Project SOP D-JRP17-WP4.SOP1 – Phenotyping scheme to differentiate emerging *Brucella* sp. from classical species

Workpackage 4

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PHENOTYPING SCHEME TO DIFFERENTIATE EMERGING BRUCELLA SP. FROM CLASSICAL SPECIES

Conventional phenotyping of new emerging Brucella was performed using available isolates from FLI. Conventional bacteriological methods were used, which are also applied for phenotyping of classical Brucella species. These methods are Gram-staining, CO_2 – requirement, hemolysis, oxidase reaction, motility, catalase reaction, H_2S production, urease-reaction, Crystal violet staining, growth on Basic fuchsin, growth on thionin, lysis by phages, agglutination in monospecific sera (A, M, R) and agglutination in a brucellosis positive control serum.

Table 1: Phenotyping results of new emerging and classical *Brucella* sp.

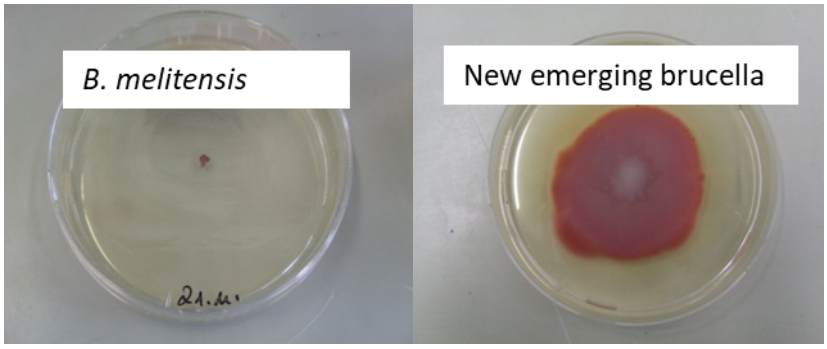
Lab number partner	Strain	Source	Gram staining	CO_2-requirement	Hemolysis	Motility at 37 °C	Oxidase	Catalase	H2S production	Urease	Disociation Trypaflavin	Thionin	Fuchsin	monospez.Serum A	monospez.Serum M	monospez.Serum R	Lysis F25 RTD	Lysis Wb RTD	Lysis Tb RTD	Lysis Tb 10^{4x}RTD	Agglutination with Brucellosis positive reference serum
09RB8471	Brucella like	frog	neg	no	neg	yes	pos	pos	pos	pos	s	pos	pos	neg	neg	neg	neg	neg	neg	neg	neg
13RB5064	Brucella like	frog	neg	no	neg	yes	pos	pos	pos	pos	r	pos	pos	neg	neg	neg	neg	neg	neg	neg	neg
14RB5420	Brucella like	frog	neg	no	neg	yes	pos	pos	pos	pos	r	pos	pos	neg	neg	neg	neg	neg	neg	neg	neg
18RB16866	Brucella like	frog	neg	no	neg	yes	pos	pos	pos	pos	r	pos	pos	neg	neg	neg	neg	neg	neg	neg	neg
20RB21708	Brucella like	frog	neg	no	neg	yes	pos	pos	neg	pos	r	pos	pos	neg	neg	neg	neg	neg	neg	neg	neg
20RB22556	Brucella like	frog	neg	no	neg	yes	pos	pos	neg	pos	r	pos	pos	neg	neg	neg	neg	neg	neg	neg	neg
20RB22606	Brucella like	frog	neg	no	neg	yes	pos	pos	neg	pos	r	pos	pos	neg	neg	neg	neg	neg	neg	neg	neg
10RB9205	Brucella like	frog	neg	no	neg	yes	pos	pos	pos	pos	s	pos	pos	neg	neg	neg	neg	neg	neg	neg	neg
10RB9206	Brucella like	frog	neg	no	neg	yes	pos	pos	pos	pos	s	pos	pos	neg	neg	neg	neg	neg	neg	neg	neg
10RB9207	Brucella like	frog	neg	no	neg	yes	pos	pos	pos	pos	s	pos	pos	neg	neg	neg	neg	neg	neg	neg	neg
10RB9208	Brucella like	frog	neg	no	neg	yes	pos	pos	pos	pos	s	pos	pos	neg	neg	neg	neg	neg	neg	neg	neg
10RB9209	Brucella like	frog	neg	no	neg	yes	pos	pos	pos	pos	s	pos	pos	neg	neg	neg	neg	neg	neg	neg	neg
10RB9210	Brucella like	frog	neg	no	neg	yes	pos	pos	pos	pos	s	pos	pos	neg	neg	neg	neg	neg	neg	neg	neg
10RB9211	Brucella like	frog	neg	no	neg	yes	pos	pos	pos	pos	s	pos	pos	neg	neg	neg	neg	neg	neg	neg	neg
10RB9212	Brucella like	frog	neg	no	neg	yes	pos	pos	pos	pos	s	pos	pos	neg	neg	neg	neg	neg	neg	neg	neg
10RB9213	Brucella like	frog	neg	no	neg	yes	pos	pos	pos	pos	s	pos	pos	neg	neg	neg	neg	neg	neg	neg	neg
10RB9214	Brucella like	frog	neg	no	neg	yes	pos	pos	pos	pos	s	pos	pos	neg	neg	neg	neg	neg	neg	neg	neg
10RB9215	Brucella like	frog	neg	no	neg	yes	pos	pos	pos	pos	s	pos	pos	neg	neg	neg	neg	neg	neg	neg	neg
10RB9216	Brucella like	frog	neg	no	neg	yes	pos	pos	pos	pos	s	pos	pos	neg	neg	neg	neg	neg	neg	neg	neg
10RB9217	Brucella like	frog	neg	no	neg	yes	pos	pos	pos	pos	s	pos	pos	neg	neg	neg	neg	neg	neg	neg	neg
09RB8910	Brucella like	frog	neg	no	neg	yes	pos	pos	pos	pos	r	pos	pos	neg	neg	neg	neg	neg	neg	neg	neg
09RB8915	Brucella like	frog	neg	no	neg	yes	pos	pos	neg	pos	r	pos	pos	neg	neg	neg	neg	neg	neg	neg	neg
06RB0264	B. microti	Common Vole	neg	no	neg	no	pos	pos	pos	pos	s	pos	pos	neg	pos	neg	pos	pos	pos	pos	pos
09RB4616	B. ceti	Dolphin	neg	no	neg	no	pos	pos	neg	pos	r	pos	pos	pos	neg	neg	neg	neg	neg	neg	pos
09RB4620	B. pinnipedialis	Seal	neg	yes	neg	no	pos	pos	neg	pos	r	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos
09RB4625	B. suis 2	Wild boar	neg	yes	neg	no	pos	pos	neg	pos	s	pos	neg	pos	neg	pos	neg	neg	neg	neg	pos
03RB0217	B. suis 1330	reference	neg	no	neg	no	pos	pos	pos	pos	s	pos	neg	pos	neg	neg	pos	n.d.	neg	pos	pos
10RB9822	B. vulpis	Fox	neg	no	neg	no	pos	pos	neg	pos	s	pos	pos	pos	neg	neg	pos	pos	pos	pos	pos
03RB0215	B. abortus 544	reference	neg	yes	neg	no	pos	pos	pos	pos	s	neg	pos	pos	neg	neg	pos	n.d.	pos	pos	pos
03RB0216	B. melitensis 16M	reference	neg	no	neg	no	pos	pos	neg	pos	s	neg	pos	neg	pos	neg	pos	n.d.	neg	neg	pos
03RB0213	B.canis RM 6/66	reference	neg	no	neg	no	pos	pos	neg	pos	r	pos	neg	neg	neg	pos	neg	n.d.	neg	neg	n.d.
03RB0215	B. ovis 63/290	reference	neg	yes	neg	no	neg	neg	neg	pos	r	pos	neg	neg	neg	pos	neg	n.d.	neg	neg	n.d.

In addition to these more classical phenotyping methods, studies were performed on the metabolic activity of new emerging *Brucella* in comparison with classical *Brucella*. Hence, we performed substrate

utilization experiments in defined minimal medium supplemented with a single carbon or other energy source. This minimal medium was modified from the medium published by Plommet containing solely salts as well as vitamins and did not significantly promote the growth of any tested Brucella strain [Plommet 1991]. Subsequent bacterial growth was determined by measuring the optical density at 600 nm (OD600) after adding various single energy/carbon sources to the minimal medium.
Based on the results of the studies and knowing that the number of new emerging brucella examined is statistically insufficient for a conclusive evaluation, we have selected methods that seem particularly suitable to us and which should also be used in the future for typing new isolates. This would allow ongoing validation of these methods for their suitability.
Any isolate that cannot be readily classified as classical *Brucella* should at least be tested for motility and agglutination with brucellosis positive reference serum or/and monospecific sera. Furthermore, lysis with Brucella specific phages is an important classification criterion. Additionally, metabolic phenotyping can be performed to determine the metabolic activity of newly isolated non-classical Brucella. Our experimental setup identified several substrates that allowed the distinction between the former *Ochrobactrum* and the atypical *Brucella* species, a feature that could potentially be used for diagnostics. Only *O. anthropi* and *O. intermedium* but none of the tested atypical *Brucella* isolates proliferated with citric acid, gluconic acid and mannitol as sole carbon and energy source in the defined minimal medium. In contrast, all tested atypical Brucella isolates but not the former O. anthropi and O. intermedium species were able to grow on adipic acid as sole carbon and energy source.

1. Motility testing

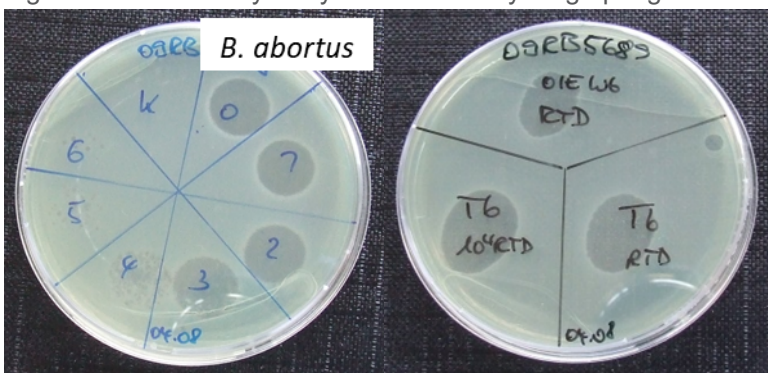
The motility test is performed on 25 ml of a semi-solid culture medium (pH: 7.0) in a Petri dish containing 25 g nutrient broth (e.g. SIFIN, order number TN1172), 28 g brucella broth (e.g. BD, order number 211088), 37 g brain heart infusion broth (e.g. Merck, order number 1.10493) and 0.05 g Triphenyltetrazolium chloride (e.g. Merck, order number 108380) per 1 liter deionized water. The Triphenyltetrazolium chloride must be added after cooling the sterilized culture medium solution to ca. 45°C. The isolate to be tested is spotted in the center of the Petri dish and incubated at 37 °C and 5% CO2. The result should be read after 24 h, 48 h and if necessary 72 h. A motile and a non-motile bacterial control should be included.
Figure 1: Result of motility testing after 48 h



2. Phage typing

Phage typing can be performed according to the specifications from the literature (Alton et al.; 1988). Different specific phages (e.g. Tbilisi phage and Weybridge phage) in different dilutions could be used. Based on phage lysis, brucella can be typed. In our study during the project, the new emerging brucella were not lysed by any of the available phages in any of the commonly used concentrations.

Figure 2: *B. abortus* lysis by Tbilisi and Weybridge phage at different concentrations



3. Agglutination with monospecific sera or brucellosis positive reference serum

Agglutination with monospecific sera is another important method to type brucella. This method is also described in the literature (Alton et al.; 1988). Based on this result Brucella species could be typed up to biovar level.
In our studies, none of the new emerging brucella isolates reacted with any of the monospecific sera. In addition, the tests with a brucellosis-positive reference serum did not show any reaction. All other classical brucella species tested reacted positively with the reference serum.

4. Metabolic phenotyping

Unfortunately, since the most suitable commercial assay methods are no longer available, metabolic phenotyping had to be performed by alternative means. As applied in this project, it is a labor-intensive and complex process. It may be possible to adapt and simplify individual parts of the study to the extent that they can be performed in a less specialized laboratory. This would require further experiments. Therefore, we show here only a brief overview of the method and expected results when using it.

Figure 3: Alternative approach for phenotyping of Brucella spp. based on metabolic data. Growth kinetics of Brucella isolates in modified(*) Plommet minimal medium substituted with a single energy source to identiy the maximum growth (maximum optical density at 600nm) of the respective isolates in the substrates of interest. Parts of this figure were created with Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license.

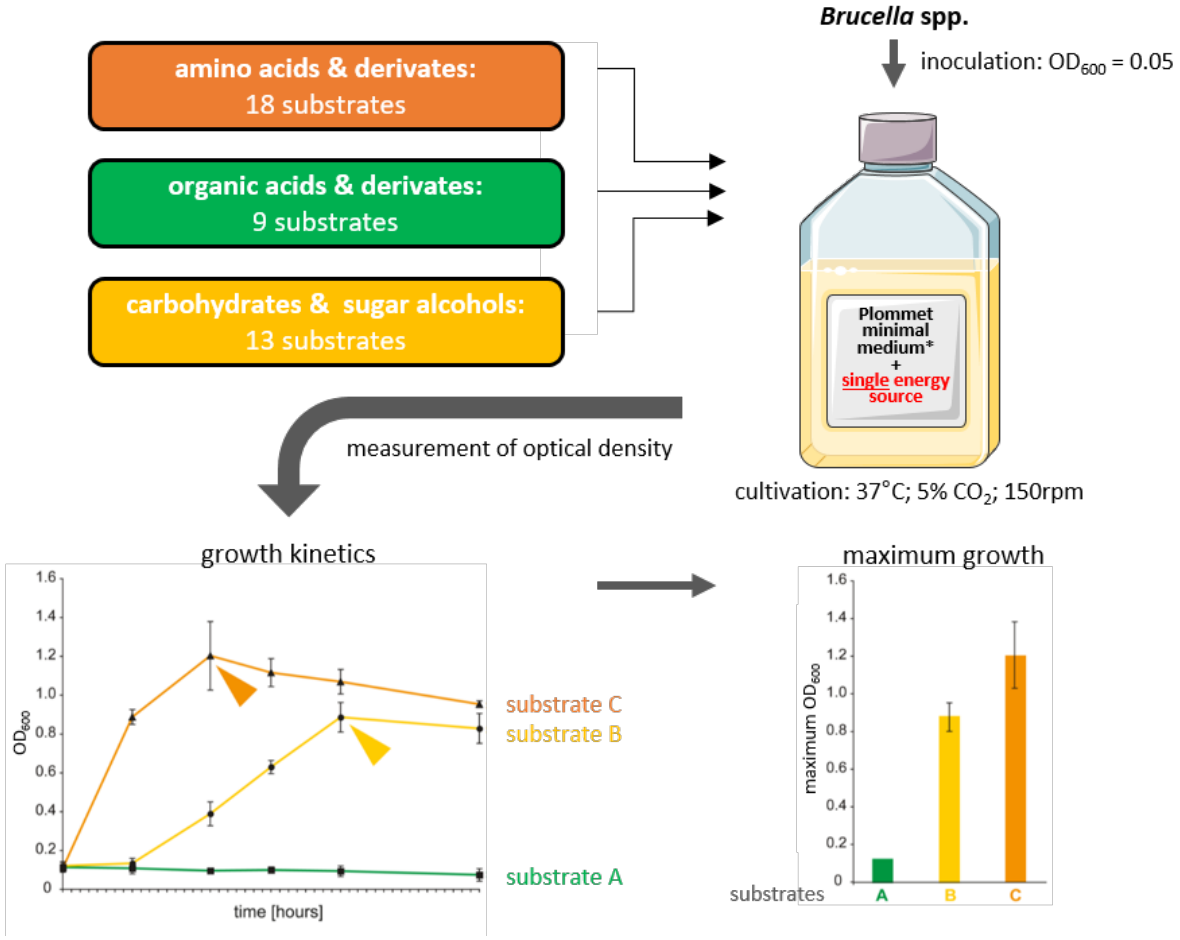


Table 2: Substrate dependent growth of typical, atypical Brucella and former Ochrobactrum spp. Growth (black) and no growth (white) is defined as OD600 >0.5 and <0.2, respectively. Poor growth (grey) is defined as OD600 between 0.2 and 0.5. C – control media.

		O. intermedium	*O. anthropi*	*B. inopinata* B01	*B. inopinata-like* B02	*B.* sp. (frog isolates)					*B. microti*				*B.* sp. NF2627	*B. vulpis* F60	*B. papionis* F8/08-60	*B. abortus* 544	*B. melitensis* 16M	*B. suis* 1330	*B. suis* 513	*B. canis* RM6/66
BfR-ID: BfR-BR-00___		736	743	173	174	507	508	510	516	528	368	747	748	751	533	656	406	001	180	549	654	083
C	Brucella broth																					
	Minimal medium																					
carbohydrates	Arabinose																					
	Fructose																					
	Fucose																					
	Glucose																					
	Maltose																					
	Mannose																					
	Rhamnose																					
	Sucrose																					
sugar alcohols	Erythritol																					
	Glycerol																					
	Inositol																					
	Mannitol																					
	Sorbitol																					
organic acids & derivatives	Gluconic acid																					
	Adipic acid																					
	Pyruvic acid																					
	Lactic acid																					
	Citric acid																					
	α-Ketoglutaric acid																					
	Succinic acid																					
	Fumaric acid																					
	Malic acid																					
amino acids & derivatives	Ectoine																					
	Alanine																					
	Arginine																					
	Asparagine																					
	Aspartic acid																					
	Cysteine																					
	Glutamic acid																					
	Glutamine																					
	Glycine																					
	Histidine																					
	Isoleucine																					
	Leucine																					
	Lysine																					
	Methionine																					
	Proline																					
	Serine																					
	Threonine																					
	Valine																					