Efficacy of next-generation sequencing in bacterial zoonoses diagnostics

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Abstract

Brucella, an extremely diverse but yet genetically highly homogenous genus of bacteria, has been a puzzle for scientists for many decades. These bacteria remain a prominent public health issue, particularly in the Balkan region. Correctly identifying and understanding the pathogen is a vital step in the epidemiology and epizootiology of any bacteria. Identification can be challenging, especially in the case of zoonotic species. This study aimed to implement fourth-generation sequencing in the typing of 11 *Brucella suis* strains kept in our archive and to compare this method to the classical and non-sequencing based molecular methods used to date. Classical biotyping is highly subjective and gave inconclusive results for 3 strains. Molecular methods used were multiplex PCR and RFLP methods since no one method can identify both species and biovar which is vital in the case of *Brucella suis* infections. Species and biovars of all the strains were successfully confirmed and in concordance with biotyping results. Oxford Nanopore long-read sequencing was used on a MinION device for next-generation sequencing (NGS). Various algorithms were implemented for genome assembly and BioNumerics 8.0 software was used for MLST identification and analysis. MLST 21 was used for biovar identification and epidemiological comparison of tested strains. The assembled genomes were 3,2 Mb in size and assembled into two chromosomes. MLST 21 analysis placed our strains into species and biovar clusters in concordance with other molecular tests used. To the extent of our knowledge, this is the first documented use of long-read sequencing in *Brucella suis* identification in this region. We conclude that bacteriological biotyping is outdated and host-specific identification in this genus is incorrect and that molecular characterisation is always the safer, faster and more suitable option. MinION sequencing proved to be a strong, accessible solution for species determination. Future study is required to determine how detailed genome information it can give, considering the error rate.

Key words: *Brucella suis*; *brucellosis; swine; horses; Nanopore MinION; NGS*

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Introduction

Brucellae belong to the α-Proteobacteria, a class of bacteria that are extremely diverse and adaptable to new habitat conditions. *Brucella* sp. is a genus currently consisting of 13 species (Ledwaba et al., 2019), in contrast to the 6 species known in 2003 (Osterman and Moryion, 2003). *Brucellae* are known to infect wild and domestic animals such as wild boars, cows, sheep, goats, dogs (Godfroid, 2002), but have also recently been cultured from marine animals, common voles, red foxes, baboons, human breast implants and fish (Al Dahouk et al., 2007; Cvetnic et al., 2017; Scholz et al., 2008a, 2008b, 2010, 2016; Whatmore et al., 2014). *Brucella suis* was the first pathogen to be used as a bioweapon in the 1950s. This goes to show that this is a genus of highly adaptable bacteria that can realistically be expected anywhere. It is important to note that human neglect and misdiagnosis are two key factors that have facilitated these resourceful bacteria in remaining a globally persistent pathogen for well over a decade, causing significant economic losses and public health issues (Pappas et al., 2006).

Brucella suis causes the chronic disease known as porcine brucellosis, which manifests as infertility and miscarriage in sows, high mortality of piglets, and orchitis in boars. *B. suis* biovars 1, 2 and 3 appear around the world wherever pigs are bred, with biovars 1 and 3 the most abundant globally (OIE Manual, Porcine brucellosis, 2009).

Brucellosis is an endemic disease in Croatia, and various *Brucella* sp. have been confirmed in swine, wild boars, cows, sheep, goats and humans (Spicic et al., 2010). *B. suis* in Croatia is present in both domestic swine and wild boar populations in all counties where pigs are bred. Croatia was the first European country where *B. suis* biovar 3 infection was detected in horses, swine and wild boars. Swine in extensive production are most affected, given the high density of animals and proximity of wild boar that facilitate the spread of the disease (Cvetnic et al., 2003, 2005; Spicic et al., 2010). The prevalence of *B. suis* bv. 2 and the established link between wildlife and outdoor breeding has been reported in other European countries, such as Hungary, Poland, and others (Szulowski et al., 2013; Kreizinger et al., 2014). The most prevalent brucellosis in Croatia is swine brucellosis, though Croatia is one of few countries having a favourable brucellosis status. However, financially and professionally well-supported control and eradication programmes should be implemented to prevent this disease from becoming an even more serious problem than in the past. Unfortunately, this is not the case (Taleski et al., 2002; Pappas, 2010).

The omnipresence of brucellosis can only be combatted through accurate and detailed pathogen identification. Classical bacteriological methods are the gold standard for identification and classification. Since these methods are time-consuming, highly subjective and dangerous, molecular methods have been in use for over two decades. In the case of Brucellae, classical multiplex PCR and genotyping technics like MLVA and MLST are the most frequently used (Spicic et al., 2010; Duvnjak et al., 2015).

Brucella genomes are highly conserved and show a high degree of similarity with less than 6% nucleotide sequence variation, which is attributed to the recent origin of the genus (Bergey's Manual, 2018). They have two chromosomes and no plasmids. They have ribosomal gene clusters carrying around 3200 protein-coding genes according to Sanchez-Jimenez et al. (2015). Also, Meyer and Shaw (Bergey's Manual, 2018) noted that *Brucella suis* bv. 3 is unique, with a single chromosome 3.2 Mbp long.

The global leader in next-generation sequencing technology is the Illumina platform (Illumina, Inc., San Diego, CA, USA), which uses synthesis-based sequencing (SBS) (www.illumina.com). The quality of its reads is still unmatching, library preparation takes hours and PCR is necessary, and the sequencing is not possible in the field. Further, the reads are usually small (max 300 bp) and the first results are expected after approximately 50-60 hours.

MinION™ (Oxford Nanopore Technologies (ONT), Oxford, UK) uses different technology based on *E. coli* nanopores. As the DNA molecule passes through the membrane it causes a change in membrane current, which is recorded and translated into base pairs. This enables the sequencing of very long individual DNA molecules and reading in real-time. The device itself is pocket-sized and library preparation can take just 10 minutes, making it a powerful tool for on-site real-time sequencing. The error rate is higher than for Illumina, though it enables denovo assembly of whole and complex genomes because of the long stretches it produces (Goodwin et al., 2016). Its low price and constant improvements enable this device to become a better research tool that is being used to build and explore model (Tyson et al., 2017) and non-model organisms (Quick et al., 2017).

This study was aimed at introducing fourth-generation sequencing into our lab and for typing 11 *Brucella suis* strains to the biovar level. MinION is small, fast, simple and cost-effective since barcoding is possible. It takes less hands-on time during preparation than typing analyses currently in use if one has good software solutions. It also produces data that can be used for complete genome sequencing since it sequences long DNA molecules. However, the error rate is higher than Illumina sequencing. Therefore, the objective of this study was to determine its applicability for species and biovar typing of *Brucellae*.

Materials and methods

B. suis strains

The tested strains are listed in Table 1. All strains were also tested biochemically and using classical PCR-based molecular methods.

Table 1. Tested strains according to origin and year of isolation

SAMPLE (CVI no.)	ORIGIN	YEAR
50	swine	2000
58	horse	2003
59	horse	2003
60	wild boar	2003
71	referent *B. suis* bv. 3	2004
72	referent *B. suis* bv. 4	2004
73	referent *B. suis* bv. 5	2004
76	swine	2004
105	swine	2009
196	swine	2015
213	referent *B. suis* bv. 2	2017

Methods

All strains were microbiological, cultural and biochemically tested according to Corbel et al. (1983) and Alton et al. (1988). This involved colony morphology (size, convexity, transparency, roughness), CO_2 growth requisite, susceptibility to thionine and fuchsine, production of H_2S, agglutination with specific monosera, ability to hydrolyse urea and reaction with Wb, Iz, Tb and R/C phages.

Bruce-ladder was used as the reference method to determine *Brucella* species (Lopez-Goni et al., 2008, 2011); and restriction fragment length

polymorphism (RFLP) (Cloeckaert et al., 1995; Vizcaino et al., 1997) and multiplex-suis (Suis-ladder) were used to determine *Brucella suis* biovars (Lopez-Goni et al., 2011). MLVA-16 genotyping was performed on a total of 16 gene loci (Al Dahouk et al., 2005; Le Flèche et al., 2006). *B. melitensis* 16M was used as the reference strain for comparison and verification of test quality.

Strains were grown from cryobeads on *Brucella* agar plates for 72 hours at 37°C. Bacterial cells were collected in nuclease-free water and resuspensions made with an absorbance of approximately 0.55 at 600 nm (Densimat, Biomerieux).

For PCR-based molecular methods, DNA was isolated using the QIAcube DNA Mini Kit and the QIAcube system (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

The 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 water (RNase-free water, Qiagen, Hilden, Germany), 0.5 µM each primer pair specific for the target locus (Macrogen, Netherlands) and 2 µL template DNA. The cycling regime differed from test to test but was done according to references. Amplifications were performed on the ProFlex thermocycler (Applied Biosystems, USA). For RFLP enzyme restriction, 20 µL reaction mixture contained 5 µL amplified DNA, 5U restriction enzyme, 2 µL associated buffer (Fermentas, Burlington, Canada) and 12.5 µL distilled water (DNase/RNase Free Distilled Water, GIBCO, Invitrogen, Paisley, UK). Digestion was done at 37ºC for 3 hours. Restriction products were analysed using capillary electrophoresis on the QIAxcel system (QIAGEN, Hilden, Germany) using High-Resolution DNA cartridge with size markers 100-2500 bp.

MLST and epidemiological analyses were performed using the BioNumerics software scheme (version 8.0; BioMerieux, Applied Maths, Belgium).

Genomic DNA isolation for sequencing started with centrifugation of the bacterial cell suspension at 5000 g for 10 minutes. The bacterial cell precipitate was treated according to the manufacturer's instructions using Genomic-tip 500/G (Qiagen). DNA quality and concentration were defined on DS-11 Spectrophotometer (DeNovix), Qubit using Qubit dsDNA BR Assay Kit (Invitrogen) and Tapestation 2200 using Genomic DNA Screen Tape and Reagents (Agilent). All samples were tested in triplicate on each device.

We prepared the sequencing library using the Rapid Barcoding Kit (ONT) with modifications to the RBK_9054_v2_revC_23Jan2018 protocol. We prepared a suspension of 9 µL DNA (≈ 1 µg genomic DNA) and 3 µL fragmentation mix and extended the incubation with AMPure XP beads to 10 minutes. Beads were washed with 80% ethanol and incubated for 30 seconds before removing the ethanol. After the second ethanol wash, beads were air-dried for 1 minute and resuspended in 12 µL Tris-HCl pH 7.85 without NaCl and incubated for 10 minutes. The remainder of the protocol was conducted on 10 µL obtained DNA suspension. The samples were barcoded and sequenced.

Sequencing was performed on the MinION device using the FLO-MIN106 R9.4 flowcell. The sequencing run lasted 46 hours and produced 3.29 million reads with approximately 12.98 billion bases (for 12 samples).

In-silico analysis

Base-calling was done with Guppy version 3.6.0 using "dna_r9.4.1_450bps_hac" as the configuration file and "SQK-RBK004" as the barcoding kit.

glk	dnaK	gyrB	trpE	cobQ	int_hyp	omp25	prpE	caiA	csdB	soxA	leuA	mviH	fumC	fbaA	ddlA	putA	mutL	acnA	Key
4	29	4	3	5	1	2	5	2	4	2	2	1	1	4	2	3	2	1	CVI_60
7	35	3	3	5	3	2	5	2	8	3	2	1	24	4	1	3	2	6	CVI_50
4	1	3	3	5	1	2	5	2	4	2	2	27	1	4	2	3	2	1	CVI_58
4	1	4	3	5	1	2	5	2	4	2	2	1	1	4	2		2	1	CVI_59
4	1	5	3	5	4	2	5	2	7	2	2	1	1	4	2	4	2	2	CVI_71
4	1	5	3	5	4	2	5	2	7	2	2	27	1	4	2	5	2	2	CVI_72
4	6	1	3	5	1	2	2	2	6	2	2	1	2	4	3	3	2	3	CVI_73
4	1	3	3	5		2	5	2	4	2	2		4	4	1	3	2	1	CVI_76
4	1	3	3	5	1	2	5	2	4	2	2	1	1	4	1	3	2	1	CVI_105
7	35	3	3	5	3	2	5	2	8	3	2	1	24	4	1	3	2	6	CVI_196
7	1	3	3	5	3	2	5	2	8	3	2	4	3	4	1	3	2	3	CVI_213

Figure 1. MLST 21 results

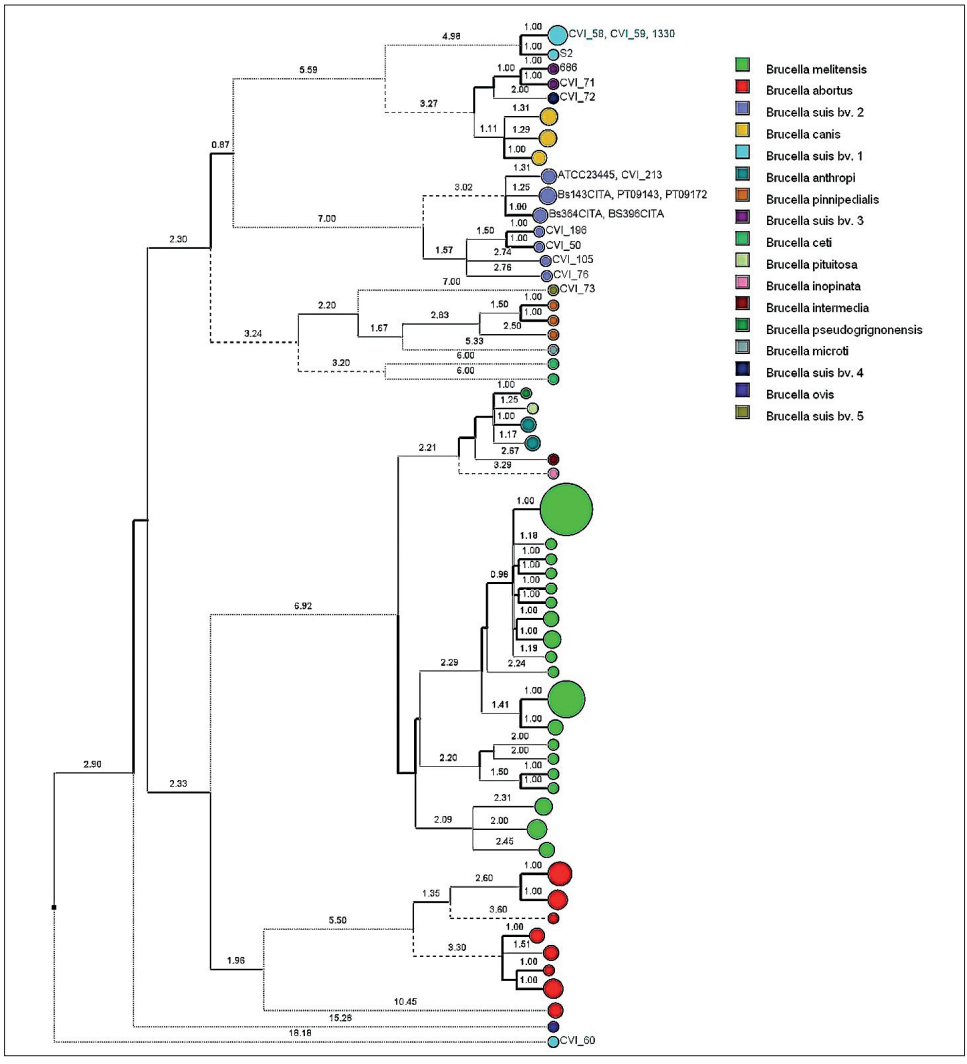

Figure 2. Dendrogram (advanced clustering method, UPGMA network creation according to character data with permutation resampling 200) showing the tested *Brucella suis* strains compared to strains present in the PubMLST *Brucella* database (colour coding by species and biovar)

For assembly, we used Raven version 1.1.5 (Vaser and Sikic, 2020), Wtdbg2 version 2.5 (Ruan and Li, 2019) and Flye version 2.7.1 (Kolmogorov et al., 2019). Ten of the eleven barcodes were successfully assembled with Raven. A complete assembly for barcode 105 was possible only with Wtdbg2. Due to the lowest coverage, the assembly of barcode 7 is a combination of the Raven and Flye assemblies, as each of them completely assembled only one contig. All barcodes were later polished with Medaka version 1.0.1 (Medaka) using the model "r941_min_high_g360" and rotated with Circulator version 1.5.5 (Hunt et al., 2015). The evaluation was performed using BUSCO version 4.0.6 (Seppey et al., 2019), Quast version 5.0.2 (Gurevich et al., 2013) and Mauve version 2.4.0 (Darling et al., 2004).

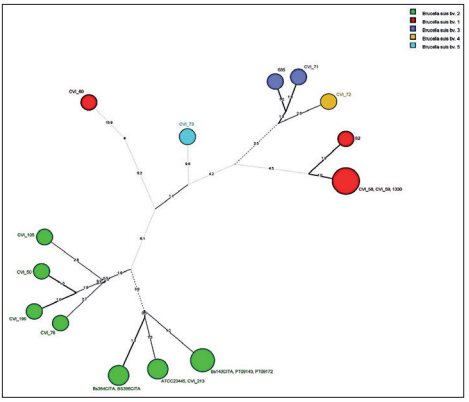

Figure 3. Planar network (advanced clustering method, UPGMA network creation according to character data with permutation resampling 200) showing the tested *Brucella suis* strains compared to *Brucella suis* strains present in the PubMLST *Brucella* database (colour coding by biovar is presented on the right of the tree; strain names are presented next to the nodes; the number of locus variants is presented with the style of the line connecting samples – the thicker the line the fewer the number of locus variants)

Results

Biotyping identified the tested strains as follows: samples CVI_58, 59 and 60 inconclusive as *Brucella suis* biovar 1 or 3, samples CVI_50, 76, 105, 196 and 213 as biovar 2, sample CVI_71 as biovar 3; sample CVI_72 as biovar 4 and sample CVI_73 as biovar 5.

Bruce-ladder identified all strains as *Brucella suis*. Since Bruce-ladder cannot be used to assign biovars, we used multiplex-suis and RFLP methods to identify the biovar. Both methods identified the strains in accordance with biotyping, except for strains 58, 59 and 60 that were identified solely as *B. suis* biovar 1.

The MLST 21 results are presented in Figure 1 as calculated via BioNumerics using the PubMLST *Brucella* database.

The epidemiological analysis of the samples according to the MLST 21 results compared with international *Brucella suis* samples in the PubMLST *Brucella* database is presented in Figures 2, 3 and 4 as a dendrogram, planar network and minimum spanning tree.

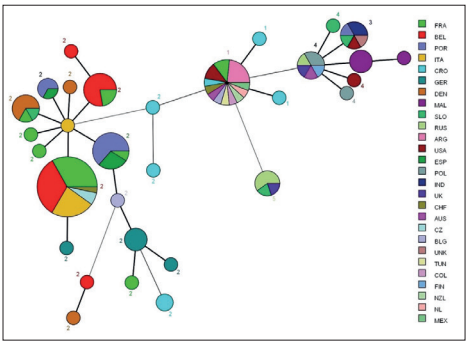

Figure 4. Minimum spanning tree (MST) showing the tested *Brucella suis* strains in relation to strains in the PubMLST *Brucella* database by country of origin and *Brucella suis* biovar (colour coding by country of origin is presented on the right of the tree; biovar characterisation is presented with numbers 1-5 next to the nodes; the number of locus variants is presented with the style of the line connecting samples – the thicker the line the fewer the number of locus variants)

DNA was isolated focusing on reads being as long as possible and DNA concentrations had to be just right to saturate enough pores but not to block

them. After isolation, we used about 600 ng for barcoding and library preparation. We used approximately 1 μg DNA for sequencing on the flowcell according to Tapestations calculations.

In silico analysis

In total, 1.69 million reads were successfully base-called across twelve barcodes. The statistics of each barcode are outlined in Table 2.

Bioinformation analyses resulted in complete, circular sequences of two chromosomes. The analysis revealed a BUSCO score of >96% and QUAST average accuracy >99.9%. Chromosome sizes differ among biovars. GC% was around 57.2%. Genomes were deposited in NCBI under accession numbers CP054945 - CP054964.

Discussion

The virulence and epidemiology of *Brucella* sp. depend most on the species involved, particularly the species biovar. The results from this study show that biotyping is not the most suitable method for biovar assignation.

Bacterial genome sequencing has opened endless questions and possibilities. The key point in the sequencing itself is the correct isolation of DNA. The kit we used was a good choice. According to Tapestation 2200 (Agilent), DNA was of good concentration and length, enabling good sequencing output and resulting in good in silico analysis.

The genome structure of the strains tested here is consistent with previous findings: all strains from all biovars tested have two chromosomes, approximately 3.2 Mbp in size. GC content was, as expected, around 57.2% per chromosome. These findings are similar to those observed by other authors (Jumas-Bilak et al., 1998).

MLST 21 identified new MLST 21 genotypes not yet present in the database (CVI_50, 60, 71, 72, 73, 105 and 196). Species identification, once compared with other isolates in the PubMLST database, gave the expected results. Strains clustered by biovar except for CVI_60 that clustered separately (Figures 2 and 3). Also, biovar 3 and 4 strains cluster closely together but can still be differentiated. Unlike MLVA typing (Duvnjak et al.,

Table 2. Read statistics of each barcode

Sample (CVI no.)	Number of bases	Number of reads	Min length	Average length	Max length
50	526585080	117194	139	4493.28	142186
58	524123652	145151	140	3610.89	158918
59	461034491	91674	148	5029.06	141499
60	500240474	164415	125	3042.55	133681
71	520117515	190734	169	2726.93	136163
72	521652873	173267	151	3010.69	179630
73	353554241	44608	129	7925.80	148159
76	307999956	126240	189	2439.80	43265
105	517836990	216689	101	2389.77	38739
196	526328311	120059	163	4383.91	112638
213	522296048	165906	150	3148.14	65018

2015), MLST 21 seems to be sensitive enough for biovar typing of *Brucella suis* strains. Separation of CVI_60 might be explained through Nanopore error rate though this assumption requires further investigation. Biovar 1 strains 58, 59 and 60 clustered with biovar 1 strains from the USA, Argentina, Mexico, Colombia, and Switzerland. Biovar 2 strains 50, 76, 105, 196 and 213 clustered with biovar 2 strains from Croatia and also with international strains, mostly from Europe. The referent strains of biovar 3, 4 and 5 (origin Slovenia) clustered with other strains of a certain biovar (Figure 4). It is also clear that the differences between biovars are minor, which might explain the reason why biovars are so difficult to identify, especially using non-molecular methods.

Except for referent strains 71, 72, 73 and 213, all strains were regionally specific and unique in the diagnostic sense (Figures 3 and 4). There may be a possibility that this is connected to the unique virulent capabilities that allow them to circulate and survive more easily in this region, though this is merely an assumption. This study was aimed at more detailed identification of strains using new, recently available techniques.

MinION sequencing still has a higher error rate than Illumina, though researchers are improving this platform weekly. Combining Illumina and MinION reads is a perfect solution, rendering long stretches of DNA with MinION that are polished by very accurate Illumina sequences. However, Guppy version 3.6.0 greatly improved base-calling and the results were substantially better and comparable to the available Illumina sequences (Amaradinghe et al., 2020).

We conclude that MinION sequencing is a must-have in bacterial species identification. It is faster, highly specific and produces a huge amount of data that can be used for chromosomal investigation, antibiotic susceptibility, strain specificities and more, especially in combination with short-read sequencing like Illumina. Since this is the first documented use of long-read sequencing in *Brucella suis* identification in this region, the results are very promising, though a future detailed study is required.

Repositories: The assembled genomes have been deposited in NCBI under accession numbers CP054945 – CP054964.

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Učinkovitost sljedeće generacije sekvenciranja u dijagnostici bakterijskih zoonoza

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Rod *Brucella* biološki je iznimno raznolik, ali genetski vrlo homogen rod bakterija te je već desetljećima znanstvenicima nepoznanica. Ove bakterije su veliki javnozdravstveni problem, a osobito na Balkanu. Pravilno prepoznavanje i razumijevanje patogena ključan je korak u epidemiologiji i epizootiologiji bilo koje bakterijske vrste, čija identifikacija može biti izazovna, osobito u slučaju zoonotskih vrsta. Cilj je ovog rada bio implementirati sekvenciranje četvrte generacije u tipizaciji 11 sojeva *Brucella suis* koje se čuvaju u našoj arhivi te ovu metodu usporediti s klasičnim i molekularnim metodama koje se trenutačno primjenjuju, a ne zasnivaju se na sekvenciranju. Klasično je biotipiziranje vrlo subjektivno i dalo je podvojene rezultate za 3 soja. Od molekularnih metoda koristili smo višestruku lančanu reakciju polimerazom (engl. Polymerase Chain Reaction, PCR) i polimorfizam duljine restrikcijskih fragmenata (engl. Restriction Fragment Lenght Polymorphism, RFLP) budući da niti jedna od metoda ne može zasebno identificirati i vrstu i biovar, a što je važno u slučaju *Brucella suis* infekcije. Vrsta i biovar svih sojeva uspješno su potvrđene i u skladu s rezultatima biotipizacije. Sekvenciranje sljedeće generacije (engl. Next Generation Sequencing, NGS) provodili smo na Oxford Nanopore MinION uređaju koji sekvencira duge lance DNK. Za sastavljanje genoma rabljeni su različiti algoritmi, a za identifikaciju i analizu rezultata MLST-a korišten je softver BioNumerics 8.0. MLST 21 je korišten za identifikaciju biovara i epidemiološku usporedbu ispitivanih sojeva. Genomi su bili veličine 3,2 Mb i sastavljeni u dva kromosoma. Analiza MLST 21 smjestila je naše sojeve u vrsne i biovarne skupine u skladu s drugim korištenim molekularnim testovima. Koliko je nama poznato, ovo je prva dokumentirana uporaba sekvenciranja dugih lanaca DNK u identifikaciji *Brucella suis* u jugoistočnoj Europi. Zaključujemo da je bakteriološka biotipizacija zastarjela i da je identifikacija biovara u ovom rodu, ovisno o domaćinu, netočna te da je molekularna karakterizacija uvijek sigurnija, brža i prikladnija opcija. MinION sekvenciranje pokazalo se kao vrlo pristupačno rješenje za određivanje vrste i biovara *Brucella suis*. Daljnja su istraživanja potrebna da bi se ustvrdilo koliko detaljne informacije o genomu može dati, imajući u vidu značajniji postotak pogreške prilikom sekvenciranja.

Ključne riječi: *Brucella suis, bruceloza, svinje, konji, Nanopore MinION, sekvenciranje sljedeće generacije*