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Lipopolysaccharide Heterogeneity in the Atypical Group of Novel Emerging *Brucella* Species

Michel S. Zygmunt,[a,b] Isabelle Jacques,[a,b,c] Nelly Bernardet,[a,b] and Axel Cloeckaert[a,b]

INRA, UMR1282 Infectiologie et Santé Publique, Nouzilly, France[a]; Université François Rabelais de Tours, UMR1282 Infectiologie et Santé Publique, Tours, France[b]; and IUT de Tours, Tours, France[c]

Recently, novel *Brucella* strains with phenotypic characteristics that were atypical for strains belonging to the genus *Brucella* have been reported. Phenotypically many of these strains were initially misidentified as *Ochrobactrum* spp. Two novel species have been described so far for these strains, i.e., *B. microti* and *B. inopinata*, and other strains genetically related to *B. inopinata* may constitute other novel species as well. In this study, we analyzed the lipopolysaccharides (LPS) (smooth LPS [S-LPS] and rough LPS [R-LPS]) of these atypical strains using different methods and a panel of monoclonal antibodies (MAbs) directed against several epitopes of the *Brucella* O-polysaccharide (O-PS) and R-LPS. Among the most striking results, *Brucella* sp. strain BO2, isolated from a patient with chronic destructive pneumonia, showed a completely distinct S-LPS profile in silver stain gels that looked more similar to that of enterobacterial S-LPS. This strain also failed to react with MAbs against *Brucella* O-PS epitopes and showed weak reactivity with anti-R-LPS MAbs. *B. inopinata* reference strain BO1 displayed an M-dominant S-LPS type with some heterogeneity relative to the classical M-dominant *Brucella* S-LPS type. Australian wild rodent strains belonging also to the *B. inopinata* group showed a classical A-dominant S-LPS but lacked the O-PS common (C) epitopes, as previously reported for *B. suis* biovar 2 strains. Interestingly, some strains also failed to react with anti-R-LPS MAbs, such as the *B. microti* reference strain and *B. inopinata* BO1, suggesting modifications in the core-lipid A moieties of these strains. These results have several implications for serological typing and serological diagnosis and underline the need for novel tools for detection and correct identification of such novel emerging *Brucella* spp.

Brucellae are Gram-negative, facultative, intracellular bacteria that can infect humans and many species of animals. The genus *Brucella* has traditionally been classified into six species, i.e., *B. melitensis*, *B. suis*, *B. abortus*, *B. neotomae*, *B. ovis*, and *B. canis*, which are reflective of animal host preference (18, 19, 24, 33, 36). The genus *Brucella* has been further expanded with a set of recently discovered species. Such species include *B. ceti* and *B. pinnipedialis*, which have been isolated from cetaceans and pinnipeds, respectively (15). *B. microti* was isolated initially from the common vole but later from the red fox and from soil (27–29). The latest validly published species is *B. inopinata*, which was isolated from a human breast implant infection and represents the most distant *Brucella* species at the phenotypic and phylogenetic levels relative to the others (11, 30). The animal or environmental reservoir of the last species is not known. New *Brucella* species will likely be described in the future, including isolates from baboons (26), isolates from wild rodents in Australia (31), and strain BO2, isolated from a patient with chronic destructive pneumonia (32). Strain BO2 and strains from wild Australian rodents have been proposed as novel lineages of the *B. inopinata* species (31, 32).

Interestingly, this group of divergent strains present phenotypic characteristics that are not characteristic of the classical *Brucella* species, such as faster growth and higher metabolic activities, and they were therefore often initially misidentified as *Ochrobactrum* spp., with the risk of compromising treatment by the use of inappropriate antibiotics and treatment duration in human cases. Some of these strains have also been shown to be untypeable with monospecific polyclonal sera commonly used to classify smooth (S) *Brucella* species as A- or M-dominant strains, suggesting possible modifications at the lipopolysaccharide (LPS) level. This in addition may compromise serological diagnosis of infections caused by these strains, because serological tests are mainly based on detection of antibodies against smooth LPS (S-LPS) and in particular its O-polysaccharide (O-PS) moiety, which is known to be the immunodominant part of *Brucella* S-LPS (12, 19, 20).

Besides the A and M determinants mentioned above, S *Brucella* strains share common epitopes on the O-PS with cross-reacting bacteria, of which the most important is *Yersinia enterocolitica* O:9 (4, 5, 12). By using monoclonal antibodies (MAbs) a number of epitope specificities on the O-PS have been reported: A, M, and epitopes shared by both A- and M-dominant strains, which have been named common (C) epitopes (3, 7, 9, 10, 13, 25, 34, 35). The C epitopes have been further subdivided, according to relative preferential MAb binding in enzyme-linked immunosorbent assays (ELISA) to A- and M-dominant strains of *B. abortus* or *B. melitensis* and to cross-reacting *Y. enterocolitica* O:9, into five epitopic specificities: C (M>A), C (A=M), C/Y (M>A), C/Y (A=M), and C/Y (A>M) (9, 35). MAbs indicated as C are specific for *Brucella*, while those indicated as C/Y cross-react with *Y. enterocolitica* O:9. The preferential binding to A- or M-dominant strains and equal binding to both strains are indicated by A>M, M>A, and A=M, respectively. It has been suggested from data from competitions between MAbs that the different O-PS epitopes are probably overlapping structures (35). The structural differences specifying these O-PS epitopes have been previously

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Address correspondence to Michel S. Zygmunt, Michel.Zygmunt@tours.inra.fr.
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partly elucidated (3, 23) and are further discussed in Results and Discussion.

Because the LPS antigenic status of strains belonging to the divergent *B. inopinata* lineage has not clearly been defined, the purpose of the present study was to investigate LPS expression in these strains as well as the distribution of the different *Brucella* O-PS epitopes identified to date by using MAbs.

MATERIALS AND METHODS

The *Brucella* strains used in this study are listed in Table 1. The strains were checked for purity, colony phase, and species and biovar characterization by standard procedures (1). Typing of strains with monospecific polyclonal sera was done as described by Alton et al. (1).

The MAbs used were produced and characterized previously (6, 8, 10). The anti-rough LPS (anti-R-LPS) MAbs used were A68/10A06/B11 (IgM), A68/24D08/G09 (IgG1), and A68/24G12/A08 (IgG3). The MAbs specific for the O-PS epitopes were 2E11 (IgG3; M epitope), 0F03 (IgM; M epitope), 12G12 (IgG1; C [A=M] epitope), 07F09 (IgG1; C [A=M] epitope), 12B12 (IgG3; C [M>A] epitope), 18H08 (IgA; C/Y [A=M] epitope), 04F9 (IgG2a; C/Y [A>M] epitope), 05D4 (IgG1; C/Y [A>M] epitope), and 16C10 (IgG3; C/Y [M>A] epitope) (Table 1). All MAbs were used as hybridoma supernatants in ELISA and Western blotting.

ELISA using whole bacteria as the antigen and Western blotting after SDS-PAGE of proteinase K-digested S-LPS preparations were performed as described previously (2, 6, 10, 14, 16). Silver staining of S-LPS gels was performed as described previously (14, 16).

RESULTS AND DISCUSSION

Serotyping performed in our laboratory with anti-A and anti-M monospecific polyclonal sera confirmed the antigenic heterogeneity of strains belonging to the divergent *B. inopinata* lineage (11, 30–32); i.e., *B. inopinata* strain BO1 showed weak agglutination with anti-M monospecific polyclonal serum, all wild rodent isolates from Australia were clearly A-dominant, and the human *Brucella* sp. strain BO2 isolate showed absence of agglutination with both monospecific polyclonal sera (Table 1). *B. microti* reference strain CCM 4915 was confirmed as being M dominant (29). ELISA data using the anti-O-PS MAbs confirmed the A-dominant status of the 12 wild rodent *Brucella* sp. strains, as previously shown for classical A-dominant *Brucella* strains such as *B. suis* 1330, used as a control in this study (Table 1). The distribution of O-PS epitopes appeared to be homogeneous within this group of Australian wild rodent isolates. However, of particular interest is that all these strains showed an absence of binding or weak binding of MAbs specific for the C epitopes, as previously reported for *B. suis* biovar 2 strains and some marine mammal *Brucella* isolates (2, 9). Therefore, the O-PS structure of the wild rodent isolates may be identical or close to that of *B. suis* biovar 2. The *Brucella* O-PS structure has been described as being constituted by homopolymers of 4,6-dideoxy-4-formamido-α-D-mannopyranose residues. O-PS from A-dominant strains is a linear α-1,2-linked polymer with about 2% α-1,3 linkages, while O-PS from M-dominant strains is a linear polymer of pentasaccharide repeating units containing one α-1,3-linked and four α-1,2-linked monosaccharide residues (3, 23). MAbs specific for the C/Y epitopes probably recognize α-1,2-linked tri- or tetrasaccharides of the O-PS (3). The α-1,3 linkage should be mainly involved in the structure recognized by MAbs specific for the M epitope, since such MAbs fail to react with *Y. enterocolitica* O:9, lacking the α-1,3 linkages, and their preferential binding to M-dominant O-PS correlates with an increased number of α-1,3-linked monosaccharide

TABLE 1 Binding in ELISA of the anti-O-PS MAbs to the *Brucella* strains used in this study

Species (biovar)	Strain	Host or source	Geographic origin	Agglutination with monospecific serum: A	M	Binding titer (maximal absorbance) by ELISA of MAb[a]: 2E11 (M)	04F03 (M)	12G12 [C (A=M)]	07F09 [C (A=M)]	12B12 [C (M>A)]	18H08 [C/Y (A=M)]	04F9 [C/Y (A>M)]	05D4 [C/Y (A>M)]	16C10 [C/Y (M>A)]
B. melitensis (1)	16 M	Goat	USA	−	+	90 (2.506)	90 (2.572)	7,290 (2.701)	2,430 (2.791)	90 (2.907)	90 (2.858)	30 (2.420)	810 (2.432)	810 (2.482)
B. suis (1)	1330	Swine	USA	+	−	<10	270 (3.930)	7,290 (5.272)	2,430 (4.573)	30 (3.234)	270 (4.368)	7,290 (4.669)	21,870 (4.317)	90 (4.250)
B. suis (2)	Thomsen	Swine	Denmark	+	−	<10	<10	10 (2.422)	10 (1.249)	<10	90 (3.356)	7,290 (3.109)	7,290 (3.211)	10 (2.533)
B. microti	CCM 4915	Common vole	Czech Republic	−	+	90 (3.691)	90 (4.162)	2,430 (4.782)	810 (4.481)	30 (2.700)	30 (3.288)	90 (3.293)	2,430 (4.481)	270 (4.414)
B. inopinata	BO1	Human	USA	−	+	<10	90 (3.170)	7,290 (3.345)	2,430 (3.444)	90 (3.076)	90 (3.512)	21,870 (3.192)	7,290 (3.230)	810 (3.441)
Brucella sp.	BO2	Human	Australia	−	−	<10	<10	<10	<10	<10	<10	<10	<10	<10
	83-211 (83-13)	Wild rodent	Australia	+	−	<10	<10	<10	<10	<10	90 (4.120)	7,290 (4.857)	7,290 (3.890)	10 (1.730)
	NF 2627	Wild rodent	Australia	+	−	<10	<10	<10	<10	<10	30 (3.472)	7,290 (4.669)	2,430 (4.617)	10 (1.748)
	NF 2629	Wild rodent	Australia	+	−	<10	<10	<10	<10	<10	90 (3.896)	7,290 (4.605)	7,290 (4.329)	10 (2.135)
	NF 2637	Wild rodent	Australia	+	−	<10	<10	<10	<10	<10	90 (3.401)	2.430 (3.960)	7,290 (3.558)	<10
	NF 2640	Wild rodent	Australia	+	−	<10	<10	10 (1.511)	<10	<10	90 (4.140)	7,290 (5.254)	7,290 (4.953)	30 (3.596)
	NF 2651	Wild rodent	Australia	+	−	<10	<10	10 (1.234)	<10	<10	90 (4.396)	7,290 (4.669)	7,290 (5.572)	10 (1.660)
	NF 2653	Wild rodent	Australia	+	−	<10	<10	10 (1.543)	<10	<10	90 (4.162)	7,290 (4.861)	2,430 (5.083)	10 (3.302)
	NF 2668	Wild rodent	Australia	+	−	<10	<10	10 (1.255)	<10	<10	30 (4.060)	7,290 (5.264)	2,430 (4.786)	10 (2.528)
	NF 2810	Wild rodent	Australia	+	−	<10	<10	<10	<10	<10	30 (3.098)	2,430 (3.844)	7,290 (3.888)	10 (1.521)
	NF 2815	Wild rodent	Australia	+	−	<10	<10	30 (2.965)	10 (1.719)	<10	90 (4.326)	7,290 (5.556)	7,290 (5.556)	30 (3.832)
	NF 2816	Wild rodent	Australia	+	−	<10	<10	10 (1.583)	<10	<10	90 (4.710)	7,290 (4.856)	7,290 (4.953)	10 (2.546)
	NF 2816b	Wild rodent	Australia	+	−	<10	<10	<10	<10	<10	90 (4.233)	2,430 (4.857)	7,290 (4.325)	10 (1.141)

[a] Results are expressed as the titers of the MAb, i.e., the highest dilutions of the MAb giving an absorbance value above 1.0. Maximal absorbance was observed mostly at a 1/10 dilution of the MAb. The epitope specificities of the MAbs are identified in parentheses.

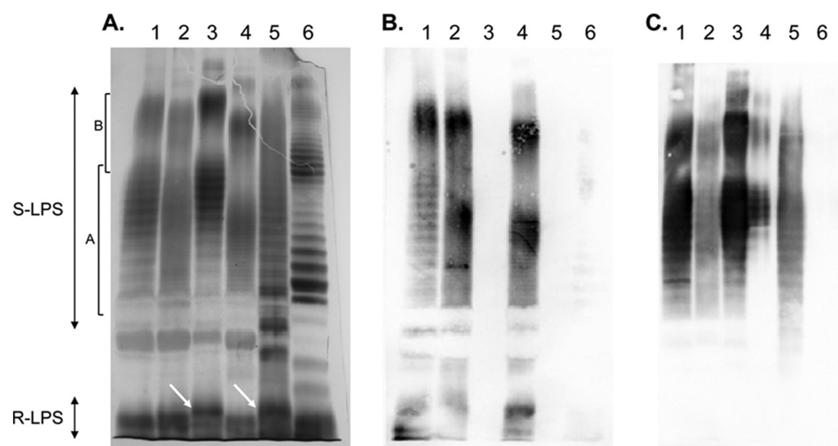

FIG 1 Silver staining (A) and Western blot profiles with MAbs A68/10A06/B11 (anti-R-LPS) and 12G12 (anti-S-LPS; C [A = M] epitope) (B and C, respectively) after SDS-PAGE of proteinase K-digested S-LPS preparations of *B. melitensis* 16 M (M-dominant reference strain) (lanes 1), *B. suis* 1330 (A-dominant reference strain) (lanes 2), *B. microti* CCM 4915 (lanes 3), *Brucella* sp. strain 83-211 (wild rodent isolate from Australia) (lanes 4), *B. inopinata* BO1 (lanes 5), and *Brucella* sp. strain BO2 (lanes 6). The R-LPS part and the S-LPS parts with short and intermediate O-chains (bracket A) and with long O-chains (bracket B) are indicated. The arrows in the R-LPS part indicate the bands of higher molecular mass observed for *B. microti* CCM 4915 and *B. inopinata* BO1.

residues. According to a recent study, *B. suis* biovar 2 also lacks α-1,3-linked monosaccharide residues in its O chain, and therefore the α-1,3 linkage may be involved in C epitope MAb recognition as well (M. V. Zaccheus et al., submitted for publication).

For most anti-O-PS MAbs, *B. inopinata* BO1 showed a MAb binding pattern in ELISA that was close to that of the M-dominant *B. melitensis* 16 M strain used as a control (Table 1). However, MAbs specific for the C/Y (A>M) epitopes showed significantly higher binding titers to strain BO1 than to strain 16 M, and in this case these were highly similar to those observed for the A-dominant strain *B. suis* 1330 (Table 1). To our knowledge, this is a new situation in the distribution of the C/Y (A>M) epitope, since the balance for this epitope between A- and M-dominant strains has always been clear before, with a usually superior binding to A-dominant strains (9). It is worth mentioning that MAbs specific for this epitope were initially classified as specific for the A epitope in early studies in the 1980s and the beginning of the 1990s (7, 14, 16, 21, 22).

Finally, the human *Brucella* sp. strain BO2 isolate proved to be the most atypical of this study, with a lack of binding in ELISA of all MAbs directed against O-PS epitopes (Table 1).

The LPS heterogeneity of strains of this study was also assessed by SDS-PAGE and Western blotting using the MAbs (Fig. 1). Silver staining of the LPS gels revealed the typical A-dominant banding pattern, with a close succession of regularly spaced narrow bands, in the S-LPS parts of all Australian wild rodent isolates (Fig. 1 and data not shown). The bimodal distribution, according to the O-chain length, of S-LPS molecules was similar to that observed for A-dominant control strain *B. suis* 1330 and as also reported for previously characterized S-LPSs of A-dominant *Brucella* strains (14, 16). This banding pattern was also confirmed by Western blotting using the MAbs specific for the O-PS epitopes and R-LPS epitopes. The latter MAbs revealed both the R-LPS part in the bottom of the gel and the S-LPS molecules, as previously reported (6). The banding pattern in silver staining of the S-LPS part of *B. inopinata* BO1 was typically M dominant, with regularly spaced doublets or triplets, as observed for control strain *B. melitensis* 16 M and for *B. microti* CCM 4915 (Fig. 1). As revealed by silver staining, there was some heterogeneity in the bimodal distribution of S-LPS molecules between these M-dominant strains. The M-dominant pattern was also revealed in Western blotting using the anti-O-PS MAbs (Fig. 1). Interestingly, in silver-stained gels *B. inopinata* BO1 displayed an additional regularly spaced banding pattern in the intermediate region between R-LPS and S-LPS that was not seen in any other *Brucella* species (Fig. 1). However, these bands were not detected in Western blotting using the MAbs of this study (Fig. 1 and data not shown). The additional bands detected by silver staining may thus constitute a distinct structural region in the LPS of *B. inopinata* BO1. None of the anti-R-LPS MAbs reacted with *B. inopinata* BO1 and *B. microti* CCM 4915, although they revealed, as expected, both R-LPS and S-LPS bands in M-dominant control strain 16 M (Fig. 1 and data not shown). Interestingly in silver staining of the LPS gel, an additional band or a shift in size of one of the R-LPS bands was observed for strains *B. inopinata* BO1 and *B. microti* CCM 4915 relative to the other strains used in this study (Fig. 1). This observation suggests a structural modification in the core-lipid A moiety of LPS of these strains, which may mask the epitopes recognized by the anti-R-LPS MAbs. To our knowledge, this kind of variation has also not been reported before for any of the classical *Brucella* species.

Brucella sp. strain BO2 showed a completely distinct S-LPS profile in silver-stained gels, with a higher regular spacing of the S-LPS bands that looked more similar to that observed for enterobacterial S-LPSs such as that from *Escherichia coli* or *Salmonella* (14, 16, 17). However, according to the typical S-LPS banding pattern, indicating in addition a multimodal distribution of S-LPS molecules, there is no doubt that this strain is smooth. As expected from the ELISA data, this strain failed to react in Western blotting with all MAbs against *Brucella* O-PS epitopes (data not shown) but showed a very weak reactivity with anti-R-LPS MAbs (Fig. 1 and data not shown).

Two genetic regions have been identified in the genomes of classical *Brucella* species as being essential for O-PS biosynthesis and translocation (20, 37). They are called *wbo* and *wbk* and encode several enzymes, such as glycosyltransferases, and proteins involved in O-PS polymerization and translocation. A blastn search (http://www

.ncbi.nlm.nih.gov/sutils/genom_table.cgi), using these genetic regions, in the genome sequences of strain BO2 (available in GenBank) failed to detect any of the classical *Brucella* O-PS biosynthetic genes (data not shown). Although the genome sequences of strain BO2 are not completely assembled, we suspect that these genes are truly absent because in the other novel strains of this study with the same genomic assembly status as BO2, these genes were detected using blastn. On the other hand, since there is clearly S-LPS production in strain BO2 as evidenced by the LPS gels of this study, another genetic region absent from the classical *Brucella* species must be involved in a new O-PS biosynthetic pathway of this strain. The molecular basis of the novel LPS variations shown in this study therefore merits further investigation.

The results of the present study have several implications for serological typing and serological diagnosis and underline the need for novel tools for detection and correct identification of such novel emerging *Brucella* spp.

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