strains, but the improved performance in the present study may also be a result of the development of the Vitek 2 database. Identification of *C. guilliermondii* and *Candida lusitaniae* was problematic, both in the study by Graf *et al*. [8] and in the present study, whereas all *C. glabrata* isolates were identified without difficulty. This is in contrast to the study by Massonet *et al*. [9], in which most difficulties were encountered with *C. glabrata*. The reason for this discrepancy is unknown. As in the study by Graf *et al*. [8], the present database was unable to separate *Candida inconspicua* and *Candida norvegensis*.

Conventional identification methods are still considered to be the reference standard for the identification of yeast isolates, but are laborious and time-consuming, and are suited better to research than to clinical laboratories. The Vitek 2 system identifies most clinically important *Candida* spp. reliably within 15 h, and appears to be an excellent alternative identification method for clinical laboratories performing fungal diagnostics.

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RESEARCH NOTE

A new variant of *Brucella melitensis*

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ABSTRACT

Brucella melitensis is highly pathogenic and constitutes a serious risk to public health. In Argentina, biovar 1 has been isolated from infected animals, but the Rev.1 strain vaccine is not authorised for use. This report describes nine atypical *B. melitensis* isolates obtained from humans. These isolates grew slowly, produced small colonies and were susceptible to penicillin and dyes, similar to the *B. melitensis* Rev.1 vaccine strain, but were inhibited by streptomycin 2.5 mg/L. The isolation of such atypical *B. melitensis* variants has never been reported from animals in Argentina, and could indicate the emergence of a new mutant variant.

Keywords *Brucella melitensis*, identification, phenotypic characteristics, Rev.1 vaccine, variant

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Although the organisms constituting the genus *Brucella* are highly homogeneous, the genus is classified currently into six nomen species: *Brucella abortus*, *Brucella suis*, *Brucella melitensis*, *Brucella canis*, *Brucella ovis* and *Brucella neotomae* [1–3]. This taxonomic scheme correlates somewhat with the preferential natural host, although the first three species can also infect other animals [4]. Differences in virulence have been observed, and the apparent rank virulence order shown in guinea-pigs seems to be similar to that in humans: i.e., *B. melitensis* >> *B. suis* >> *B. abortus* [5]. Each species is subdivided into biovars on the basis of cultural, biochemical and serological differences, but the three biovars of *B. melitensis* are distinguished solely by their immunochemical reactions with monospecific anti-lipopolysaccharide (LPS) A and M-determinant sera [6]. Isolation of atypical *B. melitensis* variants in Israel, and of dye-sensitive strains in various countries, has been reported, indicating that differences are not limited to the agglutination pattern [7,8].

The Argentine National Human Brucellosis Network (NHBN) at the ANLIS Dr C.G. Malbrán (Buenos Aires, Argentina) studied 118 *B. melitensis* isolates from humans during the period 1994–2004. The isolates were obtained from clinical laboratories in Argentine provinces, and were sent to the NHBN headquarters for characterisation. The present study reports the isolation and typing of nine *B. melitensis* isolates with atypical phenotypic characteristics that could indicate the emergence of a new mutant variant. All nine atypical isolates were differentiated from other Gram-negative organisms on the basis of morphology, motility, lactose fermentation on MacConkey agar, acid production on agar containing glucose, haemolysis on blood agar, catalase, oxidase and urease (Christensen method) reactions, and nitrate and citrate reduction. Rough/smooth phase variation was observed using obliquely reflected light, suspending a colony in acriflavine, and by staining colonies with crystal violet [6].

After the isolates were identified as members of the genus *Brucella*, their species identification and biovar were established according to the recommendations of the International Committee on Bacterial Nomenclature (ICBN), Subcommittee on Taxonomy of the Genus *Brucella* [2]. Serum and CO_2 requirements, H_2S production, and growth in the presence of thionin (20 mg/L), basic fuschin (20 mg/L), safranin O (100 mg/L), erithrytol (1 g/L), penicillin (5000 IU/mL) and streptomycin (2.5 mg/L) added to Brucella Agar (BBL Microbiology Systems, Cockeysville, MD, USA) were determined. Growth patterns on thionin blue and malachite green (2 mg/L) were investigated, both with and without the presence of CO_2 5% v/v. Urease tests (Bauer's method) and agglutination with polyclonal monospecific anti-A, -M and -R antisera were performed [6]. Susceptibility to *Brucella* phages was determined using Tb, R/C, Wb and Iz phages at 1 and 10^4 routine test dilution (RTD) [9]. Reference strains *B. abortus* 544-2, *B. suis* 1330, *B. melitensis* biovar 1 16 M, biovar 2 63/9 and biovar 3 Ether, and *B. melitensis* Rev.1, were included in each test as controls. Finally, PCR-RFLP (restriction fragment length polymorphism) analysis was performed by digesting the amplified *omp25* gene with *Eco*RV, and the amplified *rpsl* gene with *Nci*I [10,11].

Brucellosis is not a sustainable disease in humans, and the source of infection always resides in domestic or wild animals, or their derived products. However, new *Brucella* strains and species may emerge as those existing already adapt to social and agricultural changes [12–14]. In Argentina, the sheep population (*c.* 15 million) is concentrated mainly in the south and north-east of the country, while goats (*c.* 4 million) are located mostly in the north-west. Surveys revealed a 0.5–0.8% prevalence of caprine brucellosis in the north-western provinces, with the isolation of *B. melitensis* biovar 1 from infected goats, although the use of the *B. melitensis* Rev.1 vaccine has not been authorised. Ovine brucellosis caused by *B. ovis* was found in regions where sheep are located, but *B. melitensis* has only been isolated from a few sheep [15].

Of the 118 *B. melitensis* isolates from humans, 107 (90.67%) belonged to biovar 1, two (1.69%) belonged to biovar 3, and nine (7.6%) were atypical. Isolate 874 was characterised in 1986 at the Pan American Zoonosis Center (PAHO/WHO) as atypical *B. melitensis* biovar 1 (Table 1). To check its stability, a suspension containing 10^8 CFU/mL was injected into two guinea-pigs and

Table 1. Clinical and epidemiological data for the nine atypical *Brucella melitensis* isolates

Strain	Argentina province	Source	Year isolated	Gender	Age (years)	Main symptoms	Epidemiology
874	Jujuy	Blood × 2	1986	M	ND	ND	ND
290[a]	Jujuy	Bone-marrow × 1	1996	F	7	Fever, pancytopenia, arthralgias, weigh loss	Ingestion of goat cheese
429	Córdoba	Blood × 2	2000	M	ND	ND	ND
489	Catamarca	Blood × 2	2000	F	5	Fever, arthralgias, hepatosplenomegaly	Ingestion of goat cheese
497	S. del Estero	Blood × 3	2000	M	18	Fever, weight loss	Rural worker
544	Jujuy	Bone-marrow × 1	2002	F	27	Fever, weight loss, asthenia	Bolivian farmer
582	Salta	Blood × 2	2002	ND	ND	Hepatosplenomegaly	ND
605	Tucumán	Blood × 3	2002	M	8	ND	Goat handling
712	Jujuy	Blood × 2	2003	M	30	Fever, weight loss, pancytopenia	Bolivian farmer

[a]Isolated from the same patient at three different times.
ND, no data.

Table 2. Differential characteristics of the atypical *Brucella melitensis* isolates in comparison with species belonging to the genus *Brucella*[a]

			Growth on media with						Agglutination in sera[g]		Lysis by phages[h]		
Strain	CO_2 requirement	H_2S production	Thionin[b]	Basic Fuchsin[b]	Safranin O [c]	Streptomycin[d]	Penicillin[e]	Urease[f]	A	M	Tb	Wb	Iz
290 IBS	(–)	(–)	(+/–)	(–)	(–)	(–)	(–)	(–)	(–)	(+)	(–)	(–)	(+/–)
429 IBS	(–)	(–)	(+)	(–)	(–)	(–)	(–)	(–)	(+)	(–)	(–)	(+/–)	(+)
489 IBS	(–)	(–)	(+/–)	(–)	(–)	(–)	(–)	(–)	(–)	(+)	(–)	(–)	(+/–)
497 IBS	(–)	(–)	(+)	(–)	(–)	(–)	(–)	(–)	(–)	(+)	(–)	(–)	(+/–)
544 IBS	(–)	(–)	(+/–)	(–)	(–)	(–)	(–)	(–)	(–)	(+)	(–)	(+/–)	(+)
582 IBS	(–)	(–)	(+/–)	(–)	(–)	(–)	(–)	(–)	(–)	(+)	(–)	(+/–)	(+/–)
605 IBS	(–)	(–)	(+/–)	(–)	(–)	(–)	(–)	(–)	(–)	(+)	(–)	(+/–)	(+/–)
712 IBS	(–)	(–)	(+/–)	(–)	(–)	(–)	(–)	(–)	(–)	(+)	(–)	(–)	(+/–)
874 IBS	(–)	(–)	(+/–)	(–)	(–)	(–)	(–)	(–)	(–)	(+)	(–)	(+/–)	(+/–)
B. melitensis 16 M[i]	(–)	(–)	(+)	(+)	(+)	(–)	(+)	(+)	(–)	(+)	(–)	(+/–)	(+)
B. melitensis 63/9[i]	(–)	(–)	(+)	(+)	(+)	(–)	(+)	(+)	(+)	(–)	(–)	(+/–)	(+)
B. melitensis Ether[i]	(–)	(–)	(+)	(+)	(+)	(–)	(+)	(+)	(+)	(+)	(–)	(+/–)	(+)
B. suis 1330[i]	(–)	(+ +)	(+)	(–)	(–)	(–)	(–)	(+ +)	(+)	(–)	(–)	(+)	(+)
B. abortus 544[i]	(+)	(+/–)	(–)	(+)	(+)	(–)	(+)	(–)	(+)	(–)	(+)	(+)	(+)
B. melitensis Rev.1[j]	(–)	(–)	(–)	(–)	(–)	(+)	(–)	(–)	(–)	(+)	(–)	(+/–)	(+)

[a]Results obtained after incubation with 5% CO_2; all the isolates grew with i-erythritol 1 mg/mL added to the basal medium.
[b]20 mg/L; [c]100 mg/L; [d]2.5 mg/L; [e]5000 IU/L in base medium.
[f]Bauer's method, all the *B. melitensis* isolates were positive on Christensen media.
[g]A, A monospecific antiserum; M, M monospecific antiserum; No isolate was agglutinated by rough (R) *Brucella* antiserum.
[h]With routine test dilution (RTD), no isolate was lysed by R/C phage.
[i]Reference strains; [j]vaccine strain; IBS, isolated *Brucella* strain.
(–) negative; (+/–) weakly positive; (+) positive; (++) strong positive.

was recovered after 6 weeks from their spleens. The isolates had the same phenotypical characteristics as the original strain, indicating that the isolate could be considered stable following animal passage.

All nine atypical isolates were smooth, grew slowly, produced small colonies and were susceptible to penicillin and dyes, similar to the Rev.1 vaccine strain, but were inhibited by streptomycin 2.5 mg/L (Table 2). A slight increase in the growth pattern on basic fuchsin was observed when CO_2 5% v/v was present during incubation, and also when sterile equine serum 5% v/v was added to the basal medium. However, growth was inhibited by thionin blue and malachite green, even in the presence of CO_2. Of the nine atypical *B. melitensis* isolates, eight were identified as biovar 1 and one as biovar 2, based on a difference in the quantitative distribution of the A and M antigens.

In Argentina, only *B. melitensis* biovar 1 has been isolated from animals, while two cases of infection with biovar 3 have been reported in humans [16] (IX Congreso de Argentino de Microbiología, Buenos Aires, 2001, abstract 220), probably because infected animals are diagnosed mainly by serological tests. The nine patients in the present study (Table 1) were hospitalised with fever and weight loss as the main symptoms. A girl (aged 7 years, isolate 290) relapsed 6 months after completing treatment and the same strain was isolated.

PCR-RFLP of the *omp25* gene with *Eco*RV showed that all nine isolates were true *B. meliten-*

sis. Susceptibility of *B. melitensis* to dyes and penicillin has been reported previously, as well as the finding that polymorphism of the *omp2* porin gene correlates with dye sensitivity [7,17,18].

This virulent atypical *B. melitensis* variant could not have originated from the vaccine Rev.1 strain (as determined by PCR-RFLP of the *rpsl* gene with *Nci*I), although some of its phenotypic characteristics resembled the vaccine strain. The variant could have been introduced into the country via an infected animal, since brucellosis can exist in a latent form for several years [19], or it could be a new adaptation variant of *B. melitensis*.

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