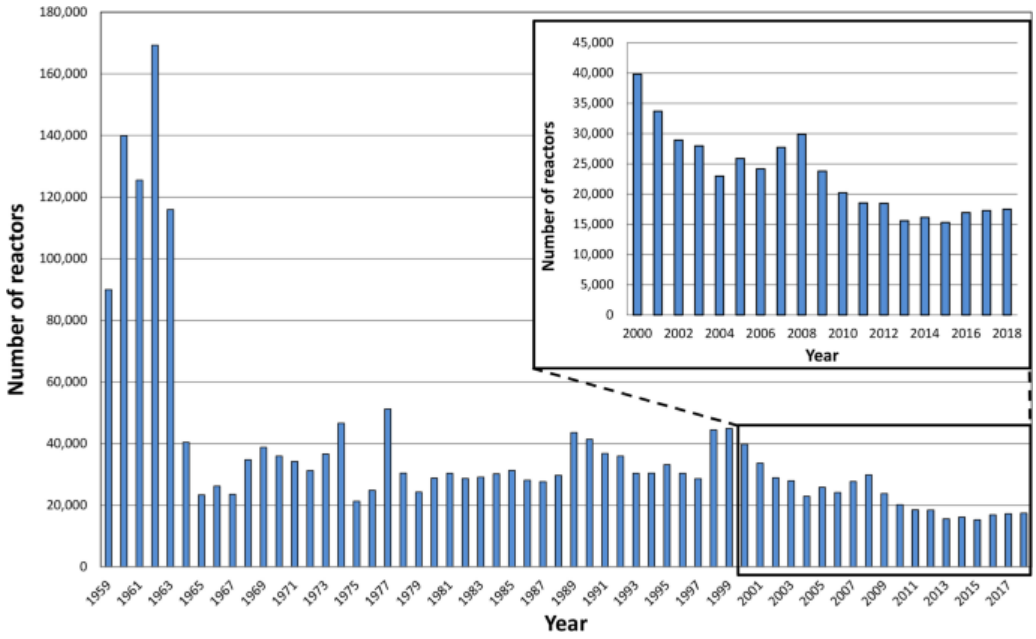

FIGURE 3.1: THE ANNUAL NUMBER OF TB REACTORS IN IRELAND, FROM 1959 TO 2018, INCLUDING MAGNIFICATION OF THE PERIOD 2000–2019. FROM ARTICLE BY MORE *ET AL* "CAN BOVINE TB BE ERADICATED FROM THE REPUBLIC OF IRELAND? COULD THIS BE ACHIEVED BY 2030?" **(141)**

3.1.2 Whole Genome Sequencing in Bovine Tuberculosis Control:

Bacterial genome sequencing by WGS is not only useful for the understanding of human TB outbreaks, but also to further understand and explore *M. bovis* infection among animals. In 2022, the TB Scientific Working Group, an independent advisory group to the DAFM, made recommendations on how best the DAFM could use WGS to assist the Irish TB eradication programme(142). The Group advocated the use of WGS to attribute or rule out sources of infections, to infer transmission chains as well as to quantify rates of transmission within and between different species including cattle, badgers and deer(142). While spoligotyping and VNTR were previously used for this purpose, these genomic techniques are limited in their resolution in comparison to WGS(142). Indeed, one study by Crispell *et al* in Wicklow, that used WGS, was able to show a close relationship between *M. bovis* isolates among cattle and wildlife(30). Crispell *et al* also noted a high level of genetic diversity among deer strains suggesting that they may be acting as a source of infection for local cattle herds (30).

A project funded by DAFM and led by UCD called BTBGenIE seeks to integrate *M. bovis* WGS with the TB eradication and surveillance programme nationally(143). The DAFM Central Veterinary Research Laboratory (CVRL) in Backweston, Co Kildare has infrastructure and capacity to perform WGS on *M. bovis* isolates recovered from cattle and badgers since 2020 (142). Currently, their *M. bovis* database comprises of >1600 sequences, of which at least 1200 are from cattle and 200 are from badgers(142). *M. bovis* isolates within the

database are from counties across Ireland but with a particular focus on isolates from Co. Clare(142).

In order to properly analyse collected data and to determine directionality of transmission, the TB Scientific Working Group noted the importance of capturing isolates over long periods of time from well sampled local populations of cattle and wildlife. They also highlighted the importance of good local epidemiological metadata including spatial, temporal and contact relationships to aid the interpretation of this data(142).

3.1.3 Animal to Human Transmission:

As demonstrated in Chapter 2, the epidemiology of *M. bovis* infection has changed in recent years, and, despite improvements in bTB control, patients continue to be diagnosed with *M. bovis* infection. A question that arises is whether these newly diagnosed cases of *M. bovis* infection are the result of disease reactivation from infection acquired earlier in life, perhaps in a time before the widespread pasteurisation of milk, or whether there is ongoing transmission between animals and humans.

Over the years, genotypic studies comparing human and animal *M. bovis* isolates have been performed worldwide using genotyping tools of varying degrees of resolution including spoligotyping, MIRU-VNTR and Restriction Endonuclease Analysis (REA) (72, 73, 75). By comparing human *M. bovis* isolates with circulating animal or cattle genotypes, such studies aimed to differentiate between newly acquired and remotely acquired infection. In more recent times, high resolution genomic analysis of both human and

animal isolates via WGS has been carried out to more effectively analyse potential transmission pathways(54).

These genomic studies have come to varying conclusions depending on the setting, with some reporting that human infection is primarily due to reactivation of latent disease, and others reporting ongoing low-level transmission between animal and human populations.

Limited genomic analysis of Irish human and animal isolates was carried out by Ojo *et al* in 2008 using spoligotyping and MIRU-VNTR typing (144). This group analysed 11 human *M. bovis* isolates collected between 1998 and 2006 in the Southwest of Ireland. Eight spoligotypes were identified among these 11 isolates. Three strains belonged to SB0140, the most common animal spoligotype in Ireland and the UK. Two human isolates were SB139 – a spoligotype previously detected from a cow in Northern Ireland. Other spoligotypes identified among human isolates, and also within animal spoligotype databases, included SB0130, SB1047 and SB0142. The researchers also identified 3 novel spoligotypes closely related to SB0140. Subsequent ten locus VNTR typing results produced different profiles for each *M. bovis* isolate. Two of the patients with strains belonging to SB140 were aged over 80 years at time of diagnosis, suggesting the predominance of this spoligotype over time. Thus, while human isolates did match circulating animal isolates, it is likely that these spoligotypes have persisted over prolonged periods of time and higher resolution genomic analysis is indicated to further understand potential clusters of infection.

To this end, this study aims to compare human *M. bovis* isolates with circulating animal strains to determine whether there is evidence of ongoing

transmission of *M. bovis* infection between animals and humans within Ireland.

3.2 Methods:

The human *M. bovis* isolates sequenced by the IMRL were compared to (i) animal *M. bovis* genome sequences that were already published by Crispell *et al* (30) and (ii) genome sequences of *M. bovis* from infected cattle herds across Ireland provided by colleagues in UCD on behalf of the DAFM group. The aim of this study was to identify clusters or possible transmission events of *M. bovis* infection between animals and humans and to examine the timelines involved. Comparing patients' isolates' sequences with the sequences of *M. bovis* strains currently circulating among Irish cattle herds and wildlife would help to distinguish between likely new or old human *M. bovis* infection.

Bioinformatic analysis of human and domestic pet *M. bovis* WGS data from the IMRL was performed using the Ridom SeqSphere+ Software and whole genome SNP analysis as detailed in Chapter 2.

Derived WGS data was compared with 45 wildlife and cattle *M. bovis* sequences previously published by Crispell *et al* in 2020(30) and 79 *M. bovis* sequences from cattle and badgers provided by the UCD and DAFM groups.

Crispell *et al* sequenced 45 animal *M. bovis* isolates originating from County Wicklow in 2014 and 2015 (30). These *M. bovis* isolates were recovered from 15 deer, 2 badgers and 28 cattle, all sourced from an area of about 100km^2(30). The WGS dataset was uploaded to the publicly available National Center for Biotechnology Information Sequence Read Archive (NCBI-SRA) and was downloaded from this site for use in this project. Their study aimed to

ascertain the prevalence of *M. bovis* infection among deer populations. *M. bovis* isolates were derived from culled deer and badger populations and also from slaughtered cattle with bTB. The research group performed sequencing using the Illumina NextSeq system and mapped reads to the *M. bovis* reference genome (AF2122/97). All isolates obtained in their study were within 35 SNPs of each other(30).

Further sequenced data provided for this study by the DAFM via UCD was derived from 74 cattle, 3 badgers and 2 unidentified animals collected between 2019 and 2020. The geographic distribution of these isolates within Ireland was not available to us.

M. bovis is a zoonotic disease and the primary means by which a human will become infected is from contact with an infected animal or consumption of their unpasteurised produce. People working within close proximity of potentially infected animals, for instance farmers, abattoir workers or veterinarians, would thus be considered at increased risk of infection.

As discussed in Chapter 2, it is possible for human-to-human transmission to occur however this is considered to be rare. This chapter will focus on possible animal to human transmission events based on WGS data and patient and animal metadata that was available for this investigation.

3.3 Results:

3.3.1 Overview of Cluster Analysis:

Among the 64 IMRL human and pet *M. bovis* isolates sequenced to date and the 123 externally derived animal isolates, 20 clusters involving 37 patients, 3 domestic pets (two cats and one dog) and 60 animal isolates were identified by cgMLST. Seventeen of these clusters contained at least one human *M. bovis* isolate. Fourteen clusters included both animal and human isolates. These clusters were determined using Ridom SeqSphere+ software with 12 allelic differences as the cluster threshold and depicted using a minimum spanning tree (MST) (Figure 3.2). This chapter will focus on those cgMLST clusters containing human and animal isolates that were <12 SNPs apart as determined following MTBseq analysis.

The genomic relatedness between *M. bovis* isolates recovered from humans, bovine, deer, pet cat and dogs, badgers and unknown animal host is shown in the MST in Figure 3.2.

Subsequent whole genome SNP analysis performed on these clusters showed that one cluster had patient isolates (n=2) that were <6 SNPS apart from each other (as described in Chapter 2).

One cluster (Cluster 1 – highlighted in the red box in Figure 3.2) involved multiple patients (n=7) with *M. bovis* isolates that were <6 SNPs apart from *M. bovis* circulating in animals suggestive of close genomic links. This cluster also involved human isolates (n=2) and a pet cat isolate that were < 6 SNP apart from each other.

Highlighted in blue boxes in Figure 3.2 are “clusters” (n=3) where human and animal isolates are within 6 – 12 SNP differences of each other.

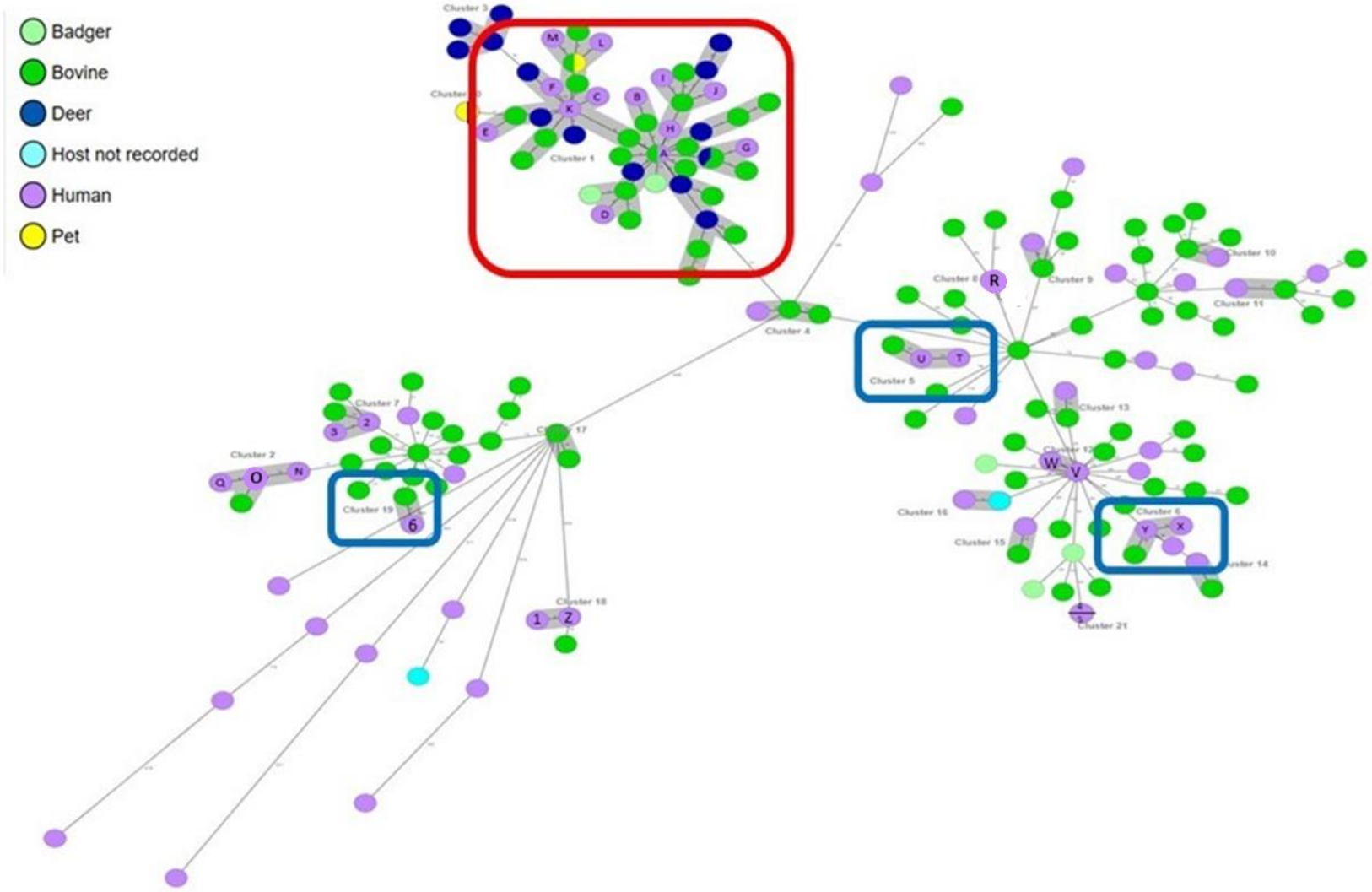

FIGURE 3.2: MINIMUM SPANNING TREE (MST) OF HUMAN AND ANIMAL *M. BOVIS* ISOLATES BY CGMLST. 20 CLUSTERS WERE IDENTIFIED USING 12 ALLELIC DIFFERENCES AS CLUSTER THRESHOLD. HIGHLIGHTED IN THE RED BOX IS A CLUSTER WHERE MULTIPLE HUMAN *M. BOVIS* ISOLATES (N=7) WERE <6 SNPS DIFFERENT FROM CIRCULATING ANIMAL *M. BOVIS* ISOLATES (CLUSTER 1). HIGHLIGHTED IN BLUE BOXES IN ARE "CLUSTERS" (N=3) WHERE HUMAN AND ANIMAL ISOLATES ARE WITHIN 6 – 12 SNP DIFFERENCES OF EACH OTHER (CLUSTER 5, 6 AND 19).

3.3.1.1 Cluster One:

Cluster One was the largest cgMLST cluster involving 13 human *M. bovis* isolates, 1 domestic pet cat isolate and 42 animal isolates including 30 bovine, 10 deer and 2 badger isolates.

Human isolates were derived from patients diagnosed with *M. bovis* infection over a 12-year period (2008 – 2020). Average age of human *M. bovis* cases was 48.5 years (21-85) and 46% were male. Of the 13 human cases, 10 presented with pulmonary infection while 3 presented with disseminated infection, 2 of whom were meat factory workers.

Forty-one of the animal isolates were obtained from publicly available WGS data from Crispell e*t al's* study and included 29 bovine, 10 deer and 2 badger isolates(30). Those isolates derived from Crispell *et al's* study will be referred to as "UCD" isolates. One animal isolate was a bovine isolate obtained from the DAFM. All but four of the UCD isolates fell into Cluster One by cgMLST.

Two human isolates within this cluster were 6 SNP differences from each other, indicating potential close epidemiological links. One pet cat isolate was found to be 3 and 5 SNPs differences respectively from two human isolates (L and M). The cat isolate was found to have zero SNP differences when compared with an animal isolate within this cluster. Seven human isolates were found to be <6 SNP differences from circulating animal isolates (Figure 3.3).

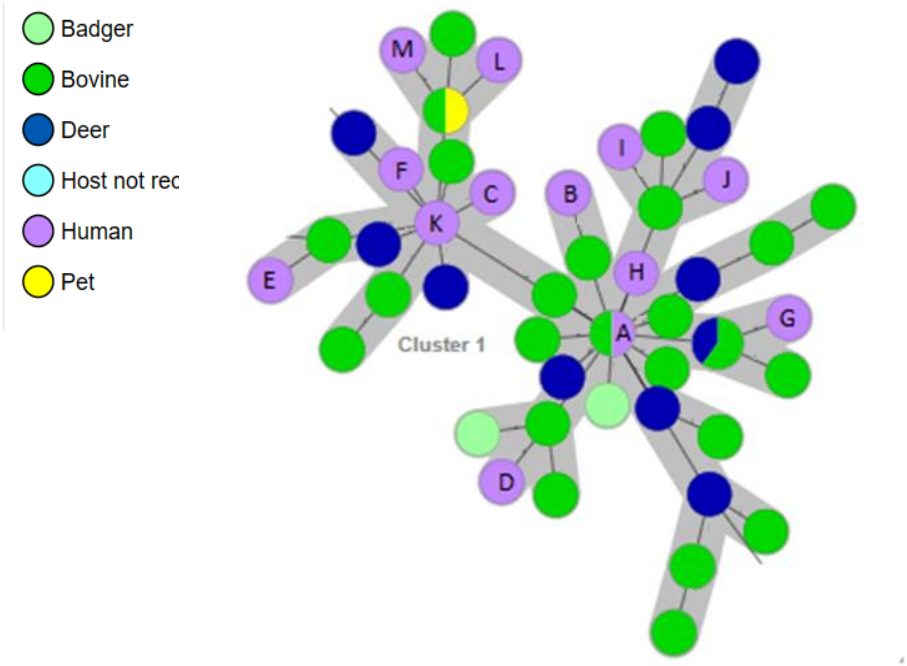

FIGURE 3.3: CLUSTER 1 MINIMUM SPANNING TREE (MST) OF HUMAN AND ANIMAL *M. BOVIS* ISOLATES CREATED BY CGMLST.
THIRTEEN PATIENT ISOLATES ARE INCLUDED WITHIN THIS CLUSTER LABELLED "A" TO "M". 30 BOVINE (GREEN), 10 DEER (DARK BLUE), 2 BADGER (LIGHT GREEN) AND ONE PET CAT (YELLOW) IS INCLUDED.

Patient isolate "K" was found to have less than 6 SNP (between 1 and 3) differences when compared with 6 animal isolate sequences, including deer isolates. Review of this patient's medical record found that the patient was diagnosed in 2008 with pulmonary disease aged between 35 – 39 years, was Irish born and had a history of unpasteurised milk consumption and contact with TB infected animals whilst living on a farm. No data was available on underlying immunosuppression, comorbidities or occupation. This patient isolate was also 11 – 12 SNP differences from 2 other patient isolates (L and M) and one pet cat isolate (yellow).

The pet cat isolate (yellow) was found to have zero SNP differences when compared with a bovine isolate. This pet cat isolate is dated from 2010. It and two animal isolates were <6 SNP (n=3-5) differences away from two patient

isolates (“L” and “M”). Patients “L” and “M” were both females, aged less than 50 years, who had both been diagnosed with pulmonary *M. bovis* infection in 2015. Individual patient isolates were 6 SNP differences apart from each other suggesting that infection had not been acquired by contact with each other but potentially via a common source. The patient chart was available for review for one of these patients and recorded relevant risk factors included previous consumption of unpasteurised milk and rare venison, living on a farm, the presence of pet dogs at home, as well as travel to TB endemic areas. Comorbidities included previous malignancy; however, the patient was not actively immunosuppressed.

Human *M. bovis* isolate “G” was found to have less than 6 SNP (n= 3-4) differences when compared 6 animal isolates (5 bovine, 1 deer). This patient was male, aged between 80 and 85 years of age and had been diagnosed with pulmonary infection in 2019. Unfortunately, no chart was available for review of potential patient risk factors.

Patient isolate “G” was 10 SNP differences apart from isolate “J” which was from a female aged in her early 50s diagnosed with disseminated infection in 2020 and had multiple comorbidities (n=7) including malignancy.

Human *M. bovis* isolate “A” was within 2 SNP differences of 2 animal isolates. This patient was a foreign-born male aged between 55 and 59 who presented with disseminated infection in 2018. Relevant risk factors for this patient included his occupation as an abattoir worker, history of unpasteurised milk consumption and underlying comorbidity of HIV infection.

Similarly, human *M. bovis* isolate "B" was 2 SNPs different from an animal isolate. This patient was an Irish male aged between 50 and 55 diagnosed with both pulmonary and extrapulmonary infection in 2018. Of note, this patient was a meat factory worker, had a number of comorbidities (n=4) and had been receiving the biologic monoclonal antibody, adalimumab, for an autoimmune condition. This patient's isolate was also between 6 – 12 SNPs from other patient isolates: "A" (n=7), "H" (n=12) and between 6-12 SNPs apart from 25 sequenced animal isolates.

Patient isolate "I" was 5 SNPs apart from 2 animal isolates. It was also within 8 – 11 SNP differences of human isolates ("A" and "J"). Information on this patient was lacking, however the patient was documented to be male, aged between 65-69 years and diagnosed with pulmonary infection in 2019.

A SNP difference relationship table of Cluster One isolates, within 12 allelic differences of each other, is depicted in Table 3.1. Boxes highlighted in red illustrate where there are < 6 SNP differences between isolates, while blue boxes highlight where SNP differences were between 6 and 12 between isolates.

TABLE 3.1 SNP DIFFERENCES BETWEEN CLUSTER ONE *M. BOVIS* ISOLATES DISPLAYED IN A RELATIONSHIP TABLE. HUMAN ISOLATES ARE LABELLED A-M. WHERE SNP DIFFERENCES BETWEEN ISOLATES ARE <6, THIS HAS BEEN HIGHLIGHT IN RED. WHERE SNP DIFFERENCES ARE BETWEEN 6 AND 12, THIS HAS BEEN HIGHLIGHTED IN BLUE

	A	B	C	D	E	G	H	I	J	K	L	M	Cat
A	**0**	7	17	9	19	10	7	8	12	15	17	17	16
B	7	**0**	22	14	24	15	12	13	17	20	22	22	19
C	17	22	**0**	24	22	25	22	23	27	18	20	20	19
D	9	14	24	**0**	26	17	12	15	19	22	24	24	23
E	19	24	22	26	**0**	27	24	25	29	20	22	22	21
G	10	15	25	17	27	**0**	15	14	18	23	25	25	24
H	7	12	22	12	24	15	**0**	13	17	20	22	22	21
I	8	13	23	15	25	14	13	**0**	8	21	23	23	22
J	12	17	27	19	29	18	17	8	**0**	25	27	27	26
K	15	20	18	22	20	23	20	21	25	**0**	12	12	11
L	17	22	20	24	22	25	22	23	27	12	**0**	6	5
M	17	22	20	24	22	25	22	23	27	12	6	**0**	3
Cat	16	19	19	23	21	24	21	22	26	11	5	3	**0**
Cow (DAFM)	5	10	20	12	22	13	10	11	15	18	20	20	19
Cow	6	11	21	11	23	14	9	12	16	19	21	21	20
Deer	17	20	20	24	22	25	22	23	27	2	14	14	11
Cow	5	10	20	6	22	13	8	11	15	18	20	20	19
Badger	6	11	21	7	23	14	9	12	16	19	21	21	20
Cow	5	10	20	6	22	13	8	11	15	18	20	20	19
Deer	4	9	19	11	21	12	9	10	14	17	19	19	18
Cow	2	7	17	9	19	10	7	8	12	15	17	17	16
Cow	4	9	19	11	21	12	9	10	14	17	19	19	18
Cow	4	9	19	11	21	12	9	10	14	17	19	19	18
Cow	3	8	18	10	20	11	8	9	13	16	18	18	17
Cow	16	21	19	23	19	24	21	22	26	17	19	19	18
Cow	3	8	18	10	20	11	8	9	13	16	18	18	17
Deer	5	10	20	12	22	13	10	11	15	18	20	20	19
Deer	17	22	20	24	22	25	22	23	27	6	14	14	13
Cow	2	7	17	9	19	10	7	8	12	15	17	17	16
Deer	3	8	18	10	20	11	8	9	13	16	18	18	17
Cow	5	10	20	12	22	13	10	11	15	18	20	20	19
Cow	6	11	21	13	23	14	11	12	16	19	21	21	20
Cow	5	10	20	12	22	13	10	11	15	18	20	20	19
Cow	5	2	20	12	22	13	10	11	15	18	20	20	19
Cow	9	14	24	16	26	17	14	15	19	22	24	24	23
Cow	17	22	20	24	22	25	22	23	27	2	14	14	13
Deer	6	11	21	13	23	14	11	12	16	19	21	21	20
Cow	4	7	19	11	21	12	9	10	14	17	19	19	16
Cow	5	10	20	12	22	11	10	5	9	18	20	20	19
Deer	14	19	17	21	19	22	19	20	24	3	11	11	10
Deer	9	14	24	16	26	15	14	9	13	22	24	24	23
Deer	10	15	25	17	27	16	15	10	14	23	25	25	24
Cow	5	10	20	12	22	11	10	5	9	18	20	20	19
Cow	8	13	23	15	25	4	13	12	16	21	23	23	22
Deer	7	12	22	14	24	3	12	11	15	20	22	22	21
Cow	7	12	22	14	24	3	12	11	15	20	22	22	21
Cow	16	21	19	23	21	24	21	22	26	1	13	13	12
Cow	7	12	22	14	24	3	12	11	15	20	22	22	21
Deer	7	12	22	14	24	3	12	11	15	20	22	22	21
Cow	8	11	23	15	25	4	13	12	16	21	23	23	20
Badger	20	23	29	27	31	28	25	26	30	27	29	29	26
Cow	16	19	19	23	21	24	21	22	26	11	5	3	0
Cow	17	22	20	24	22	25	22	23	27	12	6	4	3
Cow	17	22	20	24	22	25	22	23	27	2	14	14	13
Cow	16	21	19	23	21	24	21	22	26	1	13	13	12

3.3.1.2 Cluster Five:

Cluster 5 involved 2 patient isolates and 1 animal isolate. Only patient "U" and the bovine isolate were within 12 SNP differences of each other (n=11). Limited information was available on this patient; however, it was inferred that the patient was an Irish-born male aged between 55-59 years and diagnosed in 2008 with pulmonary infection.

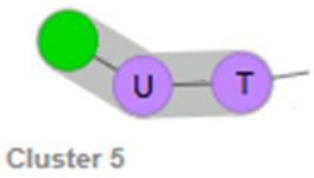

FIGURE 3.4: CLUSTER 5 MINIMUM SPANNING TREE (MST) OF HUMAN AND ANIMAL *M. BOVIS* ISOLATES CREATED BY CGMLST. THIS INCLUDES 2 HUMAN *M. BOVIS* ISOLATES LABELLED "U" AND "T" AND 1 BOVINE *M. BOVIS* ISOLATE.

3.3.1.3 Cluster Six:

Cluster 6 consisted of 2 patient isolates and one bovine animal isolate. Eleven SNP differences occurred between patient "Y" and the animal isolate. This patient was diagnosed in 2009 and was a male aged between 85-89 years. His chart was not available for review.

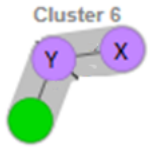

FIGURE 3.5: CLUSTER 6 MINIMUM SPANNING TREE (MST) OF HUMAN AND ANIMAL *M. BOVIS* ISOLATES CREATED BY CGMLST. THIS INCLUDES 2 HUMAN *M. BOVIS* ISOLATES LABELLED "X" AND "Y" AND 1 BOVINE *M. BOVIS* ISOLATE

3.3.1.4 Cluster Nineteen:

Cluster 19 involved a human isolate "6" and an animal bovine isolate. These sequences had 6 SNP differences between them. This patient was a male aged 40-44 years, diagnosed with extrapulmonary infection in 2011. He had limited past medical history, with only one comorbidity documented and no history of immunosuppression. Of note, the patient was a meat factory worker at the time of diagnosis.

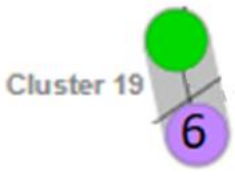

FIGURE 3.6: CLUSTER 19 MINIMUM SPANNING TREE (MST) OF HUMAN AND ANIMAL *M. BOVIS* ISOLATES CREATED BY CGMLST. THIS INCLUDES 1 HUMAN *M. BOVIS* ISOLATE LABELLED "6" AND 1 BOVINE *M. BOVIS* ISOLATE.

3.3.2 Abattoir Workers:

A relatively common occupation among patients with *M. bovis* for whom charts were available for review, was that of an abattoir or meat factory worker.

Of the 27 charts available for review, occupation was documented in 19 patients. Five patients were noted to be abattoir/meat factory workers. All of these patients were male with an average age at diagnosis of 47 years (range 31 – 62). Within this cohort, 3 patients presented with disseminated disease, one presented with extrapulmonary disease, and one presented with pulmonary disease alone.

Three of these patients were foreign born, one was born in Ireland, and one did not have country of origin documented. Two patients had significant immunosuppression, whilst 2 had a single documented comorbidity without immunosuppression and one had no medical record available to review.

One of the immunosuppressed patients had HIV, the other had an autoimmune condition and was receiving a monoclonal antibody as treatment for his condition. One of these patients reported previous consumption of unpasteurised milk. Both presented with disseminated disease. Both patients had isolates which clustered by cgMLST within Cluster 1 and were 7 SNPs apart from each other. Of note, both patients' isolates were within 2 SNP differences of animal isolates sequenced by UCD. Both patients were diagnosed in 2018.

The remaining patient isolates within this cohort of abattoir workers clustered by cgMLST within clusters 4, 10 and 19 respectively. One patient's isolate was 6 SNPs from a DAFM sourced isolate, whilst the remaining patients' isolates were

between 14 – 18 SNPs from other DAFM isolates. All 5 patients' *M. bovis* isolates were lineage La1.8.1.

3.3.3 Farmers:

Five of the patients on whom occupation was recorded in their medical notes were farmers. The average age of this cohort was 68.8 years, 4 patients were male, one patient was female. Three patients presented with pulmonary disease, one presented with extrapulmonary disease, and one presented with disseminated infection.

Country of origin was documented in two patients, both of whom were Irish born. Comorbidities were recorded in two patients, one of whom had 3 comorbidities but was not overtly immunosuppressed, the other was receiving 3 immunomodulatory therapies, including a TNF inhibitor for an autoimmune condition. Another relevant risk factor in this patient was contact with a family member with a previous diagnosis of TB infection.

Four of the five patients had isolates available for sequencing. One patients' *M. bovis* sequence clustered by cgMLST with another human isolate and an animal isolate and was found to be 6 SNP differences from the human isolate and 14 SNPs from the animal isolate. Another patient isolate clustered by cgMLST with an animal isolate however, there were 20 SNP differences between the sequenced isolates.

The two patient samples that did not cluster with other isolates on cgMLST were from patients born in 1920 and 1930, who presented with pulmonary disease. This perhaps suggests that their infection with *M. bovis* had been acquired more

remotely, perhaps even before the widespread pasteurisation of milk. However, history of previous unpasteurised dairy consumption was not available on either patient.

3.3.4 New versus Old Infection:

As described previously, 20 clusters were created by cgMLST. Three such clusters contained animal only isolates, leaving 17 clusters that included human isolates. Three clusters contained human (n=6) only isolates. The remaining 14 clusters contained a mixture of human and animal isolates. Twenty-eight patient isolates included in cgMLST analysis were from patients born prior to 1960. Thirty-three were from patients born post-1960.

Among the three "human only" *M. bovis* clusters, 5of the 6 human isolates were from patients born post-1960, one patient was born pre-1960.

Among the clusters containing a mixture of both animal and human *M. bovis* isolates:

i) Two cgMLST clusters contained a mixture of isolates from patients born pre (n=4) and post (n=11) 1960 and animal isolates (n=44).
ii) Six cgMLST clusters contained isolates from patients (n=8) born only pre-1960 and animal isolates (n=6).
iii) Six cgMLST clusters contained isolates from patients (n=8) born only post-1960 and animal isolates (n=7).

Fifteen isolates (53.6%) from patients born pre-1960 did not cluster with any other human or animal isolates. Nine isolates (27.7%) from patients born post-1960 did not cluster with any human or animal isolates. Patients born post-1960

were more likely to cluster with other human or animal isolates (p=0.0409using Medcalc N-1" Chi-squared test). Of note, five of these isolates from patients born post-1960 were of lineages other than 1.8.1, suggesting that infection was acquired outside of Ireland.

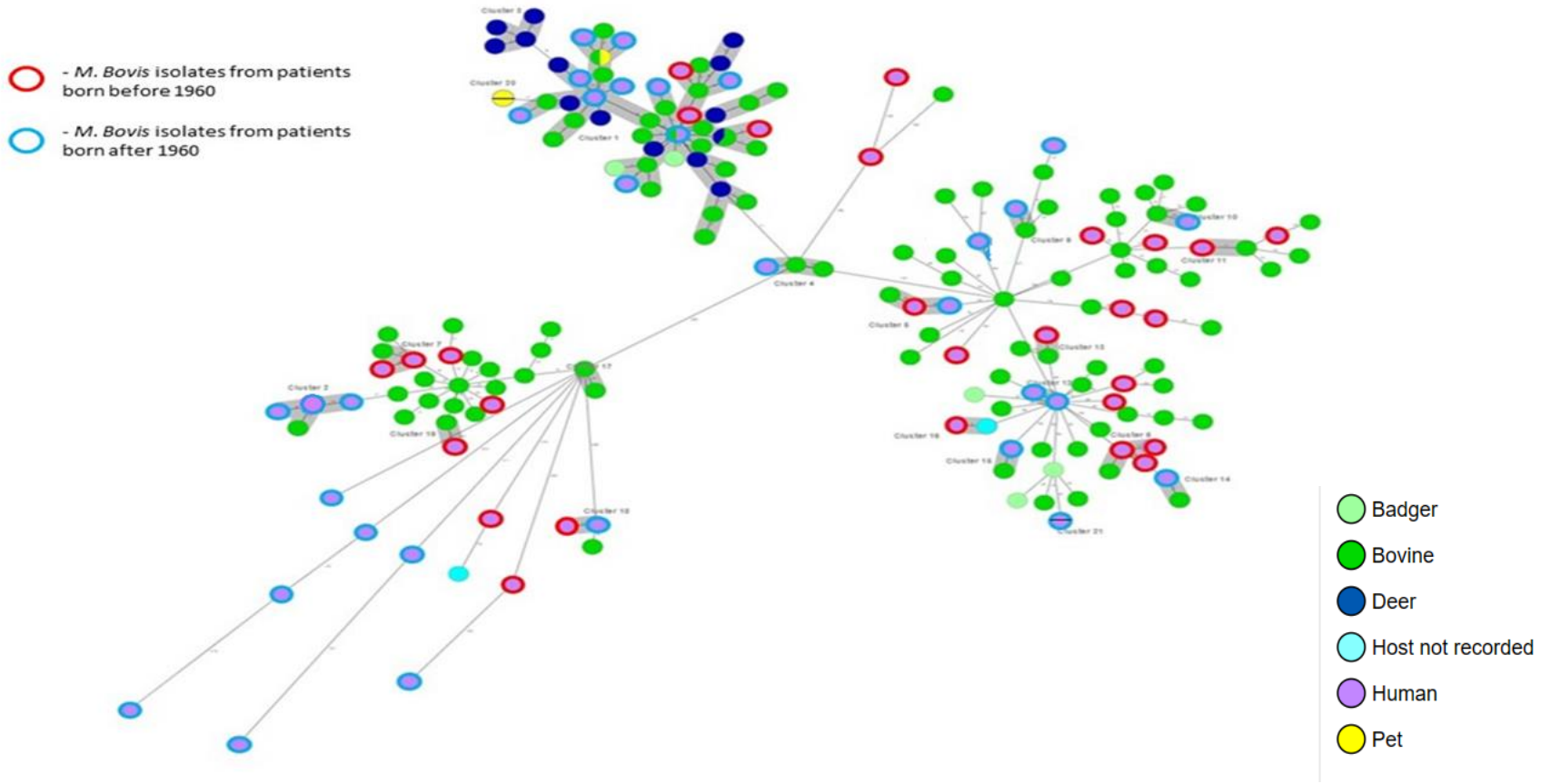

FIGURE 3.7: MINIMUM SPANNING TREE (MST) OF HUMAN AND ANIMAL *M. BOVIS* ISOLATES WITH ISOLATES FROM PATIENTS BORN PRE-1960 (N=28) ENCASED BY RED CIRCLES, AND THOSE FROM PATIENTS BORN POST-1960 (N=33) ENCASED IN BLUE CIRCLES.

3.4 Discussion:

3.4.1 Animal to Human Transmission:

Within Cluster One, it is interesting to note that the animal isolates collected between 2014 and 2015 were closely related (<6 SNP differences) to human isolates, with year of diagnosis spanning form 2008 – 2020. For instance, patient "K's" isolate was 1 – 3 SNP from 6 animal isolates. However, these were temporally remote from each other by at least 6 years with patient "K" being diagnosed in 2008 – 6 years prior to documented disease from the animals. This shows the perhaps slow evolution of the *M. bovis* genome.

The pet cat isolate in Cluster 1 dates from 2010 and so is also temporally remote from the cattle isolate to which it was identical which was from 2014. The two closely genomically related human isolates were from patients who were both diagnosed in 2015.

Two abattoir workers diagnosed in 2018, with significant underlying immunosuppression, presented with disseminated infection. Both patients had *M. bovis* isolates 2 SNPs apart from animal *M. bovis* isolates. The exact location where these individuals worked was not recorded, and while these isolates are closely related to each other (7 SNPs), it is less likely that neither patient acquired disease from each other, rather there may have been a common, likely animal, source of infection.

The isolates of these 2 abattoir workers matched closely with isolates obtained from Wicklow in 2014 and 2015. It is unclear at what time point

these patients initially encountered the disease and perhaps there was a period of disease latency before reactivation.

It is interesting that all but one animal isolate within Cluster 1 was obtained from the UCD sequenced isolates from Crispell *et al's* study conducted in 2014 and 2015. One animal isolate originated from the DAFM cohort samples. Of note, DAFM isolates represented samples from across Ireland whilst the UCD isolates represent multiple isolates from a 100km² area in Wicklow. The location in Ireland from where the DAFM isolates had been collected was not available, nor was the county patients included in this study were residing in. Comparison of spatial locations of animal and human isolates would have been useful to analyse potential transmission events.

The TB Scientific Working Group advised that in order to use WGS to infer transmission directionality of *M. bovis,* deep sampling from infected cattle and wildlife over an extended period of time, i.e. 10 years is needed(142). The importance of this is highlighted by this study. The densely sampled Wicklow isolates matched closely with a number of patients' isolates which were processed at the IMRL in SJH. Perhaps those patients living within the Wicklow area were more likely to have their isolates, recovered from clinical specimens, processed at the IMRL, SJH, due to their relative geographic proximity to Dublin.

Those animal isolate sequences received from DAFM that spanned across Ireland did cluster by cgMLST with human isolates with 3 examples of animal and human isolates within 6 – 12 SNPs of each other. However,

these isolates were perhaps less geographically dense. To further elucidate potential transmission links, further data on the county from which the animal, and indeed the human, isolates originated would be useful to further assess transmission dynamics.

The DAFM laboratories store > 3000 tissue samples from bTB cases from 2017 to date, with further archived isolates dating back to 2012 from cattle and badgers(142). A rate limiting step in the generation of this WGS data is the culture of the *M. bovis* isolates themselves(142). Comparing the DAFM bank of sequenced isolates with the human isolates may further elucidate transmission links. Denser WGS data and increased quality of any accompanying metadata would allow better understanding of transmission dynamics. Unfortunately, the DAFM biobank of *M. bovis* sequences does not extend prior to 2012 and so comparison of old versus new strains to determine whether human infection is new or the result of reactivation of latent disease is difficult. Perhaps future studies will involve retrospective analysis of *M. bovis* WGS data as the biobank will have increased significantly with time.

Among the 61 sequenced human *M. bovis* isolates, 3 pet *M. bovis* isolates and 123 externally derived animal isolates, 20 clusters were identified by cgMLST (threshold of <12 allelic differences). Fourteen of these clusters included both animal and human *M. bovis* isolates. Three of these clusters contained only human M. *bovis* isolates. *M. bovis* isolate sequences from patents born post-1960 were more likely than those from patients born pre-1960 to cluster with other human *M. bovis* isolates or circulating

animal strains. This suggests ongoing *M. bovis* transmission, with disease often being the result of recent infection from either an animal or human source rather than the result of reactivation of latent disease.

3.4.2 Disease among Abattoir Workers:

Of the 13 human *M. bovis* infections in Cluster 1, two were among abattoir workers. As both cases were significantly immunosuppressed, this not only highlights the risk that significant immunosuppression presents to abattoir workers, but also the potential risk that such abattoir workers may present to their colleagues. Both patients presented with disseminated bTB involving their lungs among other organs. Three of the abattoir workers in this study were foreign born yet had isolates that clustered with Irish animal isolates suggesting local acquisition.

Research by the Migrant Rights Centre Ireland (MRCI) estimates that immigrants account for 59% of those employed in meat production, processing and preserving(145). Their survey of 151 migrant workers within this sector painted a dismal view of the workplace conditions, with 62% reporting inadequate training on commencing employment and 58% not knowing who their Health and Safety Officer was(145). Their research also highlighted the lack of specific Health and Safety Authority (HSA) guidelines for meat factory worker safety in Ireland.

By the 24th July 2020 there had been 23 Covid-19 outbreaks in meat processing plants (MPP) involving 1047 people in Ireland(146). Studies reported that the environment within MPP such as metal work counters, low temperatures and moist air increased the risk of COVID-19 outbreaks.

Given the rapid spread of COVID-19 within this setting, it may be that respiratory transmission of *M. bovis* infection is also a risk.

This raises the question of whether the occupational risks are fully understood by meat factory workers, their doctors and indeed their employers? What pre-employment checks, if any, are carried out on these workers who may be at risk of significant disease? Additionally, is Personal Protective Equipment (PPE) appropriately used and what hygiene standards are implemented within these environments?

There is increasing concern for the risk of zTB among meat factory workers in Ireland. In 2024, Professor Joseph Keane, a Consultant Respiratory physician at SJH, wrote to the Irish Medical Journal calling on abattoirs to implement pre-employment screening for untreated HIV and to caution workers on TNF alpha inhibitors of the occupational risk of zTB(147). The letter describes *M. bovis* infection diagnosed in an abattoir worker receiving a TNF alpha inhibition in 2023 with potential WGS links to 5 other patients with *M. bovis* infection, including 3 other meat factory workers(147).

The meat industry in Ireland has an annual export value of 3.9 billion euros(145). The sector employs about 15,000 workers. There are 56 DAFM approved slaughterhouses (33 bovine, 9 pig, 10 sheep and 4 poultry) which account for >95% of all slaughtered livestock(146). DAFM veterinary and technical staff regulate and supervise operations within these settings and are supplemented by an additional 700 Temporary Veterinary Inspectors who perform antemortem and postmortem cattle inspections(146).

Additionally, there are about 180 smaller local authority approved meat plants(146). This represents a very large work force potentially at risk of disease, however thankfully *M. bovis* cases remain relatively infrequent.

3.4.3 Pets:

Cluster One included a pet cat isolate zero SNPs from a bovine isolate and <6 SNP differences from two human isolates. Unfortunately, due to the retrospective nature of this study there were no details available on whether there was an epidemiological link between either human and this cat. However, it does raise the possibility that domestic pets, particularly cats, may pose an infection risk to their owners.

Infection among cats in Ireland has been reported previously, for instance in 2014, a nosocomial outbreak of feline TB caused by *M. bovis* was reported in a Kildare Veterinary practice(148). The cluster of cases in question involved 5 domestic cats who were euthanised due to confirmed or suspected *M. bovis* infection over a 13.5-week period. Three (A, B and C) of the cats came from the same household whilst 2 cats (D and E) had attended the veterinary practice for elective surgical procedures on days where the household cats had been euthanised. Cats A and B, were known to hunt and presented with possible bite-like submandibular wounds, whilst cat C, which was not known to hunt, presented with respiratory symptoms. A dog within the household remained healthy. Cats D and E presented with surgical site infections with progression to systemic illness. Cats B and D had *M. bovis* infection confirmed on culture and cat E had a fluid swab which was positive for AFB.

The authors hypothesised that infection had likely occurred in cats A and B following a bite from an infected rodent, e.g., a mouse or vole, whilst hunting and that they had likely transmitted the infection to cat C via the respiratory tract. Cats D and E likely developed infection via contact with an infected environment. This report highlighted the need for veterinarians to be alert to the possibility of *M. bovis* infection among cats presenting with non-healing wounds and of the need for appropriate diagnostic testing and barrier nursing where cases are suspected. There were no further cases reported among animals and staff at the practice or amongst the affected households.

Among the IMRL isolates, there was a further dog and cat isolate from 2019, representing Cluster 20. As noted in Chapter One, infection in dogs is much rarer than in cats. However, in 2023, Ottewil *et al* reported an Irish case of a pet dog, on immunosuppressive treatment, acquiring cutaneous *M. bovis* infection(109). The dog likely transmitted infection to a domestic cat within the household that also fell ill with respiratory symptoms 6 months later. All but one of the human household contacts yielded a positive IGRA and TST and received treatment for latent infection. Two years following this event, a family member who had visited the household in question developed pulmonary *M. bovis* infection and their cultured *M. bovis* isolate was identical to the pet animal isolates by WGS.

3.4.4 Comparison with other Studies:

A New Zealand study typed 23 human *M. bovis* isolates collected between 1998 and 2002 and compared REA results with circulating animal strains(75). REA uses enzymes to cleave *M. bovis* DNA at specific nucleotide sequences. Gel electrophoresis of the fragmented DNA is performed, and the patterns created are used to differentiate strains (138). Fourteen human isolates matched 9 different circulating domestic and wildlife REA strains, 5 of which were REA 115, a strain common in the North Island, 2 were REA 21 and the remainder provided different REA patterns. Eight patients whose isolates matched circulating REA patterns had risk factors recorded, 5 of whom had potential animal exposure. Eight patients had isolates that matched REA patterns not seen among New Zealand wildlife, 4 of these patients had been born overseas. Their results suggested some ongoing low-level transmission of infection between animals and humans. As 80% of their cases were aged > 30 years, many cases were suspected to be reactivations of latent disease.

A study from the Asturias region in Northern Spain performed spoligotyping and MIRU-VNTR typing on 19 human *M. bovis* isolates collected between 2006 and 2015(73). Ten different spoligotype patterns were identified among these human isolates, all but one matched circulating strains among Asturian farms. Forty-seven percent of human isolates (9/19) were identical or very similar to animal isolates by MIRU-VNTR analysis suggesting potential recent transmission. One patient without any documented risk factors shared an identical MIRU-VNTR

genotype to a patient with smear positive pulmonary disease with risk factors suggesting potential human to human disease transmission. The authors commented that the rate of genotypic matches between animal and human isolates was higher than expected within this low prevalence *M. bovis* setting where effective disease control measures were in place.

In contrast to this Spanish study, an Italian study also using spoligotyping and MIRU-VNTR to compare animal and human isolates, concluded that most infections were the result of disease reactivation(72). The study analysed 9 human *M. bovis* isolates between 2011 and 2015. Five isolates belonged to spoligotype SB120, a common circulating spoligotype among Italian cattle, however only 3 of these matched circulating strains by MLVA analysis. The remainder of patients had spoligotypes not circulating in Italian cattle, 3 of whom were foreign born.

Kohl *et al* performed WGS analysis of 38 animal and 93 human *M. bovis* isolates collected between 1999 to 2017 in Germany(54). Using 12 SNPs as their threshold for possible recent transmission, cluster analysis grouped 46 strains into 13 clusters. The clusters contained animal only, human only and mixed host isolates. They noted that 32 of their 38 animal isolates grouped into clusters and that within 4 of these clusters, strains had been recovered over 9 years apart suggesting autochthonous persistent bTB infection cycles.

Only 12 of the 93 human strains grouped into clusters, 4 of these clusters involved 9 solely human isolates collected over a period of 2 years. All isolates were 1 or less SNP differences apart, with one exception,

suggesting recent putative transmission links. Other clusters involved cattle and human isolates 2 SNPs apart and another involved a racoon and human strain separated by 12 SNPs.

Unlike animal isolates, most human isolates did not cluster - suggesting that these isolates may represent infection arising from reactivation of latent disease acquired many years before. In 2003, the majority of *M. bovis* infections in Germany occurred in individuals aged over 60 years. There were no animal isolates from before 1999 to compare these human isolates to. The diversity among human isolates may also be due to travel from other countries. With only one close relationship noted between cattle and human isolates, the occurrence of transmission between cattle and humans, for instance via unpasteurised milk, seemed rare.

3.4.5 Mapping against *M. bovis* reference genome AF2122/97:

In this study, sequenced *M. bovis* isolates were mapped against an MTB reference genome. As previously stated, where MTB genomes are used as reference genomes for *M. bovis* isolates, the number of detected SNPs rise and this can lead to errors in read mapping and variant calling. To assess whether the use of an MTB genome rather than an *M. bovis* genome would impact on our SNP distance analysis, we mapped a subset of *M. bovis* isolates to the *M. bovis* AF2112/97 reference genome. Isolates from Chapter 3 clusters 1, 5, 6 and 19 were used.

There were no differences in SNP distance among isolates belonging to Cluster 5 when mapped to an MTB genome compared with an *M. bovis*

one. Isolates within Cluster 6 were 13 SNP differences apart when mapping to an *M. bovis* genome compared to 11 SNPs apart when mapping to an MTB one. Isolates within Cluster 19 were 5 SNP differences apart when mapping to an *M. bovis* genome compared to 6 SNPs apart when mapping to an MTB one.

Differences in SNP distances between isolates within Cluster 1 when mapped to an *M. bovis* reference genomes compared to an MTB genome are summarised in **Table 3.2.** Whilst there were small discrepancies among SNP distances between isolates when the mapping genome was changed to *M. bovis*, distances and number of isolates within 6 SNPs or between 6 - 12 SNPs of each other were largely comparable.

TABLE 3.1: NUMBER OF HUMAN AND ANIMAL ISOLATES < 6 SNP DIFFERENCES OR WITHIN 6 -12 SNP DIFFERENCES FROM IMRL ISOLATES (A-M) WITHIN CGMLST CLUSTER 1 WHEN MAPPED TO AN MTB REFERNCE GENOME VS AN *M. BOVIS* GENOME

	Human < 6 SNPs		**Human 6 – 12 SNPs**		**Animal <6 SNPs**		**Animal 6-12 SNPs**	
	Mapped to MTB	Mapped to *M. bovis*	Mapped to MTB	Mapped to *M. bovis*	Mapped to MTB	Mapped to *M. bovis*	Mapped to MTB	Mapped to *M. bovis*
A	0	0	6	6	18	13	13	16
B	0	0	2	3	1	1	26	25
C	0	0	0	0	0	0	0	0
D	0	0	2	1	0	0	20	5
E	0	0	0	0	0	0	0	0
G	0	0	1	1	6	6	11	7
H	0	0	3	2	0	0	25	25
I	0	0	2	3	2	1	28	26
J	0	0	2	2	0	0	4	3
K	0	0	2	0	6	6	4	3
L	0	0	2	1	2	0	2	3
M	0	0	2	1	3	3	1	0
Cat	2	1	1	2	2	2	3	1

SNP: Single nucleotide polymorphism. MTB: Mycobacterium tuberculosis. M. bovis: Mycobacterium bovis.

Chapter 4: One Health Discussion and Conclusion

4.1 One Health Approach:

"One Health" is an integrated approach which recognises the interdependence of human health, animal health and the environment and the need for engagement from all relevant sectors(2).

The WHO Roadmap for Zoonotic Tuberculosis identified 10 priorities under the following three core themes: i) the improvement of scientific evidence base, ii) the reduction of disease transmission at the animal to human interface and iii) the strengthening of multidisciplinary collaboration (2).

This Roadmap is framed within the United Nations (UN) Sustainable Development Goals 2016-2030 and the WHO End TB strategy and is supported by the Global Plan to End TB 2016-2020 which identifies those with zTB as a potentially neglected population(2). This Chapter will look at the core themes and priorities highlighted in this Roadmap and relate them to the outcomes of this study.

4.1.1 Improving Scientific Evidence Base:

The Roadmap for Zoonotic Tuberculosis identifies three priorities under this heading including i) the collecting and reporting of more complete and accurate data, ii) improving diagnosis in people, iii) addressing research gaps.

4.1.1.1 Collecting and Reporting of More Complete and Accurate Data:

Surveillance systems capable of identifying trends and risk factors are needed to better understand zTB and implement control strategies(4). The

ultimate goal of surveillance is to identify potential threats to health early on and to rapidly respond accordingly(4). Molecular techniques can further enhance this surveillance.

Unlike many lower income countries, Ireland is fortunate to have a robust public health service that tracks notifiable diseases and reports data to the HPSC and the ECDC. Additionally, the National bTB Eradication Programme ensures that disease prevalence among cattle is carefully monitored.

Nonetheless, Ireland has the highest herd prevalence of bTB and the highest rate of zTB in the EU, despite a significant and sustained financial investment(110). Further knowledge of the disease within our country is thus desirable to aid in its control and ultimate eradication.

This study strengthens the evidence base surrounding *M. bovis* infection in humans and updates our knowledge on the epidemiology of disease in Ireland, as discussed in Chapter 2. Despite the commonly held perception that human *M. bovis* infection is predominately extrapulmonary in nature and affecting patients of advanced age, this study shows that human *M. bovis* disease is predominantly respiratory in nature with a falling average age of patient diagnosis. Those affected by disease are potentially a more complex and comorbid patient cohort.

However, despite our efforts, the correlation of *M. bovis* genomic data with epidemiological data was limited due to restrictions on our ability to collect data without explicit patient consent and a lack of available clinical data. Prospective consenting of patients for inclusion in epidemiological

studies in relation to *M. bovis* could help further our understanding of the disease.

4.1.1.2 Improving Diagnosis in People:

Diagnosis of human *M. bovis* infection in Ireland is possible due to the ability to access appropriate diagnostics and CL3 laboratory facilities. Further to this, WGS has been performed at the IMRL on new isolates since 2020 and has been performed retrospectively on stored isolates. This study demonstrates that use of these tools is invaluable when it comes to comparing animal and human *M. bovis* isolate genomic sequences to assess potential transmission patterns. WGS also facilitates the detection of genomic mutations associated with drug resistance and allows determination of lineages. However, interpretation of WGS data depends on the quality of the associated epidemiological and clinical data. Due to the slow evolution rate of the *M. bovis* genome, observing few SNP differences between human or indeed animal *M. bovis* isolates may be of limited epidemiological significance. The association of genomic data with high quality patient clinical and social data and animal demographics would allow the best use of any generated WGS data.

4.1.1.3 Research Gaps:

A key question posed at the beginning of this project was whether human *M. bovis* infection is newly acquired or the result of reactivation of latent disease acquired earlier in life.

This study performed WGS analysis on human *M. bovis* isolates over a 20-year period and compared them with circulating animal isolates, as described in Chapter 3. This analysis showed close genomic links between multiple animal and human derived *M. bovis* isolates, suggesting ongoing transmission between animal and human populations. Additionally, 1 cluster was identified involving human isolates that were genomically identical to each other, suggestive of potential human-to-human disease transmission.

A key stumbling block in our analysis was the retrospective nature of this study. Relevant patient information was often either missing from charts, or, due to constraints of the consent declaration issued by the HRCDC, data that was collected had to be minimised to maintain anonymity. With the growing role of WGS in disease surveillance, efforts should be made to prospectively consent patients for use of their personal meta data alongside their isolates' WGS data to facilitate further interpretation of the generated data. Patient location would be a key point of interest when comparing animal and human isolates. Also, physicians, when reviewing patients with *M. bovis* infection, should be reminded to clearly document within the patient's medical chart, the presence or absence of relevant exposures or risk factors for disease.

4.1.2 Reducing Transmission at the Animal – Human Interface:

The occurrence of human *M. bovis* infection is dependent on the presence of infection in cattle(71) and other animal reservoirs. With rising herd prevalence of bTB it is likely that human *M. bovis* cases may continue to rise. Three priorities identified by the Roadmap for Zoonotic Tuberculosis to reduce transmission at the animal – human interface include: i) ensuring safer food ii) improving animal health and iii) reducing risks to humans.

4.1.2.1 Ensuring Safer Food:

Measures in place by the bTB Eradication Programme reduce the risk of transmission of *M. bovis* to humans via the food chain through annual herd SICCT, inspection of animal carcasses for TB-like lesions and the pasteurisation of milk. Both human and animal prevalence of *M. bovis* infection have fallen since the bTB eradication programme was introduced in 1954.

4.1.2.1.1 Consumption of Unpasteurised Dairy Produce:

There is a growing trend of consumption of unpasteurised milk. The UK Food Standards Agency (FSA) reported that the proportion of the population in the UK consuming unpasteurised milk rose from 3 % in 2012 to 10% in 2018.

The Scientific Committee of the Food Safety Authority of Ireland (FSAI) advises against the consumption of raw milk and strongly recommends the pasteurisation of all milk for human consumption to reduce the risk of human infection by contaminating pathogens. The FSAI strongly

recommends that children, pregnant women, the elderly, those who are unwell or who have a chronic condition should not consume unpasteurised milk. These views are in keeping with the European Food Safety Authority (EFSA)(149).

In 1996, the sale of raw milk in Ireland was banned by the DAFM. However, the sale of raw milk was legalised once more with the introduction of the 2006 EU Hygiene regulations(149) which provides for the sale of raw milk for direct human consumption under certain conditions.

A good practice guideline was published by the producer association, Raw Milk Ireland (RMI), in association with DAFM and FSAI in 2018. It advises that raw milk for consumption should only come from healthy animals with a low somatic cell count (<200,000 per ml)(149). Strict controls should be implemented including the testing for pathogens (*Listeria monocytogenes, Campylobacter spp., Salmonella spp.*, Coagulase positive *staphylococci* and *Enterobacteriaceae*) and the provision of shelf life and labelling information. A warning label is mandatory and reads “This milk has not been heat-treated and may contain harmful bacteria”.

Bovine and caprine herds should be screened for bTB twice a year. If bTB is detected in a herd, production of raw milk for human consumption must cease and raw milk in circulation must be withdrawn/recalled(149). Where a bTB is detected within a herd, any cheese from unpasteurised milk originating from that herd since last inspection should also be regarded as unsuitable for human consumption(150).

Of note, reliable methods for detecting viable *M. bovis* within milk or dairy products are not routinely available and so there is no practical way of assuring that unpasteurised products are free of *M. bovis*(150).

In this study, a comparative analysis has been performed on patients born pre and post 1960, reflecting that exposure to unpasteurised milk was more common prior to 1960. This comparative analysis showed that the number of risk factors per patient seemed to increase in those born post 1960, in particularly the number of immunosuppressive conditions. Additionally, in those born post 1960, the proportion of patients presenting with respiratory infection increased and the proportion of patients presenting with extrapulmonary disease decreased. Furthermore, *M. bovis* isolates derived from patients born after 1960 were more likely to "cluster" by cgMLST with circulating animal and human *M. bovis* isolates suggesting ongoing transmission of *M. bovis* between animal and human populations.

It is likely that the widespread pasteurisation of milk did not occur overnight, and it is probable that many farming families may have continued to consume unpasteurised milk for quite some time after pasteurisation regulations were introduced in 1957. Indeed, a study in 2017 found that 39% of dairy farmers drank unpasteurised milk at least once a week, with no significant difference found between younger and older farmers(151). A 1998 study reported this figure at 84%(152).

4.1.2.2 Infected Meat:

Animals entering the food chain are inspected ante and post-mortem for the presence of visible TB-like lesions. All cattle sent for slaughter must have a SICTT performed within the preceding 12 months.

Reactor cattle are slaughtered separately at the end of the day to allow a more detailed inspection. If cattle are clinically healthy at slaughter, with no visible TB-like lesions postmortem, the carcass is considered suitable for human consumption, whether or not the animal was a reactor. There are no barriers for trade of this meat within Ireland or indeed the EU (150).

Where TB lesions are identified in only one part of the carcass, that portion of the carcass is deemed unfit for human consumption, however the rest of the carcass enters the food chain. If lesions are found in two or more areas of the carcass, then the entire carcass is deemed unfit for human consumption. Oftentimes, TB lesions are limited to the lymphatic nodes within the head, thorax and abdomen. Less commonly organs such as the liver, lungs, spleen, kidneys and mammary gland may be involved (150). Involvement of the muscle mass is much rarer and typically only encountered in advanced disease. The FSAI concluded that the occurrence of viable *M. bovis* in the muscle mass of cattle is uncommon and that the current safeguards in place are adequate to protect public health, however, shall remain under review(150).

The EFSA published a scientific report in June 2013 on meat inspection which concluded that the risk of *M. bovis* infection in humans from meat was negligible as it was not considered to be a meat-borne pathogen(153).

4.1.2.2 Improving Animal Health:

Healthier animals ultimately lead to a safer and healthier food supply. Increased herd size and increased cattle age are known risk factors for *M. bovis* infection (154, 155). Poor nutrition in cattle can increase their susceptibility to infection by suppressing the cellular immune response(155).

Local bTB prevalence impacts on herd risk of bTB. bTB is more likely to occur among groups/clusters of herds, rather than among isolated herds, and contact with an adjacent herd increases the risk of infection (155). Herd bTB history and the introduction of new cattle from an outside source to a herd are also risk factors for infection(139). Of note, there is substantial movements of herds across Ireland as animals are bought or sold(156).

The dairy sector in Ireland has expanded since the removal of milk quotas in 2015(139). This expansion has been associated with an increase in herd bTB prevalence (139). Dairy herds have a higher incidence of *M. bovis* infection than beef herds(155). The physiological stress and resultant immunosuppression in dairy animals may also contribute to the risk *M. bovis* infection (154).

Good hygiene and management may reduce risk of bTB. Intensive management practices where there is increased contact between cattle within a herd is associated with an increase in bTB incidents (155). Cattle housed in cubical housing or covered yards are at increased risk of disease

(155). Rough grazing is also associated with disease, as silage can attract badgers which may act as a reservoir for infection(155).

A review by Allen *et al* identified several factors potentially contributing to the persistence of bTB in Ireland and the UK(157). Issues identified included increased reservoir population density (i.e badgers), farming practices that are potentially conducive to infection, such as large herd sizes, herd density and significant cross border cattle movement and the potential role of the climate and of environmental contamination in sustained infection cycles of bTB within the UK and Ireland.

The proximity of domestic pets to infected wildlife and farmed animals is also a concern, particularly where the animal may be immunosuppressed due to novel immunosuppressive treatments as described by Ottewill *et al(109)*. Domestic farm pets may also be at risk of developing *M. bovis* infection, raising the question of should they be screened for disease when a herd breakdown occurs on their farm?

4.1.2.3 Reducing Risk to Humans:

Occupational exposure and immunosuppression appear, from this research, to be key risk factors for the development of *M. bovis* infection in Ireland. The Roadmap for Zoonotic TB highlights farmers, veterinarians, abattoir workers, butchers and livestock traders as being at increased risk of disease.

M. bovis infection due to occupational exposure is most likely to occur as the result of inhalation or via the handling of infected animals(8). *M. bovis*

is classified as a Class 3 biological agent. This is defined as an agent that can cause severe human disease and presents a serious hazard to workers with a risk of spread to the community, but there is usually effective prophylaxis or treatment available(158).

EU Directive 2000/54/EC, transposed into Irish legislation by the Safety, Health and Welfare at Work (Biological Agents) Regulations 2013, sets out minimum requirements for the health and safety of workers exposed to biological agents (including zoonoses) at work. Employers have a duty to mitigate risks posed by biological agents, as well as to inform their workers of any risks associated with their occupation. Employers must carry out a risk assessment, prevent or reduce exposure to biological agents, provide adequate training and PPE and make effective vaccines available(159). Where an employee is found to have illness due to an occupational exposure, health surveillance should be made available to all other employees(159).

According to UK Public Health guidance, veterinary surgeons and abattoir workers are recommended to receive BCG vaccination if they are previously unvaccinated, tuberculin-negative or interferon-gamma test negative and aged under 35 years (32). Where exposure to a TB infected animal occurs in an abattoir, meat factory workers in the UK are generally not considered “contacts” as they would not have had regular close contact with an infected animal(8). Strict hygiene practices should be in place in slaughterhouses to avoid accidental ingestion, inoculation or inhalation of pathogens(8). Scratches and cuts should be covered, good

hand hygiene enforced before eating or smoking and appropriate PPE worn(8). In the UK, where an employee has been diagnosed with *M. bovis* infection, with possible occupational origin, the employer has a legal duty to report to the Health and Safety Executive(8).

The 2010 HPSC Guidelines on the Prevention and Control of Tuberculosis in Ireland recommend that unvaccinated, TST negative abattoir workers under the age of 35 years should be offered BCG vaccination(25).

Of note, in Ireland the Health Service Authority (HSA) has not issued any specific safety guidelines for meat factory workers, an issue raised by the Migrant Rights Centre Ireland's report on the industry in 2020(145).

Given a number of recent cases of *M. bovis* infection among meat factory workers in Ireland(147), as demonstrated in this study, perhaps it is time to focus on ways to mitigate this risk within this potentially vulnerable population. Increasing awareness of disease, pre-employment health checks and vaccination (where appropriate) and regular screening for latent infection in those immunosuppressed workers at increased risk of disease could be of benefit. The introduction, in Ireland, of specific safety guidelines for this workforce would also likely be beneficial.

In the UK, the National Institute for Health and Care Excellence (NICE) recommend that where *M. bovis* in confirmed in a cattle herd or other domestic animals, TST and IGRA screening are offered to unvaccinated children under 16 years of age who have regularly consumed unpasteurised milk from animals with udder TB lesions(160). Treatment for latent TB is offered to those with a positive result. Individuals with

significant exposure and within a risk group should also be assessed and screened. Groups of people considered at increased risk of TB include those: with HIV infection, previous solid organ transplant, haematological malignancy, chronic renal failure or on dialysis, those receiving TNF alpha inhibitors, injecting drug users, previous jejunoileal bypass or gastrectomy surgery and those with silicosis(160).

Furthermore, in the UK “Inform and advise” information is given to all other individuals who have been in contact with animals with TB infection who do not fall into the above risk categories. Any symptomatic contacts should be referred to the TB Clinic for further investigations(160).

In Ireland, screening is offered to unvaccinated, TST negative children under 16 years who have frequent exposure to unpasteurised milk from cattle with TB udder lesions(25). All other contacts with diseased animals are informed of the signs and symptoms of TB infection(25). Currently, in Ireland, HIV positive patients and those going on immunosuppressive therapy, particularly TNF-alpha inhibitors, are commenced on Latent TB infection treatment. Perhaps given increased risk of severe disease among the immunosuppressed, more emphasis should be placed on the screening of those at-risk groups as highlighted in the NICE guidelines.

4.1.3 Strengthening Intersectoral and Collaborative Approaches:

The Roadmap for Zoonotic TB highlights the following priorities in this area: i) increase awareness, engagement and collaboration, ii) develop policies and guidelines, iii) implement joint interventions, iv) advocate for investment.

Coordination and communication across sectors is important for understanding transmission dynamics between human and animals. Sharing of information allows identification of patients in a particular geographical area at risk of infection and facilitating responses to prevent and control disease spread(13).

Collaboration between UCD, DAFM, HPSC and the IMRL allowed the combination of multiple data sets to enhance this study's findings. It is hoped that these ties will continue to strengthen following the completion of this project and that data and expertise will continue to be shared between the IMRL and DAFM to further enhance our understanding of bTB in Ireland.

Ongoing input from our colleagues in public health is essential to act on trends or potential outbreaks detected by ongoing epidemiologic and WGS analysis of *M. bovis* infection in both animals and humans. The Guidelines for the Prevention and Control of TB Ireland were published by the HPSC in 2010 and updated in 2014. With the changing landscape of not just *M. bovis* in Ireland, but also *M. Tuberculosis,* further updates within this document are needed.

Input is also needed from our occupational health colleagues and those physicians prescribing important immunomodulatory medications. Those with occupational risk factors for infection should be explained the risk of immunosuppression particularly within the setting of zoonotic disease exposure. This not only applies to bTB but also to other zoonotic infections including *Salmonella, Brucella, Listeria, E. coli* and *Campylobacter.*

4.2 Conclusions and Recommendations:

In conclusion, this study suggests that:

i) There is likely ongoing transmission of *M. bovis* between animals, between animals (including domestic pets) and humans, and potentially between humans.

ii) Human *M. bovis* infection mayno longer be primarily attributable to reactivation of latent disease due to remote exposure to infection prior to the widespread pasteurisation of dairy produce.

iii) The epidemiology of human *M. bovis* infection appears to be changing over time. Patients are now younger, have an increased number of comorbidities and are acquiring and potentially transmitting infection via the respiratory route.

iv) Occupational exposure and immunosuppression appear to be key risk factors for the development of *M. bovis* infection in Ireland.

v) The sharing of *M. bovis* genomic databases between the IMRL, the UCD Veterinary Medicine Department and the DAFM, greatly enhances our understanding of *M. bovis* transmission in Ireland.

Of note, this study has a number of limitations. For one, the number of patients included in the study was low and access to clinical data was limited. Secondly, due to low patient numbers and limited clinical data it was not possible to perform extensive statistical analysis of our data.

Finally, *M. tuberculosis* reference genome was used for mapping rather than an *M. bovis* genome which may impact on the interpretation of SNP distances between *M. bovis* isolates.

With the above caveats in mind, recommendations arising from this study include:

Recommendation One: Irish Guidelines on Prevention and Control of TB in Ireland should be updated given the apparent changing epidemiology of disease in Ireland.

Recommendation Two: Specific HSA guidance should be issued to address the risk of disease among meat factory workers who may be exposed to infected carcases. Increasing awareness of the disease, pre-employment health checks, vaccination (where appropriate) and regular screening for latent infection in those immunosuppressed workers at increased risk of disease could be of benefit.

Recommendation Three: Promote a "One Health" approach involving animal and human health sectors. This includes continuing intersectoral collaboration between the IMRL, the UCD Veterinary Medicine Department and the DAFM. The sharing of *M. bovis* genomic databases between these services has contributed greatly to our understanding of *M. bovis* transmission in Ireland in this study.

Recommendation Four: Prospective studies are needed in this area to further understand the epidemiological links between animal and human cases of *M. bovis* infection. Efforts should be made to prospectively consent patients for use of their personal metadata along with their

isolates WGS data to allow further analysis of potential transmission events. With a growing repository of DAFM animal *M. bovis* genomic sequences and IMRL human *M. bovis* genomic sequences this will prove an invaluable resource to further understand and thus be able to better control zoonotic disease.

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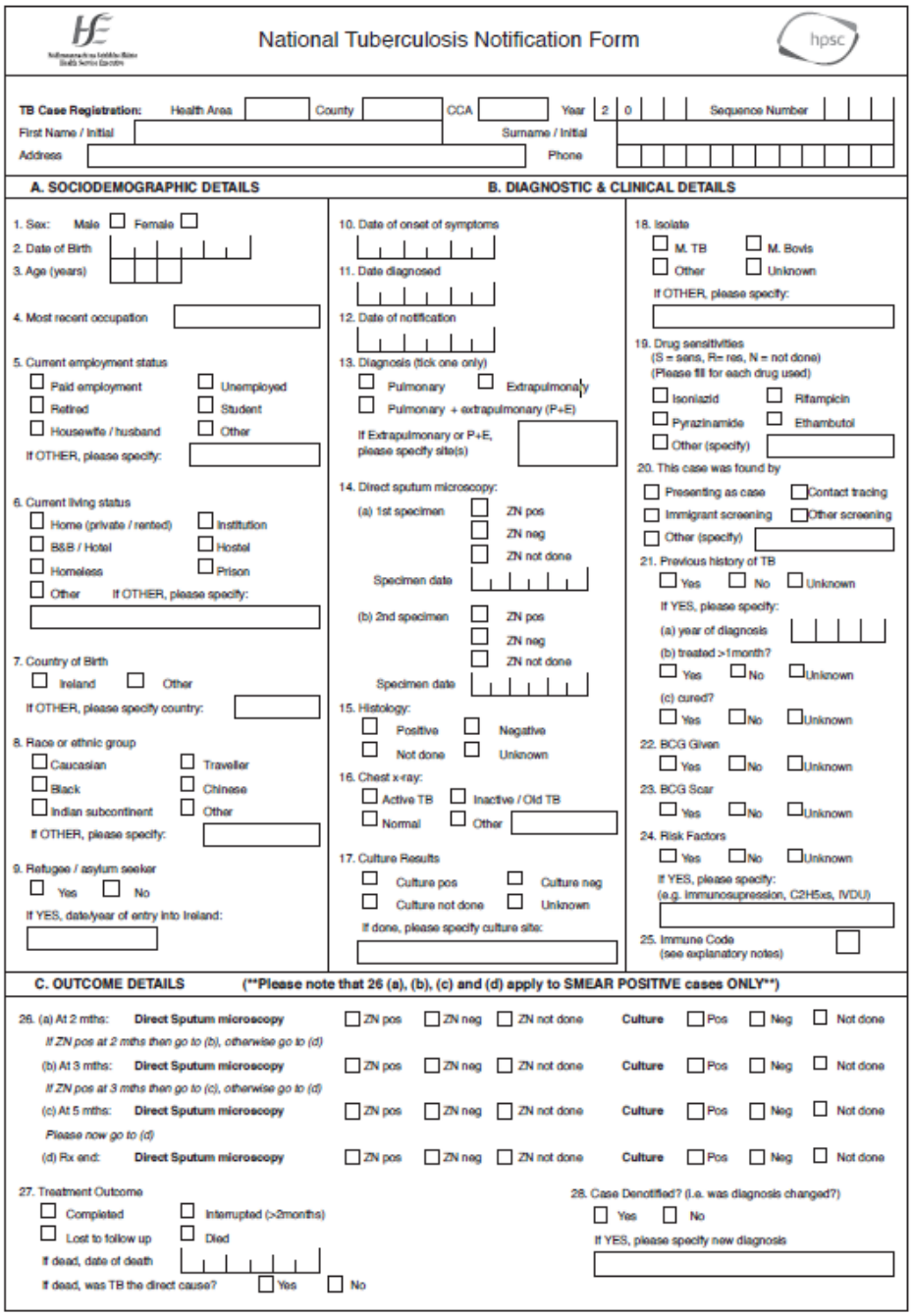
160. Publich Health England. Bovine tuberculosis: Guidance on management of the public health consequences of tuberculosis in cattle and other animals (England). 2014.

Appendices:

Appendix 1:

Tuberculosis Notification Form



National Tuberculosis Notification Form

hpsc

TB Case Registration: Health Area | County | CCA | Year 2 0 | Sequence Number

First Name / Initial | Surname / Initial

Address | Phone

A. SOCIODEMOGRAPHIC DETAILS

1. Sex: Male ☐ Female ☐
2. Date of Birth
3. Age (years)
4. Most recent occupation
5. Current employment status
   - ☐ Paid employment ☐ Unemployed
   - ☐ Retired ☐ Student
   - ☐ Housewife / husband ☐ Other

   If OTHER, please specify:
6. Current living status
   - ☐ Home (private / rented) ☐ Institution
   - ☐ B&B / Hotel ☐ Hostel
   - ☐ Homeless ☐ Prison
   - ☐ Other If OTHER, please specify:
7. Country of Birth
   - ☐ Ireland ☐ Other

   If OTHER, please specify country:
8. Race or ethnic group
   - ☐ Caucasian ☐ Traveller
   - ☐ Black ☐ Chinese
   - ☐ Indian subcontinent ☐ Other

   If OTHER, please specify:
9. Refugee / asylum seeker
   - ☐ Yes ☐ No

   If YES, date/year of entry into Ireland:

B. DIAGNOSTIC & CLINICAL DETAILS

10. Date of onset of symptoms
11. Date diagnosed
12. Date of notification
13. Diagnosis (tick one only)
    - ☐ Pulmonary ☐ Extrapulmonary
    - ☐ Pulmonary + extrapulmonary (P+E)

    If Extrapulmonary or P+E, please specify site(s)
14. Direct sputum microscopy:
    - (a) 1st specimen ☐ ZN pos ☐ ZN neg ☐ ZN not done

      Specimen date
    - (b) 2nd specimen ☐ ZN pos ☐ ZN neg ☐ ZN not done

      Specimen date
15. Histology:
    - ☐ Positive ☐ Negative
    - ☐ Not done ☐ Unknown
16. Chest x-ray:
    - ☐ Active TB ☐ Inactive / Old TB
    - ☐ Normal ☐ Other
17. Culture Results
    - ☐ Culture pos ☐ Culture neg
    - ☐ Culture not done ☐ Unknown

    If done, please specify culture site:
18. Isolate
    - ☐ M. TB ☐ M. Bovis
    - ☐ Other ☐ Unknown

    If OTHER, please specify:
19. Drug sensitivities
    (S = sens, R= res, N = not done)
    (Please fill for each drug used)
    - ☐ Isoniazid ☐ Rifampicin
    - ☐ Pyrazinamide ☐ Ethambutol
    - ☐ Other (specify)
20. This case was found by
    - ☐ Presenting as case ☐ Contact tracing
    - ☐ Immigrant screening ☐ Other screening
    - ☐ Other (specify)
21. Previous history of TB
    - ☐ Yes ☐ No ☐ Unknown

    If YES, please specify:
    - (a) year of diagnosis
    - (b) treated >1month?
      ☐ Yes ☐ No ☐ Unknown
    - (c) cured?
      ☐ Yes ☐ No ☐ Unknown
22. BCG Given
    ☐ Yes ☐ No ☐ Unknown
23. BCG Scar
    ☐ Yes ☐ No ☐ Unknown
24. Risk Factors
    ☐ Yes ☐ No ☐ Unknown

    If YES, please specify:
    (e.g. immunosupression, C2H5xs, IVDU)
25. Immune Code
    (see explanatory notes) ☐

C. OUTCOME DETAILS (**Please note that 26 (a), (b), (c) and (d) apply to SMEAR POSITIVE cases ONLY**)

26. (a) At 2 mths: **Direct Sputum microscopy** ☐ ZN pos ☐ ZN neg ☐ ZN not done **Culture** ☐ Pos ☐ Neg ☐ Not done

    If ZN pos at 2 mths then go to (b), otherwise go to (d)

    (b) At 3 mths: **Direct Sputum microscopy** ☐ ZN pos ☐ ZN neg ☐ ZN not done **Culture** ☐ Pos ☐ Neg ☐ Not done

    If ZN pos at 3 mths then go to (c), otherwise go to (d)

    (c) At 5 mths: **Direct Sputum microscopy** ☐ ZN pos ☐ ZN neg ☐ ZN not done **Culture** ☐ Pos ☐ Neg ☐ Not done

    Please now go to (d)

    (d) Rx end: **Direct Sputum microscopy** ☐ ZN pos ☐ ZN neg ☐ ZN not done **Culture** ☐ Pos ☐ Neg ☐ Not done
27. Treatment Outcome
    - ☐ Completed ☐ Interrupted (>2months)
    - ☐ Lost to follow up ☐ Died

    If dead, date of death

    If dead, was TB the direct cause? ☐ Yes ☐ No
28. Case Denotified? (i.e. was diagnosis changed?)
    ☐ Yes ☐ No

    If YES, please specify new diagnosis

Appendix 2: Project Specific Privacy Notice – St James's Hospital Tuberculosis Clinic

Trinity College Dublin
Coláiste na Tríonóide, Baile Átha Cliath
The University of Dublin

ST JAMES'S HOSPITAL

Investigating the Epidemiology of *Mycobacterium bovis* Infection in Humans

We would like to draw your attention to a research study which is being carried out by St James's hospital and Trinity College Dublin, between May 2022 and May 2024

Bovine Tuberculosis, a form of TB caused by *Mycobacterium bovis*, was a common disease in Ireland prior to pasteurization of milk and programmes to eliminate this infection in cattle herds. Despite efforts to eradicate the disease in Ireland, human infection with *Mycobacterium bovis* remains a public health concern, with 7 cases diagnosed in Ireland in 2019.

We hope our study will help us to gain a better understanding of *Mycobacterium bovis* infection and its transmission in Ireland, in order to inform public health policy. This notice is being issued to explain how to find out more information about the research study.

Who does the study involve?
The study involves analysis of microbiological samples taken from 80 to100 hospital patients who were diagnosed with *Mycobacterium bovis* infection between 2000 and 2022 (inclusive) **and** had their specimens analysed by the Irish Mycobacteria Reference Laboratory (IMRL), located in SJH.

Study Design:
1) If you were diagnosed with Mycobacterium bovis infection in the time frame noted above, your samples may be anaylsed for this study, and your SJH patient chart reviewed, where available.
2) We will extract the bacteria itself - *Mycobacterium bovis* isolates from samples provided by patients at time of diagnosis, and this bacteria will undergo further testing with whole genome sequencing (WGS). WGS is a novel technology that allows a more detailed analysis of the bacterial genomic material and allows high resolution comparison with other strains of *M. bovis*.
3) All patient information will be fully and irrevocably anonymised following analysis of the data.
4) You will not be contacted for any further information or to provide any further specimens.

There is a strong public interest in carrying out this research and the hospital has undertaken an assessment of any associated privacy risks posed to you, and taken steps to address them.

If you have been diagnosed with **bovine TB** and fit the above criteria, your samples and information may be included in this study. Please contact the researchers using the details provided below should you have any questions or concerns about this, or should you wish that your samples/information be excluded from this study. If you do not contact the study team to request not to be included, we will presume you are happy for your personal data and bio-samples to be used for this research study.

For further information please visit: www.stjames.ie/aboutus/privacynotice/

If you have any other questions or concerns about the content of this privacy notice, please contact the SJH Data Protection Officer (DPO):
Email: dataprotection@stjames.ie
Telephone: 01 410 3021

The project is a collaboration between researchers at St James's Hospital and Trinity College Dublin.

Appendix 3: Project Specific Privacy Notice – St James's Hospital Website

ST JAMES'S HOSPITAL

Trinity College Dublin
Coláiste na Tríonóide, Baile Átha Cliath
The University of Dublin

Privacy Notice

"Investigating the Epidemiology of *Mycobacterium bovis* infection in humans"

Introduction

We would like to draw your attention to a research study which is being carried out by St James's Hospital and Trinity College Dublin, between May 2022 and May 2024

Bovine Tuberculosis, a form of TB caused by *Mycobacterium bovis*, was a common disease in Ireland prior to pasteurization of milk and programmes to eliminate this infection in cattle herds. Despite efforts to eradicate the disease in Ireland, human infection with *Mycobacterium bovis* remains a public health concern, with 7 cases diagnosed in Ireland in 2019.

We hope the study will help us to gain a better understanding of *Mycobacterium bovis* infection and its transmission in Ireland to inform public health policy. This notice is being issued to explain how to find out more information about the research study.

This notice outlines the use of health information contained participants medical records for this study and the steps taken by St James's Hospital (SJH) and Trinity College Dublin (TCD), to protect your information and safeguard your rights under data protection legislation, (specifically the EU General Data Protection Regulation (GDPR) and Data Protection Acts 1988-2018).

SJH and TCD fully respect your right to privacy and actively seek to preserve your rights. Any personal information which is used for this study, will be treated with the highest standards of security and confidentiality, in accordance with data protection legislation.

In the following paragraphs we set out information in relation to the following:

- The Data Controllers.
- The Data Protection Officer (DPO).
- What Personal Data we collect.
- What we use your Personal Data for.
- How long will we hold onto your Personal Data.
- Who will have access to your Personal Data.
- International data transfers.

- Your rights.
- How we keep your Personal Data secure.
- Legal basis for processing Personal Data.
- Complaints.

The Data Controllers

SJH is the Data Controller of your medical records. SJH and TCD are joint controllers of the information taken from your medical records for this research project. A Data Controller is the legal entity which determines how and why personal data is collected and used.

The Data Protection Officer (DPO)

The hospital DPO can be contacted directly at dataprotection@stjames.ie or Tel: 01 410 3021.

What personal data is collected?

Personal data means any information about you which allows you to be identified. The study intends to use information taken from 80 to 100 patients who were diagnosed with *Mycobacterium bovis* infection between 2000 and 2020 **and** who had their microbiological specimens processed by the Irish Mycobacteria Reference Laboratory (IMRL), located in SJH. The IMRL receives microbiological specimens from hospitals across Ireland.

The following information will be accessed and shared for this study:

- Age category, gender, country of birth, co-morbidities, rural or urban dwelling, time since entry to Ireland if foreign born, occupation.
- Site of TB disease, previous history of TB diagnosis and use of immunomodulatory medications.
- Potential past exposures to *Mycobacterium bovis*: e.g. Contact with a human TB patient, travel (for >2 weeks) to or residence in a country with high TB incidence, consumption of unpasteurized milk product, occupational contact with animals, physical contactwith wild animals, pets and physical contact with any animal with TB.
- Your personal data will be **pseudonymised** during collection. This means any identifiers, e.g. name, DOB, address, will be removed and replaced with a code. The code will be kept securely by the research team until its deleted therefore fully anonymizing the data.

We will extract the bacteria itself*, Mycobacterium bovis,* from samples provided by patients at time of diagnosis, and undertake further testing with whole genome sequencing (WGS). WGS is a novel technology that allows a more detailed analysis of the bacterial genomic material and allows high resolution comparison with other strains of *M. bovis* to assess for any epidemiological links.

What we use your personal data for?

The data collected from you for this research study will only be used for this research study which we hope will allow us to better understand *Mycobacterium bovis* infection in Ireland, and why there has been a rise in human *M. bovis* infections in recent years.

How long will we hold onto your personal data?

Your personal data will be retained for 2 years during the collection and analysis phase of the study prior to being anonymized. All patient information will be fully and irrevocably anonymised following analysis of the data.

Who will have access to your personal data?

Your personal data will only be made available to the following individuals:

- M.D Candidate: Dr Mary Mansfield
- Project Investigators: Prof Tom Rogers and Dr Margaret Fitzgibbon
- IMRL Scientists: Dr Peter Flanagan and Dr Emma Roycroft

Will my personal data be transferred outside of the European Economic Area (EEA)?

No – We do not intend to transfer any personal data collected for the purpose of this study outside the EEA.

What are your Rights

If you have been diagnosed with **bovine TB** and fit criteria outlined above, your samples and information may be included in this study. Please contact us using the details provided should you have any questions or concerns about this, or should you wish that your samples/information be excluded from this study. If you do not contact the hospital to request not to be included, we will presume you are happy for your personal data and bio-samples to be used for this research study.

Under certain circumstances, by law you have the right to:

- **Right of access** – you have the right to request a copy of the information that we hold about you.
- **Right of rectification** – you have a right to correct data that we hold about you that is inaccurate or incomplete.
- **Right to be forgotten** – in certain circumstances, you can ask for the data we hold about you to be erased
- **Right to restriction of processing** – for example, if the information we have on file is inaccurate
- **Right to object** – you have the right to object to our use of your information for this research study;

If you wish to exercise any of these rights, then please submit a request to aio@stjames.ie. When submitting a request, the hospital will need information from you to confirm your identity.

How do we keep your personal data secure?

Personal data is stored confidentially and securely as required by the ICT Security Policy and Data Protection Policy. SJH is committed to ensuring that your data is safeguarded by appropriate technical and organisational security measures relevant to the processing in accordance with Article 32 GDPR requirements. When we store your personal data on our systems the data will be stored either

on SJH premises or on secure IT platforms within the EEA.

What is the legal basis for processing?

We rely on this research being in the public interest (Article 6(1)(e) & 9(2)(j) of the GDPR.

Changes to this Privacy Notice

This Privacy Notice may change from time to time and any changes to the Notice will be communicated by way of a notice on the internet/intranet and public notice boards in the hospital.

Making a complaint

In the event that you wish to make a complaint about how your personal data is being processed by us for this research project or how your complaint has been handled, you have the right to lodge a complaint directly with the Data Protection Commission.