Microbes and Infection 16 (2014) 633–639

www.elsevier.com/locate/micinf

Original article

Evaluation of a *Brucella melitensis* mutant deficient in O-polysaccharide export system ATP-binding protein as a rough vaccine candidate

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Received 2 April 2014; accepted 20 June 2014
Available online 18 July 2014

Abstract

Rough *Brucella* mutants have been sought as vaccine candidates that do not interfere with the conventional serological diagnosis of brucellosis. In this study, a rough mutant of *Brucella melitensis* was generated by the disruption of the *wzt* gene, which encodes the O-polysaccharide (O-PS) export system ATP-binding protein. *In vivo*, the mutant 16MΔ*wzt* was attenuated and conferred a level of protection against *B. melitensis* 16M challenge similar to that conferred by the vaccine strain *B. melitensis* M5 in mice. In pregnant sheep, the mutant 16MΔ*wzt* did not induce abortion. *In vitro*, 16MΔ*wzt* was more susceptible to polymyxin B and complement-mediated killing than *B. melitensis* 16M was. Most importantly, although 16MΔ*wzt* had a rough phenotype, it was able to synthesize O-PS and did not induce detectable specific antibodies in sheep. These results suggested that 16MΔ*wzt* deserved to further systematic evaluation as a vaccine for target animal hosts due to its promising features.

Keywords: *Brucella melitensis*; Rough mutant; Vaccine candidate; O-PS export system ATP-binding protein

1. Introduction

Brucellosis is an important zoonosis that induces abortion and decreased milk production in animals and causes intermittent fever, fatigue, and arthralgia in humans. Herd vaccination with *Brucella abortus* vaccine S19 and *Brucella melitensis* vaccine Rev.1 plays a major role in the prevention and elimination of animal brucellosis in many countries and regions. Although S19 and Rev.1 confer protection in cattle, sheep, and goats [1–3], both vaccines induce abortion when applied in pregnant animals, and these vaccines are composed of smooth strains that induce specific antibodies against O-PS, interfering with serological discrimination between vaccinated and infected animals [1,4,5].

To solve these problems, several strategies have been used to improve current vaccines. For safety improvement, the deletion of virulence-related genes, reduced-dose vaccination, and vaccination via the oral and conjunctival routes have been widely used [6]. To solve the problem of diagnostic interference, S19 and Rev.1 mutants with deletion of the outer membrane proteins Omp25 and Omp31 or the periplasmic protein BP26 have been generated. Methods were then developed to detect antibodies against these proteins to differentiate infected and vaccinated animals [7,8]. In addition, heterologous proteins expressed in *Brucella* vaccines, e.g., GFP, have been used as a marker, and an immunoenzymatic assay was designed to measure anti-GFP antibodies to allow for the discrimination of mice vaccinated with S19-GFP and those immunized with S19 [9]. Nevertheless,

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http://dx.doi.org/10.1016/j.micinf.2014.06.013

most studies have limited the use of current vaccines to laboratory animals.

In addition to improving current vaccines, many scientists have endeavored to design novel rough vaccines with satisfactory immunogenic properties that do not induce O-PS-specific antibodies. One of the most successful rough vaccine strains is *B. abortus* RB51, which is a highly attenuated strain that confers protective immunity and does not interfere with conventional serological diagnosis [10]. Another rough strain, *B. melitensis* B115, also confers significant protective immunity, equivalent to that provided by *B. melitensis* Rev.1 [11,12]. However, Gonzalez and Barrio et al. evaluated certain *B. melitensis* rough mutants in mouse [13] and sheep [14] models and found that the protective immunity conferred by most rough mutants did not reach the level induced by Rev.1. Furthermore, certain ewes vaccinated with the rough mutants tested positive in an enzyme-linked immunosorbent assay (ELISA) based on the smooth lipopolysaccharide (LPS) antigen. The researchers inferred that rough mutants are of limited use in developing new live vaccine candidates for animals [14].

Although there are positive and negative viewpoints on rough brucellosis vaccines, considering that O-PS is a valid target for the differential diagnosis of brucellosis and that the RB51 strain has been successfully developed as a live vaccine for animals, we believe that the development of a rough brucellosis vaccine is still promising. In the present study, we deleted *wzt*, a gene encoding the O-PS export system ATP-binding protein, which functions in the export of O-PS to the cell surface, from the genome of *B. melitensis* 16M, and studied its role in virulence, pathogenicity, and protection in animals.

2. Materials and methods

2.1. Ethics statement

All animal research was approved by the Beijing Association for Science and Technology (approval: SYXK, Beijing 2007-0001), and the animal research complied with the guidelines for laboratory animal welfare and ethics of the Beijing Administration Committee of Laboratory Animals.

2.2. Bacterial strains and growth conditions

The virulent strain *B. melitensis* 16M, the rough strain *Brucella canis* RM6/66, and the vaccine strain *B. melitensis* M5 were all kindly donated by Qianni He (Institute of Veterinary Research, Xinjiang Academy of Animal Sciences, China). These strains were originally collected and preserved in the Chinese Veterinary Culture Collection Center (CVCC). All *Brucella* strains were routinely grown in tryptic soy broth (TSB) or tryptic soy agar (TSA). *Escherichia coli* stains were grown on Luria–Bertani (LB) plates with or without ampicillin (100 mg/L) and chloromycetin (30 mg/L) (Table 1). All work with living virulent *Brucella* strains was performed in biosafety level 3 facilities at China Agricultural University.

Table 1
Bacterial strains and plasmids.

Strain or plasmid	Characteristic(s)	Source or reference
Bacterial strains		
B. melitensis 16M	Wild type, smooth, virulent	Qianni He laboratory
B. canis RM6/66	Wild type, rough, virulent	Qianni He laboratory
B. melitensis M5	Vaccine strain, smooth	Qianni He laboratory
B. melitensis 16MΔ*wzt*	*wzt* deletion mutant of 16 M	This work
B. melitensis 16M pBBR*wzt*	complementation strain of *wzt* deletion mutant	This work
Escherichia coli DH10B	F-*mcr*A Δ(mrr-hsdRMS-mcrBC) Φ80dlacZΔM15 ΔlacX74 endA1 recA1 deoR Δ(ara,leu)7697 araD139 *gal*U *gal*K *nup*G *rps*L(Strr) *nup*G	Invitrogen
Plasmids		
pEX18AP	*sac*B, *bla*, Amp^r	
pBBR1MCS	Broad-host-range plasmid, Cm^r	
pEX18Ap-*wzt*	WU0393-WU0394/WU0395-WU0396 cloned into pEX18Ap for *wzt* gene deletion	This study
pBBR*wzt*	WU0393c-WU0396c cloned into pBBR1MCS for complementation assay	This study

2.3. Animals

Six- to eight-week-old female BALB/c mice were purchased from Weitong Lihua Laboratory Animal Services Centre (Beijing, China) and bred in individually ventilated cage rack systems. Pregnant female sheep at 100–120 days of gestation were obtained from brucellosis-free regions and determined to be seronegative by the rose bengal plate agglutination test (RBT) and a standard tube agglutination test (SAT) [16]. The sheep were housed in restricted-access large-animal isolation facilities. At the end of the experiment, all animals were euthanized with an animal-culling device and disposed of according to the relevant national regulations.

2.4. Construction of the wzt deletion mutant and its complementary strain

To construct the recombinant plasmid for deleting the *wzt* gene (BMEI1416), the 5′- and 3′-fragments flanking the *wzt* gene were amplified. To construct the complementation strain of 16MΔ*wzt*, primers were designed to amplify the entire *wzt* gene. Using previously described methods [15], the *wzt* deletion mutant of *B. melitensis* 16M was constructed. This mutant was referred to as 16MΔ*wzt*, and the complementation strain was referred as 16M pBBR*wzt*.

2.5. Phenotypic characterization of the mutants

The phenotypes of 16MΔ*wzt* and its complementation strain were characterized by coagglutination with acriflavine solution and anti-sera against smooth and rough *Brucella*