Differential phenotyping of *Brucella* species using a newly developed semi-automated metabolic system

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**Abstract**

**Background:** A commercial biotyping system (Taxa Profile™, Merlin Diagnostika) testing the metabolization of various substrates by bacteria was used to determine if a set of phenotypic features will allow the identification of members of the genus *Brucella* and their differentiation into species and biovars.

**Results:** A total of 191 different amines, amides, amino acids, other organic acids and heterocyclic and aromatic substrates (Taxa Profile™ A), 191 different mono-, di-, tri- and polysaccharides and sugar derivates (Taxa Profile™ C) and 95 amino peptidase- and protease-reactions, 76 glycosidase-, phosphatase- and other esterase-reactions, and 17 classic reactions (Taxa Profile™ E) were tested with the 23 reference strains representing the currently known species and biovars of *Brucella* and a collection of 60 field isolates. Based on specific and stable reactions a 96-well "*Brucella* identification and typing" plate (Micronaut™) was designed and re-tested in 113 *Brucella* isolates and a couple of closely related bacteria. *Brucella* species and biovars revealed characteristic metabolic profiles and each strain showed an individual pattern. Due to their typical metabolic profiles a differentiation of *Brucella* isolates to the species level could be achieved. The separation of *B. canis* from *B. suis* bv 3, however, failed. At the biovar level, *B. abortus* bv 4, 5, 7 and *B. suis* bv 1-5 could be discriminated with a specificity of 100%. *B. melitensis* isolates clustered in a very homogenous group and could not be resolved according to their assigned biovars.

**Conclusions:** The comprehensive testing of metabolic activity allows cluster analysis within the genus *Brucella*. The biotyping system developed for the identification of *Brucella* and differentiation of its species and biovars may replace or at least complement time-consuming tube testing especially in case of atypical strains. An easy to handle identification software facilitates the applicability of the Micronaut™ system for microbiology laboratories.

**Background**

*Brucella* spp. are the causative agents of brucellosis, one of the major bacterial zoonotic diseases that is responsible for reproductive failure in animals leading to tremendous economic losses and for a potentially debilitating infection in man. Furthermore, *Brucella* is listed as category B bioterrorism agent.

Species and biovar classification of brucellae is historically based on natural host preference and phenotypic traits, i.e. CO₂ requirement, H₂S production, urease activity, dye-sensitivity, lysis by *Brucella*-specific bacteriophages, agglutination with monospecific antisera, and oxidative metabolic patterns [1-3]. In concordance with this biotyping scheme the genus *Brucella* (*B.*) currently comprises the six classical species *B. melitensis* bv 1-3 (predominantly isolated from sheep and goats), *B. abortus* bv 1-7 and 9 (from cattle and other Bovidae), *B. suis* bv 1-3 (from pigs), bv 4 (from reindeer) and bv 5 (from small ruminants), *B. canis* (from dogs), *B. ovis* (from sheep), and *B. neotomae* (from desert wood rats) [4]. Further, two novel species of marine origin, *B. pinnipedialis* (from seals) and *B. ceti* (from dolphins and whales) [5], and *B. microti* at first isolated from the common vole *Microtus arvalis* [6], then from red foxes...
(Vulpes vulpes) [7] and also directly from soil [8] have been added to the genus. Most recently B. inopinata sp. nov. isolated from a breast implant wound of a female patient has been described as a new species with so far unknown animal reservoir [9].

A biotyping assay useful for Brucella identification and species differentiation must consequently be able to identify the rising number of upcoming new species as well as single atypical strains which do not fit within the pre-existing scheme [10,11]. In addition, clinically relevant and closely related bacteria of other genera should be discriminated. Using commercially available rapid bacterial identification systems such as the API 20 NE® (BioMerieux, Nürtingen, Germany) which include a restricted number of biochemical tests Brucella spp. may be misidentified e.g. as Psychrobacter phenylpyruvus (formerly Moraxella phenylpyruvica) [12] or Ochrobactrum anthrophi [13].

The aim of our study was to develop a miniaturised semi-automated system for the reliable identification of members of the genus Brucella and the differentiation of its species based on comprehensive metabolic activity testing.

**Results**

The Taxa Profile™ system testing the utilization of amino acids (A plates) and carbohydrates (C plates) as well as other enzymatic reactions (E plates) [Additional files 1, 2 and 3] revealed a very high biodiversity among the closely related species and biovars of the genus Brucella (Figure 1A, [Additional files 4, 5 and 6]). The stability of metabolic profiles significantly varied between the different species and biovars, yet most of the stable markers were found in the Taxa Profile™ E plate. Differences between cultures of the same strain were most frequently observed in the species B. abortus and B. microti, and in biovar 1 of B. suis. A total of 196 out of 570 biochemical reactions proved to be both stable and discriminatory, and showed differences in the metabolism of the 23 Brucella reference strains or helped to distinguish Brucella spp. from closely related bacteria such as Ochrobactrum inopinata. In general, the broadest metabolic activity could be observed for strains of the species B. suis, B. microti, and B. inopinata. In contrast, the metabolic activity of B. ovis, B. neotomae and B. pinnipedialis was low.

The comprehensive biotyping of the reference strains resulted in clusters agreeing in principle with the present conception of the genus Brucella (Figure 1A). A subset of 93 substances which preserved the clustering of the reference strains and achieved a satisfying discrimination was consecutively selected (Figures 2 and 1B). The newly configured 96-well plate assay tested for 29 aminopeptidases, 2 phosphatases, 4 glucosidases, 1 esterase, and the metabolism of 11 monosaccharides, 3 disaccharides, 7 sugar derivatives, 15 amino acids, 11 organic acids, 1 salt, 1 amino acid derivate, 1 peptide, and 1 base. In addition, 6 classical reactions, i.e. nitrite, nitrate, pyrazinamidase, Voges-Proskauer medium, urease and H₂S production, and three controls, i.e. peptidase control, pyrazinamidase control and assimilation control were included.

Glu(pNA)-OH (ENAOH), Pyr-pNA (PYRNA) (constantly negative reaction), and H-hydroxyprolin-βNA (HP) (constantly strong positive reaction) turned out to be key substances useful for the identification of the genus Brucella and its differentiation from other bacteria [Additional file 7].

A stable negative reaction for D-threitol (D-TOL) and mostly positive reactions for L-alanine (L-Ala), D-alanine (D-Ala), propionic acid (Prop), L-proline (L-Pro), D-proline (D-Pro), and D-serine (D-Ser) could be observed in B. melitensis. B. microti which also makes use of alanine and proline could be separated from B. melitensis by a constantly negative reactivity for Prop and D-Ser. A positive myo-inositol (INOL) reaction seemed to be characteristic for most B. melitensis strains and B. inopinata. Bis-p-nitrophenyl phosphate pH 7.5 (BISPH7), p-nitrophenyl phosphate di(2-amino-2-ethyl-1,3-propanediol) pH 7.5 (PHOS7), and p-nitrophenyl-α-d-glucopyranoside pH 7.5 (aGLU7) were found positive frequently in B. suis and regularly in B. microti strains, variable in B. melitensis and mostly negative in B. abortus. Glutarate (Gluta) and mesaconic acid (Mesac) which were almost exclusively metabolized by B. microti might be helpful for further differentiation. P-nitrophenyl-α-d-glucopyranoside pH 5.5 (aGLU5) and p-nitrophenyl-n-acetyl-β-d-glucosaminide pH 7.5 (CHIT7) showed weak positive reactions in B. suis and B. canis and strong positive reactions in B. microti and B. inopinata. B. microti and B. inopinata exhibited outstanding metabolic capabilities in comparison to all other brucellae, sharing a series of reactions with O. anthropi and O. intermedius. Most remarkably, both species were strongly positive in the Voges-Proskauer reaction. The slow growing strains of the B. ovis group did not metabolize any carbohydrates except for D-glucose-L-cysteine (GLUCY), L(+)-arabinose (L-ARA), D-TOL, and adonite (ADON) and only a few amino acids. In addition, B. ovis strains were usually not able to deoxidize nitrite (NTI, nitrite reduction) and nitrate (NTA, nitrate reduction). Ac-Gly-Lys-βNA (AcGK) tested strongly positive in B. ovis and B. canis whereas Trp-βNA (W) regularly tested negative in these species as compared to all other Brucella spp. In comparison with other species B. pinnipedialis was weak in metabolizing D(-)-ribose (D-RIB), D(-)-arabinose (D-ARA), D (+)-glucose (D-GLU), L-ARA, D(+)-galactose (D-GAL),
D(+) -xylose (D-XYL), and a-D- talose (D-TAL). B. ceti and B. pinnipedialis showed significantly different carbohydrate utilization patterns. B. neotomae was the only species tested negative for d-Ala-pNA (DANA), Gly-pNA (GNA), Leu-pNA (LNA), Lys-pNA (KNA), Lys-bNA (K), and Gly-Gly-bNA (GG). Like B. neotomae the two yet unidentified strains isolated from foxes were negative for DANA and GNA. Despite of genetic consistency with the genus Brucella (data not shown) these two strains completely differed in their metabolic profile from the species described to date.

The panel of 93 discriminating reactions was re-evaluated for its usefulness in the identification of Brucella and the differentiation of its species and biovars using a broad spectrum of well characterized field strains. Both intra-assay variability were ascertained to be negligible. Results of the cluster analysis of the 113 strains investigated regarding their ability to metabolize the 93 selected substances supported our findings in the smaller collection of Brucella reference strains (Figure 3). Based on the metabolic profiles determined by the Brucella specific Micronaut™ microtiter plate, B. melitensis and B. abortus isolates fell into two distinct groups (Figure 3). B. suis (except for biovar 5) could be found in another group but the biovars 1, and 3 and 4 gathered together with B. inopinata and B. canis isolates, respectively. B. suis bv 2 could be separated by its substrate assimilation pattern. B. suis bv 5 showed metabolic traits similar to B. ovis, B. neotomae and the marine mammal strains. Each Brucella strain investigated revealed an individual metabolic profile.

Figure 1 Cluster analysis of Brucella reference strains based on biochemical reactions. Cluster analysis of the 23 Brucella reference strains based on 570 (A) and 93 (B) biochemical reactions tested with the Taxa Profile™ system (plate A, C, and E) and the newly developed Brucella specific Micronaut™ microtiter plate, respectively. Hierarchical cluster analysis was performed by the Ward linkage algorithm using the binary coded data based on the empirically set cut-off.
Using the newly developed Brucella specific Micronaut™ biotyping assay, B. abortus bv 4, 5, and 7, B. suis bv 1-5, B. ovis, B. neotomae, B. pinnipedialis, B. ceti, B. microti, and B. inopinata could be discriminated within the genus with a specificity of 100% (Table 1). In contrast, members of the three B. melitensis biovars formed a homogenous group. Although the metabolic activity of B. melitensis strains did not correlate with the classical biotyping scheme, subgroups within the species could still be defined (Figure 3). Gram-negative microorganisms other than brucellae e.g. Ochrobactrum intermedium, O. anthropi, Yersinia enterocolitica O:9, and Acinetobacter Iwoffii showed differing oxidative metabolic profiles and could clearly be distinguished from Brucella spp. in our experimental setting. Furthermore, a screening of the Micronaut-IDS database (Merlin Diagnostika) which is a widely used rapid identification system for Gram-negative and Gram-positive bacteria clearly discriminated brucellae from other bacterial taxa on the basis of four enzymatic reactions i.e. HP, PyrβNA (Pyr), urease, and NTA [Additional file 8, only clinically relevant bacteria are shown].

The biotyping results were independent of the host and the geographic origin of Brucella isolates.

Discussion
Classical phenotyping and metabolic markers of Brucella spp

Although Brucella is a monophyletic genus, apparent differences between its species do exist e.g. host specificity and pathogenicity. Nowadays, Brucella species and biovars are distinguished by a limited number of microbiological tests measuring quantitative or qualitative differences of dye bacteriostasis, hydrogen sulfide production, urea hydrolysis, carbon dioxide requirement, bacteriophage sensitivity and agglutinin absorption. For at least half a century these microbiological procedures have not changed, although various new Brucella species showing variable phenotypic traits have been detected and new diagnostic methods have been developed.

Neither the classical biochemical tests nor antigenic properties and phage-sensitivity can be considered a reliable guide to the identification of Brucella species. Contradictory results were often reported [14]. However, variations in H₂S production, CO₂ requirement, a change in dye tolerance or atypical surface antigens i.e. inconsistent A and M antigens usually do not affect the oxidative metabolic pattern of a strain [15,16]. Metabolic activities have proven to be stable parameters allowing unambiguous species identification, particularly in strains which show conflicting identities by conventional determinative methods [14,17-19]. In addition, differing metabolism may help to describe new species [6,9,20]. In our series, two strains isolated from foxes in Austria (strain no. 110 and 111) which displayed an atypical metabolic pattern could be identified.

Oxidative metabolic profiles remain qualitatively stable for long periods of time and usually show no change in characteristic patterns after in vivo and in vitro passages [21]. However, quantitative differences in the oxidative metabolic rate of monosaccharides have been observed after multiple passages in vitro [21]. Variants in the oxidative metabolic pattern found among different CFUs of the same strain have been described in varying frequencies depending on Brucella species and biovars [22]. In our experiments, B. suis bv 1 showed the highest intrastrain variability in its enzymatic activity (data not shown).

Despite the stability of the metabolic markers and their consecutive usefulness in diagnostic assays, studies describing the differences in the metabolism of Brucella spp. have not been conducted for decades as the...
Cluster analysis of 113 Brucella strains including the reference strains and two isolates of a potentially new species that originated from Austrian foxes based on 93 biochemical reactions tested with the newly developed Brucella specific Micronaut™ microtiter plate. Hierarchical cluster analysis was performed by the Ward algorithm using the binary coded data based on the empirically set cut-off.
Table 1 Specificity of the Brucella specific Micronaut™ microtiter plate.

| Species Biovars | Specificity in % |
|-----------------|------------------|
| Species         | Biovar differentiation | Species differentiation |
| 1               | 0                 | 100                     |
| 2               | 75                | 100                     |
| 3               | 90                | 100                     |
| B. abortus      | 4 100             | 100                     |
| 5               | 100               |                         |
| 6               | 0                 |                         |
| 7               | 100               |                         |
| 9               | 0                 |                         |
| B. melitensis   | 1 19              | 100                     |
| 2               | 89                |                         |
| 3               | 64                |                         |
| B. suis         | 1 100             | 74 100                  |
| 2               | 100               |                         |
| B. ovis         |                   | 100                     |
| B. canis        |                   | 60                      |
| B. neotomae     |                   | 100                     |
| B. ceti         |                   | 100                     |
| B. pinnipedialis|                   | 100                     |
| B. microti      |                   | 100                     |
| B. inopinata    |                   | 100                     |

Specificity of the Micronaut™ system to differentiate Brucella species and biovars.

classical laboratory techniques are labour-intensive and very demanding. Especially Warburg manometry which is carried out in a respirometer measuring oxygen uptake has been widely used to determine oxidative metabolic patterns in order to describe and differentiate species, biovars, and atypical strains of the genus Brucella. Formerly, manometric studies on the metabolic activity of brucellae helped to quantitatively define the species classified within the genus [23]. However, due to the demanding techniques applied only a restricted number of strains and reactions were tested and various substrates e.g. D-asparagine, L-proline, adonitol, fructose and glucose were regarded as not useful for species and biovar differentiation [23,24]. In the comprehensive setting of this study most of these substrates also proved their usefulness.

Manometric studies have confirmed that a characteristic oxidative pattern for Brucella species exists whereas specific profiles for the biovars have not yet been described except for B. suis bv 1-4 [25]. Using the Micronaut™ system we were able to discriminate B. abortus bv 4, 5, and 7, B. suis bv 1-5, B. ovis, B. neotomae, B. pinnipedialis, B. ceti, B. microti and B. inopinata with a specificity of 100%. However, differentiation among the B. melitensis biovars was impossible as, according to their oxidative metabolic activity, they form a very homogenous group. The results of the cluster analysis based on our biotyping data (Figure 3) are in general concordance with the genotyping data acquired by Multiple Loci VNTR (Variable Number of Tandem Repeats) Analysis (MLVA) [26]. Neither biotyping nor genotyping proved a biovar specific clustering in B. melitensis strains [27]. Although we tested a substantial number of biochemical reactions we may have chosen the wrong set of substrates for the differentiation of B. melitensis strains, but the separation of this species in three biovars could also be somehow artificial.

Biotyping of Brucella spp. using commercially available assays

If biological traits such as enzymatic activities are tested all potential variables must be reduced to a minimum to avoid intra- and inter-assay variations which may occur in addition to minimal biological variations. Commercial test systems offer a large number of quality controls both in the production chain and under experimental conditions.

Commercially available microtiter plates coated with various substrates to characterize the metabolic pattern of bacteria have already been used to describe new species of the genus Brucella e.g. the Biolog™ system for B. ceti [28] and the Micronaut™ system for B. microti and B. inopinata [6,9]. However, comprehensive metabolic studies including all currently known species and biovars are rare.

Using the Biolog™ GN MicroPlate system (Biolog, CA, USA) based on 44 differentially oxidized substrates, B. melitensis, B. abortus and B. suis isolates could be grouped into taxa identical with the presently recognized species [29]. However, only a restricted number of strains (n = 35) were tested and biovars were not differentiated. In a larger strain collection (n = 71) which included all biovars of the six classical Brucella species only 50% of the strains were correctly identified confirming the poor specificity of this commercially available, substrate mediated, tretrazolium identification technique [30]. López-Merino and colleagues used the Biotype 100™ carbon substrate assimilation system (bioMérieux, Marcy-L’Etoile, France) which comprises 99 carbohydrates, organic acids and other carbon substrates to discriminate B. melitensis, B. abortus, B. suis and B. canis [31]. Using the most discriminating carbon substrates i.e. D-glucose, D-trehalose, D-ribose, palatinose, L-fucose, L-malate, and DL-lactate more than 80% of the B. melitensis and B. abortus strains could be correctly identified. Similar to the Brucella specific
Micronaut™ plate designed in this study B. suis and B. canis could not always be discriminated. The limited number of field isolates tested per species may have produced inconclusive results, particularly when only reference strains were available which are well known for atypical phenotypic traits. Future studies on larger strain collections may reveal more unique metabolic profiles suitable for species and biovar differentiation and also helpful to discriminate between B. suis bv 3 and B. canis.

Nevertheless, the overall specificity for the identification of Brucella species using the Micronaut™ system reached 99%.

Experimental conditions potentially interfering with bacterial metabolism and influencing biotyping results

Many experimental parameters may influence the metabolic activity of bacteria. For instance, oxidative rates may decrease if Brucella is prepared from 48 hours rather than 24 hours cultures [25] because Brucella is able to adapt to starvation. This effect does not seem to be important in the Micronaut™ system since turbidity is measured reflecting bacterial growth within a period of 48 hours as an indirect parameter for substrate utilization. Consequently, the bacteria have plenty of time to switch on all necessary metabolic pathways. Hence, the metabolic rate of glutamic acid may differ between B. abortus and B. melitensis [32] but after 48 h the substrate is entirely metabolized by both species. For the same reason B. suis has been described as inactive in the metabolism of glutamic acid but our results revealed extensive utilization of this substrate at least for the biovars 3-5 whereas the metabolism was variable in the biovars 1 and 2.

The growth medium can also have an effect on the utilization of substrates and brucellae may operate with alternate metabolic pathways leading to discrepant stimulatory effects in different assays [30]. Therefore, a minimal medium i.e. buffered sodium chloride peptone (from potatoes) solution was used in Taxa Profile™ and Micronaut™ plates to avoid interference with other potential substrates in the culture medium.

The rates of oxidation of various compounds are also strongly dependent on intact bacterial membranes and pH values [33,34]. In our experiments, asparagines were easily oxidized by most of the Brucella spp., but aspartic acid was not (exceptions were B. suis bv 4, B. microti, and B. inopinata). Furthermore, glutamic acid was oxidized, but intermediates in the pathway, such as α-ketoglutarate and succinate (except for B. microti and B. inopinata) were usually not. Lowering the pH of a reaction mixture containing intact cells of brucellae markedly increased the oxidation rate of these metabolites e.g. L-aspartate, α-ketoglutarate, succinate, fumarate, L-malate, oxaloacetate, pyruvate and acetate [34]. Differences between Brucella species may occur in the pH range at which the bacteria are able to utilize some of the substrates and therefore labile metabolic profiles can be observed [35]. Nevertheless, such reactions may be helpful for the differentiation of species and biovars if assay conditions are stable. The effect of extracellular adjustment of the pH upon intracellular enzymatic reactions can be explained by organic acids permeating the cell more readily when undissociated than when ionized. Hence, a pH change may overcome the permeability barrier for many substrates especially of the Krebs’ cycle. For this reason our results do not easily reflect intracellular substrate utilization. In proteomic studies on intracellular brucellae and bacteria grown under stress conditions comparable to the intracellular niche of Brucella, enzymes of the TCA cycle i.e. the succinyl CoA synthetase and aconitate hydratase were found increased [36,37]. In contrast, intermediates of the TCA cycle such as citrate, isocitrate, α-ketoglutarate, succinate, malate, fumarate were not generally metabolized in vitro or showed variable metabolism in the different species such as oxaloacetic acid.

Although modelling of the intracellular niche of brucellae is not a topic of this study the Micronaut™ system might be helpful to investigate differences in the metabolic activity between the species under various growth conditions. This will allow a much deeper insight in the metabolic changes of intracellular compared to extracellular brucellae and will help to understand survival strategies of the pathogen under starvation, microaerobic and acidic conditions. In this context, a negative correlation between metabolic activity and the relative degree of virulence was observed among B. abortus strains [38]. Avirulent mutants of B. melitensis, B. abortus and B. suis that failed to replicate or survive in macrophages or animal models often had mutations in the carbohydrate metabolism [39]. In our study, B. microti which is not known to be human pathogenic was the metabolically most active species.

Independent of the method used a broad agreement can be observed for the utilization of carbohydrates by Brucella spp. whereas the results of the amino acid metabolism are more variable [3,16]. Differences in the oxidation rate of different isomers of the same amino acid have been described for short incubation periods, e.g. B. suis and B. melitensis are known to oxidize D-alanine more rapidly than the L-isomer [40] or B. abortus oxidized L-glutamic acid and L-asparagine rapidly whereas relatively slight activity was obtained with the D-isomers [38]. Differences in the metabolism rate could not be used for differentiation in our multi-substrate test. As many substrates were tested at the same time the incubation period was prolonged to 48 hours to ensure that each substrate was completely utilized.
With a few exceptions, there are only minor differences in the general utilization of D- and L-isomers of amino acids within the same species [41]. Therefore both isomers of the same amino acid were only included three times in the Micronaut™ plate, i.e. D-/L-proline, D-/L-alanine, and D-/L-serine. In our experiments, opposing metabolic activity could be observed for the different isomers of proline in B. abortus by 3, B. suis by 2, and B. canis, for the isomers of alanine in B. canis and B. neotomae, and for the isomers of serine in B. suis by 1, 2, and 4, B. ovis, B. microti and B. inopinata.

Further, substrate concentration may influence the metabolic activity of Brucella [34, 38]. Although sample volumes are different in Taxa Profile™ and Micronaut™ plates the final substrate concentration is the same. Hence, apparently contradictory results in these two test systems which could be observed in our study cannot be explained by different concentrations of the same compound.

Because of the small volumes used in the Taxa Profile™ plate turbidity could not be measured due to technical limitations. Therefore the indicator phenol red was added to colorimetrically measure respiration. In contrast, in the 96-well Micronaut™ plate turbidity as a measure of bacterial growth was determined. The measurement of respiration instead of growth is much more sensitive since bacteria may respond metabolically by respiring but not by growing [42]. Hence, this effect may have led to differing results for the utilization of the same substrate on the two platforms. However, respiration could not be used in the genus Brucella since some strains are dependent on CO₂ which catalyzes abiotic reduction of the dye.

As most metabolic pathways are encoded within the Brucella genome brucellae might present as fastidious due to slow growth. Although the genome sequence of B. microti is almost identical to that of B. suis with an overall sequence identity of 99.84% in aligned regions, phenotypically these species differ significantly which might be caused by variable gene regulations and different growth patterns [43].

Both respirometry and tetrazolium reduction assays proved that B. abortus is characteristically stimulated by L-alanine, L-asparagine and L-glutamate [30]. In contrast, the Micronaut™ results were heterogeneous for L-alanine in B. abortus strains. The differences in metabolic activity observed between these methods might be caused by the cut-off selected in our experiments. Deduced from the OD values measured with the Micronaut™ system three levels of substrate utilization could be defined: no/weak metabolic activity (−), moderate metabolic activity (+), and strong metabolic activity (+++) [Additional file 7]. The different levels of oxidative metabolic activity on amino acid and carbohydrate substrates determined by Micronaut™ agreed with the oxygen uptake levels for most substrates measured by conventional manometric techniques [25]. However, owing to the dispersion of the individual OD values, quantitative differences are of limited practical relevance. The selection of cut-offs which delineated positive and negative metabolic activity greatly contributed to the classification of the presentation of substrate utilization. Of course, the limit between two activity patterns is rather artificial.

Conclusions

The results of the comprehensive biotyping study presented evidence that species of the genus Brucella can be correctly identified by their metabolic patterns. Although a range of metabolic properties allows clustering of Brucella into species and biovars clearly defined boundaries do not always exist.

Based on a selection of 93 different substrates out of 570 initially tested, a Brucella specific 96-well Micronaut™ microtiter plate was developed and successfully evaluated in a large panel of Brucella strains comprising all currently known species and biovars. Although the Micronaut™ system still requires a biological safety cabinet throughout the procedure it is much easier to handle and does not require the preparation of specific reagents leading to quicker results than conventional microbiological methods. Hence, the Micronaut™ system may replace or at least complement time-consuming tube testing. Furthermore, an easy to handle identification software facilitates its applicability for routine use.

The newly developed Brucella specific 96-well Micronaut™ plate fulfilled the performance criteria recommended for a typing assay, i.e. typeability, reproducibility, stability and discriminatory power. Although we were not able to examine the epidemiological concordance of our biotyping results, there are definite indications that the metabolic profiles of different isolates match within the same outbreak [16]. The Micronaut™ system has also proven to be invaluable in the characterization of otherwise untypable new species. However, reference and new strains should always be tested in the same series because the differences in oxidative metabolic profiles may not only be qualitative but also quantitative.

Biodiversity of Brucella spp. also reflects taxonomic (natural and evolutionary) relationships that exist between and among the organisms sequestered and clustered within the classification scheme. Hence, the Micronaut™ system is not only a diagnostic assay it can be a striking tool in functional taxonomy of the genus Brucella.

Our results may raise the question if the widely accepted biotyping scheme based on only a few phenotypic features is sufficient to get a clear idea of the true composition of the genus Brucella and will meet future demands. The new diagnostic approach presented in this study may help to overcome these limitations.
Methods

**Brucella strains**

*Brucella* spp. were characterized by classical microbiological methods according to Alton et al. (1988) [2]. Comprehensive biochemical phenotyping was performed on the *Brucella* reference strains representing all currently known species and their biovars as well as on up to 7 field isolates per species and biotype as far as available (Table 2). The consecutively established *Brucella* specific 96-well microtiter plate was evaluated testing the reference strains and a broad range of *Brucella* isolates (a total of 113 strains) originating from various animal hosts and human patients, i.e. *B. melitensis* bv 1 (n = 8), bv 2 (n = 14) and bv 3 (n = 11); *B. abortus* bv 1 (n = 9), bv 2 (n = 2), bv 3 (n = 5), bv 4 (n = 6), bv 5 (n = 1), bv 6 (n = 3), bv 7 (n = 1) and bv 9 (n = 3); *B. suis* bv 1 (n = 6), bv 2 (n = 8), bv 3 (n = 1), bv 4 (n = 2) and bv 5 (n = 1); *B. canis* (n = 5), *B. ovis* (n = 4), *B. neotomae* (n = 1), *B. pinnipedialis* (n = 8) and *B. ceti* (n = 1), *B. microti* (n = 10), *B. inopinata* (n = 1), and two atypical strains according to the hitherto existing biotyping scheme (Table 2). Isolates of diverse geographical origin were deliberately selected to gain a large variety of strains.

### Table 2 *Brucella* strains tested for metabolic activity

| Species | Biovar | Strain | Culture collection | Host | Number of field isolates |
|---------|--------|--------|--------------------|------|-------------------------|
|         |        |        |                    |      | Taxa Profile™ (570 substrates) | Micronaut™ *Brucella* plate (93 substrates) |
| B. abortus | 1 | 544 | NCTC® 10093 | Cattle | 6 | 8 |
|          | 2 | 86/8/59 | NCTC 10501 | Cattle | 1 | 1 |
|          | 3 | Tulya | NCTC 10502 | Human | 4 | 4 |
|          | 4 | 292 | NCTC 10503 | Cattle | 5 | 5 |
|          | 5 | B3196 | NCTC 10504 | Cattle | 0 | 0 |
|          | 6 | 870 | NCTC 10505 | Cattle | 3 | 2 |
|          | 7* | 63175 | NCTC 10506 | Cattle | 0 | 0 |
|          | 9 | C68 | NCTC 10507 | Cattle | 2 | 2 |
| B. melitensis | 1 | 16 M | NCTC 10094 | Goat | 4 | 7 |
|          | 2 | 63/9 | NCTC 10508 | Goat | 5 | 13 |
|          | 3 | Ether | NCTC 10509 | Goat | 4 | 10 |
| B. suis | 1 | 1330 | NCTC 10316 | Swine | 4 | 5 |
|          | 2 | Thomsen | NCTC 10510 | Swine | 6 | 7 |
|          | 3 | 686 | NCTC 10511 | Swine | 1 | 0 |
|          | 4 | 40 | AFSSA® Ref. 40 | Reindeer | 1 | 1 |
|          | 5 | 513 | AFSSA Ref. 513 | Wild rodent | 0 | 0 |
| B. canis | RM6/66 | NCTC 10854 | Dog | 4 | 4 |
| B. ovis | 63/290 | NCTC 10512 | Sheep | 2 | 3 |
| B. neotomae | SK33 | NCTC 10084 | Desert rat | 0 | 0 |
| B. pinnipedialis | NCTC 12890 | Common seal | 7 | 7 |
| B. ceti | NCTC 12891 | Porpoise | 0 | 0 |
| B. microti | CCM® 4915 | Common vole | 1 | 9 |
| B. inopinata | BO1 | BCCN® 09-01 | Human | 0 | 0 |
| Unknown | BR® 11.1.001/002 | Fox | 0 | 2 |
| Total | 23 reference strains | | 60 field isolates | 90 field isolates |

*Brucella* reference strains and overview of field isolates tested with the Taxa Profile™ system and the newly developed *Brucella* specific Micronaut™ microtiter plate.

* NCTC: National Collection of Type Cultures.
* AFSSA: Agence Française de Sécurité Sanitaire des Aliments.
* CCM: Czech Collection of Microorganisms.
* BCCN: Brucella Culture Collection from Nouzilly.
* BR: Bundesinstitut für Risikobewertung.
* The authenticity of the *B. abortus* bv 7 reference strain has been questioned; this strain remains as a potential reference strain until an agreement will be finally reached [44].
Various strains initially tested with the 384-well Taxa Profile™ plates were re-evaluated using the newly developed 96-well plate. In addition, a limited selection of closely related and clinically relevant bacteria was tested, i.e. *Acinetobacter lwoffii* (DSM 2403), *Versinia enteroxolitica* O:9 (IP-383 RKI/Paris), *Ochrobactrum intermedium* (CCUG 24964), *O. anthropi* (DSM 6882), *Enterococcus faecalis* (DSM 2570), *Escherichia coli* (DSM 1103), *Pseudomonas aeruginosa* (DSM 1117), and *Staphylococcus aureus* (DSM 2569).

**Culture and sample preparation**

All strains were grown on *Brucella* agar for 48 h at 37°C with or without 10% CO₂ depending on the needs of the particular species. Horse serum (10%) was added to the culture medium to facilitate the growth of *B. ovis*. Colony material was harvested and solubilised in 0.1% buffered sodium chloride peptone (from potatoes) solution and in sterile 0.9% NaCl for use in profile A or C plates and profile E plates, respectively. The turbidity of the bacterial suspension was adjusted to a 2.0 McFarland standard. Each well of the 384- and 96-well plates was inoculated with 25 μl of the respective preparation, respectively. The microtiter plates were incubated at 37°C for 48 h before reading.

**Brucella phenotyping**

The metabolic activity of *Brucella* was comprehensively assessed using the Taxa Profile™ system (Merlin Diagnostika, Bornheim-Hersel, Germany) based on 384-well microtiter plates coated with various substrates. The Taxa Profile™ A microtiter plate allows testing of 191 different amines, amides, amino acids, other organic acids and heterocyclic and aromatic substrates [Additional file 1]. The Taxa Profile™ C microtiter plate enables the analysis of 191 different mono-, di-, tri- and polysaccharides and sugar derivates [Additional file 2]. Using the Taxa Profile™ E microtiter plate another 188 substrates to determine enzymatic activity were tested: 95 amino peptidase- and protease-reactions, 76 glycosidase-, phosphatase- and other esterase-reactions, and 17 classic reactions [Additional file 3]. According to manufacturer’s instructions supplementary reagents were added to some wells of the Taxa Profile™ E microtiter plates to visualize substrate utilization. In addition, the indicator phenol red was added to all wells of the Taxa Profile™ A and C microtiter plates to optimize detection. The blank value was measured for each biochemical reaction on the same plate and subtracted from measured values. In order to assess inter-assay variability five independent experiments per strain were conducted.

For evaluation of the newly developed *Brucella* specific 96-well microtiter plate three trials per strain were run independently. Intra-assay variability was assessed with the reference strains testing all substances twice within the same experiment. Since the blank values measured on extra plates proved to be constant a fixed mean value of each substrate was subtracted from the measured data.

**Data acquisition and analysis**

Turbidity and colour change were measured photometrically using a Multiskan Ascent® photometer (Labsystems, Helsinki, Finland) at a wave length of 405 nm, 540 nm and 620 nm according to manufacturer’s recommendations. Optimal OD cut-off values were empirically adapted from the preliminary test results of the 384-wells Taxa Profile™ microtiter plates.

Stable and discriminatory markers were selected to design a 96-well Micronaut™ plate (Figure 2) to identify bacteria of the genus *Brucella* and to classify their species and biovar.

Dendrograms were deduced from the biotyping data using SPSS version 12.0.2 (SPSS Inc., Chicago, IL, USA). First of all, three different character data sets were defined following the metabolic activity tested (Taxa Profile™ A ("amino acids"), C ("carbohydrates"), and E ("other enzymatic reactions"). Each character was considered as equal within the particular data set. Both the raw OD data and the binary coded data based on the empirically set cut-off were analyzed using the Pearson coefficient and the categorical coefficient, respectively. Hierarchical cluster analysis was performed by the Ward’s linkage algorithm, and a dendrogram was generated. If necessary, analysis was repeated within each cluster for further discrimination. Secondly, a separate data analysis of the 23 *Brucella* reference strains representing the currently known species and biovars was performed including all biochemical reactions of the Taxa Profile™ system or exclusively the substrates selected for the newly developed plate. Finally, the whole collective of 113 strains tested with the *Brucella* specific Micronaut™ microtiter plate was analyzed to prove the diagnostic system. An identification table presenting quantitative and qualitative metabolic activity was created [Additional file 7] and the specificity of the test system to differentiate *Brucella* species and biovars was calculated (Table 1).

**Additional material**

**Additional file 1: List of biochemical reactions tested with the Taxa Profile™ A plate.** The Taxa Profile™ A microtiter plate allows testing of 191 different amines, amides, amino acids, other organic acids and heterocyclic and aromatic substrates.

**Additional file 2: List of biochemical reactions tested with the Taxa Profile™ C plate.** The Taxa Profile™ C microtiter plate enables the analysis of 191 different mono-, di-, tri- and polysaccharides and sugar derivates.
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Authors’ contributions
SAD, HN, HT, KN, BA and AH were responsible for the study design. PB, CG, KN, SAD and HS were in charge of strain collection, selection and the biotyping work. SAD, PB and WK performed cluster analysis and checked the dataset for errors. KN, PB, SAD and HN designed the Brucella specific Micronaut™ microtitre plate. SAD wrote the report. KN, HN and WK helped to draft the manuscript. All authors read, commented and approved the final article.

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