Brucellosis – new paradigms for a classical pathogen

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Introduction

Globally, brucellosis remains one of, if not the major bacterial zoonosis, still devastating productivity of livestock and affecting mankind, either directly through infection, or indirectly through reduction of productivity among food-producing livestock. It is estimated by the WHO that more than 500,000 new cases of human brucellosis occur each year. Many nations have deployed eradication campaigns, with some countries such as the UK and several other Northern European countries successfully gaining brucellosis-free status. Even in these countries, comprehensive surveillance is an essential prerequisite to maintain this status, exemplified by recent introductions of Brucella-infected cattle to the UK, despite pre- and post-export screening.

The aetiological agent of brucellosis is a small, pleomorphic Gram-negative rod, which was first isolated from human clinical cases of undulant fever by David Bruce (1887). Later, in 1904, the Mediterranean Fever Commission under the leadership of David Bruce, identified the reservoir of infection in goats and subsequent transmission to man through consumption of unpasteurised milk. In subsequent years, similar microbes were identified in cattle (B. abortus) and swine (B. suis).

This microbe belongs within the α-2 proteobacteria clustering with Bartonella, Ochrobactrum, Agrobacteria and Rhizobia as phylogenetic neighbours. The genus was originally divided into species based on various biochemical capabilities, dyes and phage susceptibilities and host preferences (see Table). These species in some cases were further divided into biovars (see Table). Following whole genomic hybridization studies, the high degree of homogeneity among the brucellae was noted, prompting the re-classification into a single species, B. melitensis. Although justifiable according to phylogenetic criteria, this classification has proved unpopular, largely through distinct host susceptibilities and differences in host-pathogen interactions. Brucelae generally possess two chromosomes with a large replicon of 2.1 Mbp (chromosome 1) and a smaller replicon of 1.2 Mbp (chromosome 2); however, B. suis biovar 3 possesses a single replicon of 3.1 Mbp. Plasmids have not been reported within the brucellae.

Diagnostic Challenges

Clinical Diagnosis:

Human clinical disease is characterized by undulant fever, but in many instances other presentations will be predominant including spondylitis, neuropsychiatric complaints, arthralgia, arthritis, respiratory signs and in chronic cases, focal signs often with abscess formation. However, in many cases, clinical signs are protean and often non-specific. In livestock, the disease typically manifests as reproductive failure, often through abortion or weak, infected offspring, but orchitis, epididymitis and lameness can also occur. During acute infection with B. melitensis, blood cultures will yield brucellae in 70-80% of individuals. However, this is significantly reduced with infection with other Brucella species. Importantly, recovery of brucellae from clinical material is often lengthy, necessitating extended incubation periods where brucellosis is suspected.

Serology:

Serological diagnostics have evolved over time from tests for agglutinins through to numerous ELSIA formats and fluorescent antibody polarization assays. Associated with this evolution is increased specificity; however, even this new generation of assays are prone to detecting false-positive serologically reactive samples. These have been recently reviewed elsewhere. The antigen offering superior

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**Table.** Species, biovars and reservoirs of the brucellae.

<table>
<thead>
<tr>
<th>Brucella species</th>
<th>Biovar</th>
<th>Reservoir host</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brucella melitensis</td>
<td>3</td>
<td>Sheep, goats, cattle</td>
</tr>
<tr>
<td>Brucella abortus</td>
<td>7</td>
<td>Cattle</td>
</tr>
<tr>
<td>Brucella suis</td>
<td>5</td>
<td>Swine</td>
</tr>
<tr>
<td>Brucella canis</td>
<td>1</td>
<td>Dogs</td>
</tr>
<tr>
<td>Brucella ovis</td>
<td>1</td>
<td>Sheep</td>
</tr>
<tr>
<td>Brucella neotomae</td>
<td>1</td>
<td>Rodents</td>
</tr>
<tr>
<td>Brucella pinnipedae*</td>
<td>Not determined</td>
<td>Otter, seal</td>
</tr>
<tr>
<td>Brucella cetaceae*</td>
<td>Not determined</td>
<td>Dolphin, porpoise</td>
</tr>
</tbody>
</table>

* Species names not yet approved.
sensitivity is lipopolysaccharide with its α-polysaccharide side chain of homopolymers of N-formyl-perosamine [N-formylated 4-amino, 4,6-dideoxyglucose] found either in α 1-2 linkages or α (1-2) linkages together with α (1-3) usually in a 4:1 ratio. These variations result in the A and M serotype specificity of brucellae. The α-polysaccharide side chain shows distinct structural similarity with the N-formyl-perosamine homopolymer of N-formyl-perosamine of Yersinia enterocolitica O:9. This microbe is frequently found in association with swine, but also in other livestock species. It is likely that exposure to this microbe, or similar ones, may account for the serological “false alarms” encountered within brucellosis-free countries. The search for improved diagnostic antigens has become something of a search for the “Holy Grail”. Several different antigens have been assessed; however, the overall consensus of opinion is that they fail to offer diagnostic advantage over current serological assays.

Seroserology is further complicated when used in countries where the disease is endemic. Here significant proportions of the population will naturally have elevated titres against brucellae. Furthermore, brucellosis eradication efforts utilizing live attenuated vaccine strains (except RB51), will result in positive titres in recently vaccinated livestock.

**Immunological approaches:**

In order to overcome the problems outlined above, investigations have been undertaken to evaluate use of other immunodiagnostic methods. As Brucella stimulate a strong Th1 response, the potential of using elevated interferon-gamma (IFN-gamma) levels following specific stimulation has been explored. Early results look promising, with this approach clearly differentiating between brucellosis and Yersinia infections, however, no improvements in test sensitivity were offered. Further limitations arise from the undulating levels observed with IFN-gamma responses and their requirements for stimulation of blood cells within a tight time window.

**Polymerase chain reaction assays:**

Many PCR assays have been published for the detection of brucellae using various targets, including the intragenic spacer, IS711, outer membrane protein BCSP31. Typically these will detect levels of 10fg, which equates to three genomes. The limitation of this approach is the likelihood of sufficient bacteria present in blood or serum to enable detection. Brucellae are rapidly internalized by host cells, whereby they are instrumental in development of their particular replicative niche. Foci of infection are often found in lymph nodes, bone marrow and various tissues, especially reproductive tissues in livestock. Application of PCR to these samples will be much more likely to detect Brucella DNA.

**Typing methods:**

Classical biotyping has been the “gold standard” approach for distinguishing different isolates into their respective species and biovars. However, this needs highly skilled staff, large amounts of viable organism requiring containment level three facilities and lengthy incubation periods. Given the reputation of Brucella as the most frequently encountered laboratory-acquired infection, this approach is not to be undertaken lightly and is probably best reserved for reference facilities. Various microbial typing methods, including outer membrane protein typing; ribotyping; AFLP; PFGE-RFLP; insertion sequence typing; to name but a few, have been applied to the Brucella with mixed success. These approaches were able to successfully speculate isolates, but proved unable to offer differentiation down to biovar levels.

Recent application of PCR-based variable number tandem repeat typing (VNTR) or hypervariable gc-tameric gilgonucleotide finger prints (Hoof Prints) has proved to be a valuable method for traceback studies when outbreaks occur. However, on a more cautionary note, those targets most suited for epidemiological profiling will not necessarily identify isolates, but are best suited for microbial forensic typing. Selection of more slowly evolving repeats, or even use of single nucleotide polymorphisms, can however provide a molecular equivalent to biotyping.

**Brucellosis and the “omics” revolution**

Genomes of the three principal pathogenic species have now been published, while that of B. ovis is currently in progress. Comparative genomics of these three species has confirmed the remarkable homogeneity among these species, with closest similarity between B. abortus and B. melitensis. Surprisingly, only 101 unique genes were present/absent in one of these three genomes. Transcripts were detected from 54 of these using reverse transcriptase PCR, with one unique transcript to each of B. abortus and B. melitensis and a further 20 in B. suis. Thus, it is likely that the intriguing differences in host specificity may result from either subtle variations within this conserved DNA, or through differential expression of conserved genes rather than possession of unique genomic DNA.

Studies of the proteome (secretome and cellular proteome), have revealed differences between wild type and attenuated vaccine strains of B. melitensis. Notably, expression differences were demonstrated in iron regulatory proteins, sugar binding, lipid degradation and protein biosynthesis between wild type and attenuated strains. Comparative proteomic analysis of B. abortus and B. melitensis produced 312 differentially expressed proteins. Whether these are representative of the species and their significance in host specificity of these microbes remains to be determined.

Analysis of the “omic” approaches was heralded as the likely way to dissect the basis for host specificity and virulence for these microbes; however, the biological basis for these traits remains elusive. Scrutiny of the Brucella genomes for conventional microbial virulence genes has met with limited success, although genes for capsules, toxins and other conventional virulence determinants have failed to produce insights into the pathogenesis of these microbes. Surprising findings, such as the presence of genes necessary for expression of flagellum in this non-motile organism, were detected. Upon further investigation, it appears that these are transiently expressed and may indeed have a role in the pathogenesis of these microbes. Certainly the surface lipopolysaccharide plays a crucial role in virulence of the brucellae together with expression of type IV secretion system. Certain the surface lipopolysaccharide plays a crucial role in virulence of the brucellae together with expression of type IV secretion system. Microarray analysis of different Brucella species hybridized to B. melitensis 16M, again show limited diversity among species. This work identified differences between species suggestive of horizontal genetic acquisition, that appeared to be clustered into genomic islands; however, these differences alone
may not be sufficient to account for the host specificity observed within the brucellae.16

**Control strategies**

Eradication campaigns have been introduced in many countries with varying degrees of success. Typically these are based on vaccination of susceptible hosts, surveillance to detect infected livestock and their subsequent slaughter, and movement restrictions where localized infection has been detected. The combination of these methods can lead to eventual freedom from disease, although the likelihood of success is largely dependent on compliance of many different groups (farmers, import/export, veterinary workers, public health workers, scientists and government). Without this concerted, joined-up approach, chances of success are limited.

**Current challenges**

Our new challenges are the growing incidence of *B. melitensis* infection in cattle, particularly in areas such as the Middle East. Vaccine efficacy is challenged by this new threat as cattle are routinely immunized with attenuated *B. abortus* strains (either S19, or RB51). The ability of these vaccines to protect against *B. melitensis* challenge in bovines remains largely untested.

A further challenge is posed by the threat of infection from wild-life reservoirs. This is a major concern due to the huge disease burden in developing countries. Here the consequences of brucellosis impact dramatically on both human health and on the livestock on which man depends. Diagnostic capability is sporadic and disease epidemiology largely unknown. Introduction of pen-side diagnostic assays and education focused towards the spread of infection and methods to control disease in livestock and reduce transmission to man are urgently required.

**Recommended Further Reading:**


**References**


Clostridium difficile – new challenges for hyper-virulent strains

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Introduction

Clostridium difficile, a strictly anaerobic, Gram-positive spore-forming bacterium has been recognised as the cause of antibiotic-associated diarrhoea and colitis for about 30 years. The spectrum of disease – ranging from mild, self-limiting diarrhoea, to serious diarrhoea, to life-threatening pseudomembranous colitis – is generally referred to as C. difficile-associate disease (CDAD). Initially uncommon, but with a few noteworthy outbreaks, it has been steadily increasing in prevalence since the early 1990s, reaching levels in many of our hospitals that are generally higher than the better-known methicillin-resistant Staphylococcus aureus (MRSA). It is the most common cause of health-care-associated (nosocomial) diarrhoea and figures for Scotland are summarised in Figure 1. The shape of the curve is similar for most other countries in the developed world. CDAD causes much morbidity in elderly hospital patients and some mortality has been associated with the disease. The Health Protection Agency issued a press release in December 2005 highlighting the current status of the organism and disease. (In this web reference there is a link to the Healthcare Commission’s full report on: Management, prevention and surveillance of Clostridium difficile – Interim findings from a national survey of NHS acute trusts in England.)

The recent recognition of a hyper-virulent strain firstly in Canada and the USA and more recently in England, the Netherlands and Belgium has brought the organism to the attention of the general public as the latest “superbug”. For the most recent findings go to the “News and Activities” section of the European Study Group for Clostridium difficile web site.

Historical aspects

“Bacillus difficilis” was first recognised in 1935 as a normal component of the stools of young babies, and during the following 40 years it received scant attention in the medical literature. As recently as 1976 it was considered non-pathogenic. However, just a year or so later it was shown to be the cause of pseudomembranous colitis, a rare but life-threatening condition. This discovery was a result of the efforts of researchers investigating a significant increase in a previously very rare condition: antibiotic-associated pseudomembranous colitis, a disease which at that time was being linked to the use of specific antibiotics, especially clindamycin and the related lincomycin. C. difficile was subsequently recognized as the major cause of hospital-acquired, antibiotic-associated diarrhoea, a lesser form of the more rare and serious pseudomembranous colitis.

Current problems

As demonstrated in Figure 1, the disease has certainly increased over the past decade or so with a consequent increasing burden on health service providers, as a result of the need for isolating infected patients (or barrier nursing them in outbreaks) and the added treatment that many require. The costs involved in patient treatment, especially for those who experience...

Figure 1. Increasing reports of Clostridium difficile 1987-2005 for Scotland. Figures taken from Health Protection Scotland (formerly Scottish Centre for Infection and Environmental Health) Weekly Reports. Note that reporting is voluntary.
recurring disease, are considerable and in 1996 were reported to be in the region of £4000 per patient. Recent unpublished estimates from the USA are in the region of US$6000. If this wasn’t bad enough, a new dimension to the problem has become apparent in the last year or so. In 2004 a major outbreak began to unfold in the Montreal region of Quebec, and the Canadian Broadcasting Corporation news service announced “7000 sufferers and 600 deaths in Quebec”. These were four times and six times the expected levels, respectively. Because of the unusual severity of the symptoms, clinicians familiar with the disease suggested early in the outbreak that a strain of increased virulence was probably responsible for the problem. This strain is now known in North America as BI/NAP1. An apparently identical strain known in the UK as ribotype 027 has been recognised as causing an ongoing problem at Stoke Mandeville Hospital in Buckinghamshire where at least 12 deaths were reported from 150 cases in the period February – June 2004 (Personal communication, Jon Brazier). Since then, other centres have experienced problems with this 027 strain – notably in Exeter, Devon and Romford, Essex.

The disease: pathogenesis and epidemiology

There are several recent reviews of the crucial processes in the cause of the disease (pathogenesis) and briefly they conclude that:

- The normal protective properties of the gut – the colonisation resistance promoted by the normal resident bacteria of the healthy gut – are compromised by antibiotics or, less frequently, other therapeutic agents
- The colon becomes colonised with C. difficile
- C. difficile evades the immune response and multiplies, producing toxins A and B
- If a patient is susceptible – i.e. is unable to withstand the insult of the toxins, probably as a result of lowered immunity – pathology results, which is a result of destruction of colonocytes and an influx of inflammatory cells – predominantly neutrophils (see Figure 2).

It appears that the new hyper-virulent strain (BI/NAP1 or ribotype 027) has the following characteristics:

- It produces higher than normal levels of toxins in vitro and almost certainly in vivo.
- There is a deletion in the gene (tcdC) which normally negatively regulates toxin production – resulting in constant maximum level of transcription of toxins
- It belongs to ribotype 027 and toxinotype III
- It is resistant to fluoroquinolone antibiotics such as ciprofloxacin and moxifloxacin
- It produces the binary toxin
- The North American and European isolates are probably identical
- The disease that it causes is more severe with more colectomies required and more deaths attributable to it.

It is becoming apparent that the deletion in the toxin-regulating gene is not restricted to the 027 ribotype, so a family of super strains may soon become apparent.

The Secretary of State for Health was questioned in the House of Commons and in the written answer 12 other hospitals throughout England were reported to have the strain – from Truro in the South West to Newcastle and Sunderland in the North East (Reported in Hansard 27th June 2005). Currently at least nine hospitals in The Netherlands and six in Belgium are experiencing problems with the same strain. Although the 027 ribotype has been known for several years, the recently-recognised hypervirulent epidemic strain is distinct from earlier isolates in that only recently has it acquired resistance to fluoroquinolone antibiotics. At the time of writing this article (Jan 2006) none has been identified in Scotland.

Figure 2. a) A length of colon resected from a case of antibiotic-associated pseudomembranous colitis revealing the numerous plaques of pseudomembrane; b) histopathology of PMC, showing the infiltration of neutrophils through the damaged mucosal layer. Both images courtesy Paul Fineron.
Our early understanding of the disease assumed that the bacterium was of endogenous origin: small numbers of *Clostridium difficile* that were present in the colon were allowed to flourish after the normal microbiota was severely affected by antibiotic exposure. However, it soon became apparent that this was unlikely as it did not explain the occurrence of outbreaks. When carefully looked for in the healthy adult the organism is rarely encountered. One of our earliest publications on *C. difficile* in 1984 demonstrated that a single strain was responsible for an outbreak. It is now accepted that *C. difficile* is an infectious agent and is transmitted by faecal-oral transmission of spores from patient to patient, or contaminated environment to patient.

Once established in the gut, the major virulence factors of the organism – toxins A and B – are elaborated. The genes encoding and controlling these toxins are carried on a pathogenicity island referred to as the pathogenicity locus or “PaLoc”10. As well as the two genes encoding the toxins (*tcdA* and *tcdB*), there is both a positive regulator *tcdR* and a negative regulator *tcdC*. A final gene *tcdE* encodes a holing-like protein that is probably involved with export from the cell. Normally toxins A and B are produced on entry into stationary phase, and the transcription of their genes is repressed by *tcdC*. However, in the above-mentioned hyper-virulent strain, early transcription of the toxin genes is a result of a deletion in the *tcdC* gene.

**Laboratory detection**

Initially, laboratory diagnosis was based on traditional bacterial culture on a selective medium such as the cycloserine, cefoxitin, fructose agar (CCFA) developed by George et al in 197911. Confirmation that the isolates produced toxin was by demonstration of an antitoxin-neutralisable cytopathic effect in tissue culture monolayers. These techniques are highly specific and sensitive but labour-intensive and therefore expensive. However, following the widespread introduction of rapid toxin detection by immunoassay, laboratory detection moved away from culture/cytotoxin test to these more rapid methods. Culture methods have now largely been abandoned in the UK, most other countries in Europe and the USA and diagnosis is relying entirely on detection of toxin(s) in stool filtrates by an immuno method. Initially, only toxin A was detected, but most kits also now measure A and B. This followed the recognition of A-negative, B-positive strains as causing disease. Certainly, detection of toxin(s) is the cheapest option and the results are available rapidly. The improved sensitivity of many of the commercially available kits means that in most cases an extremely reliable diagnosis can be achieved. However, such methods do have a serious drawback. As no organism is isolated it cannot be “typed” to indicate whether it belongs to a hyper-virulent phenotype – one that should be dealt with more seriously than normal. It also does not permit surveillance of antibiotic resistance pattern or any other epidemiological or pathogenic marker.

**Recommendations for the diagnostic laboratory**

- Investigate all stools by a rapid immunoassay detecting toxins A and B or by tissue culture cytotoxin test from a) hospital patients with diarrhea, b) any other patients when requested by a clinician, c) patients with liquid stools from the community, when there is a recent history of antibiotic use.
- Perform cultures on i) all toxin-positive stool cultures on CCFA or preferably the CCEY agar of Brazier12 – which incorporates cholic acid to enhance spore germination (Figure 3), and ii) any toxin-negative stools from symptomatic patients who are suspected of having CDAD.
- Test for toxin production any positive colonies from symptomatic patients who are faecal-toxin-negative.
- Archive isolates periodically – for outbreak investigation and antibiotic sensitivities.
- If an outbreak is suspected, submit isolates to the reference laboratory for typing.

Reinstating a culture step will have a major financial implication for many laboratories and in reality it may be that only the first of the recommendations is possible.

**Recurrences**

Recurrent episodes of CDAD are a real problem, and as many as 25% of patients may suffer a relapse after initial resolution of symptoms13. This could be a real relapse or a re-infection with the same or a different strain of *C. difficile*; there is evidence for both occurring. The common problem of recurrent CDAD has necessitated thorough review of treatment schedules.

**Treatment options for CDAD**

Detection of *C. difficile* in the stool is not an indication to treat. Many patients, especially in...
wards for the elderly, carry the organism asymptomatically. They may pose a risk to other patients, but patients without symptoms do not require antibiotic therapy. However, in the future when other (safer) therapies may exist, treatment may be an option.

If the disease is mild, no treatment may be necessary as the symptoms may resolve naturally. For more severe disease, stopping the administration of the precipitating antibiotic is the first option. If this is not possible, or ineffective, the first line of treatment is administration of metronidazole or vancomycin: the former is used more typically these days. Various comparative studies have been done with these agents and most conclude that there is not much between them, but some say vancomycin works a little faster, and should be considered for more serious disease.

The real problem of patients suffering recurrences after apparent initial resolution with antibiotics – together with the anathema of treating a disease caused by antibiotics with another antibiotic – has promoted much research into the development of alternative types of treatments. These fall into four main areas: i) prebiotics and probiotics, ii) absorbents for toxin – to eliminate them from the gut, iii) “faecal transplants/enemas” – where stools donated from a healthy donor are placed in the bowel in an attempt to restore the normal microbiota, and iv) immunotherapy – either active or (probably more usefully) passive immunisation. Lynn McFarlane has recently reviewed “alternative treatments” for CDAD14. Currently none of these alternatives has become routine, but efforts are progressing in earnest. Anti-peristaltic drugs are strongly contra-indicated as they may precipitate toxic megacolon and perforation.

Infection control and the future?

Despite our increasing knowledge of the organism and the disease, it is unlikely that the problem will disappear in the near future. Many of our hospitals and institutions caring for the elderly are now generally highly contaminated with spores of *C. difficile* and increasing numbers of susceptible, antibiotic-treated patients are propagating the organism. Infection control methods are difficult for this disease but their importance cannot be stressed too much. They include regular surveillance, isolation or barrier nursing, personal hygiene, and intensive cleaning of affected wards to remove the bacterial spore load. Note that the spores of *C. difficile* are resistant to alcohol-based antiseptics (alcohol hand-washing gels are ineffective), and chlorine-based disinfectants are only partially effective. The use of hydrogen peroxide vapour is being proposed for room sterilisation – but only after patients and bedding have been removed! Adherence to strict antibiotic policies: restricting the use of those antibiotics such as oral cephalosporins and clindamycin, which are known to precipitate the disease, is also crucially important. However, more recently the use of fluoroquinolones has been seen to select for the new 027 strain.

Even in areas without the epidemic 027 strain, anecdotal reports suggest the incidence and severity of CDAD has been increasing in recent years. The situation is likely to worsen before it gets better. Major targets for research must include: the development of more effective, patient-friendly disinfectants with which to clean hospitals; new therapies that are not based on antibiotics – such as the use of prebiotics, immunotherapy or toxin inactivators; and more sensitive (molecular) methods such as real-time PCR for accurate diagnosis. Crucially, much more funding is required for this organism and disease, which to date has been minimal in the UK. Together with further research there should also be better surveillance and mandatory reporting of the organism.

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References

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