

SHORT REPORT

Newly identified variability in *Brucella canis* fatty-acid content is associated with geographical origin

A. BROWER¹*, N. LUCERO², O. OKWUMABUA³, K. K. GOPAUL⁴,
A. M. WHATMORE⁴, S. L. CRAVERO⁵ AND M. D. TRANGONI⁵

¹ University of Nottingham, School of Veterinary Medicine and Science, Loughborough, UK

² ANLIS, Brucellosis Service, Buenos Aires, Argentina

³ University of Wisconsin, School of Veterinary Medicine, Wisconsin, USA

⁴ Animal Health & Veterinary Laboratories Agency, Department of Bacteriology, Surrey, UK

⁵ Instituto de Biotecnología, INTA-Castelar, Buenos Aires, Argentina

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SUMMARY

This study compared the fatty-acid profiles of *Brucella canis* blood culture isolates obtained from infected dogs in the UK, Germany, Japan, South Africa, Peru, Mexico, Colombia, and Argentina, and from a human clinical case in Argentina, to a bank of isolates obtained from canine outbreaks in the USA. Analysis of a total of 42 *B. canis* isolates and one reference strain found a marked variation within the species. Fatty-acid analysis showed that only the isolates from Argentina, Colombia, and Mexico, which included the human *B. canis* isolate, contained a specific fatty acid, 19:0 cyclopropane (lactobacillic acid), w8c (*cis*-11,12-methylene octadecanoic acid), and that this fatty acid, when present, made up a large percentage of overall fatty-acid content. Prior to this study, the cellular fatty-acid 19:0 cyclopropane had been identified in all of the species of *Brucella* considered to be pathogenic to humans (*B. abortus*, *B. melitensis*, *B. suis*) except for *B. canis*. Discovering that this fatty acid not only occurs in *B. canis*, but also that it is only present in some strains of the species provides a new focus for investigations aimed at identifying the cause of reported geographical variability in human *B. canis* infection, and at finding predictors of biological behaviour and human pathogenicity within this *Brucella* species.

Key words: *Brucella canis*, canine, fatty acid, lipopolysaccharide, zoonosis.

In the 1980s chromatographic analysis of cellular fatty acids was a popular technique used to speciate bacteria, including members of the genus *Brucella*. This tool has largely been replaced by molecular techniques that more effectively discriminate species within a given genus. However, a diagnostic need to

differentiate isolates of *B. canis* within outbreaks of disease has developed, and fatty-acid analysis has been investigated as a promising tool for this new purpose [1]. *B. canis* outbreaks in large and small US dog-breeding facilities have resulted in thousands of blood culture-positive dogs and thus significant potential human exposure, yet reports of human *B. canis* infection are extremely rare in the USA. In contrast, serological studies and clinical case reports from Argentina and Mexico [2–4] suggest that human seroconversion and clinical infection with *B. canis*

* Address for correspondence: Dr A. Brower, Associate Professor of Pathology, University of Nottingham School of Veterinary Medicine, Room B23, Veterinary Academic Building, Sutton Bonington Campus, Loughborough LE12 5RD, UK.
(Email: alexandra.brower@nottingham.ac.uk)

Table 1. *B. canis* isolates used for fatty-acid comparison

Isolate no.	Dendrogram identification	Geographical origin	Source species	19:0 cyclopropane and total percentage of fatty acids
1	68	Argentina, Buenos Aires ¹	Canine	Present, 37.81 %
2	58	Argentina, Buenos Aires ¹	Canine	Present, 41.11 %
3	64	Argentina, Buenos Aires ¹	Canine	Present, 35.41 %
4	67	Argentina, Buenos Aires ¹	Canine	Present, 37.91 %
5	46	Argentina, Buenos Aires ¹	Human	Present, 37.5 %
6	66	Argentina, Buenos Aires ¹	Canine	Present, 35.42 %
7	65	Argentina, Buenos Aires ¹	Canine	Present, 28.83 %
8	42	Colombia, Medellin ¹	Canine	Present, 41.95 %
9	39	Mexico ²	Canine	Present, 40.00 %
10	49	USA, Carrollton, MO ³	Canine	Absent, 0 %
11	61	USA, Carrollton, MO ³	Canine	Absent, 0 %
12	54	USA, Carrollton, MO ³	Canine	Absent, 0 %
13	51	USA, Cashton, WI ⁴	Canine	Absent, 0 %
14	52	USA, Berryville, AR ³	Canine	Absent, 0 %
15	44	USA, Dalton, WI ⁴	Canine	Absent, 0 %
16	48	USA, Dalton, WI ⁴	Canine	Absent, 0 %
17	45	USA, Hilbert, WI ⁴	Canine	Absent, 0 %
18	43	USA, Hillsboro, WI ⁴	Canine	Absent, 0 %
19	50	USA, Kewaskum, WI ⁴	Canine	Absent, 0 %
20	47	USA, Kewaskum, WI ⁴	Canine	Absent, 0 %
21	63	USA, Kewaskum, WI ⁴	Canine	Absent, 0 %
22	59	USA, Kewaskum, WI ⁴	Canine	Absent, 0 %
23	55	USA, Kewaskum, WI ⁴	Canine	Absent, 0 %
24	53	USA, Kewaskum, WI ⁴	Canine	Absent, 0 %
25	60	USA, Kewaskum, WI ⁴	Canine	Absent, 0 %
26	62	USA, Kingston, WI ⁴	Canine	Absent, 0 %
27	56	USA, Montreal, MO ³	Canine	Absent, 0 %
28	57	USA, Soldiers Grove, WI ⁴	Canine	Absent, 0 %
29	29	USA ²	Canine	Absent, 0 %
30	30	USA ²	Canine	Absent, 0 %
31	33	USA ²	Canine	Absent, 0 %
32	36	Cornell reference strain ²	Canine	Absent, 0 %
33	25	Peru ²	Canine	Absent, 0 %
34	27	Germany ²	Canine	Absent, 0 %
35	32	Germany ²	Canine	Absent, 0 %
36	40	Germany ²	Canine	Absent, 0 %
37	37	Germany ²	Canine	Absent, 0 %
38	34	Japan ²	Canine	Absent, 0 %
39	28	Japan ²	Canine	Absent, 0 %
40	31	Japan ²	Canine	Absent, 0 %
41	35	UK ²	Canine	Absent, 0 %
42	38	South Africa ²	Canine	Absent, 0 %
43	26	South Africa ²	Canine	Absent, 0 %

Isolate obtained from: ¹ Lucero Laboratory; ² Whatmore Laboratory; ³ Massengil Laboratory; ⁴ WVDL.

occur with greater frequency in these countries. Further, these reports do not describe human contact with large outbreaks in canine breeding facilities, as is known to occur in the USA, but instead record minimal or no known exposure to dogs. Given the apparent geographical discrepancy in human

B. canis seroconversion and infection, we hypothesized that host and bacterial factors may be more significant than contact with infected dogs in the development of human *B. canis* infection. In a first step towards investigating this hypothesis, we acquired a wide geographical range of *B. canis*

Table 2. *Brucella melitensis*, *B. suis* and *B. abortus* index profile

Reference index number	Fatty acid	Total fatty acids (%)
63	16:0	13.00
82	17:0	0.67
92	18:1 w7c	19.69
95	18:0	9.04
96	11-methyl 18:1 w7c	2.17
105	19:0 cp w8c	50.23
113	20:2 w6,9c	3.17

isolates, obtained from eight countries on five continents, for comparison to those in our bank of US canine isolates, using fatty-acid profiling (Table 1).

Genomic homogeneity within the genus *Brucella* makes speciation challenging [5–7], and clear discrimination of all isolates in this study as *B. canis* was necessary prior to analysing samples for differences in fatty-acid content. Therefore, we employed several molecular discrimination techniques to ensure correct speciation. A total of 41 *Brucella* isolates obtained from dogs in the USA, UK, Germany, Japan, South Africa, Peru, Mexico, Colombia, and Argentina, a canine reference strain, and one isolate obtained from a human clinical case in Argentina were confirmed as *B. canis* after initial screening using standard biochemical tests by a combination of three molecular-typing techniques. Molecular techniques included real-time PCR analysis of species-specific single-nucleotide polymorphisms (SNPs) and variable-number tandem repeat (VNTR) typing. The SNP-based real-time PCR uses a series of probe pairs labelled with alternative fluorescent dyes which correspond to two alternative states at SNP sites that define particular *Brucella* spp. [8]. In this multiplex assay probes defining the species *B. canis* reacted with all 43 samples confirming their identity. For VNTR sequencing, a 21-locus VNTR scheme that can cluster isolates according to classical taxonomic group was used [9]. Forty of the 43 samples possessed a specific profile at six relatively stable markers that we have only found associated with *B. canis* in studies of over 1300 *Brucella* isolates characterized to date, obtained from numerous species (Groussaud & Whatmore, unpublished data). The remaining three samples (isolate nos. 38, 39, 40 from Japan) all possess a second profile identical to that of the remaining 40 with the exception that one locus (VNTR14) could not be amplified from these isolates. Again, we see this

particular profile only with isolates of *B. canis* and these data are thus consistent with the conclusion that all 43 samples represent *B. canis*. In addition, combinatorial PCR of 27 isolates from Argentina, Colombia, and USA (WVDL) with a *B. suis* strain as control also indicated that all of these isolates were *B. canis* (data not shown). Once rigorous discrimination confirmed that all 42 isolates and our reference strain represent *B. canis*, they were compared by fatty-acid analysis. The samples were subjected to gas chromatographic and dendrogram analyses of whole-cell fatty-acid methyl esters using Sherlock MIS software at the MIDI facility (Microbial ID Inc., USA) using previously published protocols [10]. Fatty-acid analysis showed that only the isolates from Argentina, Colombia, and Mexico, which included the human isolate, contained 19:0 cyclopropane, w8c (19:0 cp), and that this peak represented a high percentage of the total cellular acids, ranging between 21.83 and 41.95, in these nine isolates (Table 1). The index profile used for *Brucella* in the chromatographic analysis includes a combination of *B. melitensis*, *B. abortus* and *B. suis*, and shows 19:0 cp at 50.23% of total acids (Table 2). Among the Argentinean, Colombian, and Mexican isolates, similarity indexes to this *Brucella* profile ranged from 0.663 to 0.806, and dendrogram analysis showed distinct clustering of these isolates (Fig. 1). Thus, the *B. canis* isolates obtained from Argentina, Colombia, and Mexico produce a fatty-acid profile consistent with the *Brucella* spp. of greatest zoonotic potential (those included in the index profile). Because the database used for our analysis does not include a *B. canis* index profile, the non-Argentinian, Mexican, and Colombian *B. canis* isolates actually matched more closely with other bacterial profiles, including *Methylobacterium mesophilicum*, *Ochrobactrum anthropi*, *Sphingomonas paucimobilis* (*Pseudomonas paucimobilis*), *Methylobacterium fujisawaense*, and *Roseomonas* genomospecies 5, with similarity indexes to a given species in the range of 0.395 to 0.740. Figure 2(a, b) shows key profile and chromatographic differences between these isolate groupings. The difference in similarity indices reflects the critical presence or absence of 19:0 cp found in the isolates, and highlights the strong similarity that those from Argentina, Colombia and Mexico have with more pathogenic *Brucella* spp.

Brucella spp. lack most functional gene sequences encoding for known virulence factors [11, 12], thus a critical component of *Brucella* virulence is the lipopolysaccharide (LPS) surface antigen. Members of the

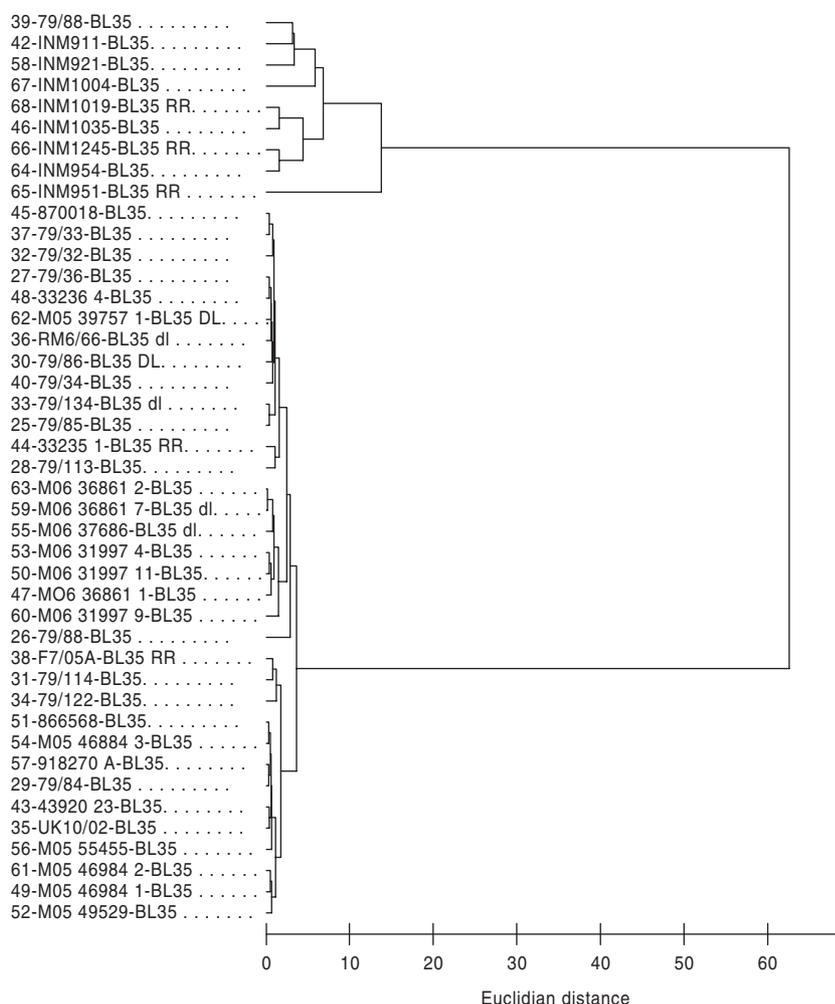


Fig. 1. Complete dendrogram derived from the cellular fatty-acid profiles of 42 *B. canis* isolates and the *B. canis* reference strain RM6/66.

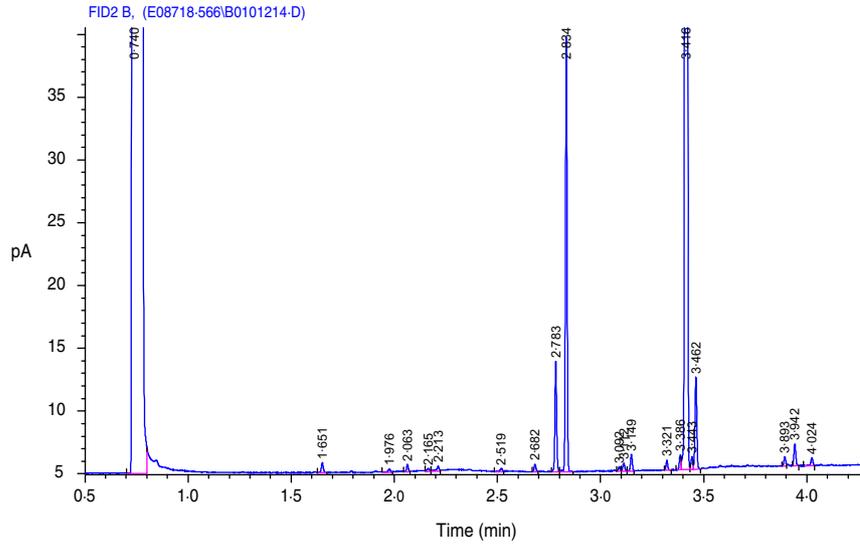
genus *Brucella* are described as having a smooth or rough phenotype, which reflects differences in the LPS of the outer cell membrane. Virulence in humans is typically associated with the smooth phenotype, which is the naturally occurring phenotype of *B. melitensis*, *B. suis*, and *B. abortus*. *B. canis* stands out among the six classical *Brucella* spp. (there are now ten *Brucella* spp. with the recognition of *B. ceti*, *B. pinnipedialis*, *B. inopinata* and *B. microti* [13]) as the one species that naturally occurs in the rough form while retaining limited zoonotic potential.

While associations between rough *vs.* smooth phenotype and the full pathogenic potential of the bacteria, and the relationship between phenotype and fatty-acid content are not fully elucidated, aspects of these relationships have been described. The LPS smooth phenotype has been linked to the ability of *Brucella* to replicate in host cells [14], it has been suggested that the heterogeneity of the lipid A domain

on *Brucella* LPS may primarily depend on fatty-acid substitutions [15], and the LPS of smooth brucellae have been shown to contain lipid A and an O-polysaccharide, while the rough species investigated had only the lipid A-core oligosaccharide [16]. These findings suggest that significant variations in fatty-acid content are likely to alter virulence. Prior to this study, 19:0 cp had been identified in all of the species of *Brucella* considered to be pathogenic to humans (*B. abortus*, *B. melitensis*, *B. suis*) with the exception of *B. canis*. When *B. ovis*, a naturally occurring rough species that is not pathogenic to humans and *B. abortus* were compared, 19:0 cp was found in both, but in higher concentrations in *B. abortus* [17]. In another study by Dees *et al.* isolates defined as either *B. canis* or *B. suis* were compared, and a percentage of 19:0 cp (range 35–50%) similar to that found our study in Argentinean, Colombian and Mexican *B. canis* isolates (range 21.83–41.95%), was

(a)

Retention time (min)	Fatty-acid peak	Total fatty acids (%)
2.834	16:0	8.97
3.149	17:0	0.36
3.416	18:1 w7c	84.04
3.462	18:0	2.05
No additional significant peaks		*19:0 cp w8c
		*0



(b)

Retention time (min)	Fatty acid peak	Total fatty acids (%)
2.870	16:0	11.42
3.189	17:0	0.51
3.457	18:1 w7c	39.44
3.505	18:0	5.20
3.530	11-methyl 18:1 w7c	1.02
*3.793	*19:0 cp w8c	*37.50
4.035	20:2 w6,9c	0.78

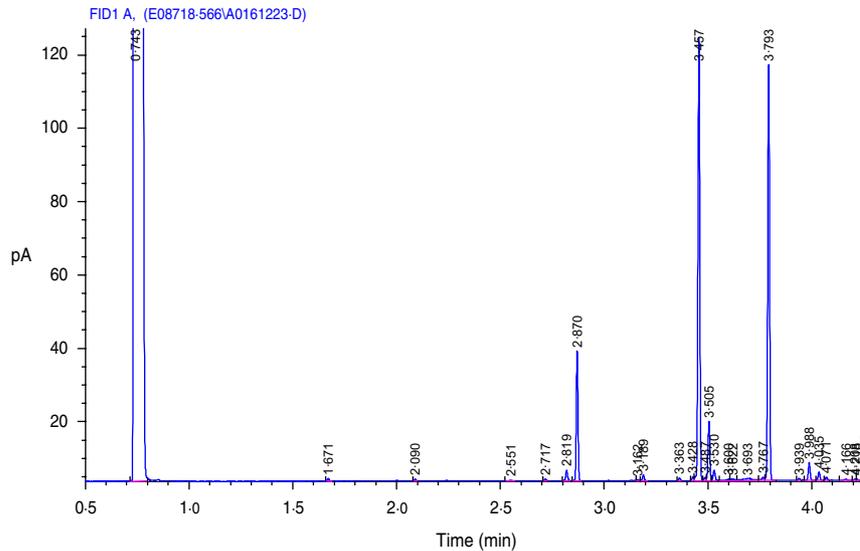


Fig. 2 [colour online]. (a) Features of non-Argentinean, Colombian or Mexican isolates showing a typical US *B. canis* profile and corresponding chromatograph (isolate nos. 10–43). (b) Features of Argentinean, Colombian and Mexican isolates (isolate nos. 1–9) showing a *B. canis* isolate profile from Argentina and corresponding chromatograph. 19:0 is indicated by an asterisk (*).

interpreted as indicative of 'rough mutants of *B. suis*', where the percentage of total cellular acids was reported at 35–50% [18]. This earlier conclusion appears to be a different interpretation of similar results to those we have presented, and highlights the difficulty in correctly speciating *Brucella* prior to the development of the types of molecular techniques employed in our study. Until now, fatty-acid composition has not been scrutinized in a large number of *B. canis* isolates speciated using methods beyond classical biochemical and morphological techniques and our findings contradict the previous conclusion that fatty-acid content as high as 41.95% 19:0 cp excludes isolates as *B. canis*.

Detailed epidemiological data, not available for many of the isolates used in this study, is needed to better understand if there is increased zoonotic potential in the 19:0 cp variants of *B. canis*. Close examination of the US outbreaks from which 19 of the 19:0 negative isolates (Table 1, isolate nos. 10–28) used in this study were obtained has indicated no clinical human infections in spite of close contact with infected dogs and their secretions. As mentioned previously, human blood culture-positive infections with canine *Brucella* are extremely rare, but the Argentinean isolate no. 5 (Table 1) was obtained from a human clinical case of canine brucellosis. In this case the patient left the hospital before the typing of the strain was completed and no further epidemiological data could be obtained. Isolate no. 1 (Table 1), also from Argentina, was recovered from a dog epidemiologically linked with the seroconversion of its owners. A paucity of isolates available for study from true human infections, and scant epidemiological data on human cases has significantly limited investigation into human *B. canis* infection. However, the historical reports, serological data, and isolate data we present in this study, and the important difference we have found between fatty-acid content within isolates of *B. canis* suggest that *B. canis* isolates from Mexico, Colombia and Argentina should be further scrutinized. Future investigations will be aimed at determining if isolate variability in fatty-acid content is epidemiologically linked to human *B. canis* infection.

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DECLARATION OF INTEREST

None.

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