Cronobacter species

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Introduction

The newly designated genus, *Cronobacter,* is composed of Gram-negative, facultative anaerobic rods which are members of the Enterobacteriaceae family and closely related to *Enterobacter* and *Citrobacter*. It is composed of *C. sakazakii, C. malonaticus, C. turicensis, C. muytjensii* and *C. dublinensis,* plus an unnamed sixth genome species. The organism has come to prominence due to its association with severe neonatal infections; necrotizing enterocolitis, septicaemia and meningitis, which can be fatal. However, these are rare in infants, and infections occur in all age groups, though fortunately with less severe clinical outcomes. This article reviews various aspects of *Cronobacter,* including its physiology, virulence, detection and control.[1]

Nomenclature and taxonomy

Initially, *Cronobacter* genus was defined as the species *Enterobacter sakazakii* by Farmer *et al.* in 1980 to honour the Japanese bacteriologist Riichi Sakazaki. They used DNA-DNA hybridization to show that yellow-pigmented *Enterobacter cloacae* were only 41% and 54% related to *Citrobacter freundii* and *E. cloacae,* respectively. These were used as representatives of the *Citrobacter* and *Enterobacter* genera, and the results warranted the organism being recognized as a separate species. As they were phenotypically closer to *E. cloacae*, they were kept in the *Enterobacter* genus. Further phenotypic analysis led to the description of 15 *E. sakazakii* biogroups, with biotype 1 being the most common. However, since the 1980s, bacterial systematics has increasingly used DNA sequencing for its analysis and for determining relatedness. Analysis of both partial 16S rDNA and hsp60 gene sequences showed that *E. sakazakii* isolates formed at least four distinct genomogroups which could be unique species. However, such a taxonomic revision required considerable further analysis for substantiation. The *Cronobacter* genus was defined first in 2007 and revised in 2008. Differentiation between the newly defined *Cronobacter* spp. is primarily based on genotypic (DNA-based) analysis and is largely supported by biochemical traits *(Table 1)*. With a

few exceptions, the former biotypes and genomogroups corresponded with the new species; as shown in *Table 1*. The division between *C. sakazakii* and *C. malonaticus* has been problematic, as the organisms are so closely related that differentiation was not always feasible, even using 16S sequencing. The definition of the two species does include the biotype profiles and recently has been re-examined using multilocus sequence typing (MLST) based on 7 housekeeping genes. Some confusion between the species may have been caused by the previous use of biotyping, with the results that a few biotype index strains were assigned the wrong species. MLST has also revealed a strong clonal nature in these species, as will be considered later.[2]

Since members of the *Cronobacter* genus were formerly known as the single species *Enterobacter sakazakii,* this name was used in publications before mid-2007. Unfortunately, there is now some uncertainty which *Cronobacter* species was referred to in many of these publications, as the strains had been identified using phenotyping rather than genotyping. However, the majority of isolated strains are usually *C. sakazakii,* and it is probable that this has been the species of major study to date.

Source

It is the occurrence of the organism in powdered infant formula which has been highlighted; however, *Cronobacter* is ubiquitous. It has been isolated from a wide range of sources, and asymptomatic human carriage has also been reported. One probable niche for *Cronobacter* is plant material, as it has been isolated from cereals, wheat, corn, soy, rice, herbs and spices,

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Table 1: *Cronobacter* spp. groupings

Cronobacter spp.	16S cluster group	Biotypes	MLST sequence type
C. sakazakii	1	1, 2, 3, 4, 7, 8, 11,13	1, 3, 4, 8, 9, 12-18
C. malonaticus	1	5, 9,14	7,10,11
C. turicensis	2	16	-
C. muytjensii	3	15	-
C. dublinensis	4	6,10,12	-
Genomospecies 1	4	16	-

vegetables, and salads. Rats and flies may be additional sources of contamination. The organism has been isolated from a range of other foods, including cheese, meats, milk powder, powdered infant formula and a large number of food ingredients. It is notable that, when *E. sakazakii* was defined, it included a strain which had been isolated from dried milk in 1960. Therefore *Cronobacter* has been present in dried milk products for many decades. As will be covered later, early surveillance studies used methods which probably under-estimated the organism's prevalence and concentration in food. The bacterium has been isolated from the hospital environment and clinical samples, including cerebrospinal fluid, blood, bone marrow, sputum, urine, inflamed appendix, neonatal enteral feeding tubes and conjunctivae.[3,4]

Physiology and growth requirements

Cronobacter can grow over a wide temperature range. The lowest is near refrigeration (~5°C) and the maximum growth temperature (44-47°C) is strain dependent. Due to interest in the organism and infant infections, growth and death rates have been determined for the five species associated with reconstituted infant formula. At room temperature (21°C), *Cronobacter* had a doubling time of 40-94 minutes. Decimal reduction times and z-values varied considerably between strains, i.e. D_{55} 2-49 minutes, z-values 2-14°C. Early studies indicated the organism was very thermotolerant. However, subsequent work clarified that the organism was less thermotolerant than *L. monocytogenes*. Nevertheless, the organism can survive spray drying. The organism's tolerance to drying has been well noted, and it can survive for two years desiccated in infant formula and then grow rapidly on reconstitution.

Cronobacter strains grow well at 37°C on nonselective media as well as the selective media, VRBA, MacConkey Agar and Deoxycholate Agar. Some strains produce two colony types (glossy and matt). About 80% of strains produce a nondiffusible, yellow pigment on Tryptone Soya Agar at 25°C, and this led to the early description of 'yellow-pigmented *Enterobacter cloacae*'. Pigment production is temperature dependent, and even fewer strains produce it at 37°C. The organism probably colonizes plant material, and the yellow carotenoid-based pigmentation may protect it from sunlight-generated oxygen radicals. The organism often produces a novel heteropolysaccharide comprising glucuronic acid, D-glucose, D-galactose, D-fucose and D-mannose. This material has been patented for use as a thickening agent in foods. This capsular material, induced under nitrogen-limited conditions, could facilitate the organism's attachment to plant surfaces. Combined with a tolerance to desiccation, this gives the organism an armoury to colonize plant material and maybe survive harsh, environmental

(Courtesy of Nottingham Trent University)

Figure 1: False colour image of *Cronobacter sakazakii* attached to an enteral feeding tube.

conditions. However, these traits may also contribute to the organism's presence in starches used in the manufacture of infant formula and persistence during the manufacturing process. The organism attaches to surfaces, forming biofilms that are resistant to cleaning and disinfection agents, and it has also been found as part of the mixed flora biofilm in enteral feeding tubes from neonatal intensive care units *(Figure 1)*.

Cronobacter infections

Cronobacter infections are not unique to neonates. They occur in all age groups, albeit with a greater incidence in the more immunocompromised very young and elderly. Neonates, particularly those of low birth-weight, are the major identified group at risk, with a high mortality rate. In the USA, the reported *Cronobacter* infection incidence rate is ~1 per 100,000 infants. This incidence rate increases to 9.4 per 100,000 in infants of very low birth-weight, i.e. <1.5kg. The first neonatal deaths attributed to *Cronobacter* were in 1958. Since then, according to the FAO/WHO, around the world, there have been 120 documented cases of neonatal and infant *Cronobacter* infections and at least 27 deaths. This undoubtedly is an under-estimate.

Fatal infant infections have followed cases of necrotizing enterocolitis (NEC), septicaemia and meningitis. Infections in older age groups are principally bacteraemias as well as urosepsis and wound infections. Necrotizing enterocolitis is noninvasive (and is multifactorial), whereas in septicaemia and meningitis, the organism has attached and invaded, presumably through the intestinal epithelial layer. Due to the understandable sensitivity towards neonatal infections, this aspect of the bacterium has been emphasized more than infections in other age groups. Necrotizing enterocolitis is a common gastrointestinal illness in neonates and can be caused by a variety of bacterial pathogens. The incidence of NEC is 2-5% of premature infants and 13% in those weighing <1.5kg at birth. It is 10 times more common in infants fed formula milk compared with those fed breast milk. Necrotizing enterocolitis due to *Cronobacter* has a high mortality rate; 10-55% of cases.[5]

In *Cronobacter* meningitis, there is gross destruction of the brain, leading sadly to either death (40-80% of cases) or severe neurological damage. The pathogenesis of the meningitis is different to *Neisseria meningitidis* and neonatal meningitic *Escherichia coli,* and is similar to that of the closely related bacterium, *Citrobacter koseri*. A number of outbreaks of *Cronobacter* spp. have been reported in neonatal intensive care units. Infections have been directly linked to reconstituted powdered infant formula which may have been contaminated intrinsically or during preparation and administration. A common feature in some of these outbreaks is the opportunity for temperature abuse of the prepared feed, which would permit bacterial growth. It is pertinent to note that the bacterium is isolated from the tracheae and has been recovered from the feeding tubes of neonates fed breast milk and ready-to-feed formula, not infant formula. Therefore, wider sources of the organism during an outbreak need to be investigated, not just the use of powdered infant formula. Infants can be colonized by more than one strain of *Cronobacter,* and therefore multiple isolates need to be characterized in epidemiological investigations.

Virulence mechanisms

Cronobacter are opportunistic pathogens, and as yet, few virulence factors have been identified. The organism can invade human

intestinal cells, replicate in macrophages and invade the blood-brain barrier, and this varies between species. Of particular interest are *C. sakazakii, C. malonaticus* and *C. turciensis,* which are the only *Cronobacter* spp. that have been isolated from neonatal infections. Based on the clinical outcome of an outbreak in France (1994), it was proposed that certain variants of *C. sakazakii* were more virulent, and this appears to have been confirmed by MLST. However, the basis of the variation in virulence is unknown. *Cronobacter* produce an enterotoxin, and as with neonatal meningitic *E. coli*, outer membrane protein A probably has a role in the organism penetrating the blood-brain barrier, though the mechanism leading to the destruction of the brain cells is unknown and could, in part, be a host response.

Detection and typing methods

Early methods for detection of *Cronobacter* were based on general Enterobacteriaceae isolation procedures. However, it is now recognized that these had their limitations due to assumptions regarding upper temperature ranges, pigment production and identification using phenotyping schemes. Current international methods use greatly improved procedures and are the result of inter-laboratory trials and a deeper knowledge of the organism. As previously covered, *Cronobacter* has a notable resistance to osmotic stresses. This physiological trait, which may be linked to its ecology, has also been used in the design of selective media. Modified Lauryl Sulphate Broth (0.5M NaCl) and Cronobacter Screening Broth (10% sucrose). The use of chromogenic media to differentiate *Cronobacter* from other Enterobacteriaceae present in the sample has greatlly improved the performance of newer isolation methods.

Unlike most other members of the Enterobacteriaceae, *Cronobacter* possess the enzyme α-glucosidase, and this is exploited as a diagnostic feature in chromogenic media. *Brilliance™* Enterobacter sakazakii Agar (DFI), developed by Druggan, Forsythe and Iversen, was the first medium to incorporate a substrate for this enzyme, 5-bromo-4-chloro-3-indolyl α-D-glucopyranoside (X-α-gluc). *Cronobacter* hydrolyze this colourless chromogen to produce characteristic blue/green colonies for presumptive identification on the plate. Sodium thiosulphate and ammonium iron (III) citrate are included to differentiate hydrogen sulphide-producing Enterobacteriaceae, such as *Salmonella*, which appear grey/brown. Enterobacter sakazakii Isolation Agar is recommended in ISO/TS 22964:2006(E) for the detection of *Cronobacter* in milk and milk products. It also uses X-α-gluc as a means to differentiate *Cronobacter* from Enterobacteriaceae, while sodium deoxycholate and crystal violet are incorporated to inhibit the growth of Gram-positive organisms.

Chromogenic Cronobacter Isolation (CCI) Agar has recently been developed for the detection of *Cronobacter* in food, animal feed and environmental samples and is based on *Brilliance* Enterobacter sakazakii Agar (DFI) formulation. Optimization of peptones reduces yellow pigment production by the *Cronobacter*, which can bleach colony coloration, and an increased chromogen concentration improves the detection of *Cronobacter* with weak α-glucosidase activity.

A number of methods for the recovery of *Cronobacter* from powdered infant formula have been developed. Since the organism is normally present at such low numbers (<1cfu/g), a large volume of material is tested. The Codex Alimentarius Commission (2008) requirement is to test 30 10g quantities. Therefore presence/absence testing is applied rather than direct enumeration. Due to the stressed state of the cells, the initial step involves resuscitation with Buffered Peptone Water, followed by enrichment, then plating on a differential (i.e. chromogenic) agar. Since numerous methods use phenotyping as the confirmation test, they are more applicable for *Cronobacter* genus detection rather than any specific species. Commercial companies producing phenotyping kits have been updating their databases due to the taxonomic revision, for example, the former *E. sakazakii* Preceptrol™ strain ATCC® 51329 has been reclassified as *C. muytjensii* not *C. sakazakii*. The range of some previous *E. sakazakii* DNA-based PCR probes to the *Cronobacter* spp. need to be re-evaluated, meanwhile new species-specific *Cronobacter* probes have also been developed. The FDA method has been revised with the inclusion of chromogenic media, and the revision of the ISO method is underway.

Since the organism is ubiquitous, typing schemes are required both for epidemiological and environmental investigation. As given above, initially, 15 biogroups of *Cronobacter* were defined, with biogroup 1 being the most common. These divisions, however, are not specific enough for epidemiological investigations. Instead, initial procedures used plasmid profiling, chromosomal restriction endonuclease analysis and multilocus enzyme electrophoresis. This was followed by the application of Random Amplified Polymorphic DNA (RAPD) ribotyping and, more recently, pulsed-field gel electrophoresis (PFGE), and Multiple-Locus Variable-number tandem repeat Analysis (MLVA). To date, PFGE with two restriction enzymes (*Xba*1 and *Spe*1) is the most common method. The technique is widely employed and can be used for transnational investigations, as per PulseNet, since the gel results can be electronically analyzed.

Typing *Cronobacter* to understand its diversity has led to the development of a multilocus sequence typing (MLST) scheme which is available online (www.pubMLST.org/cronobacter/). The scheme for *C. sakazakii* revealed stable clones, some of which could be traced over a 50 year period, from a wide range of countries and sources. Of particular interest were three sequence types. One (ST8) was primarily composed of clinical isolates, another (ST4) was a mixture of clinical and powdered infant formula isolates, and the remaining type was principally nonclinical in origin (ST3). This may reflect different ecologies of the organism. The scheme will be of considerable use in the future for choosing representative *Cronobacter* strains when undertaking further studies.

Control measures

The International Commission for Microbiological Specifications for Foods has ranked *Cronobacter* as 'severe hazard for restricted populations, life threatening or substantial chronic sequelae or long duration'. Subsequently, it has been given the same ranking as more familiar food and waterborne pathogens, such as *Listeria monocytogenes, Clostridium botulinum* types A and B and *Cryptosporidium parvum*. As referred to above, although the bacterium is ubiquitous, due to the limited routes of infection, controlling neonate infections has focussed on the microbiological quality of powdered infant formula, especially breast milk substitutes. Powdered infant formulas are not necessarily sterile but conform to international microbiological specification guidelines. Due to the raised awareness of the organism, the guidelines from 1979 were revised by the Codex Alimentarius Commission in 2008

for products with an intended use with infants aged 0-6 months. In the earlier 1979 guidelines, *Salmonella* was the only named pathogen requiring testing in powdered infant formula (sampling plan n=60 c=0 m=0/25g), as well as sampling plans for coliforms (n=5 c=1 m=<3 M=20). The 2008 revision has *Cronobacter* spp. also specified (n=30 c=0 m=0/10g). The microbiological criteria have not been applied to follow-on formulas. These decisions were reached in part following a number of expert meetings, including three by the Food and Agriculture Organization/World Health Organization (FAO/WHO) in 2004 (Geneva), 2006 (Rome) and 2008 (Washington).

Spray-drying does not act as a sterilizing step as the organism can survive the drying process. Nevertheless, there will be a considerable reduction in viability, and the surviving cells may be severely damaged. As well as testing the powdered formula, environmental samples are taken from the production environment as well as from ingredients (especially starches and plant-derived material). In addition, production facilities and processes are designed to control enteric pathogens, especially *Salmonella*.

The first and second FAO/WHO meetings reviewed the organisms associated with neonatal infections, those found in powdered infant formula and also those that had been epidemiologically linked. Subsequently, *Salmonella* and *Cronobacter* were designated Category A (Clear evidence of causality), and other named Enterobacteriaceae and *Acinetobacter* were in Category B (Causality plausible, but not yet demonstrated). The prevalence of *Cronobacter* in powdered infant formula has been determined many times and varies between 2-14%. There are no published reports of *Cronobacter* in powdered infant formula exceeding 1 cell/g. In fact the likely level is ~1 cell/100g. Hence the need to consider opportunities for extrinsic bacterial contamination and multiplication.[6,7]

In order to reduce the number of intrinsic bacteria and limit bacterial growth, the FAO/WHO (2004 and 2006) expert committees proposed that powdered formula be reconstituted at temperatures no cooler than 70°C, and that it is used immediately (within 3h) rather than stored. As stated earlier, a common feature in a number of outbreaks has been a lack of adequate hygienic preparation and temperature control of the reconstituted infant formula.

A second outcome from the FAO/WHO meetings was the production of an online risk model; http:// www.mramodels.org/ESAK/default.aspx. The model allows the user to compare the level of risk between different levels of contamination and reconstitution practices. The model was based on growth and death kinetic data for a limited number of *Cronobacter* strains and has recently been extended to cover all organisms in Categories A & B; *Cronobacter* spp., *Salmonella*, other named Enterobacteriaceae and *Acinetobacter* spp.[8]

It should be noted that not all neonate infections have been linked to contaminated powdered infant formula. Breast milk can also contain the bacterium, and the *C. malonaticus* type strain was isolated from a breast abscess. The organism has also been isolated from hospital air, human intestines and throats. So, control of microbiological content of powdered infant formula will not necessarily totally remove the risk of neonate infection by this bacterium.

Final comments
The FAO/WHO 2004 expert committee recommended that research should be promoted to gain a better understanding of the ecology, taxonomy, virulence and other characteristics of *Cronobacter*. This has largely been undertaken by groups around the world. By understanding the organism better, improved detection systems have been designed and commercialized.

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Recommended reading

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Note: Readers should read the original articles for matters relating to international policies, and scientific details as these have been necessarily summarized in the article above. For a full copy of this article with associated references please email: stephen.forsythe@ntu.ac.uk.

Mycobacterium bovis in cattle: here today, where tomorrow?

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Mycobacterium bovis, the causative bacterium of bovine tuberculosis (bTB), is a member of the *Mycobacterium tuberculosis* complex (MTC) of bacterial strains, including at least seven species and subspecies, which cause similar disease in mammalian hosts. Cattle are considered to be the true hosts of *M. bovis*, but this bacterium causes disease in a broad mammalian host range, including humans. In Great Britain (GB), there are over 8000 new human cases of tuberculosis each year. Around 99% are the result of infection by the specifically human-adapted *M. tuberculosis*, with only 24 cases (0.5% of the culture-confirmed cases) attributable to *M. bovis* in 2007, a trend that has remained stable, if not declined, for the last ten years[1]. However, prior to the gradual introduction of wide-scale milk pasteurization in the 1930s, it was reported that there were approximately 2500 deaths annually in GB due to bTB. Indeed, most cases of human *M. bovis* infection in GB today are still being diagnosed in individuals born in the UK before 1960. In humans, *M. bovis* may cause tuberculosis of essentially the same symptoms and pathology as that caused by *M. tuberculosis*. The two diseases can be distinguished only by bacterial culture or molecular characterization of the isolated micro-organism.

Today, the overall risk to human health posed by *M. bovis* in GB remains low due to the cattle test and slaughter programme, milk pasteurization and meat inspection. Specific groups, such as farmers, animal handlers, vets and those who regularly consume raw milk products, are at some risk, but without these controls, *M. bovis* could once again present a significant public health problem. However, the impact of *M. bovis* on the GB cattle population is an entirely different matter. Bovine TB remains one of the most difficult animal health problems facing the farming industry today. There were some 5000 new incidents (breakdowns) of bTB in GB herds in 2008, an increase of 19.6% on those recorded in 2007. Post-mortem or culture evidence of *M. bovis* infection was detected in about 55% of these incidents, the so-called "confirmed" breakdowns. Just over 40,000 animals were slaughtered under the control programme in 2008[1]. The burden of this infection is carried largely in herds in the regions of the South West and West of England and Wales (*Figure 1*), but spread from these endemic areas into neighbouring parts of the North and East regions has been observed over recent years. Scotland is the only region of GB which is officially recognized as bovine tuberculosis free by the European Commission.

The control of bovine TB in cattle
The cattle test and slaughter scheme
Compulsory routine herd tests for bTB are carried out at a frequency of between 1-4 years, depending on the local incidence of confirmed herd breakdowns. The routine screening test is the SICCT (single intradermal comparative cervical test), commonly referred to as the 'skin test' or 'tuberculin skin test' and broadly similar to the Mantoux and Heaf skin tests used for TB screening in humans. The testing procedure involves the simultaneous injection of a small amount of *M. bovis* and *M. avium* tuberculins (purified protein derivative (PPD); a crude extract of bacterial cell wall antigens) into two sites of the skin of the animal's neck, followed by a comparative measurement of any swelling (delayed-type hypersensitivity reaction) which develops at the two injection sites after 72 hours. If the swelling caused by the *M. bovis* tuberculin is larger than that caused by the *M. avium* tuberculin by a defined amount, the animal is considered a 'reactor' and compulsorily slaughtered.

The use of the comparative measurement increases the specificity of the test, and thus reduces the number of false positives due to exposure to environmental mycobacteria, which may cross-react with *M. bovis*. However, when post-mortem tests confirm the presence of *M. bovis* infection in at least one skin test reactor, the original and subsequent skin tests are read under a more severe interpretation to enhance the sensitivity of the testing regime, at the expense of specificity. Cattle herds in which reactors are disclosed are placed under movement restrictions until no further reactors are identified at retests.

The gamma-interferon test, an *in vitro* test which also measures a cell-mediated immunity against *M. bovis*, is applied to SICCT-negative animals, in specific circumstances, to maximize the probability of detecting any infected animals which for any reason escape detection by the SICCT. It can be applied in herds in high risk areas suffering from a chronic breakdown, in attempt to clear infection, and in herds in low risk areas, in an attempt to rapidly remove infection and prevent local disease establishment.
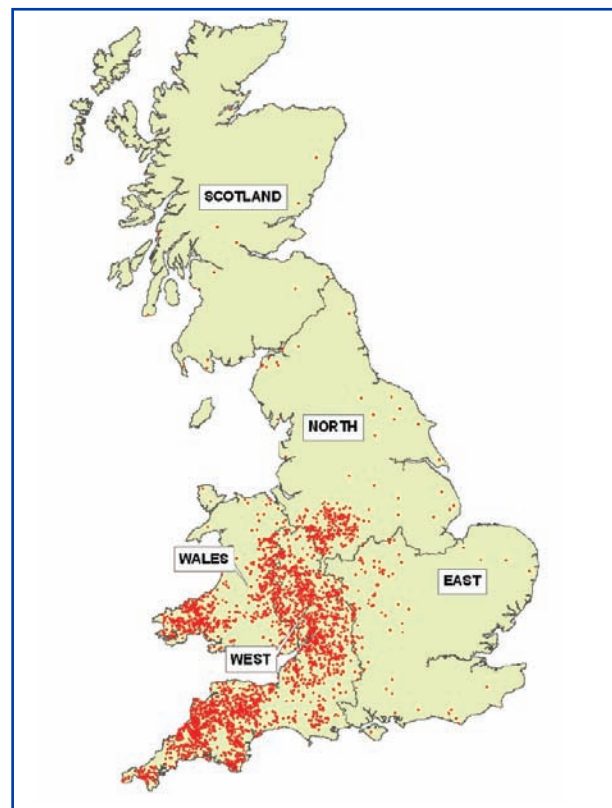

Figure 1. The geographic distribution of confirmed bovine TB incidents (red) in Great Britain in 2008
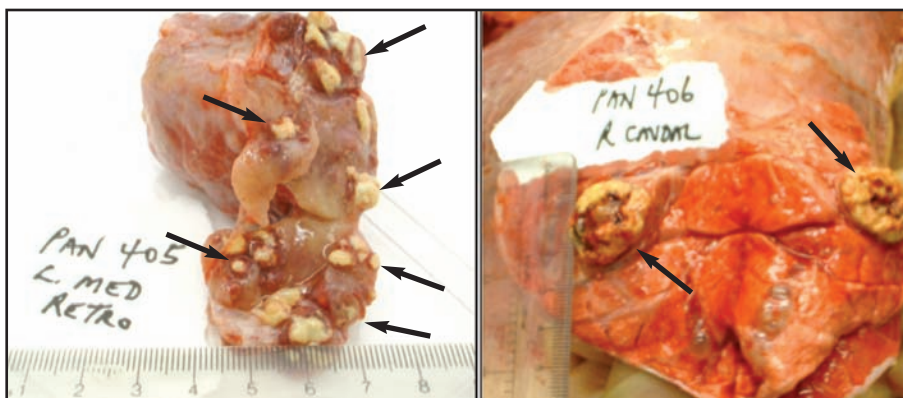
**Figure 2. 'Visible lesions' caused by *M. bovis* observed at post-mortem
a) multifocal areas of necrosis and mineralization in a bovine left medial retropharyngeal lymph node
b) bisected granuloma in the right caudal lung lobe with areas of necrosis and mineralization.
Photos courtesy of Linda Johnson, Pathology, VLA, Weybridge**

Any TB breakdown where *M. bovis* has been isolated or visible lesions detected in slaughtered cattle will trigger skin testing of neighbouring cattle herds and tracings of cattle moved into or out of the herd before the onset of the breakdown, in order to assess the potential origin and spread of the infection.

The inconvenience and cost of movement restrictions to these farmers and to the taxpayer can be significant, particularly in those herds with chronic infections. The cost to the taxpayer of the bTB testing and control programme in 2008/09 was just over £40 million pounds for testing, with almost a further £53 million pounds paid to British farmers for the removal of reactors[2].

It is also now a statutory requirement, with some exceptions, that all cattle over 42 days old moving out of a 1 or 2 yearly-tested herd must have a negative skin test within 60 days prior to movement. This measure aims to reduce the transmission of bTB between herds by the movement of infected individuals or groups of animals, since it is known that the movements of cattle out of areas with a high burden of bTB is a principal predictor for disease occurrence outside these areas[3].

Post mortem examination at the slaughterhouse (meat inspection)
In addition to tuberculin skin testing of herds, the carcases of cattle sent to slaughter are routinely inspected by the Meat Hygiene Service for gross pathological evidence of bTB. This activity provides a cost-effective, supplementary, continuous bTB surveillance method, which is particularly important in areas where routine TB testing of cattle herds is less frequent.

Laboratory testing for M. bovis
Fresh or frozen tissue samples of cattle presenting with typical visible lesions of TB at routine meat inspection *(Figure 2)* and from slaughtered test reactors are submitted to the Veterinary Laboratories Agency (VLA) for bacteriological culture and molecular typing of any isolates, providing valuable data for the epidemiological investigation of breakdowns.

Bacterial culture
All bacteriology is conducted in a containment level three laboratory. Homogenized cattle tissue samples are routinely cultured on modified Middlebrook 7H11 Agar, but additional media can be used in certain circumstances and for nonbovine samples to enhance the accuracy of

detection. *Mycobacterium bovis* will grow on Stonebrinks and Lowenstein Jensen (LJ) base, but most strains are inhibited by the addition of glycerol or enhanced by the addition of pyruvate to Lowenstein Jensen *(Figure 3)*. *Mycobacterium bovis* is a slow-growing micro-organism, and so cultures are incubated for up to six weeks at 37°C and then examined for growth. The parallel use of a liquid culture system (BBL™ MGIT™, BD, USA) has also been recently adopted by the VLA to increase the speed of results. A fluorescence signal, released by the depletion of dissolved oxygen in the culture, indicates the presence of mycobacteria. In the absence of colony morphology, molecular methods are used to confirm growth of *M. bovis*.

Microscopic examination
The histological examination of tissue samples can also prove useful in the diagnosis of bTB, particularly for the rapid provisional diagnosis of suspect cases of bTB first detected during commercial slaughter of cattle. Various conditions can cause gross granulomatous lesions in the organs and lymph nodes of cattle which may not always be a result of infection with *M. bovis*. Since confirmation by bacterial culture takes up to 6 weeks, the observation of characteristic acid-fast bacilli in processed sections of lesioned tissues may be used as an indication of likely *M. bovis* infection in the source herd, so that the appropriate control actions can be instigated pending characterization of the causative organism by culture and molecular techniques.

Molecular typing of M. bovis strains
Some bacterial species, such as *Escherichia coli* and *Neisseria meningitidis*, have a striking amount of intra-specific genetic variation, generated through mechanisms which enable the uptake of replacement DNA sequences from unrelated bacterial species. There is little evidence for the replacement of genetic material in members of MTC, with the exception of *M. canetti* [4]. The resulting absence of genetic variation means that common molecular typing methods, which characterize DNA sequence variation at a panel of loci, are uninformative for *M. bovis*.

Figure 3. Growth of *M. bovis* on bacterial culture media: Middlebrook 7H11 Agar (left), Stonebrinks (centre), Lowenstein Jensen base (right). Photos courtesy of Jemma Brown, TB Diagnosis, VLA, Weybridge.

Mycobacterium bovis is most frequently characterized and differentiated by spacer-oligonucleotide typing or 'spoligotyping'. This method measures variation in the number of unique spacer sequences in the 'Direct repeat' (DR) genomic region, unique to members of MTC. A polymerase chain reaction (PCR) is applied directly to amplify heat-killed isolates of *M. bovis*. The PCR products are loaded onto a membrane containing hybridized unique spacer oligonucleotides, which are subsequently developed by an enhanced chemiluminescence system and the resulting pattern interpreted. Unique patterns within *M. bovis* represent unique spoligotypes, and isolates with identical patterns are deemed to be of the same spoligotype.

The majority of all *M. bovis* spoligotypes isolated from cattle in GB are SB0140 (32% in 2008; light blue in *Figure 4*) and SB0263 (24% in 2008; dark red in *Figure 4*). Additional discrimination can be obtained with another typing method; variable number of tandem repeats (VNTR). This method detects variation in the number of repeat units found at multiple loci. The VNTR profile of an isolate is represented as a string of integers, reflecting the number of repeats detected at each locus. Using the combined results of these two typing methods, 81 unique *M. bovis* 'genotypes' in cattle were identified in 2008[5].

Characterizing *M. bovis* isolated from cattle is particularly useful, when combined with our knowledge of the distribution of *M. bovis* spoligotypes and genotypes in GB, for rapidly identifying the source herd of infected animals introduced to a herd through medium or long distance cattle movement.

Bovine TB in badgers
Most mammals are susceptible to infection by *M. bovis* to a varying degree, but the Eurasian badger (*Meles meles*) is considered the most important maintenance host in wildlife and a significant vector of *M. bovis* infection for cattle in the UK and Ireland. In 1997, following the publication of the Krebs report, which cited 'compelling' evidence that badgers are involved in bTB transmission to cattle[6], an Independent Scientific Group on Cattle TB (ISG) was formed to advise the government on how best to tackle the problem of bTB, including the contribution that badger culling could make to control of the disease in cattle, through reliable scientific evidence. The primary activity of the group was to oversee the Randomized Badger Culling Trial (RBCT) conducted between 1998 and 2005 in ten areas of high bTB incidence in England.

The RBCT demonstrated spatial associations of *M. bovis* infection between cattle and badgers and confirmed that transmission occurs between the two host species. Reactive badger culling (on and near farmland where recent outbreaks of bTB had occurred in cattle) was apparently associated with a 20% increase in new cases of cattle herd infection, although the validity of this conclusion has been disputed[7]. This part of the trial was suspended by Ministers in 2003.

Pro-active badger culling (annual culling across all accessible land) in trial areas was shown to result in a 23% reduction in the number of new cases of cattle herd infection during the trial (during-trial period). However, this beneficial effect was offset by a 24% increase in the number of new cases of cattle herd infection in the surrounding unculled areas. Badgers live in territorial social groups at reasonably high density in GB. Culling was observed to disrupt this territorial system, causing them to range more widely, a
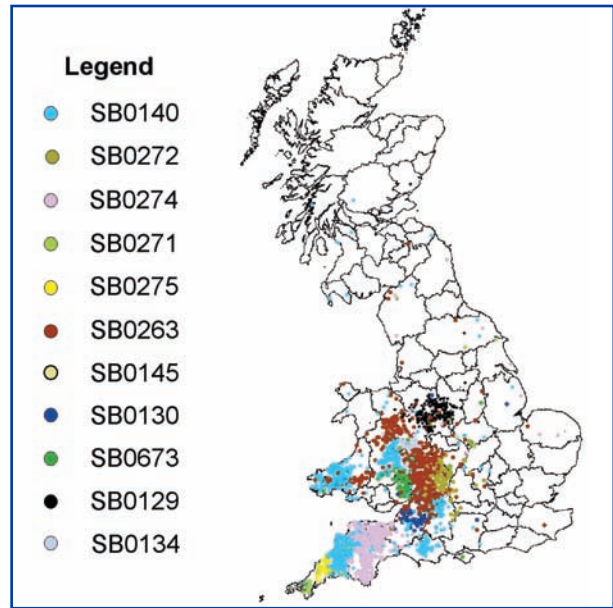

Figure 4. Geographic distribution of the most frequently (>1% of the total) isolated spoligotypes in 2008 (VLA, 2008).

phenomenon referred to as 'perturbation', thus increasing the potential for both badger to badger and badger to cattle transmission. As a result, the prevalence of *M. bovis* in remaining badgers increased, as did the incidence in cattle herds on neighbouring lands around culled areas (2km^2 buffer area). The ISG published their Final Report to Ministers in June 2007, concluding that badger culling as conducted in the RBCT cannot meaningfully contribute to the future control of cattle TB[8].

The decision whether to include badger culling in control and eradication programmes is taken independently by the devolved governments of England, Wales and Northern Ireland. In July 2008, the Secretary of State for Environment, Food and Rural affairs in England announced that, "in line with the advice I have received from the Independent Scientific Group – our policy will be not to issue any licences to farmers to cull badgers for TB control". Given the scientific guidance, practicality and cost of culling on a potentially larger scale, it was considered that badger culling "cannot meaningfully contribute to the future control of cattle TB" (http://www.defra.gov.uk/corporate/about/who/ministers/statements/hb080707.htm).

Further analysis, including data following the cessation of culling (post-trial period) within the proactive culling trial areas has shown that beneficial reductions in bTB incidence have increased in magnitude to a statistically significant 30% reduction over the whole period. No detrimental effects were observed in neighbouring lands during the post-trial period[9]. The impact of culling on bTB incidence is changing over time. Ongoing analyses of the long-term effects will provide further evidence as to whether badger culling could be considered once more in England.

The future of bovine TB in United Kingdom
The bTB eradication plan for the United Kingdom 2010 has been approved by (and will receive financial support from) the European Commission. An effective eradication programme for TB in cattle requires a holistic, multifaceted approach, incorporating enhanced infection

surveillance in cattle, with different approaches for high and low incidence areas, and control strategies which prevent infection or re-infection of clean herds by exposure to purchased infected cattle, neighbouring cattle or local wildlife reservoirs. The latter may be achieved through badger removal, badger vaccination and/or herd biosecurity measures. Although the principles of bTB eradication are common to all UK governments, there are differences in the specific tools adopted and priorities addressed by England, Wales and Northern Ireland in order to reach this objective, reflecting the variation in economic, stakeholder, political and disease profiles of the regions. The TB Eradication Group for Wales was set up in 2007 and has executed initiatives, such as TB Health check (a one-off test of all cattle herds in Wales, irrespective of routine testing frequency) and plans to roll out simultaneous badger culling and cattle control in an intensive action pilot area (IAPA) in 2010. Initial recommendations of the TB Eradication Group for England relating to cattle movement have already been implemented, with further recommendations on strategies to help bTB-affected farm businesses trade more effectively expected shortly.

Further fundamental changes have been agreed with the Secretary of State for implementation in 2010, including changing the areas on which testing frequencies are set to reflect risk rather than historical incidence. A Badger Vaccine Deployment Project to assess and maximize the viability of using an injectable badger vaccine is expected to commence in England in 2010.

Eradication is recognized as an expensive and long-term goal. It takes time for any new measures to result in a reduction in disease prevalence. International efforts, such as the Australian Brucellosis and Tuberculosis Eradication Campaign (BTEC), took 27 years to eradicate bTB and achieve Officially Tuberculosis Free (OTF) status for their national cattle herd. However, the current economic and emotional burden is unsustainable by both the taxpayer and the farming industry. An increasing reservoir of *M. bovis* in cattle and wildlife also compromises public health, and the HPA has recommended further investigation into the epidemiology of *M. bovis* of human cases likely to have been infected recently in this country[1]. However, with the focus now on strategies towards 'eradication' of bTB from the cattle population, hopefully the threat from *M. bovis* to human health will remain firmly in the history books.

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Terms
A herd disclosing reactor animals is 'confirmed' for infection of *M. bovis* by the detection of 'visibly lesioned' (VL) tissues at post-mortem examination and/or the isolation of the *M. bovis* organism by bacterial culture. In a herd in which VL tissues are detected by meat inspection at the slaughterhouse, infection by *M. bovis* is confirmed by the detection of acid-fast rods, by microscopic examination of VL tissues and/or the isolation of the *M. bovis* by bacterial culture.

Sensitivity is the ability of a test to correctly identify infected animals as positive (the higher the sensitivity the lower the probability of false negatives).

Specificity is the ability of a test to correctly identify noninfected animals as negative (the higher the specificity the lower the probability of false positives).

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