Veterinary Microbiology 180 (2015) 146–150

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Short communication

Characterisation of *Brucella suis* isolates from Southeast Europe by multi-locus variable-number tandem repeat analysis

Sanja Duvnjak*, Ivana Račić, Silvio Špičić, Maja Zdelar-Tuk, Irena Reil, Željko Cvetnić

Croatian Veterinary Institute, Department for Bacteriology and Parasitology, Laboratory for Bacterial Zoonoses and Molecular Diagnosis of Bacterial Diseases, Savska cesta 143, 10000 Zagreb, Croatia

ARTICLE INFO

Article history:
Received 12 November 2014
Received in revised form 23 July 2015
Accepted 14 August 2015

Keywords:
Brucella suis
MLVA
Genotyping
Croatia
SE Europe
Infectious disease

ABSTRACT

Porcine brucellosis is a common bacterial zoonosis which can cause significant financial losses. Its diverse and often complicated factors have hampered efforts to control disease spread.

The aim of the study was to assess the epidemiological situation of porcine brucellosis primarily in Croatia and its relationship to genotypes present in other, mostly European countries. One hundred and seven *Brucella suis* strains isolated from swine, hares, cattle, humans, wild hares, a wild boar and a mare originating mainly from Croatia (112), but also a few from Slovenia, Bosnia and Herzegovina, Serbia and Macedonia (15) were tested using classical microbiological testing, Bruce-ladder, RFLP, Multiplex-suis and genotyped using multi-locus variable-number tandem repeat analysis (MLVA).

We determined 43 *Brucella suis* genotypes. Strains were grouped according to phylogenetic and geographic relationships, revealing both regional specificity and uniqueness and suggesting possible sources and modes of spread among animals. Our study also confirmed problems with Bruce19 locus that may hinder comparisons of new types with those in the international database.

Forty-one novel genotypes were identified and deposited into the international database. Our study supports the idea of wild animals as a source of disease in domestic animals and also gives evidence to hypothesis of cross-border animal trafficking between former Yugoslavian countries. It also highlights the need to expand such research across more of southeast Europe, especially to countries with poorer social and economical situation in order to prevent a realistic outbreak and for better understanding of the biology of this pathogen.

1. Introduction

Brucellosis is a common, global bacterial zoonosis which causes significant economic losses and is a possible public health issue (Pappas et al., 2006).

Brucella suis causes the chronic disease known as porcine brucellosis, which manifests as infertility and miscarriage in sows, high piglet mortality and orchitis in boars. *B. suis* biovars 1, 2 and 3 appear around the world wherever pigs are bred, and biovars 1 and 3 are the most abundant globally (OIE Manual, Porcine brucellosis, 2015). In Europe, biovar 2 is the most frequent cause of infections in domestic pigs and wild boars, and wild boars are considered the main source of infection (Godfroid et al., 1994; Leuenberger et al., 2007).

In Croatia, *B. suis* is present in both domestic and wild populations in virtually all counties where pigs are bred. In fact, Croatia was the first European country where *B. suis* biovar 3 infection was detected in horses, swine and wild boars. The animals most frequently infected are swine in extensive production, since the high density of animals and proximity to wild boar facilitate spread of the disease (Cvetnic et al., 2003, 2005; Spicic et al., 2010). The prevalence of *B. suis* bv. 2 and established link between wild life and outdoor breeding was also reported in other European countries like Hungary, Poland, and others (Szulowski et al., 2013; Kreizinger et al., 2014)

In recent years, multi-locus variable-number tandem repeat analysis (MLVA) has proven effective at local strain differentiation, particularly when used with the MLVA-16 combination of minisatellites (Panel 1) and macrosatellites (Panel 2).

The aim of the present study was to assess the epidemiological and geographical relationships of strains isolated from wild and domesticated animals over the past 50 years in Croatia, Bosnia and Herzegovina, Serbia and Macedonia, and their relationship to genotypes present primarily in Europe. The results of the study

* Corresponding author. Fax: +385 1 6190 841.
E-mail addresses: marjanovic@veinst.hr (S. Duvnjak), racicivana@gmail.com (I. Račić), spicic@veinst.hr (S. Špičić), zdelar-tuk@veinst.hr (M. Zdelar-Tuk), reil@veinst.hr (I. Reil), cvetnic@veinst.hr (Ž. Cvetnić).

http://dx.doi.org/10.1016/j.vetmic.2015.08.013
0378-1135/

may improve our understanding of brucellosis and our ability to monitor and control its spread, perhaps increasing the possibility of eradication.

2. Materials and methods

2.1. B. suis strains tested

A total of 127 *Brucella suis* strains were examined in this investigation. Most of Croatian strains (112 strains: 87 swine, 14 wild hare, 5 hare, 3 human, 1 wild boar, 1 mare and 1 bovine) were gathered during routine testing after slaughter and during 4 major outbreaks in 6 counties (17 swine isolates from Sisak-Moslavina County in 2009; 8 swine isolates from Vukovar-Srijem County in 2010; 17 swine isolates from Zagreb and Bjelovar-Bilogora County in 2011 including two isolates from the same year: a swine isolate from Sisak-Moslavina County and a wild boar isolate from Vukovar-Srijem County; 21 swine isolates from 2012 in Bjelovar-Bilogora County). Twenty-eight isolates were isolates from Croatia but with unknown date: 14 from wild hares, 4 hare, 9 swine and 1 human. These isolates originated from 5 counties (Osijek-Baranja, Sisak-Moslavina, Zagreb City, Zagreb, and Varaždin Counties). The isolates from Croatia dating before and early 2000s are 2 human, a mare and a swine isolates from Bjelovar-Bilogora and Sisak-Moslavina Counties in 1991 and 1992 as well as old archive cattle and swine isolates. Isolates originating from other southeast European countries are: 3 isolates from Macedonia (2 swine and 1 human) from late 1980s and early 1990s.; three isolates from Serbia from 2006 originating from 1 cattle and 2 swine; and four strains from Slovenia originating from 2 hares and 2 of unknown origin. All isolates belong to different animals. In some cases, more isolates from one animal were available. However, they were considered to be of the same strain after biotyping and were not all genotyped. In this study we also tested 5 reference strains, originating from Slovenia, present in our archive representing 5 different *B. suis* biovars.

2.2. Methods used

Strains were confirmed as *Brucellae* using classical microbiological biotyping that included microscopic, cultural and biochemical testing (Corbel et al., 1983; Alton et al., 1988). Among molecular tests, Bruce-ladder was used as the reference method to determine *Brucella* species (Lopez-Goni et al., 2008); and restriction fragment length polymorphism (RFLP) (Cloeckaert et al., 1995; Vizcaíno et al. 1997) and multiplex-suis ("Suis-ladder") were used for determining *Brucella suis* biovars (Lopez-Goni et al., 2011). MLVA-16 genotyping was done on a total of 16 gene loci (Al Dahouk et al., 2005; Le Flèche et al., 2006). Loci were divided into three panels. *B. melitensis* 16 M was used as the reference strain for comparison and verification of test quality.

DNA was isolated using the commercially available QIAcube DNA Mini Kit and the QIAcube system (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Supernatant (2 or 5 μl) was used in DNA-based tests. The same PCR reaction mixture was used for all molecular tests: 20 μl reaction mixtures consisting of 10 μl HotStarTaq Master Mix (Qiagen, Hilden, Germany), 6 μL of water (RNase-free water, Qiagen, Hilden, Germany), 0.5 μM of each primer pair specific for the target locus (Invitrogen, Paisley, UK) and 2 μL of template DNA. The cycling regime differed from test to test but was done according to references. Amplifications were performed in a Fast Thermal Cycler 9800 (Applied Biosystems, USA). For RFLP enzyme restriction, 20 μl reaction mixture contained 5 μl of amplified DNA, 5U of restriction enzyme, 2 μl of associated buffer (Fermentas, Burlington, Canada) and 12.5 μl distilled water (DNase/RNase Free Distilled Water, GIBCO, Invitrogen, Paisley, UK). Digestion was done on 37 °C for 3 h. Restriction products were analysed using capillary electrophoresis on the QIAxcel system (QIAGEN, Hilden, Germany) with size markers in the bp ranges of 15–500, 50–1000 or 100–3000, depending on expected band sizes.

2.3. Data analysis

Band sizes from MLVA-16 results were translated into the number of individual repeats (Le Flèche et al., 2006). Results were presented in the form of 16-digit numerical codes based on version 3.6 *Brucella* allele assignment table (available at http://mlva.u-psud.fr). Polymorphism was calculated using the Hunter-Gaston Diversity Index (HGDI) (Hunter and Gaston, 1988; Le Flèche et al., 2006). The obtained codes were analysed using the categorical coefficient and Unweighted Pair Group Method with Arithmetic Mean (UPGMA) using BioNumerics software (version 7.5; Applied Maths, Belgium). Data was compared to results from different countries deposited in the Brucella2012 database (available at http://mlva.u-psud.fr).

3. Results

We analysed 127 *Brucella suis* isolates from various domestic and wild animals and humans from southeast Europe to assess the epidemiological and geographical relationships of strains isolated over the past 50 years in Croatia, Bosnia and Herzegovina, Serbia and Macedonia, and their relationship to genotypes present in Europe.

Biotyping identified 127 *B. suis* strains: 39 strains of biovar 1, 83 of biovar 2, 3 of biovar 3, and 1 each of biovars 4 and 5 (including 5 reference strain, one of each biovar)(Table 1).

Table 1
Results of biovar testing according to host and origin.

Biovar	Host	Origin
B. suis biovar 1	40 (31.5%) 18 hare (45%), 14 swine (35%), 4 human (10%), 1 cattle, mare, reference, unknown (10%)	Croatia, Macedonia, Slovenia
B. suis biovar 2	84 (66%) 78 swine (92.8%), 3 hare (3.6%), 1 cattle, reference, unknown (3.6%)	Croatia, Serbia, Slovenia
B. suis biovar 3	1 reference	Slovenia
B. suis biovar 4	1 reference	Slovenia
B. suis biovar 5	1 reference	Slovenia