Antigenic, Immunologic and Genetic Characterization of Rough Strains \textit{B.abortus} RB51, \textit{B.melitensis} B115 and \textit{B.melitensis} B18

Rosanna Adone\textsuperscript{1*}, Michele Muscillo\textsuperscript{2}, Giuseppina La Rosa\textsuperscript{2}, Massimiliano Francia\textsuperscript{1}, Michela Tarantino\textsuperscript{1}

\textsuperscript{1}Dipartimento Sanità Pubblica Veterinaria e Sicurezza Alimentare, Istituto Superiore di Sanità, Roma, Italy, \textsuperscript{2}Dipartimento Ambiente e Connessa Prevenzione Primaria, Istituto Superiore di Sanità, Roma, Italy

Abstract

The lipopolysaccharide (LPS) is considered the major virulent factor in \textit{Brucella} spp. Several genes have been identified involved in the synthesis of the three LPS components: lipid A, core and O-PS. Usually, \textit{Brucella} strains devoid of O-PS (rough mutants) are less virulent than the wild type and do not induce undesirable interfering antibodies. Such of them proved to be protective against brucellosis in mice. Because of these favorable features, rough strains have been considered potential brucellosis vaccines. In this study, we evaluated the antigenic, immunologic and genetic characteristics of rough strains \textit{B.abortus} RB51, \textit{B.melitensis} B115 and \textit{B.melitensis} B18. RB51 derived from \textit{B.abortus} 2308 virulent strain and B115 is a natural rough strain in which the O-PS is present in the cytoplasm. B18 is a rough rifampin-resistant mutant isolated in our laboratory. The surface antigenicity of RB51, B115 and B18 was evaluated by testing their ability to bind antibodies induced by rough or smooth \textit{Brucella} strains. The antibody response induced by each strain was evaluated in rabbits. Twenty-one genes, involved in the LPS-synthesis, were sequenced and compared with the \textit{B.melitensis} 16M strain. The results indicated that RB51, B115 and B18 have differences in antigenicity, immunologic and genetic properties. Particularly, in B115 a nonsense mutation was detected in \textit{wzm} gene, which could explain the intracellular localization of O-PS in this strain. Complementation studies to evaluate the precise role of each mutation in affecting \textit{Brucella} morphology and its virulence, could provide useful information for the assessment of new, attenuated vaccines for brucellosis.

Introduction

In \textit{Brucella} spp., as in many other gram-negative bacteria, the smooth lipopolysaccharide (S-LPS) is an important component of the outer membrane, strongly involved in pathogenesis mechanisms. Its precise role as a virulence factor is not yet clear. It has been suggested, however, that the LPS molecule may play a key role in the invasion and intracellular multiplication of \textit{Brucella} spp. as well as in protecting the cell against complement-mediated lysis. Moreover, the LPS is the immunodominant antigen to which the majority of antibodies resulting from either infection or vaccination are directed [1–4].

The S-LPS molecule has three sections: the lipid A, the core oligosaccharide and the distal O-poly saccharide chain (O-PS or O-antigen). The O-PS is a homopolymer of N-formyl-perosamine. \textit{Brucella} strains carrying complete S-LPS have a smooth (S) phenotype, so termed after the smooth texture of the colonial surface, while \textit{Brucella} devoid of O-PS have a rough (R) phenotype.

\textit{B.abortus}, \textit{B.melitensis}, \textit{B.suis}, \textit{B.neotomae} and \textit{B.microtus}, as well as the recently isolated \textit{B.pinnipedialis} and \textit{B.ceti} species, express a smooth phenotype, while \textit{B.abortus} RB51, \textit{B.melitensis} B115, \textit{B.suis} and \textit{B.canis} are typically rough strains [5,6].

Smooth-to-rough phase variation can spontaneously occur in \textit{Brucella} smooth strains as result of environmental factors but the molecular mechanism responsible for such variation has not yet been defined [7–9]. Owing to the lack of antigenic O-PS, true R-mutants neither induce anti O-PS antibodies which could interfere with a serologic diagnosis of brucellosis, nor react with anti-O-PS antibodies [5,10]. In addition, these mutants show outer membrane morphological and physiological changes resulting in the uptake of crystal violet and the autoagglutination in acriflavine solution [5]. With the exception of \textit{B.suis} and \textit{B.canis}, which are rough but virulent, R-mutants are less virulent than the wild type.

Because of these features, \textit{Brucella} R-mutants have been considered potential brucellosis vaccines [11,12]. The strain RB51 has replaced the S19 as vaccine for brucellosis in cattle in many countries [12]. RB51 is a spontaneous R-mutant derived from the virulent strain \textit{B.abortus} 2308 after a series of passages in selective media [10]. It expresses no O-PS on its cell surface, and therefore induces no diagnostically undesirable antibodies, mainly directed against this antigen [10,13,14]. Nevertheless, it produces anti-RB51 antibodies, as detected by specific serologic tests [13,15]. Genetic analysis showed that RB51 carries the genetic element IS711, spontaneously inserted into the \textit{wboA} gene [16]. Complementation of RB51 with \textit{wboA}, has been shown to increase O-PS expression without, however, restoring the smooth phenotype, suggesting the presence of additional mutations responsible for its rough morphology [17–20].
**Materials and Methods**

**Bacterial strains and growth conditions**

*B. melitensis* B115 and *B. abortus* 99 were provided to us by the Veterinary Laboratories Agency (VLA) of Weybridge (U.K.), while the *B. abortus* RB51 vaccine strain was isolated from the commercial vaccine "RB-51 CZV", (Cooper-Zelhtia Veterinaria, SA, Spain). *B. melitensis* 16M was isolated in our laboratory after several passages on Brucella agar medium supplemented with rifampin. B18 showed different antigenic and immunological properties compared to other strains – despite its rough morphology, it induced detectable anti-O-PS antibodies in laboratory animals [29].

In this study, we compared the antigenic and immunologic characteristics of RB51, B115 and B18. A molecular analysis, for each strain, of the 21 genes known to be involved in LPS synthesis was also performed. The genes of the three rough strains under study were PCR-amplified and sequenced, and the results compared to a smooth reference strain *B. melitensis* 16M the sequences of which had been published in GenBank [30]. This preliminary study would ultimately allow an in-depth investigation of potential correlations between immunologic characteristics and genetic makeup, necessary for the development of novel *Brucella* vaccines.

**Ethics statement**

Experiments with mice, according to National (D.L. 116/92) and European (86/609/EEC) regulations, were previously authorized by the National Authority (Decreto 225/2009-B). Bovine serum samples collected from *B. abortus* 99-naturally-infected cattle were provided by the Istituti Zooprofilattici Sperimentali, responsible for the serological surveillance of herds as prescribed by the Italian brucellosis eradication plan. For these sera, no ethical approval was sought, as they were obtained from animals that contracted the infection naturally.

**Rough Strains**

*B. melitensis* B115 is a natural, stable, rough strain, the phenotype of which has been evaluated according to classical criteria [5]. Many reports confirmed the lack of surface O-PS. Additional studies, however, demonstrated the presence of detectable O-antigen in the cytoplasm [21,22].

The mechanism of LPS synthesis is largely unknown, but genetic studies indicate that it is similar to that existing in some gram-negative bacteria. Several genes have been proven to be involved in the biosynthetic pathways of lipid A, core, and O-PS [7,11,16,19,23,24]. Most of these genes are clustered in two genetic regions, *wrk* and *uso*. R-mutants can result from mutations affecting O-PS precursor synthesis, its polymerization and export, or from a variety of defects in the inner core polysaccharide [11,25,26].

In preliminary studies conducted in our laboratory to assess the potential value of B115 as vaccine, B115 was unable to induce antibodies to O-PS in mice, even when administered at high dosages. Nevertheless, it elicited specific anti-B115 antibodies and was able to confer good protection [27,28]. *B. melitensis* 18 is a rough, stable, rifampin-resistant mutant of *B. melitensis* isolated in our laboratory by several passages on Brucella agar medium supplemented with rifampin. B18 showed different antigenic and immunological properties compared to other strains – despite its rough morphology, it induced detectable anti-O-PS antibodies in laboratory animals [29].

In this study, we compared the antigenic and immunologic characteristics of RB51, B115 and B18. A molecular analysis, for each strain, of the 21 genes known to be involved in LPS synthesis was also performed. The genes of the three rough strains under study were PCR-amplified and sequenced, and the results compared to a smooth reference strain *B. melitensis* 16M the sequences of which had been published in GenBank [30]. This preliminary study would ultimately allow an in-depth investigation of potential correlations between immunologic characteristics and genetic makeup, necessary for the development of novel *Brucella* vaccines.

**Antigenic characterization and antibody response evaluation**

The smooth or rough phenotype of *Brucella* strains was determined by classical criteria, such as crystal violet staining, acriflavine agglutination (0.1% w/v) and agglutination with monospecific *Brucella* sera to A and M antigens [5]. Cell-surface expression of O-PS was tested by evaluating the ability of each strain to bind antibodies induced by smooth or rough *Brucella* strains, in Complement Fixation Tests (CFTs). Bacterial suspensions were prepared with whole cells of *B. abortus* 99 smooth strain, *B. melitensis* B115, *B. abortus* RB51 and *B. melitensis* B18. All bacterial strains were grown at 37°C on TSA, adjusted to a concentration of 10^8 CFU/ml (OD 600 = 0.170) and then heat-inactivated. Before their use as antigen in CFT, an equal amount of fetal calf serum (FCS, Gibco) was added to RB51 suspensions as previously described [13,15]. This was done in order to neutralize possible nonspecific reactions due to the anticomplementary activity characteristic of rough phenotype brucella strains. B18 required the same treatment to avoid the anti-complementary activity. In contrast, *B. melitensis* B115 was used as is, since it showed no anticomplementary activity in a previous study [27]. To evaluate antibody response to *Brucella* strains, three groups of five NZW rabbits were inoculated subcutaneously (s.c.) with 10^8 CFU/rabbit of viable RB51, B18 and B115, respectively. Blood samples were collected by heart puncture at 7, 15, 30, 60, 90, and 140 postinoculation days (PID). Blood was allowed to clot for 12 hours at 4°C and then centrifuged. Serum samples were divided into 1 ml aliquots and stored at −20°C until use.

The presence of anti-O-PS antibodies was evaluated by the CFT and the Rose Bengal Plate Test (RBPT), using *B. abortus* 99 smooth strain as antigen, according to Alton et al. [5]. To detect antibodies directed against antigens other than O-PS, the CFT was performed using B115 as rough antigen, as previously described [27]. Anti-O-PS antibodies were obtained from serum samples taken from *B. abortus* 99-naturally-infected cattle, provided to our laboratory by the Istituti Zooprofilattici Sperimentali. Antibodies directed against antigens other than O-PS were obtained from serum samples collected from RB51-vaccinated cattle, previously provided by Dr. Olsen (United States Department of Agriculture Ames, IA, USA) for a collaborative study to validate a specific serologic test [13]. CFTs were performed in microtitre U-bottom plates as described elsewhere [13,15,27].

**Genetic characterization of *Brucella* strains: DNA preparation, PCR assay and sequence analysis**

*B. melitensis* strains were cultured for 48 h at 37°C on TSA slopes. The pellet was resuspended in 467 μl TE/sodium buffer (50 mM Tris, 50 mM EDTA, 100 mM NaCl, pH 8) and digested for 1 hr at 37°C with 3 μl of 20 mg/ml proteinase K and 30 μl of 10% SDS. DNA was purified twice with phenol-chloroform using Phase Lock Gel Heavy tubes (Eppendorf AG, Hamburg, Germany). Nucleic acids were precipitated using isopropanol/sodium acetate and the pellet resuspended in 50 μl of nuclease-free water. A total of 21 genes involved in LPS synthesis (17 located in chromosome I and 4 in chromosome II) were analyzed for each *Brucella* strain. The *Brucella* genes under study and the primers used for PCR amplifications, designed using the Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), are listed in Table 1. A PostgreSQL database was previously created to characterize *Brucella* rough strains.
**Table 1.** Primers used for PCR and sequencing analysis of genes, involved in the LPS synthesis, examined in this study.

| PCR | Primers | Sequence (5′→3′) | Specificity (gene) | Locus* | Putative role** | Amplicon size (bp) |
|-----|---------|------------------|-------------------|--------|-----------------|-------------------|
| 347 | 1153    | AGTGGAGACAAATCTGGAATG | wboB | BMEI 0997 | Mannosyltransferase | 1686 |
| | 1154    | ATCTCTGGATTCGTTGGAGAAG | | | | |
| 348 | 1156    | GGGTTATAGCGGATAACACG | wboA | BMEI 0998 | Glycosyltransferase | 1297 (2139 in RB51) |
| | 1157    | CCCGGGCATTTAAAGATGAGAAGC | | | | |
| 349 | 1159    | ATGAGCGGACATATAAAGGTG | wa | BMEI 1326 | Protein of unknown function | 2253 |
| | 1160    | GCCTGTAGTGAGAAAAGAC | | | | |
| 350 | 1162    | ATGGCTACATCCAGCTGCACT | wbkE | BMEI 1393 | Glycosyltransferase | 1287 |
| | 1163    | TGCAGAATTTTGGTGCTG | | | | |
| 351 | 1165    | CGCAACCAAAAATCCGACA | manA\textsubscript{OM} | BMEI 1394 | Perosamine synthesis | 1256 |
| | 1166    | GAGGATAGATTTCTGACGAGTCG | | | | |
| 352 | 1168    | GCAAACCAAAAGGGGAAAGC | manB\textsubscript{OM} | BMEI 1395 | Perosamine synthesis | 1592 |
| | 1169    | TCGAAAGCTCTGAAACACGA | | | | |
| 353 | 1171    | GCGGATGAAATCAGATGTT | manC\textsubscript{OM} | BMEI 1396 | Perosamine synthesis | 1466 |
| | 1172    | GTGGCACATCATATGGGACAC | | | | |
| 354 | 1174    | CCTCTGTGATCTCGGAGA | wbkA | BMEI 1404 | Glycosyltransferase | 1271 |
| | 1175    | ATTTGAAAAATCTGGGCAAC | | | | |
| 355 | 1177    | TGTAATGCTCCAGAGCAAG | gmd | BMEI 1413 | Perosamine synthesis | 1257 |
| | 1178    | ACCCTGATGACGGGATCAAG | | | | |
| 356 | 1180    | GCTGGAAGGCGAATACTGCT | per | BMEI 1414 | Perosamine synthesis | 1297 |
| | 1181    | CACCAGAATGGCGTACCATC | | | | |
| 357 | 1183    | AGGCTCTCCACAGGATCTTA | wzm | BMEI 1415 | ABC transporter permease | 995 |
| | 1184    | ATGAGCGGCTGATCTGACGATG | | | | |
| 358 | 1185    | TGCTAGGCTGAGGCTGCTC | wzt | BMEI 1416 | ABC transporter permease | 915 |
| | 1186    | AGAGGGACCTGCAAGCAGC | | | | |
| 359 | 1189    | AACTCGGGAATGGGCAAGCTAT | wbkB | BMEI 1417 | Unknown | 934 |
| | 1190    | CAAAACGCCTGATTTTGGTG | | | | |
| 360 | 1192    | CGTACCAAGATTGGAATGCTC | wbkC | BMEI 1418 | Formyltransferase | 890 |
| | 1193    | CTGCTGGACAAAAACCTCA | | | | |
| 361 | 1195    | GTTACGGGAGGAAATGGCACA | wbf (ex wecA) | BMEI 1426 | Undecaprenyl-P-\(\alpha\)-N-acetylglucosaminyl | 1213 |
| | 1196    | CGGCCATGGAAAAGAGGATA | | | | |
| 362 | 1198    | CCACACTCTCTCCTGCTGTC | wbd | BMEI 1427 | Unknown | 2042 |
| | 1199    | GGTCTGATGAGGGGATGTCG | | | | |
| 363 | 1201    | CACTTGGCTTGGATTGAG | pgm | BMEI 1886 | Phosphoglucomutase | 1956 |
| | 1202    | TCACTGGGCTTCTGCTCA | | | | |
| 364 | 1204    | ACAGGGAGAATTTCGCGGAATGA | wz | BMEI 0053 | Transport ATPase | 976 |
| | 1205    | ATGGGTGCTGACATCTGACC | | | | |
| 366 | 1210    | GGCAATACAGGTGTTGGTGATGG | manB\textsubscript{core} | BMEI 0899 | Phosphomannomutase | 1577 |
| | 1211    | GCCTGGTCCAGTGATGGT | | | | |
| 367 | 1213    | TGGAATTCTGCAGGATGACACA | manC\textsubscript{core} | BMEI 0900 | Unknown | 1526 |
| | 1214    | TTCCATGGCTGGATATCAGT | | | | |
| 368 | 1216    | GATGGGGAGCTTCCGTTCTG | wz**** | BMEI 1134 | Amidase (?) | 1290 |

GeneBank AE008917 (chromosome I) and AE008918 (chromosome II); **Moriyon et al. (2004). doi:10.1371/journal.pone.0024073.t001

keep track of all primers, PCRs and samples, each of which was assigned an identification code (ID). A convenient web interface allows internet connection to this database, available to registered users at https://cosmos.bio.uniroma1.it. PCR amplifications were carried out using the TripleMaster PCR System and the High Fidelity Buffer (Eppendorf AG, Hamburg, Germany) following the suggested protocol for high fidelity PCR. About 100 ng of genomic DNA were used as template for each reaction. Thirty amplification cycles were performed, each comprising denaturation at 95°C for 2 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. Ten µl of each reaction mixture were analyzed by electrophoresis on a 2% agarose gel stained with ethidium bromide.
Following amplification, PCR products were purified using a Montage PCRm96 Micro-well Filter Plate (Millipore, Billerica, Mass). Bidirectional sequencing was performed using the amplification primers listed in Table 1. Purified products were sequenced by Macrogen Inc. (10F World Meridian Center 60-24 Kunchun-Ku, Kasan-Dong, Seoul, Korea 153-023) using an ABI3730 XL DNA Analyzer (Applied Biosystems, Renta, USA). The forward and reverse ABI files were aligned and assembled into a single consensus sequence using MEGA software version 5 (http://www.megasoftware.net/). All sequences were submitted to BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi/) and nucleotide and amino acid changes identified through comparison with the corresponding B. melitensis 16M sequences available in GenBank (AE008917 for chromosome I and AE008918 for chromosome II).

Results

Surface antigenicity and antibody response in rabbits

The rough phenotype of RB51, B115 and B18, tested by the classical methods of crystal violet staining and autoagglutination reaction with acriflavine, was confirmed after passages in culture media. No reversion to smooth phenotype occurred after passages in mice and rabbits (data not shown).

In CFTs performed on whole-cell antigen suspensions of RB51, B18 and B115, B. abortus 99 smooth strain, tested as control, bound only anti-O-PS antibodies. RB51 and B115 bound antibodies induced by the rough strain RB51, and did not react with anti-O-PS antibodies induced by the smooth strain B. abortus 99.

In contrast, the rough strain B18 was able to react with both kinds of antibodies – anti-O-PS and anti-RB51 - giving the same titres obtained with homologous antigens used separately.

The antibody response induced by Brucella strains in rabbits was evaluated by CFTs and RBPT, up to 140 PIDs. As expected, vaccination with RB51 did not induce antibodies to O-PS. At PID 7, 80% of rabbits had seroconverted to the rough antigen B115, reaching 100% at PID 15. Specific antibodies were still present in 60% of RB51-inoculated rabbits at PID 140.

Similarly, no antibodies to O-PS were detected in sera from rabbits inoculated with B115. Antibodies to B115 were detected in 80% of these rabbits at PID 7, and in 100% at PID 15. At PID 140, 80% of rabbits were still seropositive.

In the group vaccinated with B18, 80% of rabbits had reacted with both antigens by PID 7, giving similar titres. By PID 15, both kinds of antibodies had appeared in 100% of the animals, that had remained seropositive until PID 90. At pid 140, however, anti-O-PS antibodies were detected in 80% of rabbits, while only 40% had antibodies to B115.

Molecular analysis

The results of the molecular analysis performed on 21 genes involved in LPS synthesis are presented in Table 2. Consensus sequences of B115, RB51 and B18 strains were compared to the available B. melitensis 16M genome (AE008917 for chromosome I and AE008918 for chromosome II) in order to detect mutations. The presence of each mutation was confirmed after passages in mice, thus confirming the genetic stability of mutants (data not shown).

Of the 21 genes examined, three showed no nucleotide mutations compared to the genomic sequences of strain 16M (manC, manA, wbkC) in any of the three strains analyzed. Six genes presented only silent mutations, with no resulting change to the amino acid sequence of the corresponding protein (rfaE, gnd, per, rfaF, wzt, manBOAg). Most genes showed a number of missense mutations (rfaB, wboA, wzt, wzm, wbkB, manCore, and wob****). Two nonsense mutations were also detected, one already known, that is, the interruption of the wboA gene by an IS711 element in B. abortus RB51; the other, a point mutation at position 236 in the wzm gene for the B115 strain, that changes the amino acid tryptophan (W) into a stop codon in position 79 of the protein, resulting in a polypeptide chain that ends prematurely and a truncated protein product.

Discussion

In the present study, we evaluated the antigenic, immunologic and genetic characteristics of rough strains B. abortus RB51, B. melitensis B115 and B. melitensis B18. We amplified and sequenced 21 genes involved in LPS synthesis, in order to detect genetic mutations which could ultimately be correlated with the different properties of these strains. All sequences were compared with those of B. melitensis 16M reference strain.

RB51 and B115 are well known strains. Their antigenic, genetic and immunological characteristics, however, have not been fully assessed. B18 is a rough, rifampin-resistant mutant of B. melitensis isolated in our laboratory [29].

In Brucella strains of smooth phenotype, LPS is synthesized as two separate components, lipid A-core and the O-PS. The O-PS is a homopolymer of N-formyl-perosamine units [24,31]. Several genes involved in LPS biosynthesis have been recognized, most of them clustered in the wbk and wbo genetic regions [11]. The wbo region encodes two putative glycosyltranferases, wbaA and wboB.

The wbk contains genes coding for enzymes necessary for N-formylpemerosamine synthesis (gmd, per), its formylation (wbkC) and polymerization (wbkE, wbkF), for bactoprenol priming (wbkB and wbkF) and the ABC transporters for the O-PS translocation (wzm, wzt). It also contains genes involved in the synthesis of mannosone (manAOAg, manBOAg and manCcore), which probably act coordinately with gmd and per, and independently with other genes [18,32,33]. Finally, three genes have been shown to be involved in core synthesis - rfa, manB and wob**** [26].

These genetic regions, central for LPS synthesis in smooth strains, may be expected to be absent or different in rough strains. And indeed, B. ovis is naturally devoid of the wbo region [34]. The wbk region, however, is present both in B. ovis and in B. canis, which are natural rough strains [25]. The disruption of wkbB and manBOAg does not generate rough mutants [24,26].

Transposon mutagenesis and complementation studies, generating different rough mutants, confirmed the crucial role of these genes [24,26].

B. abortus rough strain RB51 has no O-PS exposed on its cell surface but it is not completely devoid of O-PS: it has been demonstrated that RB51 produces low levels of M-like O-antigen [35]. Genetic analysis revealed that RB51 carries an IS711 element spontaneously inserted into the wboA gene, which maps outside of the main wbk region [16]. The wboA encodes for a glycosyltranferase that is essential for the polymerization of the O-antigen [17]: disruption of this gene in B. abortus, B. melitensis and B. suis resulted in rough mutants that were unable to synthesize the O-PS [36]. The complementation of RB51 with functional wboA (RB51WboA) increased O-PS expression, but the resulting low levels of O-PS were present in the cytoplasm, and not on the surface [19,20]. It has been suggested that other LPS biosynthetic genes may have been modified affecting the export of O-PS and S-LPS to the bacterial surface or the appropriate coupling of the O-antigen to the core-LPS or both. In mice, the RB51WboA strain induced detectable antibodies to the O-antigen and showed an enhanced vaccine efficacy against the B. abortus 2308 virulent strain, without affecting its attenuation characteristic [19,20].
Table 2. Mutations detected in LPS-synthesis genes of *B. melitensis* B115, *B. abortus* RB51 and *B. melitensis* B18.

| PCR Gene | D | B. melitensis B115 | B. abortus RB51 | B. melitensis B18 |
|----------|---|--------------------|----------------|------------------|
|          |   | nucleotide position | protein position | nucleotide position | protein position | nucleotide position | protein position | nucleotide position | protein position |
| 347      |   | T to C (347)       | L to S (116)     | M                 | T to C (347)     | L to S (116)     | M                 |
|          |   | T to C (447)       | G to G (149)     | S                 |                   |                   |                   |
|          |   | A to G (690)       | A to A (230)     | S                 |                   |                   |                   |
|          |   | T to C (756)       | Y to Y (252)     | S                 |                   |                   |                   |
|          |   | G to A (971)       | R to Q (324)     | M                 |                   |                   |                   |
| 348      |   | 842 bp insertion   | frameshift       | N                 | C to A (943)     | H to N (315)     | M                 |
|          |   |                   |                 |                   | T to G (1086)    | S to S (362)     | S                 |
| 349      |   | C to T (278)       | S to L (93)      | M                 | C to T (278)     | S to L (93)      | M                 |
|          |   |                   |                 |                   | T to G (651)     | A to A (217)     | S                 |
|          |   |                   |                 |                   | A to C (801)     | Q to H (267)     | M                 |
| 340      |   | A to G (903)       | R to R (301)     | S                 | A to G (395)     | D to G (132)     | M                 |
|          |   |                   |                 |                   | T to C (397)     | F to L (133)     | M                 |
|          |   |                   |                 |                   | C to T (817)     | L to F (273)     | M                 |
|          |   |                   |                 |                   | A to G (903)     | R to R (301)     | S                 |
|          |   |                   |                 |                   | A to G (903)     | R to R (301)     | S                 |
| 350      |   | C to T (216)       | L to L (72)      | S                 | None             |                   |                   |
| 351      |   | None              |                 |                   |                   |                   |                   |
| 352      |   | C to T (1141)      | L to F (381)     | M                 | G to A (210)     | A to A (70)      | S                 |
| 353      |   | A to G (903)       | R to R (301)     | S                 | A to G (395)     | D to G (132)     | M                 |
|          |   |                   |                 |                   | T to C (397)     | F to L (133)     | M                 |
|          |   |                   |                 |                   | C to T (817)     | L to F (273)     | M                 |
|          |   |                   |                 |                   | A to G (903)     | R to R (301)     | S                 |
|          |   |                   |                 |                   | A to G (903)     | R to R (301)     | S                 |
| 354      |   | C to T (690)       | Y to Y (230)     | S                 | T to C (1144)    | S to S (48)      | S                 |
|          |   |                   |                 |                   |                   |                   |                   |
| 356      |   | C to T (690)       | Y to Y (230)     | S                 | T to C (1144)    | S to S (48)      | S                 |
| 357      |   | G to A (360)       | V to V (120)     | M                 | C to A (360)     | V to V (120)     | M                 |
|          |   |                   |                 |                   | T to C (455)     | L to P (152)     | S                 |
|          |   |                   |                 |                   |                   |                   |                   |
| 358      |   | A to C (512)       | D to A (171)     | M                 | None             |                   |                   |
| 359      |   | None              |                 |                   |                   |                   |                   |
| 360      |   | None              |                 |                   |                   |                   |                   |
| 361      |   | G to A (48)        | G to G (16)      | S                 | None             |                   |                   |
| 362      |   | C to T (263)       | T to I (88)      | M                 | A to C (615)     | P to P (205)     | S                 |
|          |   | G to A (1300)      | V to I (434)     | M                 | C to T (920)     | M to T (307)     | M                 |
|          |   | A to G (1338)      | K to K (446)     | S                 | A to G (1338)    | K to K (446)     | S                 |
|          |   | G to A (1675)      | E to K (559)     | M                 | G to A (1757)    | G to D (586)     | M                 |
|          |   | A to G (1819)      | K to E (607)     | M                 | A to G (1819)    | K to E (607)     | M                 |
| 363      |   | A to G (752)       | H to R (251)     | M                 | A to G (752)     | H to R (251)     | M                 |
|          |   |                   |                 |                   | A to G (752)     | H to R (251)     | M                 |
|          |   |                   |                 |                   | C to T (1527)    | S to S (509)     | S                 |
| 364      |   | T to C (672)       | S to S (224)     | S                 | None             |                   |                   |
| 366      |   | C to T (279)       | G to G (93)      | S                 | C to T (279)     | G to G (93)      | S                 |
|          |   |                   |                 |                   | C to T (1335)    | R to R (445)     | S                 |
| 367      |   | None              |                 |                   |                   |                   |                   |
| 368      |   | T to A (388)       | S to T (130)     | M                 | A to C (751)     | S to R (251)     | M                 |
|          |   | A to C (751)       | S to R (251)     | M                 |                   |                   |                   |
|          |   | T to C (758)       | I to T (253)     | M                 | C to T (1155)    | S to S (385)     | S                 |
**Table 2.** Cont.

D: direction of transcription of the gene; T: type of mutation; M: missense; S: silent; N: nonsense. Mutations identified are referenced to *B.melitensis* 16M. (AE 008917 for chromosome I and AE008918 for chromosome II).

doi:10.1371/journal.pone.0024073.t002

*B.melitensis* B115 is a natural rough strain, whose phenotype has been found to be very stable, even after passages *in vivo* [5,37]. Based on its *rpoB* nucleotide sequence, it has been classified as *B.melitensis* biotype 1 [38]. Many studies confirmed the absence of surface O-PS in this strain. Interestingly, however, low-titre anti-O-PS antibodies have been detected in rabbits immunized with live or killed B115 [37] and O-PS specific monoclonal antibodies obtained when mice were immunized with B115 [39,40]. Immunogold labelling using O-PS specific monoclonal antibodies revealed that the O-PS was present in the cytoplasm and that some of the expressed O-antigen was lipid-bound and associated with the cell wall [21,22]. In previous studies conducted in our laboratory, however, B115 didn’t induce antibodies to O-PS in mice, even when it was given twice at high dosages, while detectable anti-B115 antibodies were produced [27,28].

Several hypotheses have been formulated to explain the rough phenotype of *B.melitensis* B115 despite its O-chain expression [39]. Two genes, *wzm* and *wzt*, have been identified as encoding proteins with high similarity to several two-component ABC transporters. Their involvement in O-PS translocation across the inner membrane was confirmed by gene replacement. The deletion of these two genes resulted in an inability to express LPS. *B.melitensis wzm/wzt* mutants, generated in *B.melitensis* 16M by allelic replacement strategy, resulted in colonies of rough phenotype with intracellular accumulation of free O-PS, not bound to the lipid A-core molecule [24,41].

When functional *wzm* and *wzt* genes cloned into plasmids were supplied, the wild-type phenotype was restored. In contrast, in B115, the same complementation failed to restore the smooth phenotype. The possibility has, thus, been raised, that additional mutations may be present, affecting the LPS O-side-chain transport or ligation to the lipid A-core molecule [24].

In this study, we tested the surface O-PS expression of RB51, B115 and B18 by evaluating their ability to bind antibodies directed against O-PS or against antigens other than O-PS. This was done by CFTs, using whole-cell suspensions of RB51, B115 and B18 as antigens.

CFT results confirmed that RB51 whole cells bind homologous antibodies and fail to react with antibodies directed to O-PS. Similarly, whole cells of B115 did not bind anti-O-PS antibodies, as previously indicated [42], but did react with antibodies induced by the rough strain RB51. Interestingly, however, B18 was able to bind both kinds of antibodies.

Moreover, like RB51, the strain B18 needed to be previously incubated with FCS to neutralize anticomplementary activity causing non specific reactions in CFTs. In contrast, B115 was naturally devoid of anticomplementary activity and did not require the addition of FCS.

Our data thus confirm that, despite the rough phenotype in common, the three strains under study - RB51, B115 and B18 - present differences in their surface antigenicity, including the expression of O-PS on the cell-surface of the B18 strain.

The antibody response induced by RB51, B18 and B115 was tested in three groups of rabbits, respectively. Antibodies to O-PS were detected using the *B.abortus* S99-based CFT [3], while specific antibodies induced by rough *Brucella* strains were detected by a B115-based CFT [43].

The CFT was used as a serological test because of its ability to detect antibodies induced by rough *Brucella* strains with satisfactory sensitivity and specificity, as previously demonstrated [13,27].

The results confirmed that B115 and RB51 fail to elicit anti-O-PS antibodies, while both induce specific antibodies still detectable at PID 140. The lack of anti-O-PS antibodies in B115 and RB51-inoculated rabbits was confirmed by *B.abortus* 99-based RBPT. The production of specific antibodies induced by B115 and RB51 in rabbits was similar to that observed in mice in a previous study [27].

All B18-inoculated rabbits produced both anti-O-PS antibodies and specific anti-B18 antibodies, in similar titres. At PID 140, 80% of sera still contained low levels of antibodies to O-PS, while specific anti-B18 antibodies were detected only in 40% of rabbits. This may have been due to a relatively low sensitivity of the B115-based CFT in detecting anti-B18 antibodies.

Serological results confirmed the absence of surface O-PS in RB51 and B115 and the presence of the O-PS antigen in B18. We didn’t investigate the presence of intracellular O-PS in B18. Our antigenic results, however, suggest that it may be exposed at surface.

These data also indicate that intracellular O-PS in B115 is incapable of eliciting detectable O-PS response. This is not surprising; a similar phenomenon has been reported for the Δ*pgm* rough mutant, which harbours small amounts of cytoplasmic O-antigen but fails to induce antibodies to O-PS in mice [4]. The nature of this lack of response remains to be determined.

The genetic analysis of RB51, B115 and B18, performed by comparing the sequences of 21 genes involved in LPS-synthesis with those of the *B.melitensis* 16M strain, indicated the presence of some mutations, most of them missense mutations.

The nucleotide and amino acid differences detected in the genes examined are shown in Table 2. Passages in mice confirmed the stability of all mutations.

Sequence analysis of B115 genes revealed high homology with *B.melitensis* 16M, with few genetic differences detected - six point mutations, four of the silent type, one missense, and one nonsense. Silent mutations were present in genes *manCOAg*, *per* and *manBcore*. A missense mutation was detected in the *pgm* gene. This mutation was also present in RB51 and B18. The gene *pgm* plays a central role in O-PS biosynthesis since it codes for the enzyme phosphoglucomutase which is responsible for the interconversion of glucose-6-phosphate to glucose-1-phosphate, and is thus essential to the biosynthesis of glucose or galactose in *B.abortus* [3].

A nonsense mutation was detected in the *wzm* gene. Since this gene was found to be responsible for the incorporation of the O-PS to the periplasmic side of the membrane, such mutation may be linked to the rough morphology of B115. In addition, this mutation - detected only in B115 - can therefore be used as a marker for the molecular identification and differentiation of B115.

In RB51, beyond the well known insertion in gene *wboA* [16,17], we detected several genetic differences between the RB51 strain and the published *B.melitensis* 16M genome (Table 2). Silent mutations were detected in *wbkF*, *gmd*, *per*, *wbkF*, *wza* (0053) and *manBcore*. Missense mutations were detected in eleven genes: *wboB*,
characterization of Brucella Rough Strains

**References**

1. Lapaque N, Moriyon I, Moreno E, Górriz JP (2003) *Brucella* lipopolysaccharide acts as a virulence factor. Curr Opin Microbiol 8: 60–66.

2. Moreno E, Moriyon I (2002) *Brucella melitensis*: a nasty bug with hidden credentials for virulence. Proc Natl Acad Sci USA 99: 1–3.

3. Ugalde J, Cahener C, Feldman MF, Ugalde RA (2000) Identification and characterization of the *Brucella abortus* phosphoglucomutase gene: role of the *Brucella* lipopolysaccharide in virulence and intracellular multiplication. Infect Immun 68: 5716–5723.

4. Ugalde JE, Comerci DJ, Leguizamón MS, Ugalde RA (2003) Evaluation of *Brucella abortus* phosphoglucomutase (gpm) mutant as a new live rough-phenotype vaccine. Infect Immun 71: 6269–6269.

5. Alton GG, Jones LM, Angus RD, Verger JM (1983) Techniques for the brucellosis laboratory. Institut National de la Recherche Agronomique, Paris, France.

6. Foster G, Osterman BS, Godfroid J, Jaques I, Cloekaert A (2007) Characterization of the *Brucella melitensis* wbkF gene and a silent mutation in determining the *B. melitensis* 16M reference strain.

7. Braun W (1947) Bacterial dissociation. Bacteriol Rev 11: 75–114.

8. Henry BS (1933) Dissociation in genus *Brucella*. 2: 1008–1016.

9. Moriyon IM, Grillo MJ, Monreal D, Gonzalez D, Marin C, et al. (2004) Rough morphology [25]. It has later been shown, however, that they were highly conserved not only in the classical S-type *Brucella* species, but also in the rough strains *Bovis* and *Bratia*, suggesting that other genes should be responsible for the rough morphology [23].

10. Our results indicate that the rough strains RB51, B115 and B18, characterized by different antigenic and immunologic properties, show differences in genes involved in LPS synthesis. We were able to identify the specific genes affected by such mutations. These findings may provide a valuable basis for further investigations (e.g., complementation studies) to assess the precise role of each mutation in determining the *Brucella* phenotype and its characteristics, essential for the development of attenuated and immunogenic vaccines.

**Author Contributions**

Conceived and designed the experiments: RA. Performed the experiments: RA MM GLR MF MT. Analyzed the data: RA MM GLR MT. Contributed reagents/materials/analysis tools: MM GLR. Wrote the paper: RA.
34. Vizcaíno N, Caro-Hernández P, Cloeckaert A, Fernández-Lago L (2004) DNA polymorphism in the omp25/omp31 family of Brucella spp.: identification of a 1.7-kb inversion in Brucella cetaceae and of a 15.1-kb genomic island, absent from Brucella suis, related to the synthesis of smooth lipopolysaccharide. Microbes Infect 6: 821–834.

35. Cloeckaert A, Zygmunt MS, Guilloteau LA (2002) Brucella abortus vaccine strain RB51 produces low levels of M-like O-antigen. Vaccine 20: 1820–1822.

36. Winter AJ, Schurig GG, Boyle SM, Sriranganathan NM, Bevins JS (1996) Protection of BALB/c mice against homologous and heterologous species of Brucella by rough strain vaccines derived from Brucella melitensis and Brucella suis biovar 4. Am J Vet Res 57: 677–683.

37. Jones LM, Diaz R, Taylor AG (1973) Characterization of allergens prepared from smooth and rough strains of Brucella melitensis. Br J Exp Pathol 54: 492–508.

38. Marianelli C, Ciuchini F, Tarantino M, Pasquali P, Adone R (2006) Molecular characterization of the rpoB gene in Brucella species: new potential molecular markers for genotyping. Microbes Infect 8: 860–865.

39. Cloeckaert A, Zygmunt MS, Nicolle JC, Dubray G, Limet JN (1992) O-chain expression in the rough Brucella melitensis B115: induction of O-polysaccharide-specific monoclonal antibodies and intracellular localization demonstrated by immunoelectron microscopy. J Gen Microbiol 138: 1211–1219.

40. Cloeckaert A, Zygmunt MS, Dubray G, Limet JN (1993) Characterization of O-polysaccharide specific monoclonal antibodies derived from mice infected with the rough Brucella melitensis strain B115. J Gen Microbiol 139: 1551–1556.

41. Halling SM, Detilleux PG, Tatum FM, Judge BA, Mayfield JE (1991) Deletion of the BCSP31 gene of Brucella abortus by replacement. Infect Immun 59: 3863–3868.

42. Diaz R, Jones LM, Leong D, Wilson JB (1968) Surface antigens of smooth Brucellae. J Bacteriol 96: 893–901.

43. Adone R, Francia M, Ciuchini F (2000) Brucella melitensis B115-based complement fixation test to detect antibodies induced by Brucella rough strains. J Appl Microbiol 105: 567–574.