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# Variable-number tandem repeat markers for identification of *Brucella abortus* 82 and 75/79-AV vaccine strains

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ABSTRACT

In Russia, live vaccine strains *Brucella abortus* 82 and *B. abortus* 75/79-AV have been widely and extensively utilized for specific prophylaxis of cattle brucellosis. To differentiate these vaccine strains from each other and laboratory collection of other vaccine ($n = 4$), reference ($n = 15$) and field *Brucella* strains ($n = 61$), the multiple-locus variable-number tandem repeat (VNTR) analyses (MLVA) were used with 12 loci containing tandem repeats from 134 bp to 8 bp recently described for *Brucella* spp. The results obtained by MLVA typing revealed that the vaccines are genetically closely related. Three sufficiently stable *in vitro* VNTR loci were chosen in order to discriminate, in single tube Multiplex PCR, products of the vaccine strains from the other *Brucella* strains. Our results demonstrated that MLVA in Multiplex PCR format is a rapid, easy, economical and efficient tool for identification of vaccine strains and will be validated in future large scale typing of *Brucella* isolates circulating in Russia.



## 1. Introduction

*Brucella* species are zoonotic pathogens that cause animal and human brucellosis and pose a serious public health threat and result in economic losses due to abortion and the slaughtering of infected agricultural animals.

Most studies conducted confirm that the highest potential for prevention of brucellosis infection is the use live vaccines [1–12]. The first effective live vaccine strain, *Brucella abortus* 19, was attenuated during subculture in the USA [1]. Later, live, attenuated *B. abortus* 19-BA was prepared in Gamaleya's Institute as a derivative of the *B. abortus* 19 strain and used for immunization of over forty million people in the Former Soviet Union (FSU), leading to the substantial reduction of human brucellosis [3–6]. Live attenuated *B. abortus* 19 strain had been used in the FSU since the early 1950s as an effective vaccine to prevent cattle brucellosis [3,4]. But due to interference with conventional serological assays, in 1974 it was replaced by live *B. abortus* 82 vaccine strain, which was officially approved in 1988 for use in veterinary practice in Russia and other countries of the FSU. Unfortunately, this vaccine possesses a high level of residual virulence and can cause abortion in pregnant cattle [7].

Search for improved vaccine strains has led to the *B. abortus* 75/79-AV vaccine strain, which was isolated in 1979 by I.P. Nikiforov from a cow and then successively passaged 5 times through pregnant guinea pigs and heifers without causing abortion thus providing promising data for vaccination of cattle [8]. At present, in the Russian Federation about 2.2 million cows and heifers are immunized per year with *B. abortus* 82 and 75/79-AV vaccine strains (Table 1).

Currently, no data is available on molecular-genetic characteristics of the vaccines and their differentiation from other vaccine, reference and field *Brucella* strains based on the conventional, unstable, time-consuming phenotypic characteristics [13], which poses a problem in veterinary practice. Therefore, there is a need for the use of more efficient DNA-based methods for identifying more stable markers.

Variable-number tandem repeat (VNTR) sequences are used as genetic markers for genotyping of many bacterial species, especially for genetically homogenous pathogens such as *Bacillus anthracis*, *Yersinia pestis*, *Mycobacterium tuberculosis* and *Brucella* species [14–20]. Multiple-locus variable-number tandem repeat analysis (MLVA) relies on PCR amplification using primers specific for the flanking regions of the VNTRs and on determination of the

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amplicon sizes, which reflect the numbers of the amplified VNTR copies. Their use provides typing results in a portable numerical format, adequate for successfully typing *Brucella* strains [21–25]. The MLVA typing system provides *Brucella* species differentiation and much greater discrimination power at the strain level among *Brucella* isolates compared to other DNA-based typing techniques [20–25].

In this study, we evaluated the MLVA typing technique using VNTR genetic markers in single tube Multiplex PCR format for differentiation of *B. abortus* 82 and 75/79-AV vaccine strains from the other *Brucella* strains. In addition, genetic stability *in vitro* of the markers used in the assay was confirmed, which is very important for evaluation of MLVA typing.

## 2. Materials and methods

### 2.1. B. abortus vaccine strains

The collection of *Brucella* strains from Gamaleya's Brucellosis laboratory used in the studies was divided in three groups: vaccines, references and field strains. The Russian *Brucella* vaccine, reference and field strains are listed in Tables 1 and 2.

### 2.2. Brucella MLVA-12 genotyping assay

*Brucella* DNA was obtained by silica-based purification method [26]. MLVA genotyping assay was performed as recently described by Le Fleche et al. [22]. PCR amplification was performed in a total volume of 25 μL containing 5–10 ng of DNA, PCR Buffer containing 67 mM Tris–HCl pH 8.6, 2.5 mM $MgCl_2$, 16.6 mM $(NH4)_2SO_4$, 1 U of Taq DNA polymerase, 200 μM of each dNTPs and 6 pM of each direct and reverse flanking primers for 12 VNTR loci.

Twelve VNTR loci containing tandem repeats (from 134 bp to 8 bp) were chosen: BRU211, BRU1322, BRU73, BRU1543, BRU1250, BRU588, BRU548, BRU339 proposed by Le Fleche et al. [22] and VNTR-24, VNTR-26, VNTR-5A, VNTR-12B described by Whatmore et al. [23]. Primer sequences and genomic locations for the 12 VNTR's in the *Brucella* MLVA were described [22,23].

Amplifications were performed in thermocycler MC-2 ("DNA-Technology", Russia). An initial denaturing step at 94 °C for 5 min was followed by 30 cycles of denaturation at 94 °C for 30 s, primer annealing at 60 °C for 30 s and elongation at 72 °C for 60 s. The final extension step was performed at 72 °C for 5 min. Three microliters of the amplification products were loaded on a 4% standard agarose gel for analyzing tandem repeats and run under a voltage of 8 V/cm for 110 min. Gels were stained with ethidium bromide (0.5 μg/mL), visualized under UV light and photographed. A 100-bp ladder O'Gene Ruler ("FERMENTAS", Lithuania) were used as molecular size markers depending on the tandem repeats unit length. Amplicon size estimates were converted to a number of repeat units using Bionumerics software.

## 3. Results

### 3.1. PCR screening for VNTR markers for B. abortus 82 and 75/79-AV vaccine strains

VNTRs are essentially DNA segments, and though many are polymorphic, others do not show any variation. For initial PCR screening, 12 VNTRs were included in the MLVA and the reference strains of *B. melitensis* 16M, *B. suis* 1330, and *B. abortus* 544 with known amplicon sizes were used for discrimination from the vaccine strains.

Results showed that identical amplicon sizes for *B. abortus* 82 and 75/79-AV vaccine strains were presented in all but one of the 12 loci (VNTR-12B). Genetic polymorphism was detected at 7 of the 12 loci across the 2 vaccines and 3 reference strains used for screening. Deletion polymorphism of the vaccine strains in comparison to the reference strains was detected at two loci, BRU211 and VNTR-24, containing large sequence repeats 63 bp and 40 bp. On the other hand insertion polymorphism of the vaccine strains was observed at five loci with shot repeats 8 bp: BRU1543, BRU1250, BRU588, VNTR-5A and VNTR-12B.

**Table 1**
Characterization of Russian *B. abortus* vaccine strains used in this study.

| Russian vaccine strains biovar 1 | Phenotype, properties and *in vivo* virulence | Cattle vaccination per year | Authors and references |
|---|---|---|---|
| 1. *B. abortus* 82 | SR-form, reduced agglutinogenic properties, immunological and anti-epizootic effectiveness, abortions observed, low virulence | About 2 million cattle in 31 regions of Russia | Prof. K.M. Salmakov All-Russian Research Veterinary Institute, Kazan, Russia [7,10] |
| 2. *B. abortus* 75/79-AV | RS-form, immunological and anti-epizootic effectiveness, no abortions and virulence observed | About 200–250 thousand cattle in Altay and Astrahan regions | I.P. Nikiforov, Prof. K.V. Shumilov, The Altay State Agrarian University, Barnaul, Russia, FGU "VGNKI", Moscow, Russia [8,9] |
| 3. *B. abortus* KV 17/100 | R-form, killed vaccine with adjuvant | Less than 1 thousand cattle in the Saratov region | Prof. K.V. Shumilov, FGU "VGNKI", Moscow, Russia [9] |
| 4. *B. abortus* 19 | S-form, agglutinogenic, the standard USDA vaccine strain | About 20 thousand cattle | Dr. John Buck and Buck Cotton, USA [1,4] |
| 5. *B. abortus* 104M | S-form, agglutinogenic, virulent | Less than 1 thousand cattle<br>Human vaccination per year | X.S. Kotlayrova, P.A. Vershilova, Gamaleya Research Institute for Epidemiology and Microbiology, former USSR [2,4] |
| 6. *B. abortus* 19-BA | S-form, a derivative of *B. abortus* 19, widely used in former USSR for human vaccination, low virulence | About 2–5 thousand persons per year | Prof. P.A. Vershilova, Gamaleya Institute, Moscow, former USSR [3–6] |