La gestione delle prove dirette per Brucella nei piani di eradicazione

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Direzione Operativa Diagnostica Generale

- Struttura Complessa presso la Sede Centrale di Roma
- Centro di riferimento (bi)-regionale per agenti zoonosici Speciali (inclusi alcuni agenti di classe di rischio 3: Brucella spp., M. bovis)
- Gestisce prove dirette per Brucella per l'IZSLT

Capacità di Laboratorio

- Riferimenti per le prove colturali e molecolari:

OIE Terrestrial Manual 2012. BOVINE BRUCELLOSIS. CH. 2.4.3. (1.b; 1.c; 1.d)

Version adopted by the World Assembly of Delegates of the OIE in May 2009.

- La Struttura esegue prove colturali dirette per Brucella secondo POS accreditata (ISO/IEC 17025 Standard)
- Validazione interna (Manual OIE, Ch 1.1.15 OIE);
- In seguito, evidenza di mantenimento validità con circuito interlab CRN, IZS Abruzzo e Molise

Approccio alla validazione del metodo

- Validazione secondo principi ISO & OIE Manual, fin dal 2003.

Attualmente:

- Ch. 1. 1.15: Principles and methods of validation of diagnostic assays for infectious diseases (2013)

Validazione del metodo

- **Valutazione caratteristiche performance della prova** (D-Se & D-Sp): Preparazione di campioni POSITIVI e NEGATIVI di Riferimento (n=30 e n=90) nel 2003
- Tabella di contingenza 2X2
- D-Se, D-Sp (e 95% I. C.)
- Accettabilità: D-Se: >=95%; D-Sp>=98%

1.b Cultures

- Basal media: Brucella medium base+5% equine serum
- Selective media:

-Farrell's Medium (VCN supplement)

Farrell's medium (Farrell,1974), which is prepared by the addition of six antibiotics to a basal medium. The following quantities are added to 1 litre of agar: polymyxin B sulphate (5000 units = 5 mg); bacitracin (25,000 units = 25 mg); natamycin (50 mg); nalidixic acid (5 mg); nystatin (100,000 units); vancomycin (20 mg).

Incubation: 8-10 days

(Feti e campioni da aborti: alte concentrazioni di Brucella spp.)

Colture primarie (a 7 gg) di feto ovino abortito, 12/2014,
su Farrell’s medium (*B. melitensis* 3)

Alternative selective solid medium

-Modified Thayer-Martin's medium (for some B. abortus & B. melitensis biotypes)

GC medium base, Hb 10g/L, colistin (7.5 mg/litre), vancomycin (3 mg/litre), nitrofurantoin (10 mg/litre), nystatin (100,000 I U/ litre = 17.7 mg) and amphotericin B (2.5 mg/litre)

Selective enrichment

-Brucella broth/Tryptose (Trypticase)-soy broth+5% equine serum for enrichment:

supplemented with an antibiotic mixture of at least amphotericin B (1 µg/ml), and vancomycin (20 µg/ml) (all final concentrations).

The enrichment medium should be incubated at 37°C in air supplemented with 5–10% (v/v) CO_2 for up to 6 weeks, with weekly subcultures onto solid selective medium

Cultures (OIE Manual of Standards, 2009)

- Singoli reattori, casi sospetti o dubbi:

- Media di arricchimento incubati at 37°C in 5-10% (v/v) CO_2 fino a 6 settimane (operativamente di solito 3), con:

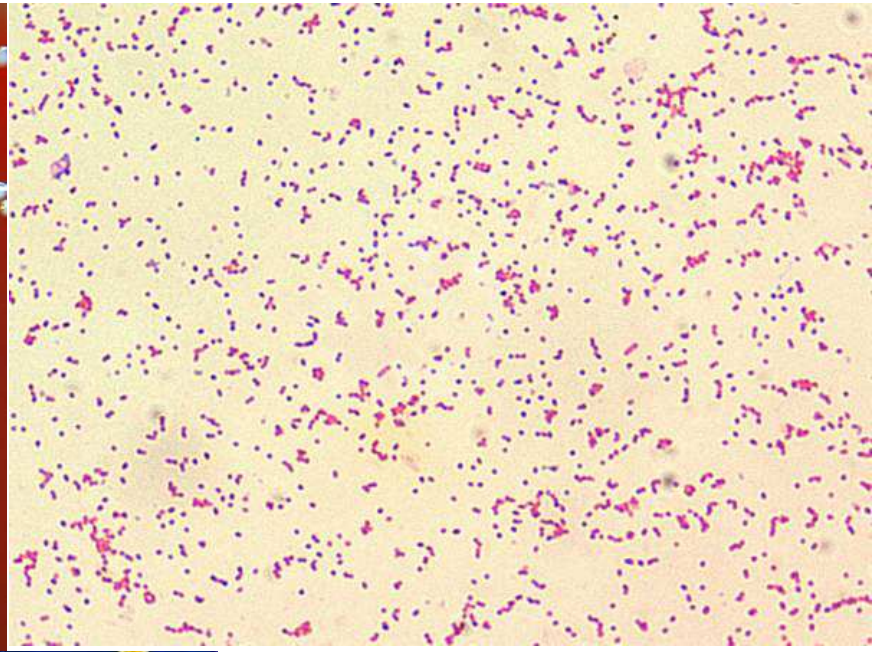
In parallelo

-subcolture media selettivi solidi

-PCR testing

Entrambe ad intervalli settimanali

Caratteristiche fenotipiche macroscopiche e microscopiche di Brucella spp.



Identificazione degli isolati a livello di Genere

1.c Identification and typing

- Test biochimici differenziali (min. CAT, OX, URE), oppure

Gallerie di test biochimici differenziali (es. API 20NE)

- slide agglutination: acriflavina, siero polivalente, o anche monovalenti A e M)
- **PCR testing** (Brucella spp.):

  -End-point PCR:

- specifici primers per amplificazione di sequenza di 223 bp di antigene immunogenico di 31 kDa di Brucella abortus **(BCPS31,** Baily et al., 1992; primers: Elfaki et al., 2005)

  -Real-Time PCR (Bounaadja et al., 2009)

Nucleic acid recognition methods…

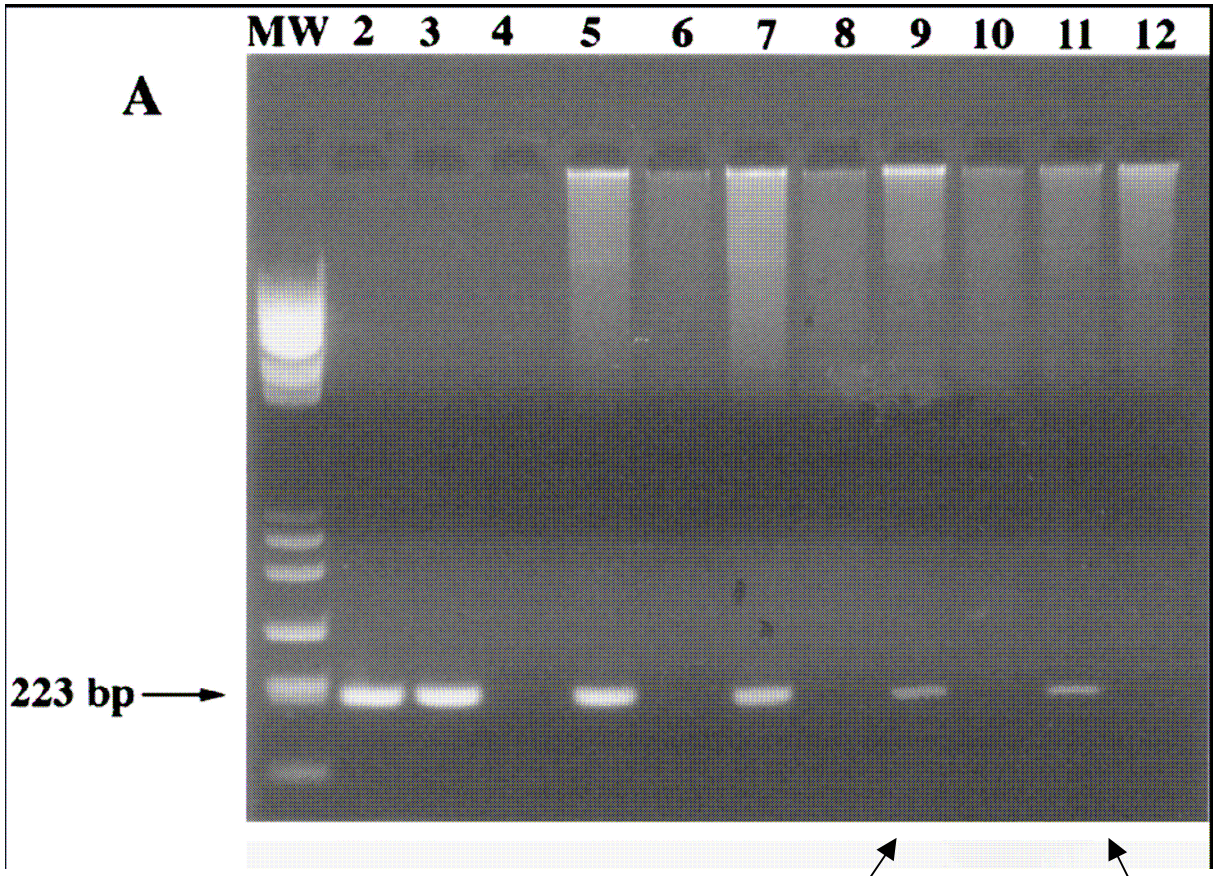
- **1. d) Nucleic acid recognition methods**

"The PCR, including the real-time format, provides an additional means of detection and identification of Brucella sp. (Bricker, 2002; Bricker et al., 2003; Bricker & Halling, 1994; 1995; Garcia-Yoldi et al., 2006; Hinićet al., 2008; Ocampo-Sosa et al., 2005). (….)"

Capitolo Brucellosi dell'OIE Manual non aggiornato dal 2009:

non cita la Ref. Rael-Time PCR del EU-RL/OIE-RL

BCPS31 PCR (223 bp amplicon**)**



Altri approcci molecolari

- Per l'evidenziazione di sequenze nucleotidiche specifiche per *Brucella spp*. da colture (arricchimento) e materiali biologici (es. Latte, tessuti mammari, linfatici):

Real-Time PCR basata su IS 711 locus

Detection & ID by Real-Time PCR

Veterinary Microbiology 137 (2009) 156–164

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Real-time PCR for identification of *Brucella* spp.: A comparative study of IS*711*, *bcsp31* and *per* target genes

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ABSTRACT

Culture is considered as the reference standard assay for diagnosis of *Brucella* spp. in humans and animals but it is time-consuming and hazardous. In this study, we evaluated the performances of newly designed real-time PCR assays using TaqMan® probes and targeting the 3 following specific genes: (i) the insertion sequence IS*711*, (ii) *bcsp31* and (iii) *per* genes for the detection of *Brucella* at genus level. The real-time PCR assays were compared to previously described conventional PCR assays targeting the same genes. The genus-specificity was evaluated on 26 *Brucella* strains, including all species and biovars. The analytical specificity was evaluated on a collection of 68 clinically relevant, phylogenetically related or serologically cross-reacting micro-organisms. The analytical sensitivity was assessed using decreasing DNA quantities of *Brucella* ovis, *B. melitensis* bv. 1, *B. abortus* bv. 1 and *B. canis* reference strains. Finally, intra-assay repeatability and inter-assay reproducibility were assessed. All *Brucella* species DNA were amplified in the three tests. However, the earliest signal was observed with the IS*711* real-time PCR, where it varied according to the IS*711* copy number. No cross-reactivity was observed in all three tests. Real-time PCR was always more sensitive than conventional PCR assays. The real-time PCR assay targeting IS*711* presented an identical or a greater sensitivity than the two other tests. In all cases, the variability was very low. In conclusion, real-time PCR assays are easy-to-use, produce results faster than conventional PCR systems while reducing DNA contamination risks. The IS*711*-based real-time PCR assay is specific and highly sensitive and appears as an efficient and reproducible method for the rapid and safe detection of the genus *Brucella*.

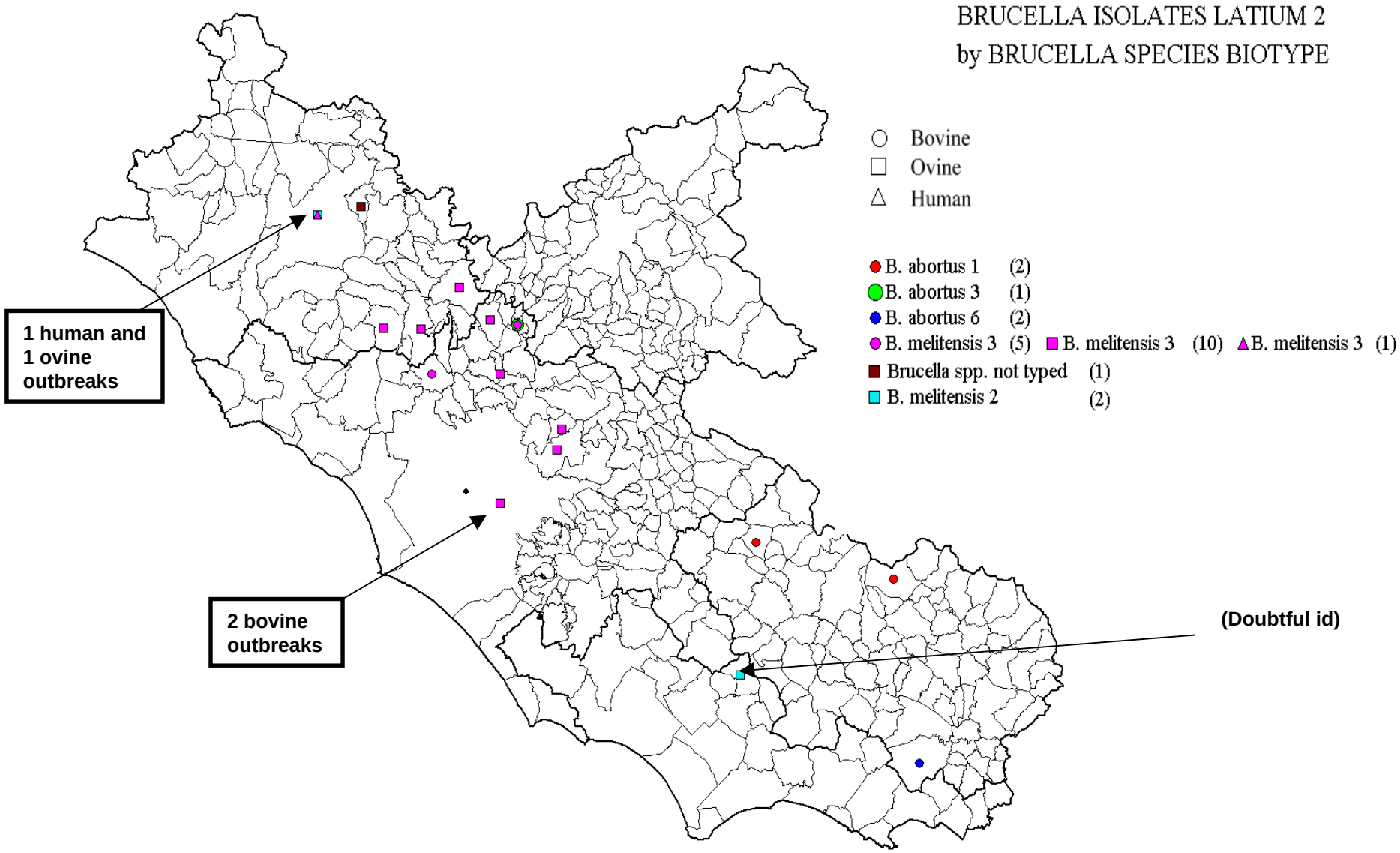
Parametri per la Validazione (Real-Time) PCR

- I parametri considerati nel processo di validazione del metodo di PCR Real Time per la ricerca del gene target IS711 in Brucella spp. sono:
- Specificità analitica (Analytical Specificity, **Asp**, Ch. 3.6.3, 1.2)
- Limite di rivelazione (LOD) o Sensibilità analitica (Analytical Sensitivity, **Ase**, Ch. 3.6.3, 1.3)
- Robustezza
- Sensibilità Diagnostica
- Specificità Diagnostica

Tipizzazione a livello di specie e biotipo

- Attualmente la DO Diagnostica Generale non investe in risorse per la successiva caratterizzazione.
- Isolati inviati a CRN Brucellosi, IZS Abruzzo e Molise, Teramo

Brucella isolates, Latium



Brucella suis

- 5 biovars, di cui solo le prime tre ritenute infettanti il suino
- In Europa B. suis 2 è descritta in alcune popolazioni di cinghiali dell'Europa dell'Est e anche in MS (Italia, Portogallo, Francia)
- La situazione epidemiologica non è chiara nella maggior parte dei MS, ma si sospetta che molte popolazioni di cinghiali mantengano l'agente in EU

Allevamento suino semi-brado, *B. suis* 2

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Short communication

The presence of *Brucella ceti* ST26 in a striped dolphin (*Stenella coeruleoalba*) with meningoencephalitis from the Mediterranean Sea

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ABSTRACT

Brucella spp. was isolated from brain, lung and intestinal lymph nodes of a dead adult male striped dolphin (*Stenella coeruleoalba*) found stranded on the Tyrrhenian coast (Tuscany, Italy) of the Mediterranean Sea in February 2012. *Brucella* spp. was associated with moderate to severe lesions of meningoencephalitis. A co-infection by *Toxoplasma gondii* was also demonstrated at brain level by means of molecular and histopathologic methods. The *Brucella* isolate was further characterized based on a fragment-specific polymerase chain reaction (PCR) approach, consisting of a set of five specific PCRs, targeting specific chromosomal IS*711* locations for marine mammal *Brucellae*, as described previously. The isolate was thus classified as *Brucella ceti* I; V fragment-positive (or *B. ceti* dolphin type), according to previous studies. Multi Locus Sequence Analysis demonstrated that the isolate belongs to Sequence Type 26, while *omp2* (*omp2a* and *omp2b* genes) sequence analysis further confirmed the isolate belonged to this group of strains. This is the first report of *Brucella* spp. from marine mammals in the Mediterranean Sea, and represents a further observation that this strain group is associated with hosts of the Family *Delphinidae*, and particularly with the striped dolphins, also in the Mediterranean area, thus constituting a further biological hazard of concern for this vulnerable subpopulation.

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