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Brucella taxonomy and evolution

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Abstract

Taxonomy and nomenclature represent man-made systems designed to enhance understanding of the relationship between organisms by comparison of discrete sets of properties. Initial efforts at bacterial taxonomy were flawed as a result of the previous use of nonsystematic approaches including common names resulting in confusing and inaccurate nomenclature. A decision was made to start afresh with bacterial nomenclature and to avoid the hazards experienced in the taxonomic classification of higher organisms. This was achieved by developing new rules designed to simplify classification and avoid unnecessary and confusing changes. This article reviews the work of a number of scientists attempting to reconcile new molecular data describing the phylogenetic relationship between *Brucella* organisms and a broader family of organisms with widely variant phenotypes that include human virulence and host range against a backdrop of strict regulatory requirements that fail to recognize significant differences between organisms with similar nomenclature.

Keywords

biotyping; *Brucella*; evolution; speciation; subcommittee; taxonomy

A challenge faced by *Brucella* researchers has been the accurate identification of new isolates within the genus while preserving sufficient, and not excessive, biosafety and biosecurity requirements. Assignment to previously recognized species can result in the imposition of significant safety and regulatory requirements while classification of unique species broadens the genus and threatens to encompass additional genera and unnecessarily subject work with these organisms to strict regulatory requirements. The problem faced by scientists is the development of accurate taxonomic relationships without undue regulatory burden that unnecessarily threatens to restrict research. Differences in opinion amongst scientists must be reconciled, and hopefully performed with the best of scientific and taxonomic intent [1]. Although it is clear that extraneous factors influence the choices made, it is essential that the choices made ‘do no harm’ to public health, investigators, the scientific community at large and bacterial taxonomy, in that order of importance. The following section describes the taxonomic history of the genus *Brucella* and provides an example of the work of scientists who, in their efforts to understand the virulence and

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evolution of these organisms and to mitigate against the threat posed by weaponization, struggle against a background including honest concern, ignorance and sweeping regulatory controls, with the potential to prevent scientific progress and threaten personal integrity.

Taxomony of the *Brucella* species

The genus *Brucella* resides within the family Brucellaceae (family III) with *Mycoplana* and *Ochrobactrum*, of the order Rhizobiales in the class Alphaproteobacteria of the phylum Proteobacteria [2]. Members of the class Alphaproteobacteria include families of organisms that are either mammalian or plant pathogens or symbionts. Among the organisms affecting mammals in the Alphaproteobacteria are the genera *Bartonella*, *Rickettsia* and *Ehrlichia*, all of which are spread by vector-based transmission. The small genome size of these organisms is consistent with obligate intracellular survival, although this property does not necessarily define insect vector-based transmission (i.e., *Coxiella*). Features of *Brucella* that distinguish it from most genera within the order Rhizobiales include infection of mammalian cells, a feature shared only with *Bartonella*, and a streamlined genome, in relation to the plant pathogens. However, major differences exist between *Bartonella*, an obligate intracellular pathogen, and *Brucella*, a facultative intracellular pathogen. First, the genome of *Brucella* spp. is 50–100% larger than *Bartonella* spp. genomes and has preserved more of the metabolic functions shared by the plant pathogens. Persistence in the soil for up to 10 weeks is consistent with the ability to metabolically utilize plant-based molecules [3]. The recent identification of *Brucella microti* in soil points to an environmental niche shared by all three genera in this family [4].

The relatively large genome size of *Brucella* organisms suggests the potential to exist in different environments that may well include adaptation to a number of different hosts. Differences between host species may reflect differences in cell surface structures (cell wall) and optimal growth conditions, as well as specialized mechanisms for uptake and intracellular growth of mammalian pathogens. As a result, the ability to invade mammalian hosts is a feature that may have been acquired at least in part by both *Bartonella* and *Brucella* and may be expected to exhibit nucleotide composition (i.e., G + C) that is distinct from genes conserved from progenitor organisms. Several candidates exist to fulfill this role, including genes encoding biosynthesis of polysaccharides, secretion systems, adhesins and invasins [3,5,6]. However, it is possible that genes involved in uptake or invasion of mammalian cells were present in progenitor organisms, and lost from the plant pathogens. In this case, the genes would not exhibit distinctive nucleotide compositions, and would require more direct approaches for identification. Evaluation of the genomes of several *Brucella* spp. indicates the loss of gene function via pseudogene formation during adaptation to the intracellular lifestyle [7]. More recently, horizontal gene transfer unique to the *Brucella* spp., associated with important virulence determinants, appears to be associated with adaptation to the intracellular lifestyle [8]. In the most dramatic example, inactivation of genes involved in nutrient acquisition and utilization, cell envelope structure and urease in the genome of the nonzoonotic pathogen *Brucella ovis* is suggested to have played a role in narrowing tissue tropism and host range [9]. However, these studies do not distinguish between coevolution with a primary host or adaptation at a later date.

Speciation & host range

Speculation concerning the origin of *Brucella* species has rightly focused on the apparent adaptation to specific hosts. Coevolution of *Brucella* species with their preferred hosts is an obvious starting place based on currently observed host preferences. Yet, this simple interpretation is not consistent with the overall genetic variation observed between host species and the limited variation observed between *Brucella* spp. Obviously, host and agent

do not necessarily evolve at the same rate, but the overall similarities observed among host-adapted *Brucella* species either argues for limited genetic flexibility or more recent adaptation. Using a molecular clock based on single nucleotide polymorphisms in 13 different *Brucella* genomes representing the original six species, Foster and coworkers concluded that most *Brucella* species diverged from a common ancestor (similar to *B. ovis)* in the past 86,000–296,000 years [10], a date that certainly precedes domestication of livestock hosts, but is nowhere close to the time of divergence of the host species [11]. In summary, the divergence of the *Brucella* spp. did not involve extensive coevolution with primary hosts, but does reflect adaptation to and ultimate preference for these hosts.

However, it must be pointed out that the host preference described for *Brucella* species is not as rigorous as it may sound. Experimentally, *Brucella* organisms may infect animals other than their primary host either experimentally or under natural conditions. However, such infections appear to be self-limiting. Furthermore, in districts where there is overlap in the distribution of cattle and goats or cattle and swine, serious *Brucella* infection, including abortion storms, result only from infection of the preferred species. In the best-studied example to date, *Brucella suis* infection in cattle was observed as a result of contact with feral swine. Despite microbe shedding in the milk of infected animals, the infection was not contagious and normal healthy calves were delivered from infected cows [12]. Thus, the concept of host-specific adaptation remains a valid topic for ongoing research [13].

Brucella primarily targets the reproductive system, resulting in shedding in the milk and transmission. Direct contact with contaminated animal products is the only documented route of natural transmission and is consistent with experimental findings, suggesting the absence of vector-based transmission [14]. These properties would have been favorable for transmission among high-density populations of herding animals that may have been present prior to domestication. Exposure of other species, including carnivores and scavengers (including rodents), may have resulted from exposure to contaminated carcasses or other detritus. Thus, it seems appropriate to conclude that transmission of *Brucella* may have always occurred via direct contact or following exposure to environmental organisms.

Genome organization

It has been speculated, based on comparisons with closely related relatives, that *Brucella* or its predecessor was a free-living organism that evolved into an animal parasite. The exact steps in this process are unknown, but involve loss, acquisition and modification of traits. As all these events may be reflected in the genome, it is important that as many genomes as possible are sequenced and analyzed [3,5,6,15,16]. At this time, 38 genome sequences are available for analysis. Comparison of the sequenced genomes reveals similar sizes, overall nucleotide composition and gene synteny [3,6,8]. An interesting feature of the genomes is the observed separation across two chromosomes in some species [17]. This is a property shared with neighboring genera, and suggests the capture and modification of a megaplasmid or separation of the original chromosome into separate units [18]. Support for the contention that the two chromosomes were derived from one chromosome was based on the observation that *B. suis* biovar 3 contains a single chromosome. However, the characterization of plasmid replication functions and origin of replication on chromosome II are consistent with a plasmid-derived origin, and the presence of a single chromosome in *B. suis* biovar 3 is best explained by recombination between the rRNA loci.

Despite the plasmid origin of the smaller chromosome, essential genes are located on both chromosomes and their distribution between the two chromosomes is similar in the sequenced species. Support for the plasmid-based origin of chromosome II may also be found in closely related genera in which linear plasmids and megaplasmids exhibit similar

gene arrangements. The genomes share similar GC content, a similar proportion of coding regions and equivalent housekeeping gene distribution between chromosomes. Numerous transposons, insertion elements and phage remnants suggest a vigorous contribution to evolution. *B. suis* was shown to have numerous accessory metabolic functions on chromosome II, including an unexpected capacity to utilize plant-derived compounds. This is a feature that is generally conserved among all *Brucella* species and may enhance survival in the endoplasmic reticulum of the host.

Despite an evolutionary divergence and/or host-specific adaptation, orthologous characteristics relevant to virulence do not appear to have undergone substantial change within the genus. Although there are several examples of phage-mediated and other insertion/deletion events that may account for differences in virulence and host specificity, their contribution to virulence is not obvious and will require evaluation. As a result, the contribution of small sequence changes (single nucleotide polymorphisms) in orthologous functions remains the primary potential source of distinction.

Current scientific disputes

Brucella taxonomy and nomenclature has experienced a great deal of interest resulting from the apparent failure to reconcile genetic diversity with the broad array of phenotypes used to identify species and subspecies within the genus [19]. Since their identification in the late 19th and early 20th centuries, *Brucella* spp. have been identified based primarily on the host species from which they were isolated and in which they cause serious and ongoing infection. *Brucella melitensis* was confirmed as the cause of disease in British military personnel stationed in Malta by David Bruce in 1887 [20]. However, credit goes to Themistocles Zammit for demonstrating that the source of human infection was goats' milk [21,22]. Similarly, over the following decades *Brucella* species were found to be associated with additional hosts, including *Brucella abortus* in cattle [23], *B. suis* in swine [24], *Brucella canis* in dogs [25], *B. ovis* in sheep [26] and *Brucella neotomae* in the desert wood rat [27]. Although each has been summarily classified as a class III biohazard, there are definite differences in the severity of disease caused by these agents when compared in a single host such as humans. *B. abortus*, *B. melitensis* and *B. suis* are considered to be serious public health risks and categorized as select agents, *B. ovis*, *B. neotomae* and *B. canis* are not. This is due in part to the infrequency with which human and animal infection have been observed as well as demonstrated differences in virulence. During the 20th century a system of phenotypic analysis was developed that was used to confirm species identification as well as subdivide them for improved diagnosis and epidemiological tracking [28,29]. This was based on a number of phenotypic properties, including growth in the presence of various dyes, antibiotics and metabolic substrates, susceptibility to bacteriophage, and dye- or antibody-based agglutination. Phenotypic differences were used to effectively identify the origin of infection via epidemiological trace-back, helping to end the chain of infection [30,31]. The system proved valuable, but the advent of molecular analysis brought the promise of improved identification strategies.

However, despite the initial promise, these molecular methods did nothing to improve diagnostic capability. Instead, the earliest molecular evidence indicated that the species and biovars were indistinguishable using available methods with DNA homologies exceeding 95% [32,33]. Consistent with overall similarity, evaluation of genome architecture, conserved and potentially variable gene functions, including 16S ribosomal RNA and outer membrane proteins, as well as multilocus tandem repeat sequences, all pointed to a genus with little overall divergence. By the late 1980s the work of Verger encapsulated the idea that *Brucella* was a monospecific genus, *Brucella melitensis*, comprised of six biovars distinguished using previously recognized species designations to simplify transitional

nomenclature [34]. Despite the accuracy of Verger's work, redesignation was not received with enthusiasm [35], primarily owing to the concerns of clinical and veterinary laboratories with a record of success using the original species/biovar designations for epidemiological purposes, as well as a desire to avoid confusion among researchers regarding the origin and hazard associated with a particular agent. The concern was that critical distinctions between species would not be readily recognized by regulatory agencies and individuals lacking experience, leading to significant delays in experimentation. In addition to these practical considerations, this new classification scheme appeared to completely overlook, or at least scientifically underestimate, the importance of genetic divergence in explaining the difference between organisms with regard to host preference and evolution. For this reason, a return to the original nomenclature was agreed on by the international *Brucella* taxonomy and nomenclature subcommittee of the International Committee on Systematics of Prokaryotes (ICSP) with majority support from the International Brucellosis Research Conference in Pamplona, Spain, in 2003 [36].

With the advent of genomic sequencing applied to *Brucella* spp., the nature of their genetic divergence is being revealed. Variable number tandem repeat sequences throughout the genome provide hypervariability due to recombination between and within the repeats that is not provided in other sequences. Multilocus, variable number, tandem repeat sequence analysis is the most frequently used approach and confirms both a clear distinction between species, as well as the close genetic relationship represented by biovars within a species or clade [19,37,38]. However, an added strength of this approach is the exceptional level of geographic distinction observed [19].

Multilocus sequencing has also demonstrated a useful role in phylogenetic studies and global epidemiology [15,19]. The approach uses the DNA sequences from a combination of conserved housekeeping genes that experience slow evolution with generally neutral substitutions. The use of multiple loci is advantageous in that it protects against skewed results from sudden changes that may occur in a single gene locus while retaining an historical record of changes occurring in these conserved loci. Using this approach, Whatmore confirmed that four originally identified species (*B. melitensis*, *B. abortus*, *B. ovis* and *B. neotomae*) represent distinct clusters or clades of organisms. *B. suis* isolates form a much more diverse cluster with *B. suis* biovar 5 distinctly separate from the others and more closely related to the marine mammal isolates and *B. neotomae*, perhaps consistent with the broad diversity of phenotypes (including host range) observed. *B. suis* biovars 3 and 4 appear to have arisen from biovar 1 along with *B. canis*, consistent with a close relationship reported using other typing schemes, including phenotypic analysis. Taken together, these results indicate the continued improvement in our knowledge of the history and evolution of this genus and the valuable tool they represent for evaluating the origins of virulence and host-specific adaptation. A similar profile has been reported based on whole-genome sequencing [39].

Possible expansion of the genus

In parallel with improved diagnostic capability, improved detection methods have resulted in the identification of new species within the genus *Brucella.* This started with the identification of marine mammal isolates in dolphins [40] and in seals [41], which has since been proposed to be three separate genetic lineages (dolphin, seal and porpoise) that group with *B. neotomae* and *B. suis* biovar 5 [19,38,42–44]. This was followed by the identification of *B. microti*, initially in voles, then foxes and finally in the soil [4,45,46]. DNA sequence analysis and comparison of orthologous genes reveals a separate clade for *B. microti*, but a close relationship with *B. suis*. However, numerous phenotypic differences separate these two organisms and belie their close DNA homology. For example, a change

in 23S ribosomal DNA sequence may be the cause of differential growth [15]. The isolation of *Brucella inopinata* BO1 from a breast implant and BO2 from a human lung broadens the group significantly owing to the first observed divergence in 16S ribosomal DNA sequence among *Brucella* spp. [47]. Finally, a close genetic relationship has been confirmed between *Brucella* and *Ochobactrum* spp. based on conserved gene sequence homologies and the presence of the conserved internal spacer sequence 1 and resulted in a call to group *Brucella* within the genus *Ochrobactrum*, despite obvious differences in virulence between these two genera [19].

Activities of the *Brucella* Taxonomy Subcommittee

The function of the *Brucella* nomenclature and taxonomy subcommittee is to assemble available information pertaining to the genus *Brucella* and, using the rules and statutes found in the code, maintain a rigorous classification scheme that is stable and avoids confusion according the International Code of Nomenclature of Bacteria [101]. According to the code, new information must be assessed in order to determine its potential impact on the current taxonomic scheme so that accuracy is maintained and research progresses. Perhaps most importantly, this should be performed in a way that does not hinder the research or endanger investigation (i.e., 'to do no harm'). This principle is encapsulated in rule 56a of the committees activities part 5 "a name whose application is likely to lead to accidents endangering health or life or both or of serious economic consequences", or in Latin, '*nomen confusum*' [48], as previously cited in a recent review by Whatmore of available literature of *Brucella* evolution [19].

Use of rule 56a part 5, in the past, may be found in the minutes of the 2003 meeting in Pamplona in which it was decided that the differences between the *Brucella* spp. are significant and warrant designation as a polyspecific genus. The nature and chronology of the committee deliberations is described within the minutes of the Prague, Nimes and Pamplona meetings of the subcommittee [35,36,49,50]. For a thorough presentation of the major arguments in support of this view, I recommend the article by Whatmore that carefully outlines the justification resulting in the return to the original polyspecific *Brucella* nomenclature [19]. In addition it should be noted that the majority opinion of participants of the International *Brucella* Research Conference in Pamplona Spain (2003) was that designation as a monospecific genus posed a safety risk to personnel and the public, as well as to continued research. However, the summary does not include the fact that returning to the polyspecific genus is generally supported on the scientific grounds that the *Brucella* species represent separate clades with biovars clustering within each clade.

Another recent request for a re-evaluation of the broader family of organisms suggested that the *Brucella* be reclassified within the genus *Ochrobactrum*. Resistance to this latest proposal was expressed on several levels. First and foremost is the concern that such a designation violates rule 56a part 5. The *Ochrobactrum* spp. are opportunistic pathogens requiring no more than BSL2-level containment for experimentation. By contrast, the *Brucella* spp. are all BSL3-level agents and handling requires extensive training of personnel. CDC or US Department of Agriculture approval of facilities and Department of Justice screening of personnel may include psychological testing, as warranted by the Lieberman–Collins bill working its way through the US Congress. Broadening either genus at this time would dramatically hamper research to meet regulatory requirements, and frankly has little support from the *Brucella* clinical, veterinary or research communities. However, despite these practical considerations it is important that the best science is followed and progress unhindered. In order to adhere to this approach, such drastic taxonomic changes must be rigorously justified, otherwise such changes violate the first principle of bacterial taxonomy, which includes the creation of stable nomenclature and

avoidance of useless names that may cause error or confusion. Although it cannot be overlooked that the identification of new *Brucella* spp. broadens the genus, the information available at this point does not appear to be able to meet this standard and any suggestion to re-evaluate the broader family of Brucellaceae or inclusion of *Brucella* within another genus in this family must await additional findings and better understanding of significance of the observed similarities.

Conclusion & future perspective

It is obvious that there are significant differences among the *Brucella* species. Although they appear to be relatively small when compared with differences observed in other genera, one must consider the system in which these changes occurred. Although endowed with the ability to persist in the environment, *Brucella* are normally found in association with their preferred hosts. This close association has apparently resulted in adaptive changes over time. Identification of these changes may be used to identify important interactions that contribute to invasion, persistence, transmission and even virulence, and emphasize the need for more complete comparative genomics. Coevolution of these organisms with their preferred hosts for the most part does not appear to fit a simple pattern and, therefore, adaptation or speciation appears to have occurred rapidly and relatively recently.

Overall, the issues discussed underscore the need to sequence additional *Brucella* genomes before significant changes to *Brucella* taxonomy should be considered. A proposal outlining the minimal standards for designating a new species is planned for submission in order to provide a more rigorous and up-to-date classification scheme. The value to researchers will include increased safety by properly designating organisms that present a danger to investigators, as well as avoiding unnecessary and confusing changes to nomenclature, and providing insight into species-specific adaptation, evolution, host–pathogen interaction and virulence.

Given these changes I feel compelled to offer thanks to the members of the previous subcommittees for their work and vigor in attempting to keep the workings of the committee transparent and up to date. The return to the classical nomenclature should not be viewed as a criticism of their decisions, but rather as recognition on the part of the research community of the limitations, misunderstandings and even dangers associated with the newer designations. However, most importantly, it is an effort like previous subcommittees to remain active with regard to the latest information while maintaining, as best as possible, the spirit of taxonomic distinction and rigor.

Executive Summary

- In order to institute an improved approach to species identification, a manuscript will be submitted to the *International Journal of Systematic and Evolutionary Microbiology* (*IJSEM*) for publication suggesting the level of experimental data necessary to establish a new species.
- Introduction of new species should be performed only following evaluation according to a predetermined set of analysis prior to acceptance. The current recommendation suggests that a minimum data should be provided including:
  - Biotyping, as described by Alton
  - Multilocus variable number tandem repeat sequence analysis at 15 or more loci
  - Multilocus sequencing from at least nine distinct loci

- Review by at least two members of the Taxonomy subcommittee, in order to prevent unnecessary confusion

- The items listed represent the minimum amount of publishable data necessary to assign a new species designation and may be expected to avoid the issues associated with the first principle of bacterial nomenclature (i.e., do no harm).
- It is important to point out that investigators may want to consider the analysis described above for any working stock organism in order to confirm its designation.
- The need for a system that recognizes significant differences in phenotypic properties, host specificity and virulence, along with a demand for a useful system capable of making clear distinction between isolates with regard to their possible source and potential risks forced the re-evaluation of taxonomic classification of *Brucella* as a monospecific genus.
- DNA sequence analysis has provided the tools necessary to confirm that the *Brucella* species represent distinct lineages or clades. The overall intent of the changes suggested is to provide support for changes in taxonomic classification, its relation to the evolution of the *Brucella* genus and species, and the potential significance to the study of host–pathogen interaction, virulence and immunology.
- It has taken several years to recognize the limitations or dangers that may result from a nomenclature that recognizes a monospecific genus. Of course the return to the original nomenclature has its own drawbacks, most notably the need to reconcile extant databases. However, this is a limited problem when one considers the potential benefits, including renewed interest with regard to genus and species evolution, adaptation to specific hosts, factors affecting transmission and improved diagnostic tests to aid in epidemiologic tracking.

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