Lipopolysaccharide as a target for brucellosis vaccine design

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Abstract

The gram-negative bacteria of the genus *Brucella* are facultative intracellular parasites that cause brucellosis, a worldwide-distributed zoonotic disease that represents a serious problem for animal and human health. There is no human-to-human contagion and, since there is no human vaccine, animal vaccination is essential to control brucellosis. However, current vaccines (all developed empirically) do not provide 100% protection and are infectious in humans. Attempts to generate new vaccines by obtaining mutants lacking the lipopolysaccharide O-polysaccharide, in purine metabolism or in *Brucella* type IV secretion system have not been successful. Here we propose a new approach to develop brucellosis vaccines based on the concept that *Brucella* surface molecules evade efficient detection by innate immunity, thus delaying protective Th1 responses and opening a time window to reach sheltered intracellular compartments. We showed recently that a branch of the core oligosaccharide section of *Brucella* lipopolysaccharide hampers recognition by TLR4-MD2. Mutation of glycosyltransferase WadC, involved in the synthesis of this branch, results in a lipopolysaccharide that, while keeping the O-polysaccharide essential for optimal protection, shows a truncated core, is more efficiently recognized by MD2 and triggers an increased cytokine response. In keeping with this, the *wadC* mutant is attenuated in dendritic cells and mice. In the mouse model of brucellosis vaccines, the *B. abortus wadC* mutant conferred protection similar to that provided by S19, the best cattle vaccine available. The properties of the *wadC* mutant provide the proof of concept for this new approach and open the way for more effective brucellosis vaccines.

Brucellosis: a world wide important zoonosis that requires the use of animal vaccines

Brucellosis is a bacterial zoonosis caused by members of the genus *Brucella*, a group of gram-negative bacteria that behave as facultative intracellular pathogens of mammals. There are several highly homologous *Brucella* spp. [1, 2] among which *B. abortus* preferentially infects cattle, *B. melitensis* sheep and goats, and *B. suis* swine and wildlife. Brucellosis is a main cause of abortions and infertility in these animals and a grave disease in humans that requires a long and costly antibiotherapy. The number of domestic animals susceptible to brucellosis in the world is estimated in 4,000,000,000 and of these less than 20% are in countries free of the disease. In the USA, Australia and most EU countries, bovine brucellosis has been eradicated after heavy investments and many years of vaccination and culling. Sheep and goat brucellosis has proved more intractable, largely because of the difficulties of animal management under the extensive breeding conditions of semiarid areas. Human brucellosis is always a consequence of the animal disease. Because of the structural weaknesses, there are no reliable data on the global incidence of the human disease. Figures vary widely (from <0.01 to >200 per 100,000), reflecting the difficulties in recognizing a disease that, although grave, has no pathognomonic symptoms and is thus underreported [3,4]. Because of this, WHO ranks brucellosis among the top seven "neglected zoonoses", a group of diseases that are both a threat to human health and a cause of poverty [5,6].

Vaccination is critical to control and eradicate ruminant brucellosis [7-9]. Since there is no safe human vaccine [10], animal vaccination is also the best way to lessen human infections. Vaccines *B. abortus* S19 and *B. melitensis* Rev 1 are effective against *B. abortus* in cattle and *B. melitensis* in goats and sheep [7-9]. These vaccines were empirically obtained long ago and they do not provide 100% protection, are virulent for humans and Rev 1 is resistant to streptomycin (an antibiotic of choice to treat brucellosis [11]). Moreover, both trigger an antibody response to the O-polysaccharide (O-PS)[1] of the outer membrane smooth lipopolysaccharide (S-LPS; see below) and, because O-PS carries the diagnostically important epitopes, this circumstance is a cause of false positive results in serodiagnostic tests and complicates eradication.

Attempts to overcome the drawbacks of current brucellosis vaccines

Because of the drawbacks of classical vaccines, new ones have been investigated. To solve the problem of the diagnostic interference, vaccines S19 or Rev1 have been deleted in outer membrane proteins Omp25 and Omp31 or in the periplasmic protein BP26, and associated tests detecting antibodies to these proteins in infected animals proposed [12,13]. Among the many attenuated *Brucella* mutants described in the literature [10], only a handful has been actually tested in the natural hosts. These include attenuated mutants in genes related or potentially related to *Brucella* intracellular life, such as VirB (a type IV secretion system) [14], stress proteins HtrA [15] and Asp24 [14] and the periplasmic catalase KatE [16]. Similarly, PurE (purine synthesis) [17] and cytochrome bd (respiration) [14] metabolic mutants have been investigated looking for vaccine strains that, while showing a reduced multiplication *in vivo* would still stimulate immunity. Nevertheless, none of these vaccine candidates has proved adequate either because of their high residual virulence, protection lower than that

[1] Abbreviations used: Kdo, 2-keto, 3-deoxyoctulosonic acid; LPS, lipopolysaccharide; O-PS, O-polysaccharide, PAMP, pathogen-associated molecular pattern; S, smooth; VLCFAs, very long chain fatty acids.

afforded by S19 or Rev1 or, for the antigen-deleted vaccines, a diagnostic sensitivity of the associated test lower than that achieved with the O-PS tests. Also, mutants devoid of the O-PS (i.e. rough mutants) have been extensively investigated [18-21] (see rough vaccines below).

Brucella as a silent parasite: a concept useful in the development of new vaccines

Studies carried out with smooth brucellae show that their virulence resides in their ability to control their intracellular trafficking and adapt to the intracellular niche as well as on the inability of the innate immune system to effectively recognize the presence of these bacteria during the first encounter [22]. This failure is caused by the absence of marked pathogen-associated molecular patterns (PAMPs) in some outer membrane components [22-25] of these three *Brucella* species. Accordingly, typical antigen-presenting cells like dendritic cells and macrophages that carry the TLR receptors for outer membrane PAMPs fail to detect brucellae before they reach the multiplicative intracellular niche. The consequence is an exceedingly low initial release of those innate immunity mediators that are crucial for the timely activation of the Th1 response necessary to control intracellular brucellae. Therefore, an understanding of the molecular peculiarities of *Brucella* S-LPS and the interaction with innate immunity receptors could be useful for the development of new brucellosis vaccines.

The S-LPS of gram-negative bacteria (also known as endotoxin) consists of three sections: lipid A, core oligosaccharide and O-PS. The typical lipid A is made of a hexaacylated glucosamine disaccharide carrying predominantly C12 to C14 acyl chains in ester, amide and acyl-oxyacyl bonds (Figure 1). This structure is conserved in many gram-negative groups and carries a PAMP readily recognized by the TLR4-MD2 receptor-coreceptor complex. Experiments with *Escherichia coli* C12-C14 hexaacylated lipid A show that lipid A interacts with a large hydrophobic groove in MD2, with five acyl chains deep inside, the remaining chain in a hydrophobic interaction with TLR4 and the bisphosphorylated glucosamine disaccharide tilted outwards. In this way, the lipid A phosphate groups contribute to receptor multimerization by interacting with positively charged residues in TLR4 and MD2 [26]. Subsequent signaling to the nucleus of the host cell triggers potent proinflammatory responses that, when unchecked, lead to endotoxic shock. Although purified lipid A has a potent endotoxic activity by itself, the innermost part of the core oligosaccharide is also part of the LPS PAMP. Molecular modeling and X-ray diffraction show that it forms a compact entity with an unusually high partial density [27]. This tight packing and the dense negative charge created by the 2-keto, 3-deoxyoctulosonic residues (Kdo) and phosphates (Figure 1) make the inner core a unique structure targeted by polycationic bactericidal peptides and the C1q complement component of innate immunity [24]. Moreover, these features indicate that the inner core contributes to the interaction with the positively charged amino acid residues that outside the hydrophobic groove in MD2 take part in LPS recognition [28]. The O-PS, on the other hand, is highly variable and, although often essential for the virulence of gram-negative pathogens, does not contribute to the LPS PAMP.

In contrast to most LPS, *Brucella* LPS displays low endotoxicity, is recalcitrant to bactericidal peptide binding and activates complement poorly [23]. Indeed, all these properties indicate an altered PAMP that could account at least in part for the stealthy behavior towards innate immunity [22]. Early attempts to modify this *Brucella* molecule can be traced to the late 30's of the past century [7] but they were focused on the removal of the antigen section causing diagnostic troubles in vaccinated animals (the O-PS) (see above). The O-PS is involved also in *Brucella* virulence: it contributes to complement resistance and, more important, critically

modulates bacterial entry into cells so that its removal causes attenuation. Nevertheless, vaccines devoid of this LPS section (the so-called *Brucella* rough vaccines) do not completely solve the diagnostic problems and are considerably less protective than S19 or Rev 1 [19,21] possibly because their severely affected entry into host cells results in over attenuation. On the other hand, low endotoxicity, reduced bactericidal peptide binding and poor complement activation speak of peculiarities in the sections that in typical LPS carry the PAMP triggering TLR4-MD2 mediated responses. Therefore, it is plausible that an understanding of the structure and genetics of the core and lipid A should offer possibilities to uncover the brucellae to innate receptors, thereby promoting protective Th1 responses.

Making *Brucella* LPS visible to innate immunity

A first objective of this research is lipid A. *Brucella* lipid A is a diaminoglucose disaccharide substituted with C16, C18, C28 and other very long acyl chain fatty acids (VLCFA) [29] (Figure 1) and comparative studies with other lipid A molecules have established the connection between this structure and low endotoxicity and TLR4 recognition [30]. Similar comparative analyses also show a poor recognition of *B. abortus* LPS by MD2 (Figure 2A). Based on the interaction between MD2 and the canonical, C12-C14 hexaacylated lipid A (see above), it can be postulated that lipids A with VLCFAs are not accommodated in the MD2 hydrophobic groove and are thus poor MD2-TLR4 activators. If this hypothesis were correct, a first approach to generate new vaccines would be to introduce a canonical PAMP in *Brucella* lipid A by blocking the incorporation of VLCFA onto the lipid A diaminoglucose backbone. Research is in progress in the laboratories of the authors to test this possibility.

A second target in *Brucella* LPS for the design of new brucellosis vaccines is the core oligosaccharide. The reduced affinity of bactericidal peptides for *Brucella* LPS could be accounted for by the low density of negatively charged groups that is perceived in the determination of the zeta potential[2] of *B. abortus* LPS aggregates [31], and this same feature could also contribute to a low affinity for MD2. In keeping with this, qualitative chemical analyses indicate that this core contains Kdo as the only or major negatively charged sugar plus glucose and mannose and, noteworthy, glucosamine [29]. Intriguingly, measurement of the zeta potential in the presence of polymyxin B reveals that the negatively charged groups in *Brucella* core-lipid A sections are not accessible to this potent polycationic lipopeptide. This is in contrast with the results obtained with the LPS of *Ochrobactrum* sp. LPS (Figure 2B), a *Brucella* phylogenetic relative that has a similar lipid A. Taken together, these biophysical and chemical analyses suggest that the negative charge in *Brucella* core and lipid A associated with Kdo and lipid A phosphates is largely counterpoised by core amino sugars and, moreover, that the structure is such that sterically hinders access to Kdo and lipid A.

For a better understanding of these phenomena, we are carrying out an exhaustive search for LPS core genes. A method extensively used in this kind of research is random mutagenesis and crystal violet screening for rough *Brucella* mutants (for a review see [20]). This strategy, however, has only identified some metabolic genes providing core precursors (*manA*$_{core}$, *manB*$_{core}$ and *pgm*) plus one glycosyltransferase gene (*wadA*) and, moreover, the

[2] Amphipathic lipids or glycolipids like S-LPS aggregate in aqueous solutions and their charged groups express at the surface of aggregates an electrical potential extending into the bulk of the solution that modulates the binding or repulsion of polycationic peptides. This electrical potential can be measured as the electrophoretically effective potential (zeta potential) (see [31]).

corresponding mutants not only carry core defects but also lack the O-PS. These mutants are not effective vaccines [21] and, since the phenotype caused by the O-PS loss overshadows that of the defect in the core, they are not useful for a precise analysis of the role of the latter. As an alternative approach, we have carried out an exhaustive genomic analysis of brucellae using as references the LPS genes in other α-2 *Proteobacteria* (the phylogenetic group to which brucellae belong) in search for putative LPS glycosyltransferases. In addition to the expected *waaA* (Kdo transferase gene, highly conserved in *Proteobacteria*) and *wadA* (see above) genes, we identified several ORF candidates. Two of these ORFs (ORFs BAB1_0351 and BAB1_1522) have been analyzed so far by creating the corresponding in frame deletion mutants in *B. abortus*, *B. melitensis* and *B. suis*. These mutants are noteworthy because, in any of these three species, they keep the O-PS despite carrying a less (ORF BAB1_0351) or more (ORF BAB1_1522) disrupted core structure as revealed by electrophoretic and Western blot analysis with monoclonal antibodies to the wild-type core (Figure 2C). Accordingly, these ORFs have been respectively named *wadB* and *wadC*. These results can only be interpreted to mean that the *Brucella* LPS core is branched, and that one of the branches supports the O-PS and requires the WadA glycosyltransferase while synthesis of the other branch requires, at least, WadB and WadC. The structural analyses in progress support this interpretation and place the core glucosamine in the WadB-WadC-dependent branch.

We published recently an analysis of the biological properties of the *wadC* mutant of *B. abortus* (*Ba*Δ*wadC*) [32]. We found that *Ba*Δ*wadC* is unable to multiply in bone marrow derived dendritic cells. Moreover, infection in these cells triggers a strong IL12 response, which could result in a protective Th1 response. As expected, the key aspects of this bacterial phenotype are reproduced by the LPS purified from *Ba*Δ*wadC* which, for example, triggers higher cytokine levels than the wild-type LPS in a TLR4-dependent manner (Figure 2D). Furthermore, in accordance with our hypothesis, the core defect increases the interaction with MD2 (Figure 2A). Finally, in agreement with the observations *in vitro*, *Ba*Δ*wadC* is attenuated in the standard brucellosis BALBc model (not shown).

The characteristic of the mutant prompted us to assess the protection against virulent *B. abortus* in the same mouse model. As shown in Table 1, the average protection induced by *Ba*Δ*wadC* was similar to that obtained with S19, the reference vaccine. As indicated above, S19 carries mutations in at least twenty-four genes potentially related with its attenuation, including defects in outer membrane proteins and erythritol metabolism [33]. Therefore, it is remarkable that a single mutation only deleting one or a few sugars has the same effect on immunogenicity that those multiple and not reproducible defects.

Future directions

Although the above results offer the proof of concept for the notion that PAMPs can be introduced or restored in these intracellular bacteria as the basis for new vaccine design, it is clear that additional research is necessary in at least two aspects. First, safety in the natural hosts should be considered. Since ruminants are particularly susceptible to brucellae during pregnancy, attenuated vaccines should be cleared before sexual maturity is reached. Therefore, in addition to protection, this aspect must be the subject of careful evaluation. Indeed, additional mutations in other outer membrane molecules, or in metabolic steps on a *wadC*-disrupted background could be used to obtain a safer vaccine that would still carry the core disruption as a way to bolster immunity. Alternatively, this mutation could be

generated in S19 to take advantage of its known safety while bolstering its immunogenic properties. Preliminary results obtained in mice with *wadC*-deleted S19 show that this is indeed a feasible approach to improve S19. Second, unless 100% protection is achieved against heavy challenges like those occurring when animals abort, an optimal brucellosis vaccine should be such that infected and vaccinated animals are readily differentiated. For this purpose, the removal of the O-PS (the relevant diagnostic moiety of S-LPS) has already been explored and the limitations of this approach shown [19,21]. An alternative is based on the introduction of antigen tags into the *Brucella* vaccines, an approach that has been tested in S19 with promising results [34].

Economical studies demonstrate that, when animal productivity and human health are considered together, brucellosis control and eradication is one of the most profitable measures that can be taken to improve the human condition in those areas of the world where the disease is endemic [35]. Although brucellosis control should be primarily focused on the species affecting humans, infections in sheep by the non-zoonotic species *B. ovis* is emerging as a problem in those areas where Rev 1 use is discontinued after *B. melitensis* eradication. Thus, a specific and safe vaccine against *B. ovis* is also necessary. This species naturally lacks the O-polysaccharide but it conserves the *wadC* gene and is thus susceptible to a genetic manipulation in its LPS PAMP. The results summarized here are the proof of concept for the development of new vaccines based on modification of those *Brucella* structures hampering recognition by innate immunity. If this concept is confirmed in the natural hosts, there will be possibilities for improving the S19 and Rev 1 vaccines or developing new ones against cattle, sheep and goat brucellosis. Likewise, there will be a rational approach to develop vaccines for swine, buffaloes, camels, yacks and reindeer, all of them domestic or semi-domestic species important for the daily living of a large fraction of the human population in the world and for which no vaccine is available.

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Literature cited

[1] Moreno E, Cloeckaert A, Moriyón I. *Brucella* evolution and taxonomy. Vet Microbiol 2002;90:209–27.

[2] Chain PS, Comerci DJ, Tolmasky ME, Larimer FW, Malfatti SA, Vergez LM, et al. Whole-genome analyses of speciation events in pathogenic brucellae. Infect Immun 2005;73:8353–61.

[3] Dean AS, Crump L, Greter H, Schelling E, Zinsstag J. Global burden of human brucellosis: A systematic review of disease frequency. PLoS Negl Trop Dis 2012;6:e1865.

[4] Seimenis A, Morelli D, Mantovani A. Zoonoses in the Mediterranean region. Ann Ist Super Sanita 2006;42:437–45.

[5] Maudlin I, Weber S. The control of neglected zoonotic diseases: a route to poverty alleviation. WHO/SDE/FOS/2006.1. Geneva: 2006.

[6] Seimenis A. Zoonoses and poverty - a long road to the alleviation of suffering. Vet Ital 2012;48:5–13.

[7] Nicoletti P. Vaccination. In: Nielsen KH, Duncan JR, editors. Animal Brucellosis, Boca Raton: CRC Press; 1990, pp. 283–99.
[8] Garin-Bastuji B, Blasco JM, Grayon M, Verger JM. *Brucella melitensis* infection in sheep: present and future. Vet Res 1998;29:255–74.
[9] Blasco JM, Molina-Flores B. Control and eradication of *Brucella melitensis* infection in sheep and goats. Vet.Clin.North Am.Food Anim Pract. 2011;27:95–104.
[10] Hoover DL, Nikolich MP, Izadjoo MJ, Borschel RH, Bhattacharjee AK. Develpment of new *Brucella* vaccines by molecular methods. In: López-Goñi I, Moriyón I, editors. *Brucella*: Molecular and Cellular Biology, Norfolk,U.K.: Horizon Scientific Press Ltd; 2004, pp. 369–402.
[11] Ariza J, Bosilkovski M, Cascio A, Colmenero JD, Corbel MJ, Falagas ME, et al. . Perspectives for the treatment of brucellosis in the 21st century: the Ioannina recommendations. PLoS.Med. 2007;4:e317.
[12] Jacques I, Verger JM, Laroucau K, Grayon M, Vizcaíno N, Peix A, et al. Immunological responses and protective efficacy against *Brucella melitensis* induced by bp26 and omp31 B. melitensis Rev.1 deletion mutants in sheep. Vaccine 2007;25:794–805.
[13] Fiorentino MA, Campos E, Cravero S, Arese A, Paolicchi F, Campero C, et al. Protection levels in vaccinated heifers with experimental vaccines *Brucella abortus* M1-luc and INTA 2. Vet Microbiol 2008;132:302–11.
[14] Kahl-McDonagh MM, Elzer PH, Hagius SD, Walker JV, Perry QL, Seabury CM, et al. Evaluation of novel *Brucella melitensis* unmarked deletion mutants for safety and efficacy in the goat model of brucellosis. Vaccine 2006;24:5169–77.
[15] Phillips RW, Elzer PH, Robertson GT, Hagius SD, Walker JV, Fatemi MB, et al. A *Brucella melitensis* high-temperature-requirement A (htrA) deletion mutant is attenuated in goats and protects against abortion. Res Vet Sci 1997;63:165–7.
[16] Gee JM, Kovach ME, Grippe VK, Hagius S, Walker JV, Elzer PH, et al. Role of catalase in the virulence of *Brucella melitensis* in pregnant goats. Vet Microbiol 2004;102:111–5.
[17] Cheville NF, Olsen SC, Jensen AE, Stevens MG, Florance AM, Houng HS, et al. Bacterial persistence and immunity in goats vaccinated with a *purE* deletion mutant or the parental 16M strain of *Brucella melitensis*. Infect Immun 1996;64:2431–9.
[18] Monreal D, Grilló MJ, González D, Marín CM, de Miguel MJ, López-Goñi I, et al. Characterization of *Brucella abortus* O-polysaccharide and core lipopolysaccharide mutants and demonstration that a complete core is required for rough vaccines to be efficient against *Brucella abortus* and *Brucella ovis* in the mouse model. Infect Immun 2003;71:3261–71.
[19] Moriyón I, Grilló MJ, Monreal D, González D, Marín CM, et al. Rough vaccines in animal brucellosis: structural and genetic basis and present status. Vet Res 2004;35:1–38.
[20] González D, Grilló MJ, de Miguel MJ, Ali T, Arce-Gorvel V, Delrue RM, et al. Brucellosis vaccines: assessment of *Brucella melitensis* lipopolysaccharide rough mutants defective in core and O-polysaccharide synthesis and export. PLoS One 2008;3:e2760.
[21] Barrio MB, Grilló MJ, Muñoz PM, Jacques I, González D, de Miguel MJ, et al. Rough mutants defective in core and O-polysaccharide synthesis and export induce antibodies reacting in an indirect ELISA with smooth lipopolysaccharide and are less effective than Rev 1 vaccine against *Brucella melitensis* infection of sheep. Vaccine

2009;27:1741–9.
[22] Barquero-Calvo E, Chaves-Olarte E, Weiss DS, Guzmán-Verri C, Chacón-Díaz C, Rucavado A, et al. *Brucella abortus* uses a stealthy strategy to avoid activation of the innate immune system during the onset of infection. PLoS One 2007;2:e631.
[23] Lapaque N, Moriyón I, Moreno E, Gorvel JP. *Brucella* lipopolysaccharide acts as a virulence factor. Curr.Opin.Microbiol 2005;8:60–6.
[24] Moriyón I. Against Gram-negative Bacteria: The LPS case. In: Gorvel JP, editor. Intracellular pathogens in membrane Interactions and vacuole biogenesis, Landes Bioscience / Eurekah.com, Georgetown,Texas; 2003, pp. 204–30.
[25] Palacios-Chaves L, Conde-Álvarez R, Gil-Ramírez Y, Zuñiga-Ripa A, Barquero-Calvo E, Chacón-Díaz C, et al. *Brucella abortus* ornithine lipids are dispensable outer membrane components devoid of a marked pathogen-associated molecular pattern. PLoS One 2011;6:e16030.
[26] Park BS, Song DH, Kim HM, Choi BS, Lee H, Lee JO. The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. Nature 2009;458:1191–5.
[27] Kastowsky M, Gutberlet T, Bradaczek H. Molecular modelling of the three-dimensional structure and conformational flexibility of bacterial lipopolysaccharide. J. Bacteriol. 1992;174:4798–806.
[28] Gruber A, Mancek M, Wagner H, Kirschning CJ, Jerala R. Structural model of MD-2 and functional role of its basic amino acid clusters involved in cellular lipopolysaccharide recognition. Journal of Biological Chemistry 2004;279:28475–82.
[29] Iriarte M, González D, Delrue RM, Monreal D, Conde-Álvarez R, López-Goñi I, et al. *Brucella* LPS: structure, biosynthesis and genetics. In: López-Goñi I, Moriyón I, editors. *Brucella*: Molecular and Cellular Biology, Norfolk,U.K: Horizon Scientific Press Ltd; 2004, pp. 159–92.
[30] Lapaque N, Takeuchi O, Corrales F, Akira S, Moriyón I, Howard JC, et al. Differential inductions of TNF-alpha and IGTP, IIGP by structurally diverse classic and non-classic lipopolysaccharides. Cell Microbiol. 2006;8:401–13.
[31] Velasco J, Bengoechea JA, Brandenburg K, Lindner B, Seydel U, González D, et al. *Brucella abortus* and its closest phylogenetic relative, *Ochrobactrum* spp., differ in outer membrane permeability and cationic peptide resistance. Infect Immun 2000;68:3210–8.
[32] Conde-Alvarez R, Arce-Gorvel, Iriarte M, Manček-Keber, Barquero-Calvo, et al. The lipopolysaccharide core of *Brucella abortus* acts as a shield against innate immunity recognition. PLoS Pathog 2012;8:e1002675EP–.
[33] Crasta OR, Folkerts O, Fei Z, Mane SP, Evans C, et al. Genome sequence of *Brucella abortus* vaccine strain S19 compared to virulent strains yields candidate virulence genes. PLoS One 2008;3:e2193.
[34] Chacón-Díaz C, Muñoz-Rodríguez M, Barquero-Calvo E, Guzmán-Verri C, Chaves-Olarte E, et al. The use of green fluorescent protein as a marker for *Brucella* vaccines. Vaccine 2011;29:577–82.
[35] Zinsstag J, Schelling E, Roth F, Bonfoh B, de SD, Tanner M. Human benefits of animal interventions for zoonosis control. Emerg Infect Dis 2007;13:527–31.

Table 1. Protection against *B. abortus* infection in BALB/c by vaccination with *Ba⊿wadC* or *B. abortus* S19

Vaccine Strain	CFU challenge (mean±SD log CFU/spleen)	CFU vaccine (mean±SD log CFU/spleen)
*Ba*Δ*wadC*	1,58 ± 0,04	3,90 ± 0,07
S19	1,30 ± 0,35	4,06 ± 1,11
Control PBS	6,14 ± 0,25	---

Three groups of 5 mice were inoculated subcutaneously with 1 x 10^5 colony forming units (CFU) per mouse, or sterile saline as a control. Four weeks after vaccination, each group was challenged by intraperitoneal injection of 5 x 10^4 colony forming units of virulent *B. abortus* (nalidixic acid sensitive) per mouse. Two weeks later, the number of colony forming units of the challenge and vaccine strains in the spleens was determined. The counts were done on BAB and BAB supplemented with 25 µg/mL of nalidixic acid to differentiate the challenge from the *Ba*Δ*wadC* vaccine strain, and on BAB and BAB supplemented with 1 mg/mL of erythritol to differentiate the challenge from the S19 vaccine.

Figure 1. Lipid A and core oligosaccharides of *E. coli*, *Ochrobactrum intermedium* and *B. abortus*. The *E. coli* lipid A is a bisphosphorylated glucosamine disaccharide carrying amide and ester-linked acyl or acyloxyacyl groups no longer than 16 carbons, and the core oligosaccharide (only the inner section is depicted) carries up to five negatively charged groups. In contrast, the lipid A of *O. intermedium* and *B. abortus* is made of a diaminoglucose disaccharide carrying amide-linked acyl or acyloxyacyl groups of up to 32 carbons (28 in the figure). As compared to the *E. coli* counterpart, the core oligosaccharide of these two bacteria shows a reduced charge that results both from a reduction in the number of charged groups (particularly in *B. abortus* that lacks glucuronic acid) and the presence of amino sugars. Although the precise structure of *B. abortus* core is knot known, genetic and biophysical evidence (see Figure 2) shows that it is a structure with a lateral branch hampering access to the inner negative groups of Kdo and lipid A.

Figure 2. A branch in the core oligosaccharide of *B. abortus* LPS hampers recognition by innate immunity. (A), Disruption of the *B. abortus* LPS core increases interaction with MD2. The experiment measures the amount of free MD2 upon incubation with wild type *B. abortus* (Ba-parental LPS), *B. abortus wadC* mutant (BaΔ*wadC* LPS) or *Salmonella typhimurium* LPS as a control. **(B),** *B. abortus* inner core charged groups are not accessible to polycations. The experiment shows how polymyxin B (a highly charged polycationic antibiotic) neutralizes the charge (zeta potential) of *O. anthropi* but not of *B. abortus* LPS; **(C),** The LPS of the *B. abortus wadC* mutant has an intact O-polysaccharide and an altered core; left panel, SDS-polyacrylamide electrophoresis of the LPS of Ba-parental and of BaΔ*wadC*; right panel, Western blot analysis of the LPS of Ba-parental, mutant BaΔ*wadC* and the complemented control (BaΔ*wadC*-comp); **(D),** Disruption of the *B. abortus* LPS core increases IL-12 release by dendritic cells in a TLR4-dependent manner (data are from references [32] and [31]).