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Brucellosis: Evolution and expected comeback

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ABSTRACT

Brucellosis is a serious infectious disease which causes great direct and indirect economic loses for animal holders worldwide such as the reduction of milk and meat production through abortions/culling of positive reactors, the expense of disease control/eradication and farmers compensation. Although the disease was eradicated from most of the industrial countries, it remains one of the most common zoonotic diseases in developing countries being responsible for more than 500,000 new cases yearly. Brucella is considered to be a bioterrorism organism due to its low infectious doses (10–100 bacteria), capability of persistence in the environment, rapid transmission via different routes including aerosols, and finally due to its difficult treatment by antibiotics.There are many reasons to believe that a new comeback of brucellosis may occur in near future. This expectation is supported by the recent discovery of new atypical Brucella species with new genetic properties and the recent reports of (man to man) disease transmission as will be discussed later. The development of new concepts and measurements for disease control is urgently required. In the present review, the evolution of Brucella and the different factors favoring its comeback are discussed.

1. Introduction

Brucellosis is a serious infectious disease affecting different mammalian species including man. Natural infection of farm animals occurs mainly through ingestion of food or water contaminated by uterine discharges, aborted feti or fetal membranes and even through licking the genitalia of diseased animals. In addition, infected males can also spread the infection among females through natural mating and artificial insemination. Brucellae can pass through intact or injured skin and through all mucous membranes [1].

Direct and indirect contact with diseased animals or foodstuffs of animal origin represents the major source of infection to humans. It was thought that the infected human are the dead end of the infection, however, human to human transmission was recorded recently [2]. Ice cream and homemade cheese play an important role in the spread of the disease among human as they are prepared in a way which does not eliminate viable Brucella bacilli [3].

Investigation of burned cheese rests found in the old Roman city (Herculaneum) which was suddenly destroyed in August 79 AD by the volcanic eruption (Vesuvius) revealed the presence of bacterial colonies morphologically resemble Brucella, which may be the first sign of brucellosis in the old ages [4]. In 1884, Dr. Bruce was able to differentiate between brucellosis (Malta fever) and typhoid outbreaks affected Malta. Three years later, he isolated the causative agent of Malta fever and named the bacterium *Micrococcus melitensis*. In 1897, Dr. Bang studied the disease in Denmark and could isolate *Brucella abortus* strains from aborted cattle. He noticed that the pathogen can also infect sheep, goat and horses, the disease became known as (Bang's disease). Later on, in 1918, Evans could detect the connection between animal and human cases after he isolated an organism from human aborted foetus which was closely related to Bruces's organism. In the year 1938, it was possible to differentiate among the caprine, bovine and swine forms of Undulant fever caused by *B. melitensis*, *B. abortus* and *B. suis,* respectively. Since 1884 till now, brucellosis represents a continuous re-emerging zoonoses worldwide [4–6].

Brucella is a Gram-negative, non-motile coccobacilli. It belongs to alpha-Proteobacteria, which include in addition to Brucella other members such as Agrobacterium, Rickettsia, Rhodobacterium, and Rhizobium. However, recently atypical motile Brucella isolates were isolated from diseased frogs [7].

Brucella was considered to be a facultative intracellular pathogen in most references; however, they were re-designated as facultative extracellular intracellular pathogens due to their evolutionary relationship to other alpha-Proteobacteria. Brucellae are stealth microbes which prefer induction of chronic rather than acute infections [8].

Due to the high genomic homology among the typical Brucella species, it was supposed in the 1980 s that Brucella is a monospecific genus (*Brucella melitensis*) which has 6 biovars distinguished according

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to their host prevalence, the different Brucella species were renamed e.g. *Brucella abortus* was called *Brucella melitensis* biovar *abortus*. However, this classification did not survive the new data delivered by molecular biological genotyping tools [9,10].

Through the modern molecular tools it was possible to prove that *B. melitensis, B. abortus, B. ovis* and *B. neotomae* represent 4 related clones of one organism while *B. suis* (including *B. suis* biovar 5) forms a distinct cluster from them but closely related to the marine mammals Brucella species isolated from dolphin, seal and porpoise. Meanwhile, *B. suis* biovars 3 and 4 seem to be evolved from *B. suis* biovar 1 and *B. canis*. These relationships were confirmed by the data delivered by whole genome sequencing [9,10].

However, after the discovery of the new Brucella species, the old debate arose again. Positioning of the recently detected atypical Brucella species (specially *B. microti* and *B. inopinata*) was problematic due to their clear distinction from the classical ones on phenotypic and genetic levels. Both *B. microti* and *B. inopinata* are fast growers and highly active metabolically. They have a unique *16S rRNA* gene with 5 different nucleotide sequences when blasted with the highly conserved corresponding gene of the other Brucella species. The genetic diversity among the different species of Brucella is clearer than the diversity between the closely related genera Brucella and Ochrobactrum. Trials to group both Brucella and Ochobactrum spp together were carried out through the fusion of current Brucella species in one species with subspecies and biovars (e.g. *B. melitensis* subsp. *abortus* biovar 1) and in the same time to translocate all species of Ochrobactrum into the genus Brucella. However, these trials failed as the Brucella spp. are obligatory pathogens while the Ochobactrum spp. are opportunistic pathogens. This close phenotypic relationship is best seen when blasting the genomes of both *B. microti* and Ochrobactrum. This closeness lead to the false identification of *B. microti* in the past as a new member in genus Ochrobactrum [9–11].

At the time, at least 12 Brucella species are known (Table 1). Due to its great economic and zoonotic importance, it is important to identify field isolates of Brucella not only at their species level but also their genotypes. This enables the detection of hidden foci of Brucella and to tract the sources of infection in the population. As an example, genotypic analysis of different *B. abortus* field strains isolated from cattle, bison and elk showed that the cattle isolates are closely related to elk isolates but completely divergent from those of bison [12]. Genotyping of the field isolates enables also the differentiation between infected animals/veterinarians due to accidental exposure to vaccinal strains (*B. abortus* S19 and RB51) from those infected with field strains although the *B. abortus* genome is highly conserved among various *B. abortus* biovars including S19 *B. abortus* smooth vaccinal strain which is closely related to strain 2308 [10,13]. Proper genotyping differentiates among vaccinal strains from other field genotypes using specific primers targeting the *ery* locus (for S19) or the *wbo*A gene (for RB51) [14].

Similarly, genotypic investigation of the field isolates in Germany enabled the detection of the source of human infections there. It was long believed that the human infections in Germany are related to tourisms in the Middle East countries, however, the genotypes of *B. melitensis* isolated from German patients were more related to the clades present in Southeast Europe, Turkey, Afghanistan, Turkmenistan, Far East and Southeast Asia with a clear genetic diversity from those originating from Middle East [15]. Genotyping of animal field isolates is also important for public health issues. As an example, *B. suis*, the etiological agent of swine brucellosis, consists of 5 biovars [1–5], while biovar 2 is rarely zoonotic, biovars 1 and 3 are extremely pathogenic to humans [16]. The close relationship between *B. canis* and *B. suis* enabled *B. suis* to reemerge recently among dogs causing sever reproductive problems in dogs and health hazards to humans in contact with diseased dogs. Even cattle, horses, sheep and deer in contact can catch the infection with *B. suis* also [17,18].

2. Evolution of brucellosis

Blasting the genomes of *B. melitensis, B. abortus, B. suis, B. neotomae* and *B. canis* against that of *B. ovis* reveals an overall DNA homologies of 95% indicating that they all were diverged from a common ancestor very close to the *B. ovis* 86,000–296,000 years ago[10]. This occurred as a result of the infection of wild mammals with the *B. ovis* ancestor

Table 1
List of different Brucella species and their natural hosts.*

Brucella species	Colony type	Natural host**	Zoonoses	Year of first isolation
B. melitensis (bv1-3)	Smooth	Goat and sheep	+ + +	Bruce (1893)
B. abortus (bv 1–6, 7, 9)	Smooth	Cattle	+ +	Schmidt (1901)
B. suis biovar***				Huddleson (1929)
1–3	Smooth	Pig	+ +	
2	Smooth	Wild boar, Hare	+	
4	Smooth	Reindeer, Caribou	+ +	
5	Smooth	Rodent	–	
B. ovis	Rough	Sheep	–***	Buddle (1956)
B. neotomae	Smooth	Desert rat	+	Stoenner and Lackman (1957)
B. canis	Rough	Dog	+	Carmichael and Bruner (1968)
B. ceti (B. delphini)	Smooth	Dolphins	+	Foster et al. (2007)
B. pinnipedialis (B. phocae)	Smooth	Seals	+	Foster et al. (2007)
B. microti	Smooth	Wild voles	(?)	Scholz et al. (2008)
B. inopinata	Smooth	Human	+ +	Scholz et al. (2009)
B. papionis	(?)	Baboons (*Papio* spp.)	(?)	Whatmore et al. (2014)
B. vulpis	(?)	Red foxes (*Vulpes vulpes*)	(?)	Scholz et al. (2016)
N.N.****	Smooth	Frog	(?)	Soler-Lloréns et al. (2016)

* Different Brucella species and their natural hosts according to [4,5,7,39,41–46].

** The host susceptibility range of Brucella species is not extremely narrow. Nearly all Brucella species can infect other mammals beside their primary host with the exception of *B. ovis*. In such cases, the infection is mostly mild and even self-limiting.

*** Different *B. suis* biovars vary in their zoonotic potential, while biovars 1, 3 and 4 are more pathogenic to human than *B. abortus* but less than *B. melitensis*, other *B. suis* biovars have obviously limited potential to infect humans. The reason why the *B. ovis* is not zoonotic in opposite to the rest of Brucella species is attributed to the fact that the genome of *B. ovis* contains a high percentage of pseudogenes and other mobile genetic elements compared to the rest Brucella species due to genome degradation in parallel with narrowing of the host susceptibility scope of *B. ovis*. This genomic degradation and re-arrangement lead to the deletion of the genomic island 2, which is responsible for lipopolysaccharide biosynthesis in addition to the inactivation of essential genes regulating nutrient uptake and utilization. All of these factors, beside the inactivation of genes responsible for the synthesis of the envelop outer membrane proteins, lead to the loss of the ability of *B. ovis* to invade humans and many other mammalian species [25].

**** An intermediate trait between the soil associated ancestor of Brucella species and the known host adapted Brucella species. No data are yet available about its zoonotic capability.

following their contact with infected sheep before the human could domesticate farm animals [10,13]. Later on, about 7500–22,500 years ago, *B. canis* got separated from its common ancestor shared with its closely related *B. suis* bv4 strains following feeding wild canids on infected swines with *B. suis* ancestor [10,13,19].

The Brucella ancestor was most likely a free living bacterium with one chromosome which evolved into an animal parasite with two separate chromosomes, a large sized chromosome and a smaller plasmid originating one. However, some Brucella species still have only one chromosome, others kept their ancestor accessory genes responsible for utilization of plant derived nutrients such as *B. suis* which possess transport and metabolic activities similar to those of certain soil-plant-associated bacteria. Wide co-localization of genetic loci can be seen in *B. suis* chromosome 1 and the genome of *Mesorhizobium loti* which is a plant symbiont, indicating an evolutionary relationship between Brucella and the plant pathogens and symbionts [11,20].

Over the years, the genome of Brucella species carried out independent complex genomic recombinations and translocations of DNA loci between both chromosomes. While all *B. melitensis*, *B. abortus* and *B. suis* (biovare1) genomes consist of two chromosomes with a size of 1.1 Mb and 2.2 Mb, the small chromosome of *B. suis* (biovare 2 and biovare 4) is clearly larger in size (1.35 Mb). Meanwhile their large chromosome is clearly smaller in size (1.85 Mb) instead of 2.2 Mb. On the other hand, the *B. suis* (biovare 3) strains differ also in genomic structure from other *B. suis* biovares [21].

Brucella is mostly classified as a facultative intracellular pathogen (others consider it a fac. ext./int. cellular pathogen). This may be attributed to the larger genome size in Brucella (50–100% larger than Bartonella genomes) which enables Brucella from having and sharing more metabolic functions with their related plant pathogens such as the ability to persist in soil or in other environments for long period with the ability to utilize plant based molecules [11,20].

It is believed that Brucella species evolved independent from the evolution of their hosts as Brucella phylogeny does not appear to reflect the phylogeny of Brucella species preferred hosts. During their evolution, the Brucella carried out genome reductive evolutionary processes (Domino theory for gene death) which was necessary during evolution of Brucella to get adapted to the parasitic lifecycle. During this process many genes lose their function and become either deleted or pseudogenized. This usually occurs in a stepwise manner, therefore it is called (Domino theory). One of the disadvantages of this process is that the microbe become obligatory pathogenic to compensate the lost resources [22].

Also, sometimes the inactivation or deletion of several genes during evolution may affect the bacterial virulence passively, the inactivation of the genes responsible for nutrition acquisition and utilization lead to the limitation of the virulence of *B. ovis* and narrowing its tissue tropism and host range [23–25]. In the same way, the deletion of the gene encoding an autotransporter protein from the *B. abortus* strain 19 lead to their natural attenuation property [21].

A frameshift of the GAD open reading frame lead to the impairment of the GAD system in classical Brucella species (*B. abortus, B. melitensis, B. suis, B. canis, B. ovis* and *B. neotomae*) and the closely related Ochrobactrum members but not in the newly discovered Brucella species (B. *microti, B. ceti, B. pinnipedialis* and *B. inopinata*) which kept their original open reading frame [26,27].

With the exception of *B. microti* and the closely related *B. suis* 1330 strins, all Brucella species own a highly pseudogenized ketoadipate pathway reflecting gene death “domino theory”. In contrast, only *B. microti* and *B. suis* 1330 strains own an intact ketoadipate functional pathway similar to that of the *Ochrobactrum*. This pathway is highly conserved in soil bacteria and fungi, it enables the bacteria from the utilization of plant-derived substances. In parallel to the gene death, new genes were acquired through horizontal gene transfer. These new genes are not shared with the ancestor of the Brucella such as the shared anomalous regions (SARs).

For disease induction, the Brucella must adhere to, invade and survive inside the mammalian cells. The required genetic elements to fulfill these processes seem to be acquired through genetic conversion by both Bartonella and Brucella later in the evolution [23–25]. These genes encode mostly Brucella virulence factors as the *T4SS*, *omp*31, *hpa*E and acid resistance genes. They are mainly clustered in the 15 genomic regions of Brucella genome (a genomic island), also in the regions 4, 7 and 14. The acid resistance genes enable their survival in the acidic environment in the stomach and in the phagosome. It is also possible but less likely that the progenitor organisms had such genes but lost them later in the plant pathogens [26,28,29].

The ancestor precursor of Brucellaceae carried out the first evolutionary step through acquiring the *VirB T4SS* (type IV secretion systems) which allowed Brucella to adapt to a pathogenic niche. This was accompanied by genome reduction and adaptation to enable intracellular survival and multiplication within host cells even the macrophages such as acquiring genes needed for gaining ions from the hosts. This step includes genome reduction in Brucella with at least 30% compared to Ochrobactrum which represents about 900 orthologous genes (OGs) which are present in Ochrobactrum but not in the closely related Brucella. These deleted genes encode mainly proteins involved in metabolism, utilization or biosynthesis of nutrients. At this stage the Ochrobactrum were separated from the rest of the group which are soil bacteria capable of inducing opportunistic infection in immunocompromised vertebrate hosts without being able to multiply in their hosts [26].

The following step in the evolution of Brucella involved the change to a perosamine-based O-antigen which is needed for intracellular replication (Refining LPS) and to avoid strong immune response following infection. While most field isolates of Brucella possess a smooth lipopolysaccharide (LPS) in which the O-antigen is formed of a homopolymer of N-formylperosamine. Both *B. abortus* and *B. ovis* strains can spontaneously change from smooth to rough LPS through the excision of GI 2 which carry *wbo*A and *wbo*B genes [21,30].

Normally, field isolates of *B. canis* and *B. ovis* are rough types. This can be clarified as most genes required for the synthesis of O-antigen are acquired by horizontal gene transfer to the *wbk* region (region 16, carried on a genomic island). The *wbk* region shows deletion and truncation in *B. canis* and *B. ovis*. The acquisition of these genes was necessary for the conversion of Brucella species to persist as an intracellular parasite. Shifting of the life pattern of Brucella required genetic adaptation to survive in limited metal environment. Therefore, many of the genes acquired by Brucella facilitate metal ion transport, mainly iron, magnesium and nickel to the bacterial cells. These genes become activated in response to metal limitations in the surrounding environment. e.g. the production of siderophore in iron limited environment such as in ruminant placenta, also the production of *MgtBC* transporter in response to Mg limitations. These systems enable the survival of Brucella inside the mammalian macrophages. The role of nickel in Brucella virulence is not clear. Mutations in *nik*ABCDE operon decrease the urease activity in affected strains without any negative effect on bacterial virulence. At this stage *B. inopinata* got separated from the rest. Some *B. inopinata* strains (e.g. BO1 and *B. inopinata*-like strain BO2) differ from other Brucella strains in being resistant to invasion by Brucella bacteriophages and in having different antigenic characteristics. In addition, they are fast growers when cultured on bacteriological media, a character which is shared with *B. microti* isolates. A possible reason could be the presence of an unusual spacer region in their *23S rRNA* gene which is present in other fast growing strains of *B. inopinata.* [21,26].

The following step in the evolutionary path toward virulence was gaining the facility to modify the host immune response. This was achieved through the use of the Toll interleukin receptor (TIR) domain proteins located at the 21 Kb sized genomic island 3 (GI 3). The TIR domain contains various proteins which play a role in Brucella survival inside the host through disturbing and modifying the Toll-like receptors

(TLRs) signaling pathway of the host immune system (they inhibit both TLR2-/TLR4-mediated NF-κB activation pathway) [31].

It was common during the evolutionary trip of Brucella typical strains to acquire different foreign DNA fragments by horizontal gene transfer which are absent in atypical strains. These fragments are distributed all over the genome in 13 regions and encode various proteins mostly of unknown function. At this step *B. microti* was separated from the rest of the Brucella species [21,26].

From the evolutionary side of view, *B. microti* stands in the midway between Ochrobactrum anthropic and *B. suis* 1330. Sequences alignment of both *B. microti* and *B. suis* 1330 is almost identical (homology of 99.84%). The major genetic difference was found to be induced through the lysogenization with a lambdoid bacteriophage in *B. microti*. The insertion of the 11.742 bp DNA fragment did not lead to any phenotypic changes in *B. microti*. [29].

Another surprising difference between *B. microti* and other Brucella organisms is the sequence heterogenicity of the *23S rRNA* gene which clarifies the fast growth nature of *B. microti*. [29].

The data obtained through genome analysis of various Brucella and *O. anthropi* genomes revealed that all Brucella spp. and *O. anthropi* share 4 conserved genes which are only functioning in *O. anthropi* and *B. microti* but are impaired in all other Brucella species [29,32].

3. The comeback of brucellosis

Although Brucellosis was eradicated from farm animals in most developed countries, an expected comeback of brucellosis in the near future is possible due to many reasons including:

1. Urbanization and the alteration of human socio-demographics. The human population increases worldwide, the human and the domestic animals are coming in closer contact with wild animals. As no vaccination policy is applied in Brucella free countries, the domestic animals are very susceptible to Brucellosis (naïve). The re-introduction of the disease through contact with infected wild animals will have a catastrophic effect and causes storms of abortion. In addition, financial crisis and civil war in many countries lead not only to stoppage of infectious disease control programs but also to migratory waves/refugee from developing countries. The immigrates suffer usually from poor nutrition, bad hygiene and over crowdedness which lead to spread of infectious diseases.
2. Although every Brucella species is bound to a specific host, their pathogen-host relationship is not exclusive. The growing population lead to intensive breeding of farm animals and it is common to have mixed livestock farming strategy which facilitates cross species infections [33,34].
3. All currently-used serological screening tests were originally developed and validated for use in cattle. When applied to other species, they were shown to be inaccurate, unpredictable and need re-validation [35].
4. Many new Brucella species were discovered in the last few years mainly the marine Brucella spp. which are capable of infecting terrestrial mammal as cattle, sheep, piglets and human. This complicates the running control programs. Experimental work with the newly discovered *B. microti* showed that *B. microti* owns the most potential pathogenic capability among all known Brucella spp. It can even replicate inside the macrophages. Experimental infection of mice's with 10^5 was able to kill 82% of infected animals within 7 days. Recent researches suggest a zoonotic potential of *B. microti*. Experimental infection with Brucella strains isolated from frogs and cold blooded animals revealed high potential to invade and survive in mammalian host for about 3 months [7,36].
5. The newly characterized Brucella species have a high genetic flexibility. Many of these isolates are mobile, fast growers, able to survive in the soil, more resistant to high acidity and unfavorable environmental condition and show high capacity for adaptation to new non-mammals hosts such as amphibians and are high active metabolically. They can adapt themselves very quickly to their environment to extend their host range [7].
6. Possible transmission of these unique properties of the atypical Brucella species to the widely spread typical Brucella spp. via mobile genetic elements (e.g. bacteriophages, transposons, pathogenicity islands, etc) will have a catastrophic effect on animal husbandry and public health worldwide.
7. The newly discovered Brucella species in the last 20 years show great genetic diversity even more than that exists among thousands of isolates of the classical Brucella species discovered throughout the Twentieth century. These atypical Brucella species have a close genetic relationship with soil bacteria. Genome analysis studies showed that *B. microti* lies in the midway between saprophytic soil bacteria and the pathogenic Brucella species. This enables them from gaining new genetic properties from the environmental soil bacteria [7].
8. Role of soil as primary habitat for some Brucella types such as *B. microti* which has a nonliving natural reservoir outside its mammalian host. It can survive up to 6 months in the soil, which indicates an environmental niche shared by all members of family Brucellaceae. Its frequent isolations from different animal species worldwide indicate that *B. microti* could possibly be an emerging pathogen and could release a pandemic of brucellosis. It is also possible that *B. microti* can multiply in the soil outside the mammalian host due to the presence of functional ketoadipate pathway [29,39].
9. Possible potential role of the lungworms, cestodes and other parasites in transmission of marine Brucellosis which will open the gate for new routes of transmission [37]. The role of some ectoparasites such as stomoxys in the transmission of terrestrial mammal Brucella was previously suspected [38]. Climatic changes (global warming/water scanty/dissertation) lead to the spread of insects/parasites (and therefore insect borne diseases) to new regions.
10. Brucella is a robust pathogen, with a multiple routes of infection. It can resist inside and outside the mammalian hosts for a long time even under unfavorable conditions. It persists in the food up to 15 months even under unfavorable conditions as acidity and temperature between 11 and 14 °C. or for 2–3 days under 37 °C. Brucella may also survive in aborted infected feti and contaminated manure for more than 2 months in winter or few hours if exposed directly to sunlight [3]. The presence of functional glutamate decarboxylase dependent system (GAD system) in *B. microti* allows it survival at very low pH levels. The system is activated if the bacteria is exposed to very low pH values (≤ 2.5) in order to overcome the harmful effect of acid stress. The presence of GAD system has a great diagnostic importance as a PCR target for characterization of atypical Brucella species [27].
11. Brucellae are stealth microbes which tend to chronicity rather than causing acute fatal infection. Brucella keeps its victims alive to maintain their survival. Throughout their evolution, Brucella developed dynamic strategies to escape recognition and attacks by the immune system, to modulate the acquired immune response of the host, and to escape intracellular inactivation. This makes the treatment of brucellosis very difficult. In addition, the GAD system enables oral infection (survival in the stomach) and the later survival when being engulfed by macrophages [8,27].
12. Brucella was always considered to be an animal pathogen with a high zoonotic impact and that infected humans are the dead end of the disease. However, it was proven recently that man to man infection is possible. This may be related to the continuous improvement in the diagnostic and epidemiological tools, or to the continuous adaptation of the organism to their hosts [3,7].
13. Brucella is an ideal bioterrorism/biological weapon due to its low infectious doses, persistence in the environment/host, rapid transmission via different routes including aerosols, and difficult

treatment by antibiotics. Any scape of the organism from military storage or use in terroristic attack will have catastrophic effect. Till now there is no human vaccine against brucellosis [40].

14. Brucella vaccinal strains may accidentally induce human outbreaks. Human brucellosis caused by Brucella RB51 vaccinal strain shed in cow's milk was reported by CDC in September 2017 in Texas state [41].

4. Conclusions

In conclusion: between 1968 and 2007 no new Brucella species were discovered. Since 2007 many new species were detected some of them are highly zoonotic. There are many reasons to worry about possible comeback of brucellosis. Efforts must be done to develop human vaccines against brucellosis and to adapt our Brucella control programs to the new situation.

Competing interests

The authors declare no competing interests.

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