

# Typing of *Brucella* field strains isolated from livestock populations in Italy between 2001 and 2006

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## Summary

The identification of species and biovars of *Brucella* field strains isolated in outbreaks is essential to fully understand the epidemiology of the disease and to trace sources of infection, thereby improving the outcome of brucellosis eradication programmes. It is important to identify the presence of *Brucella* strains in livestock populations and to determine the presence of new strains that might previously have been considered exotic. In this study, 732 *Brucella* strains isolated from livestock were submitted for typing by the Italian *Istituti Zooprofilattici Sperimentali* to the National Reference Laboratory for brucellosis between 2001 and 2006. Animal species affected, biovars typed and spatial distribution of isolates are discussed. *Brucella* field strains were identified using both conventional bacteriological methods and molecular techniques. Species identification was performed using the AMOS (*AbortusMelitensis OvisSuis*)-polymerase chain reaction. For biovar identification, amplification of *omp2a*, *omp2b* and *omp31* genes was performed, followed by restriction fragment length polymorphism. Final biovar identification was performed by growth in the presence of basic fuchsin and thionin, using the slide agglutination test with *Brucella* A- and M-specific antisera.

## Keywords

*Brucella*, Brucellosis, Epidemiology, Isolation, Italy, Polymerase chain reaction, Typing.

## Tipizzazione di ceppi di campo di *Brucella* isolati da animali d'allevamento in Italia tra il 2001 e il 2006

### Riassunto

L'identificazione di specie e biovariante dei ceppi isolati in focolai di brucellosi è uno strumento essenziale per comprendere pienamente l'epidemiologia della malattia e fornire il supporto al rintraccio delle fonti di infezione, migliorando in tal modo i risultati di un programma nazionale di eradicazione della brucellosi. È necessario identificare quali sono i ceppi di *Brucella* spp, presenti negli animali d'allevamento e verificare inoltre l'eventuale comparsa di nuovi sierotipi precedentemente considerati esotici. In questo studio sono stati identificati 732 ceppi di *Brucella* spp., isolati presso i laboratori degli Istituti Zooprofilattici Sperimentali in Italia ed inviati al centro di Referenza Nazionale per Brucellosi dal 2001 al 2006. Vengono illustrate: le specie animali infette, le biovarianti identificate e la distribuzione spaziale degli isolati. I ceppi di *Brucella* spp. sono stati identificati utilizzando sia metodi convenzionali che metodi molecolari. L'identificazione di specie è stata effettuata utilizzando l'AMOS (*abortus-melitensis-ovis-suis*) polymerase chain reaction. L'identificazione delle biovarianti è stata effettuata mediante amplificazione di *omp2a*, *omp2b* e *omp31*, seguita da RFLP. L'identificazione finale delle biovarianti è stata poi effettuata mediante crescita dei ceppi su agar tionina e agar fucsina e agglutinazione su vetrino con antisiero anti-A e anti-M.

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### Parole chiave

*Brucella*, Brucellosi, Epidemiologia, Isolamento, Italia, Polymerase chain reaction, Tipizzazione.

## Introduction

A national brucellosis eradication programme has been implemented in Italy for both cattle (2) and for sheep and goats (1). A crucial factor for the success of an eradication campaign is the implementation of an effective surveillance system. The principal purposes of such a system are the determination of needs for immediate or longer range actions in response to diseases and, through data analysis, the determination of priorities for such longer range actions, design of alternative actions and determination of their likely costs and benefits (11). The passive collection of data, such as the identification of strains involved in brucellosis outbreaks, is one of the major components of a surveillance system. The identification of species and biovars of *Brucella* field strains isolated in outbreaks is an essential tool to better understand the epidemiology of the disease and to offer support for trace-back of infection sources. It is also essential to identify the presence of *Brucella* strains that can affect livestock populations and new strains that were previously considered to be exotic. The knowledge of the prevailing species and biovars of *Brucella* present in a country is therefore an important epidemiological tool when formulating policies and strategies for the eradication of the disease in animal populations. Consequently, this study considered those *Brucella* strains isolated from cattle, water buffalo, sheep and goats that were submitted for typing by the Italian *Istituti Zooprofilattici Sperimentali* (Italian State veterinary laboratories) to the National Reference Laboratory for brucellosis between 2001 and 2006.

The aim of this paper is to provide an overview of the *Brucella* strains isolated from livestock in Italy during the above five-year period, with the aim of providing information that will contribute to the national brucellosis eradication programme by providing a better

understanding of the current epidemiology of brucellosis in Italy.

## Materials and methods

### Bacterial isolates and reference strains

Although epidemiological investigations are compulsory in each brucellosis outbreak recorded in Italy, the isolation of *Brucella* is not performed systematically in all infected herds or flocks.

Specimens used were transported in accordance with the World Health Organization (WHO) guidelines (14). The collection of data, such as animal species and region of origin, was standardised. All laboratories were required to complete a standard form giving relevant epidemiological data on the brucellosis outbreak in which the *Brucella* strain submitted for typing was isolated. The standard form was available on the National Brucellosis Reference Centre website ([www.izs.it](http://www.izs.it)). Reference strains of some *Brucella* species, as well as the *Brucella* polyvalent and monospecific *Brucella* A- and M- antisera were supplied by the Food and Agriculture Organization (FAO)/WHO Collaborating Centre for Reference and Research on Brucellosis (Veterinary Laboratories Agency [VLA] in Weybridge). Some strains were also supplied by the Health Protection Agency with a National Collection of Type Cultures (NCTC) bank number. All *Brucella* field isolates were subcultured in *Brucella* agar base and stored using the Microbank system at  $-80^{\circ}\text{C}$ .

### Isolation procedures

In all laboratories involved, the culturing of samples was performed in accordance with the techniques described in chapters 2.3.1. and 2.4.2. of the *Manual* of the World Organisation for Animal Health (*Office International des Epizooties*: OIE) (15).

### Identification procedures

Colonies typical of *Brucella* species were subcultured onto serum dextrose agar from which subsequent growth was examined by

Gram stain and catalase, oxidase, and urease tests (15).

### Molecular identification

The *AbortusMelitensisOvisSuis* (AMOS) polymerase chain reaction (PCR) is a multiplex PCR designed to detect selected biovars of four species of *Brucella* (3). The assay exploits the polymorphism arising from the species-specific localisation of the genetic element IS711 in the *Brucella* chromosome. Individual biovars within a species are not differentiated (4). The PCR mix used was a PCR Master Mix by Promega.

Amplifications were performed for 33 cycles in a thermal cycler GeneAmp® PCR System 9700 (PE Applied Biosystems) at the annealing temperature of 60°C. Amplicons were checked by fluorescence after electrophoresis in a 1% agarose gel in the presence of ethidium bromide.

Three different PCRs were used to amplify outer membrane protein genes of *Brucella*, omp2a, omp2b and omp31. The amplicons of omp2a, omp2b and omp31 genes were digested by endonucleases (Pst I, Hinf I, Taq I, Ava II, Nco I) and the products of digestion were checked by fluorescence after electrophoresis in 3% agarose gel in the presence of ethidium bromide.

The specific biovar pattern was obtained crossing the results of the single omp restrictions (5, 13).

### Complementary tests

Tests performed to assist PCR in the final biovar identification were as follows:

- H<sub>2</sub>S production
- growth on selective agar: basic fuchsin (20 µg/ml) and thionin (20 µg/ml)
- CO<sub>2</sub> requirement (7).

Final biovar identification for *Brucella abortus* biovars 1, 2 and 4 and *B. abortus* biovars 3, 5, 6 and 9 was performed by growth in the presence of basic fuchsin and thionin using the slide agglutination test with *Brucella* A- and M-monospecific antisera (VLA, Weybridge).

## Results

The total number of strains examined is given in Table I. In total, 732 strains submitted from 13 regions in Italy were analysed. Strains isolated from livestock were obtained from cattle (301 isolates), water buffalo (*Bubalus bubalis*) (63 isolates), and sheep and goats (368 isolates). Strains submitted were identified as *B. abortus* (244 isolates), *B. melitensis* (479 isolates) and *B. ovis* (9 isolates). The geographic distribution of *Brucella* strains isolated from cattle and water buffalo is presented in Figure 1. The strains isolated from cattle were *B. abortus* biovars 1, 3 and 6. *B. melitensis* biovar 3 was also isolated from cattle in several regions. The same strains were isolated from water buffalo. In line with the distribution of the water buffalo population in Italy, most strains were isolated in Campania region.

The geographic distribution of *Brucella* strains isolated from sheep and goats is presented in Figure 2. All *B. melitensis* strains isolated were biovar 3. *B. ovis* was isolated in Lombardy, Piedmont and Abruzzo. *B. abortus* biovar 3 was also isolated from sheep in Calabria.

## Discussion

Knowledge and understanding of the prevailing species and biovar of *Brucella* that infect livestock is crucial when formulating policies and strategies for the control of brucellosis in animal populations. The additional knowledge provided by this study on the epidemiology of brucellosis in Italy may be useful for that purpose. However, it should be stressed that the isolation of *Brucella* is not performed systematically in all brucellosis outbreaks in Italy; results of this study may therefore be affected by bias and distortions. Nevertheless, given the length of the period under study and the number of strains analysed, this present study provides a useful overview of the current epidemiology of the disease in Italy, even if it is not fully representative.

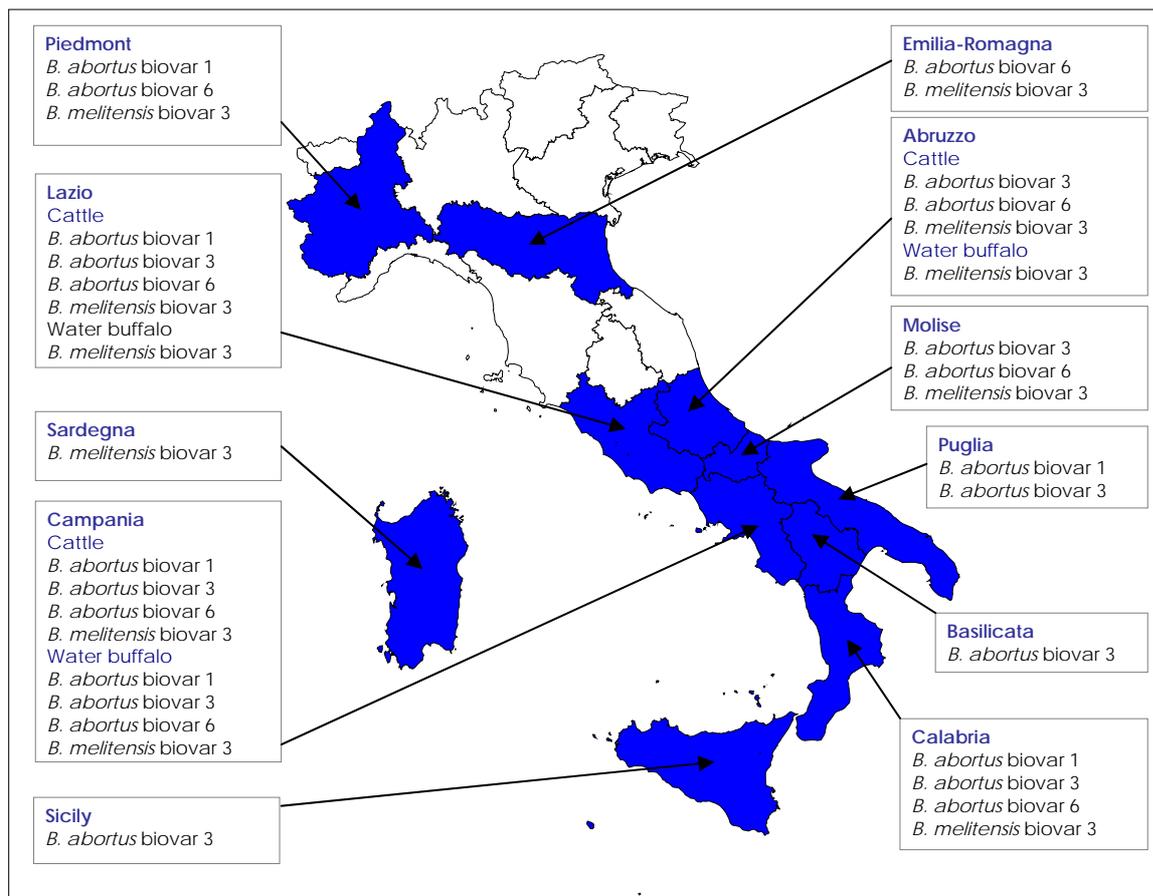


Figure 1  
Geographic distribution of *Brucella* strains isolated from cattle and water buffalo in Italy between 2001 and 2006

### Strains isolated from cattle and water buffalo

The isolation of biovars 1, 3 and 6 of *B. abortus* in cattle and water buffalo (Table I) is in agreement with previous reports on the *B. abortus* biovars isolated from cattle in Italy. Farina (9) reported the isolation of six out of the eight known *B. abortus* biovars in Italy, namely: biovars 1, 2, 3, 4, 6 and 7. Other authors have also reported the isolation in Italy of *B. abortus* biovars 6 and 7 in cattle (8). The absence of isolation of biovars 2, 4 and 7 in the last five years, as reported in the present study, may suggest that these biovars may have been eradicated from the country, which would now make them exotic. The geographic distribution of *B. abortus* biovars isolated did not show any particular regional clustering.

A high number of *B. melitensis* isolates has been recorded in this study, both in cattle and

water buffalo. The percentage of *B. melitensis* isolates of the total number of strains submitted for typing was 36.4% for cattle and 45.6% for water buffalo (Table I). This suggests that *B. melitensis* infection in cattle and water buffalo is emerging as an important animal and public health problem in Italy. *B. melitensis* can be shed in milk by infected cows. Infection among farm workers, slaughterers and veterinarians may occur through handling infected animals or organs after slaughter (12). Moreover, *B. melitensis* infection in cattle is particularly problematic because *B. abortus* vaccines do not protect effectively against *B. melitensis* infection and the *B. melitensis* Rev. 1 vaccine has not been fully evaluated for use in cattle (6). This should be taken into consideration when implementing bovine and water buffalo brucellosis control programmes using vaccination in areas in which *B. melitensis* is endemic in sheep and goats.

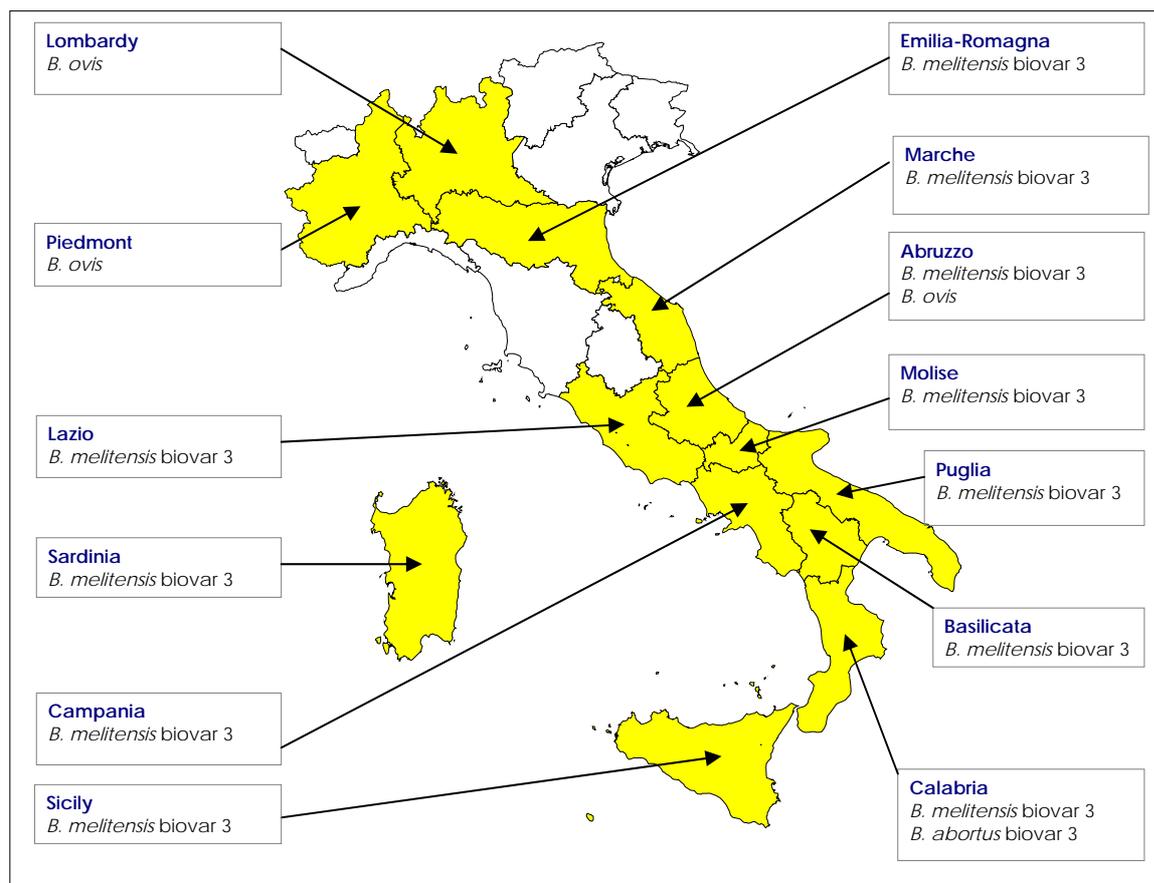


Figure 2  
Geographic distribution of *Brucella* strains isolated from sheep and goats in Italy between 2001 and 2006

### Strains isolated from sheep and goats

*B. melitensis* biovar 3 represents 95.7% of the total number of strains isolated in sheep and goats in Italy over the past five years. Farina reported the isolation of all the three known biovars of *B. melitensis*, of which over 90% were biovar 2 (9).

*B. abortus* has been isolated from sheep in Calabria. *B. abortus* abortions in sheep and goats have seldom been reported in the past

and the possibility of shedding the strain in milk has been documented in sheep (10). It has not been reported further in the last few years. Nevertheless, an evaluation of risk factors, including husbandry practices and exposure potential, should be made to determine the need to test sheep and goats that may have been exposed to cattle infected with *B. abortus*. The same should apply for cattle exposed to *B. melitensis*-infected sheep and goats.

Table I  
*Brucella* strains submitted to the National Reference Centre for typing by 13 regions in Italy between 2001 and 2006

<i>Brucella</i> strain	Biovar	Cattle ( <i>Bos taurus</i> )	Water buffalo ( <i>Bubalus bubalis</i> )	Sheep/goats ( <i>Ovis aries</i> / <i>Capra hircus</i> )	Total
<i>B. abortus</i>	1	53	15	–	68
	3	61	16	13	90
	6	80	6	–	86
<i>B. melitensis</i>	3	107	26	346	479
<i>B. ovis</i>	–	–	–	9	9
Total		301	63	368	732

*B. ovis* has been isolated in sheep in two northern regions of Italy (Piedmont and Lombardy) (Fig. 2) and in a central region (Abruzzo) (Fig. 2) which suggests the presence of the infection in the Italian sheep population, but this has never been actively investigated since *B. ovis* infection is not considered a priority.

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