# Antigenicity of the *Brucella* Cell Wall

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The members of *Brucella* spp. as Gram-negative bacteria are characterized by their sandwich-structured cell envelopes composed of the lipopolysaccharide (LPS)-covered bacterial outer membrane and the inner cytoplasmic cell membrane with a thin peptidoglycan layer between them in the periplasmic space.

brucellosis | serology | false positive serologic results (FPSR) | Gram-negative bacteria | lipopolysaccharide (LPS) | smooth (S) colony types | rough (R) colony types | cell-free DNA (cfDNA) | next generation sequencing

## 1. Introduction

Human and animal brucellosis and its health and economic consequences have been known for millennia. The pathogens behind the diseases, the *Brucella* species, were first described by Bruce, along with the rapid development of the first serologic diagnostic probe detecting *Brucella* infection by Wright at the end of the 19th century [1][2][3]. Since then, the genus *Brucella* has expanded to more than 30 known species, including isolates from exotic hosts, such as cetaceans or the surface of human breast implants [4], demonstrating the physiological and genetic flexibility of the bacteria.

Besides the ability to exist in a wide variety of hosts, this flexibility provides environmental persistence (against extreme temperature, pH, and humidity) outside of any host [5], as well as the ability of intracellular localization within the host organism [6][7][8] for most *Brucella* species. By completing the picture with the mild toxicity of the *Brucella* endotoxin—three orders of magnitude lower than the respective *E. coli* molecule [9]—the difficulties relating to the recognition and diagnosis of brucellosis are subsequently compounded.

## 2. Issues with the Antigenicity of the *Brucella* Cell Wall

Almost all members of the genus *Brucella* follow this scheme; however, with serious biological, biochemical, and subsequent serological consequences, similarly to other Gram-negative bacteria [10], there are some mutant *Brucella* species lacking the vast outer lipopolysaccharide layer [11]. These mutants were identified as R (rough)-type *Brucellas* in contrast with the S (smooth)-type species, based on the visual characterization of bacterial colonies grown on solid media. The virulence of R-type mutants is radically weakened due to the mutations not detailed here [11], making some of them applicable as *Brucella* vaccines.

According to their low prevalence, their importance from the standpoint of brucellosis serology is generally low. However, their application as vaccines, while providing opportunity for monitoring vaccination, could simultaneously prove to be an obstacle in the serologic distinction between vaccinated and infected populations.

The prevalence of the S-type *Brucella* species is substantially higher, hence false positivity in *Brucella* serology is mainly contributed by S-types. Therefore, in the following chapters, the biochemical and antigenic character of smooth *Brucella* spp. is discussed.

The description of the cell envelope will proceed from the internal parts of a bacterium (cytoplasm) to the extracellular space. Biological roles, structures, and cellular mechanisms involved in the generation of cytoplasmic proteins will not be detailed, only their antigenic characteristics will be discussed below as applicable [12][13][14]. The cytoplasm is surrounded by the inner membrane, built as a double layer (bilayer) from phospholipids and directly covers the cytoplasmic space, and is thus also referred to as the cytoplasmic membrane. There are various membrane proteins wedged into the inner membrane but, similarly to the protein content of the cytoplasm, only their antigenic characteristics will be considered [13]. At the outer side of the inner membrane, researchers find the periplasm: a structured, gel-like space with a peptidoglycan membrane distinctly different from the peptidoglycan membrane of Gram-positive bacteria, as it is not multilayered and is thus substantially thinner and more fragile. There are proteins dissolved in the periplasmic fluid, including enzymes (hydrolases, antibiotic-degrading enzymes, etc.), heavy metal neutralizers, carrier proteins, and bacterial toxin subunits. As in the case of cytoplasmic and inner membrane proteins, only their antigenic characteristics will be detailed as relevant to this research [15].

The outermost part of the envelope is the cell wall composed of a phospholipid membrane monolayer and an associated lipopolysaccharide (LPS) layer. The outer membrane also anchors the periplasmic peptidoglycan layer by specific proteins: the outer-membrane proteins (OMPs). Some OMPs belong to the porin protein family providing molecular communication across the cell wall with their typical β-barrel (a tube-like structure of antiparallel β-folds) structure [16][17][18]. A detailed presentation of OMPs and their potential in *Brucella* serology will be provided below. Recent studies have proven that lipopolysaccharide (LPS) is inhomogeneous: a mixture of full-length polysaccharide chains (S LPS) and truncated forms (R LPS) clustered around OMPs [10][19].

The asymmetric composition of the outer membrane can lead to the extremely increased hydrophobicity of the bacterium particle in all cases where the LPS becomes thinner due to mutations, causing the tendency for spontaneous congealing—similarly in the case of rough mutations.

The long (built from dozens of monosaccharides) polysaccharide chains of the LPS form a protective fur-like layer, making it difficult for hydrophobic molecules to penetrate the outer membrane and enter the periplasm. This strong fur coat lures the host immune system during the early phase of the infection and protects the *Brucella* cells from monocyte phagocytosis later, as referred below.

The LPS consists of three main elements distinct in composition, structure, and function. These elements, in order, starting from the interior of the bacterium cell toward the extracellular space are: (i) the O-specific polysaccharide

(OPS), (ii) the core oligosaccharide (COS), and (iii) the so-called lipid A. **Figure 1** presents the physical assembly of the main components only, without the detailed biochemical and biophysical processes (enzyme reactions, biochemical pathways, cell trafficking routes, and their elements [20]), resulting in the final composition and structure.

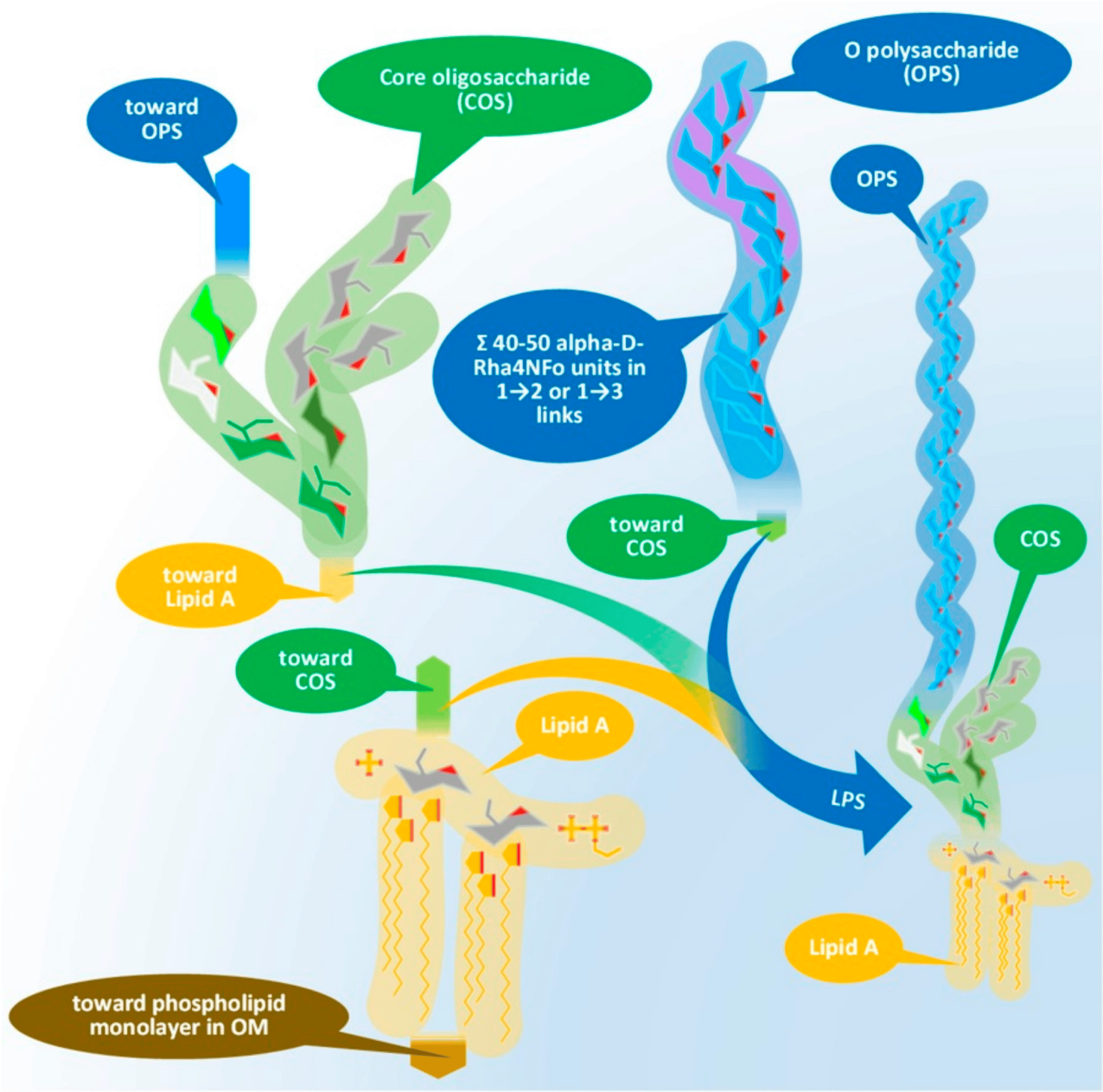


**Figure 1.** Physical assembly of LPS of Gram-negative bacteria demonstrated by the elements of *Brucella* LPS. Chemical compositions of the main components will be discussed later. LPS: lipopolysaccharide, OPS: O-specific polysaccharide, COS: core oligosaccharide, OM: outer membrane.

Lipid A, similar to phospholipids, consists of a polar head and a hydrophobic base. It is the key feature enabling the building of the asymmetric membrane bilayer of the outer membrane (OM). It is a conservative structure among Gram-negative bacteria, as detailed in **Figure 2** and in the short discussion following the figure.

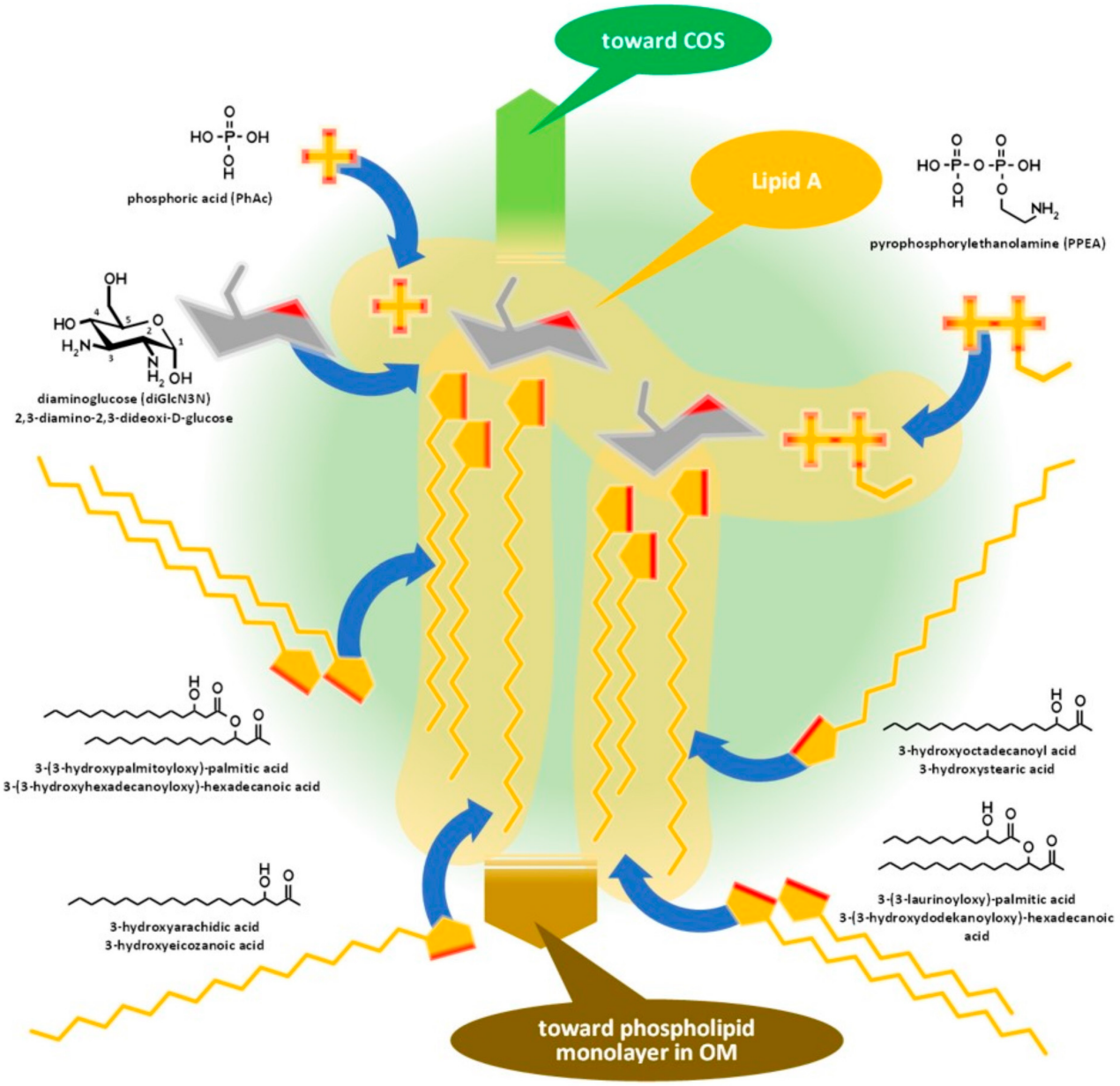


**Figure 2.** Assembly of lipid A of Gram-negative bacteria demonstrated by the composition of *Brucella* lipid A. COS: Core oligosaccharide, OM: outer membrane.

**Figure 3** and its discussion presents the core oligosaccharide (COS), which, as its name suggests, is a core that links together subunits of LPS with different structures and functions, connecting the lipid A and the O-specific polysaccharide. Molecular diversity of the COS is high among bacterial species, including the strains of species.

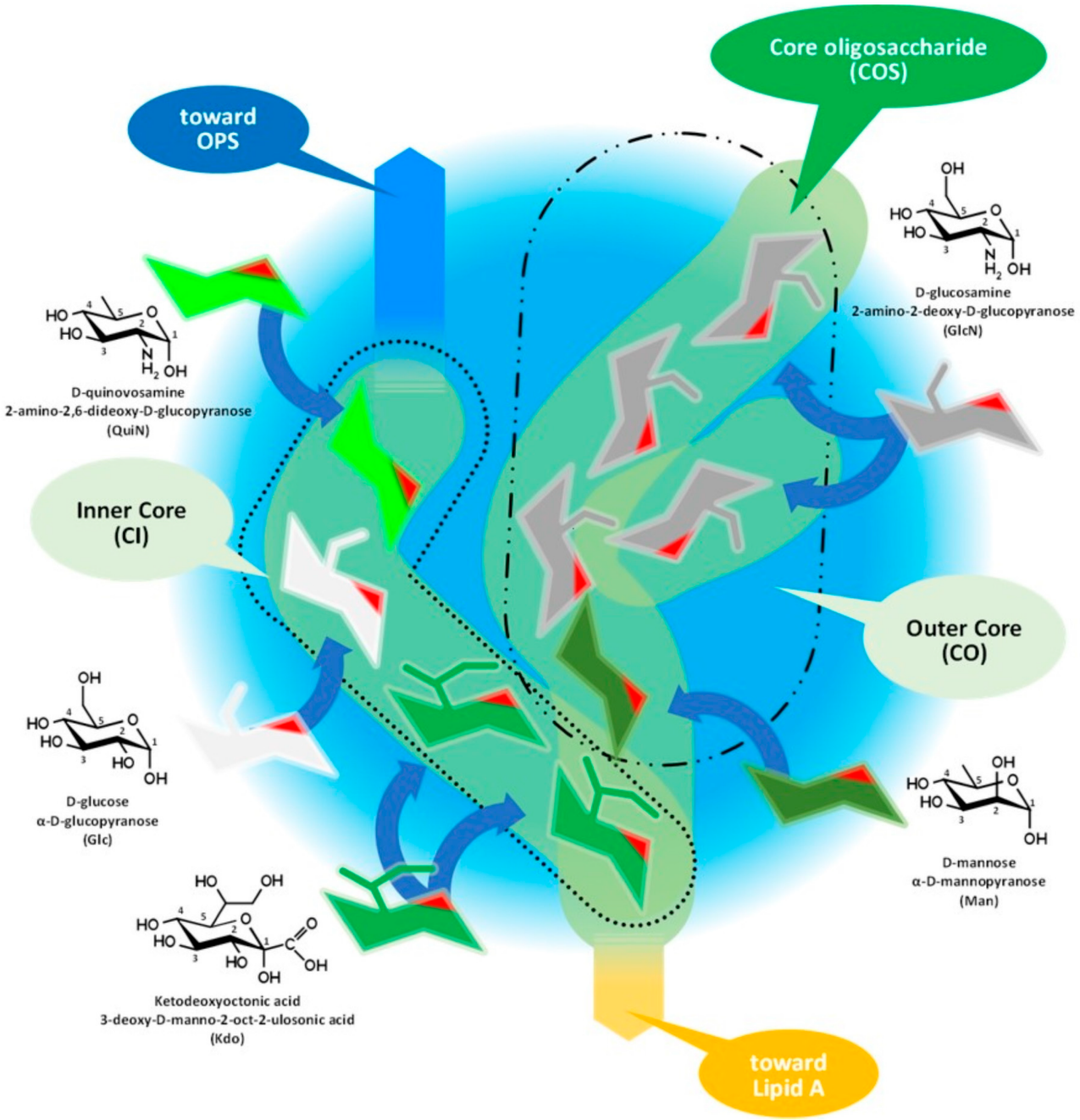


**Figure 3.** Assembly of core oligosaccharide of Gram-negative bacteria demonstrated by the composition of *Brucella* COS. OPS: O-specific polysaccharide.

The outmost part of the LPS, the O-specific polysaccharide (OPS), detailed by **Figure 4** and **Figure 5**, is also known as O-specific side chain or O antigen. The OPS is composed of a high number of repeating subunits built of 2–7 sugar components of various compositions. The pattern of subunit repetitions is characteristic of bacterial species, resulting in high antigen variability, which can serve as the basis for the serological grouping of Gram-negative bacteria. The number of sugar moieties (which is dramatically reduced in rough mutants) in the O-specific polysaccharide chain determines the morphology of the bacterial colony, i.e., smooth or rough colony types with and without lengthy OPS chains, respectively. LPS which is lacking OPS due to a low degree of polymerization could be referred to as lipooligosaccharide—LOS.

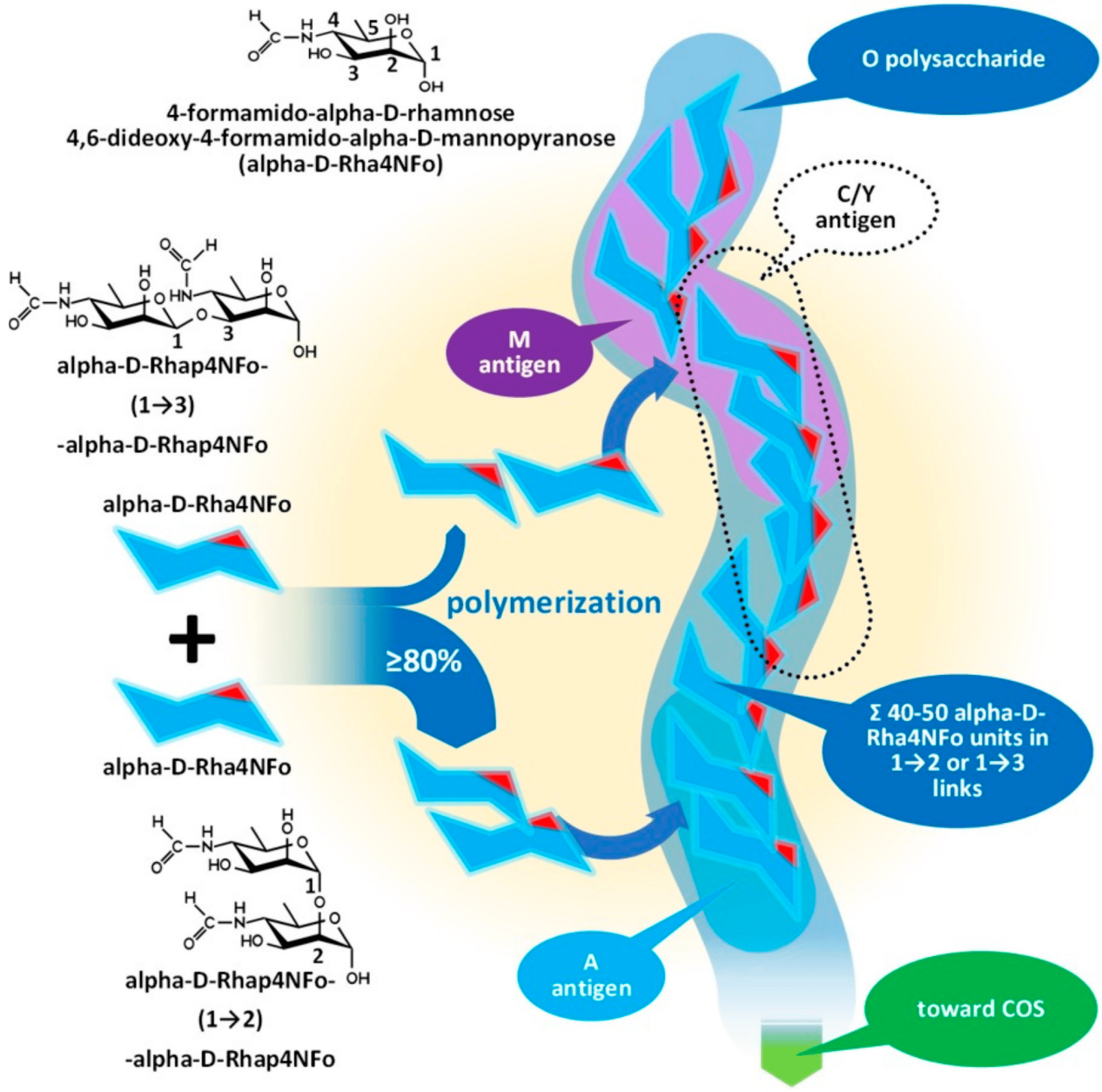


**Figure 4.** Assembly of the O-polysaccharide of Gram-negative bacteria demonstrated by the composition of *Brucella* OPS. COS: Core oligosaccharide.

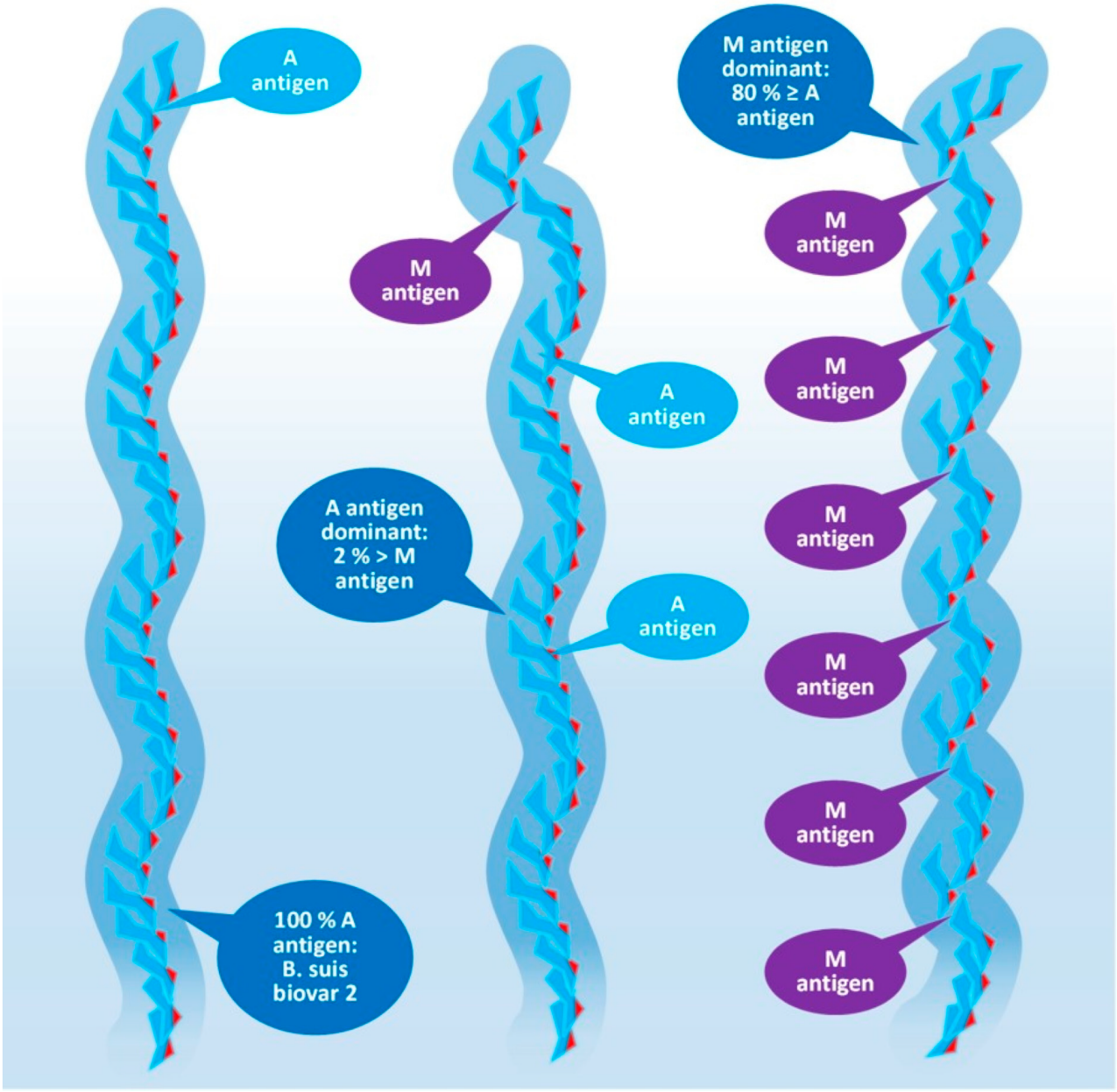


**Figure 5.** Composition of the O-polysaccharide of *Brucella* species with a different antigenic character.

Lipid A is usually built from a bisphosphorylated disaccharide with a rather conservative structure (the hydrophilic head), acylated with various fatty acid chains with variable lengths and branching, providing the hydrophobic base that noncovalently sticks to the hydrophobic side of the phospholipid monolayer in the OM.

Although it seems that lipid A structures, being the most deeply buried part of the LPS and embedded into the OM, have minor importance from a serologic point of view, as main determinants of bacterial endotoxicity—especially the low endotoxicity of brucellae, which amplify the stealthing ability of the bacteria [9][21][22]—some discussion of their antigenicity is required.

Lipid A of brucellae is the most hydrophobic membrane anchor among Gram-negative bacteria. The typical length of fatty acid chains in *Brucella abortus* is 16–18 with some very long (28–30) substituents [23][24][25] in contrast with the typical C12–C14 chains of enterobacteria [21][26]. The six long fatty acid substituents make it difficult to release the endotoxin for *Brucella*, while *Y. enterocolitica* lipid A, with only four and substantially shorter fatty acid chains, is a good source of free endotoxins.

The intracellular lifestyle that S-type brucellae enjoy in the endoplasmic reticulum [22] can be supported both by the firm anchorage of the LPS through lipid A—a useful feature to prevent dissolution in a lysosome as it generally happens with R-type bacteria —and by the attenuated host immune response [7][8][22]. Although diverse explanations have been published on the exact role of the LPS in such attenuations, data demonstrating the existence of S LPS-MHC II complexes [22][27][28][29] suggest a contribution by the extremely hydrophobic lipid A.

The core oligosaccharide has branched chains with two structurally different subunits, as **Figure 3** demonstrates on the structure of *Brucella* COS, which are: (i) the inner core (CI) and (ii) the outer core (CO). Researchers should mention that this classification of the subunits could be obsolete in the light of recent studies. The composition of the CI consists of rare sugar moieties, such as the characteristic ketodeoxyoctonic acid (Kdo) and heptose sugars or—such as in the case of *Brucella*—D-quinovosamine (QuiN) [30].

The terminal Kdo attaches the COS to the lipid A through a ketosidic bond, is sensitive to weak acids, and provides a link to the outer core as well. In the CO, the number of hexose monomers is variable with six sugar moieties as a maximum. Depending on the number of monomers, the CO could contain further small branches—in the case of *Brucella*, through the side chain of a D-glucosamine. A terminal moiety of a CI (such as in the case of *Y. enterocolitica* serotype O:3 and *Brucella* spp.) or a CO (*E. coli* and *Salmonella* spp.) branch attaches the COS to the OPS [21][30][31][32][33][34].

Investigations of the so-called deep rough mutants, such as the D31m4 of *E. coli* [35], determined the minimum length of the full COS in viable Gram-negative bacteria: two heptoses, usually Kdo monomers, should be attached to lipid A [35]. Rare exceptions exist, as in the case of *Helicobacter pylori*, with only one Kdo linked to lipid A.

The diverse composition and the branched structure of COS could serve as a good basis for antigenic differentiation; however, utilizing this diversity is difficult for serologic applications in the case of S-type bacteria as the majority of COS remain cryptic under the OPS layer. The mentioned diversity of the OPS chain length and the inhomogeneous cell surface location of the different OPS molecules, that is the clustering of R LPS molecules around OMPs [10][19], increases the theoretical possibility of an immune response against COS motifs on the intact cells and serologic detection of antibodies involved in such a response.

The O-specific polysaccharide as the outer layer of the cell wall is the main antigen determinant of the LPS and provides a solid, yet relatively unreliable, basis for the serologic differentiation of Gram-negative bacteria species and strains [36]. The molecular and topological diversity of the OPS is theoretically high: more than five dozen kinds

of sugar moieties were identified as constituents of the polymer in various numbers, proportions, and clusters, built into linear or branched chains, and linked to even non-sugary substituents [27][37].

More than 180 different O-serotypes of *E. coli* have been identified before the high throughput genotyping era until 2005 [37] due to this variability, and, paradoxically, the high molecular diversity results in a lower antigenic diversity in several cases [37]. *E. coli* O35 and *Salmonella enterica* O62 or *E. coli* O98 and *Yersinia enterocolitica* O11,24 or *E. coli* O8 and *Klebsiella pneumoniae* O5 and current subject, *Brucella* spp., *E. coli* O157:H7 and *Y. enterocolitica* O9 have identical or nearly identical antigens; in the last case, identical enough to present false positive serologic results.

The O-specific polysaccharide of the *Y. enterocolitica* O9 and the brucellae (see **Figure 4**) is characteristically a homopolymer of 4,6-dideoxy-4-formamido-alpha- D-mannopyranose (alpha-D-Rha4NFo) sugar moieties, which are linked to each other through α(1→2) and/or α(1→3) bonds in different proportions. As is shown in **Figure 5**, the prevalence of α(1→2) and α(1→3) bonds are different in the strains of *Brucella* spp. with an average proportion of the α(1→3) links between 0 and 20 percent. The first saccharide monomer of the OPS always has a reducing end and is linked to the next sugar through an α(1→2) bond. The serologic importance of the terminus of the OPS is high [38].

Principally, the serologic differentiation of Gram-negative bacterial strains is based on antibody recognition of repeating oligomeric saccharide motifs in the OPS rather than individual sugar moieties. As in the case of brucellae, whose serologic division was among the earlier classification attempts targeting bacterium species (carried out in 1932 by Wilson [39]), it may have been the very first one, preceding the serotyping of *E. coli* in 1944, or *Salmonella* spp. in 1935, during the extensive and pioneering efforts of Kaufman [40][41][42][43]. The early serotypes, M after *Brucella melitensis* and A after *Brucella abortus*, provided the basis for the later developed *Brucella* M and A antigen grouping and led to the recent stage of knowledge summarized in **Figure 4** and **Figure 5** [38][44][45].

*Brucella* OPS could carry the antigens A, M, C/Y, and C. According to recent knowledge [44], the A antigen represents an alpha-D-Rha4NFo moiety, which is linked between two alpha-D-Rha4NFo saccharides through α(1→2) bonds; or, suggesting a more simple determination: A antigen is two alpha-D-Rha4NFo moieties linked together by an α(1→2) bond (see **Figure 4**). The M antigen represents a cluster of four alpha-D-Rha4NFo moieties with a central link of an α(1→3) bond and the two saccharides on the termini of the cluster is linked by α(1→2) bonds; or, with a more simple determination: two A antigens linked together with an α(1→3) bond. As it is shown in **Figure 5**, at least one bacterium exists in the genus *Brucella* with uniform antigenicity: *B. suis* biovar 2 contains exclusively A antigens [46]. With similar rare exceptions, near the cap of the brucellae OPS, usually an α(1→3) bond can be found [38].

This molecular-based antigen division provides more sensitive typing since the determination of M and A dominant strains based on A/M ratios could identify strains between two extremis: the 100% A dominant *B. suis* biovar 2 and the *B. melitensis* 16M containing 21% of M [47].

The very existence of the C/Y and C antigens is still debated. Theoretically, they are characterized as overlapped A and M antigens with different proportions, that is, C/Y (as a common antigen of some members of the genus *Brucella* and *Y. enterocolitica* O:9) has more A than M and in C antigen A = M [48]—if researchers retain the fish and bait metaphor, they are baits for fish with the largest mouths—consequently with a lower target sensitivity.

Due to its chemical nature, which is abundantly composed of saccharides, and the rather narrow repertoire of sugar moieties occurring across bacterial families, from a serological point of view, the outermost polysaccharide component of the protective LPS layer seems to be overly uniform in the case of smooth Gram-negative bacteria; consequently, the application of isolated antigens with S LPS origin in serological tests almost automatically provides false positives. The baits used are evidently far from perfection.

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