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Genetic relatedness of *Brucella suis* biovar 2 isolates from hares, wild boars and domestic pigs

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ABSTRACT

Porcine brucellosis generally manifests as disorders in reproductive organs potentially leading to serious losses in the swine industry. *Brucella suis* biovar 2 is endemic in European wild boar (*Sus scrofa*) and hare (*Lepus europeus*, *Lepus capensis*) populations, thus these species may play a significant role in disease spread and serve as potential sources of infection for domestic pigs. The aim of this study was an epidemiologic analysis of porcine brucellosis in Hungary and a comparative analysis of *B. suis* bv. 2 strains from Europe using multiple-locus variable-number tandem repeat analysis (MLVA). MLVA-16 and its MLVA-11 subset were used to determine the genotypes of 68 *B. suis* bv. 2 isolates from Hungary and results were then compared to European MLVA genotypes. The analyses indicated relatively high genetic diversity of *B. suis* bv. 2 in Hungary. Strains isolated from hares and wild boars from Hungary showed substantial genetic divergence, suggesting separate lineages in each host and no instances of cross species infections. The closest relatives of strains from Hungarian wild boars and domestic pigs were mainly in the isolates from German and Croatian boars and pigs. The assessment of the European MLVA genotypes of wild boar isolates generally showed clustering based on geographic origin. The hare strains were relatively closely related to one another and did not cluster based on geographic origin. The limited relationships between geographic origin and genotype in isolates from hares might be the result of cross-border live animal translocation. The results could also suggest that certain *B. suis* strains are more adapted to hares. Across Europe, isolates from domestic pigs were closely related to isolates originating from both hares and wild boars, supporting the idea that wild animals are a source of brucellosis in domestic pigs.

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1. Introduction

Brucella suis is the causative agent of porcine brucellosis, an important infectious disease in the swine industry. The species has traditionally been divided into five biovars, out of which biovars 1, 2 and 3 are responsible for brucellosis in domestic pigs. These biovars differ from each other in their geographical distribution, host range and

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pathology (OIE, 2013). In Europe, the most common biovar is *B. suis* bv. 2, but infections by strains from biovars 1 and 3 have also been reported (Gennero et al., 2004; Godfroid et al., 2005; Cvetnić et al., 2005, 2009). A primary difference in the host spectrum of these biovars is that biovar 2 rarely causes infection in humans (Teyssou et al., 1989; Paton et al., 2001; Lagier et al., 2005; Garin-Bastuji et al., 2006), while biovars 1 and 3 represent significant threats to human health (Godfroid et al., 2005; OIE, 2013). Brucellosis in humans manifests as nonspecific influenza-like disease, but infections can develop into abscesses in bones and organs (especially in spleen, liver, heart-valves and brain), causing osteoarticular complications, or in rare cases death if left untreated (Godfroid et al., 2005; WHO, 2006). In swine, the disease affects mainly the reproductive system, resulting in infertility in both genders and abortion in females. Other symptoms include arthritis and spondylitis (Godfroid et al., 2005). In wildlife the reservoirs of *B. suis* bv. 2 are wild boars (*Sus scrofa*) and hare species (*Lepus europeus*, *Lepus capensis*) (Godfroid et al., 2005; Gyuranecz et al., 2011). Brucellosis manifests in wild boars and hares as inflammation and abscesses in the reproductive organs, but it may also cause miliary abscesses in the lymph nodes, liver, spleen, kidneys, urinary bladder, joints and brain (Godfroid et al., 2005; Gyuranecz et al., 2011). The consumption of aborted fetuses and fetal membranes or contaminated food is one route of transmission, but the bacteria may also spread through venereal, conjunctival–mucosal and transplacental routes (EFSA, 2009). Wild boars and hares serve as potential sources of infection for outdoor reared domestic pigs (Godfroid and Käsbohrer, 2002; Köppel et al., 2007; Muñoz et al., 2010; Wu et al., 2011; Godfroid, 2012; Grégoire et al., 2012; Wu et al., 2012; Leiser et al., 2013), and could spread the disease with cross border and long distance movements of infected animals possibly due to migration or live animal translocations (Godfroid and Käsbohrer, 2002; Godfroid et al., 2005; Godfroid, 2012; Grégoire et al., 2012).

The identification of the agent on the level of biovars is based on conventional microbiologic and molecular genetic techniques (e.g. biochemical tests, conventional and real-time PCR) (Leiser et al., 2013; Scholz and Vergnaud, 2013). Multiple-locus variable-number of tandem repeats analysis (MLVA) is a current genotyping method that enables the differentiation of closely related isolates of the same biovar, despite their genetic homogeneity; thus it is suitable for epidemiological investigations and to some extent for phylogenetic analysis as well (Le Flèche et al., 2006; Al Dahouk et al., 2007; Kılıç et al., 2011; Leiser et al., 2013; Scholz and Vergnaud, 2013). Also, MLVA is highly efficient for clustering strains and it is useful in determining if there is a geographic pattern of genotypes (Scholz and Vergnaud, 2013; Kılıç et al., 2011; Ferreira et al., 2012).

The *Brucella* MLVA-16 assay, originally developed by Le Flèche et al. (2006) and modified by Al Dahouk et al. (2007), consists of three groups of genetic markers, panel 1 (Bruce06, Bruce08, Bruce11, Bruce12, Bruce42, Bruce43, Bruce45, Bruce55), panel 2A (Bruce18, Bruce19, Bruce21) and panel 2B (Bruce04, Bruce07, Bruce09, Bruce16, Bruce30). Panel 1 (i.e. MLVA-8 subset) comprises 8 minisatellite markers (with repeat unit sizes between 12 and 134 bp) most appropriate for species-level identification, and panel 2 includes microsatellite markers with higher discriminatory power. Three microsatellite markers with repeat unit sizes between 3 and 8 bp belong to panel 2A, and the microsatellite markers with the highest discriminatory power are grouped in panel 2B, with repeat unit sizes of 8 bp. The subsets MLVA-8 and MLVA-11 (panels 1 and 2A) are suitable for the comparison of strains from different regions (Kılıç et al., 2011).

The aim of the study was to examine with MLVA *B. suis* bv. 2 strains from Hungary and to place their genotypes in context with MLVA data from other samples from Europe (Le Flèche et al., 2006; García-Yoldi et al., 2007; Abril et al., 2011). Ultimately, we hoped to gain an understanding of the genetic relatedness of this pathogen from Hungary to other isolates from throughout the continent to determine the epidemiological and geographic relationships of isolates from different hosts.

2. Materials and methods

2.1. *B. suis* biovar 2 strains from Hungary

Overall 68 *B. suis* bv. 2 strains were examined by MLVA from Hungary, including 55 isolates from wild boars, 8 isolates from domestic pigs (from 6 counties) and 5 isolates from European brown hares (*L. europeus*, from 3 counties). Of the isolates from wild boars, 45 originated from the same region (Somogy County, southwestern Hungary), collected during a monitoring program from 2011 to 2013, after an outbreak in domestic pigs in the area. The remainder of the wild boar samples ($n = 10$) originated from four other counties from central and northeastern parts of the country (Fig. 1; Table S1). The *B. suis* bv. 2 strains were identified by the "Bruce-ladder" and "Suis-ladder" multiplex PCR assays (López-Goñi et al., 2008, 2011).

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetmic.2014.05.031.

2.2. MLVA-16 assay and data analysis

Singleplex VNTR amplifications were performed as described previously (Le Flèche et al., 2006; Al Dahouk et al., 2007) on all 68 *B. suis* bv. 2 strains isolated in Hungary. The reference strain Thomsen (ATCC 23445) was used as a standard during the amplifications to confirm appropriate fragment size estimations. The number of repeats were estimated from the amplicon sizes; measured with electrophoresis or fragment analysis. Amplicons were separated in 2% or 3% agarose gels (depending on their expected sizes) and the band sizes were estimated by the Kodak MI SE program (Kodak Inc., Rochester, NY). Fragment analysis was performed on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA) in cases when VNTRs had small differences in their allele sizes or multiple alleles were detected (Bruce04, Bruce09 and Bruce19). Amplicons for the fragment analyses originated from multiplex PCRs performed under the same

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