



IZSAM G.CAPORALE
TERAMO



Brucellosi

Centro di Referenza Nazionale



Reference Laboratory
Brucellosis

Nuovi strumenti di epidemiologia molecolare per lo studio delle brucellosi in Italia

Giornata di studio sulla brucellosi. Teramo 26-27 febbraio 2015

Massimo Ancora - m.ancora@izs.it

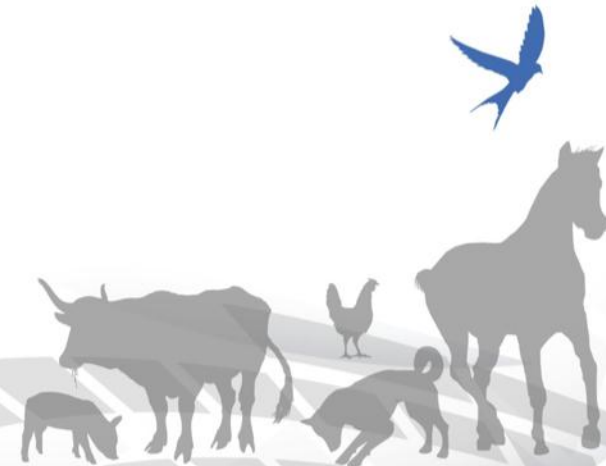




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Focus

- ✓ **Principi di tipizzazione molecolare**
- ✓ **Tools molecolari: stato dell'arte**
- ✓ **Prospettive future**

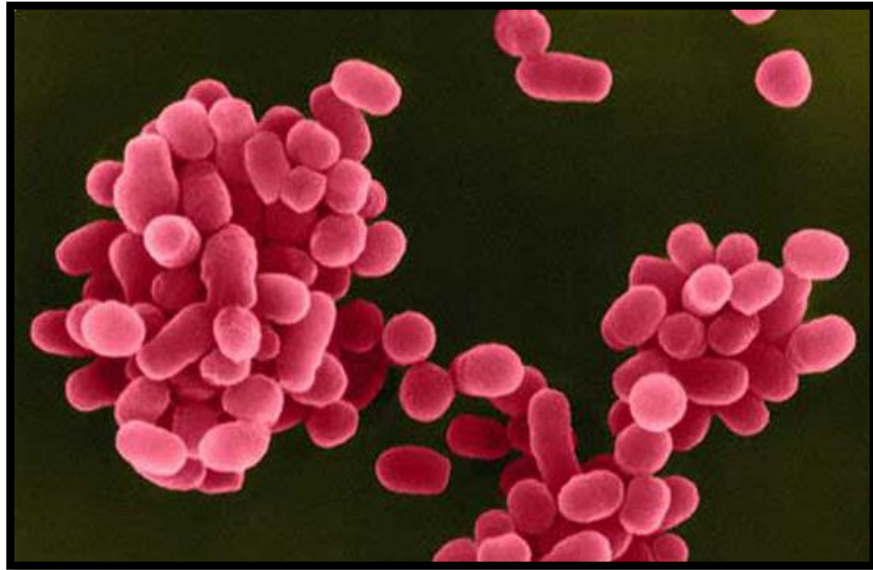


Brucellosi

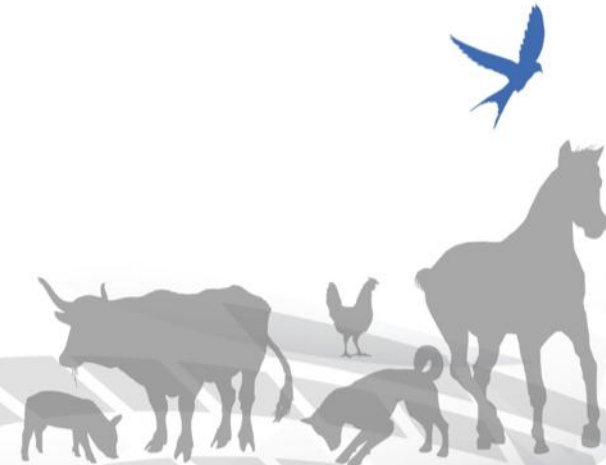
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Sir David Bruce



SEM x 29,650 © [Dr Dennis Kunkel](#), University of Hawaii



Classificazione

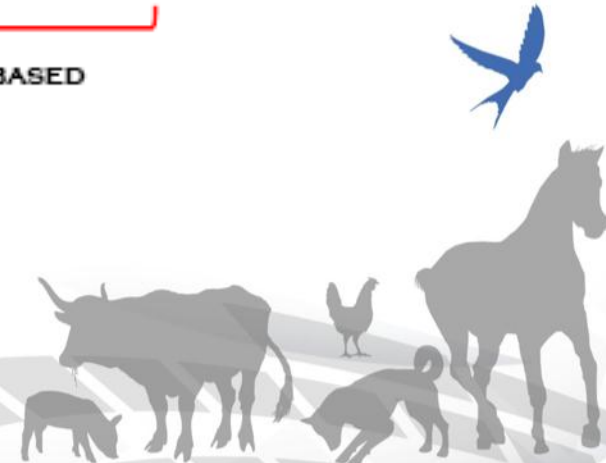
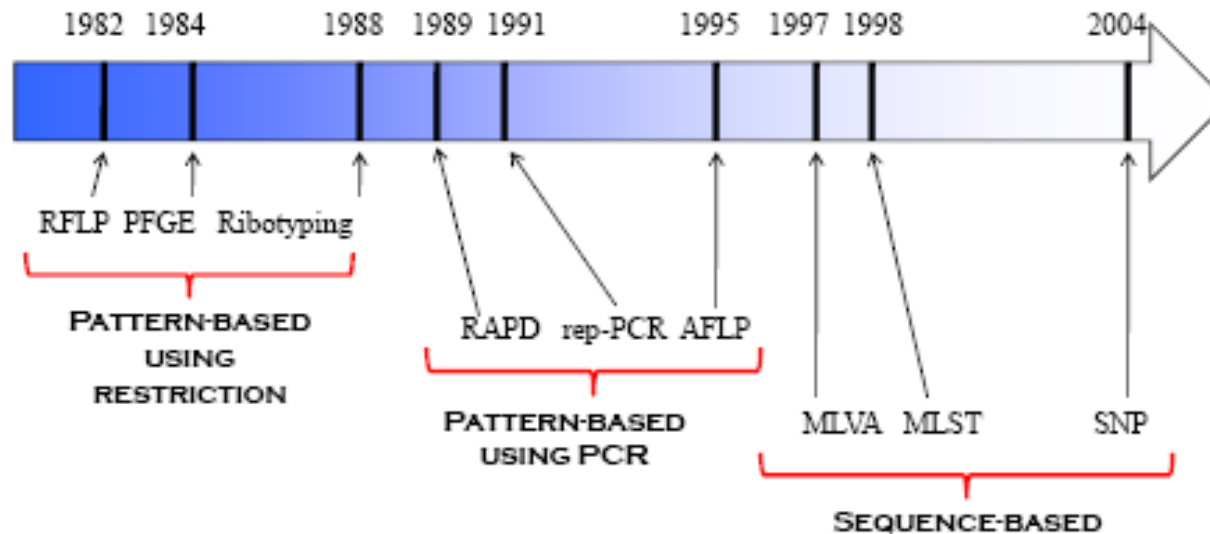
Species	Biovars	Preferred hosts
'Classical' species		
<i>B. melitensis</i>	1–3	Sheep, goats, camels
<i>B. abortus</i>	1–6, 7, 9	Cattle, bison.
<i>B. suis</i>	1–5	Pigs, hares, reindeer, rodents
<i>B. canis</i>		Dogs
<i>B. neotomae</i>		Desert rat
<i>B. ovis</i>		Sheep
Newly described species		
<i>B. pinnipedialis</i>		Seals
<i>B. ceti</i>		Cetaceans
<i>B. microti</i>		Voles
<i>B. inopinata</i>		Unknown

O'Callaghan D. and Whatmore A.M., 2011



Tipizzazione molecolare

Un po' di cronistoria:



Brucella typing

Metodi su base enzimatica:

Pulsed-field gel electrophoresis (PFGE)

Allardet-Servent, A. *et al.* (1988): "DNA polymorphism in strains of the genus *Brucella*." *Journal of bacteriology* 170.10 4603-4607.

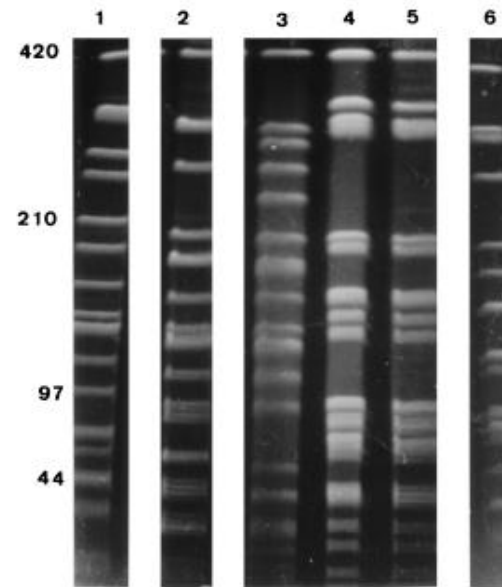
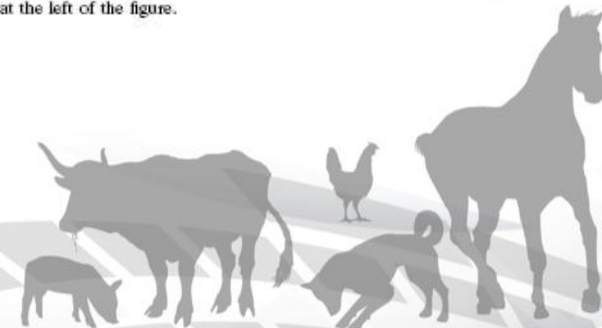


FIG. 1. PFGE. *SpeI* digestion of genomic DNA from six *Brucella* species reference strains. Lane 1, *B. melitensis* 16M; lane 2, *B. abortus* 544; lane 3, *B. ovis* 63/290; lane 4, *B. suis* 1330; lane 5, *B. canis* RM/666; lane 6, *B. neotomae* 5K33. Size markers in kilobases are at the left of the figure.



Brucella typing

Metodi su base enzimatica:

Insertion sequence based typing IS711

Ouahrani, S. *et al.* (1993). Identification and sequence analysis of IS6501, an insertion sequence in *Brucella* spp.: relationship between genomic structure and the number of IS6501 copies. *Journal of general microbiology*, 139(12), 3265-3273.

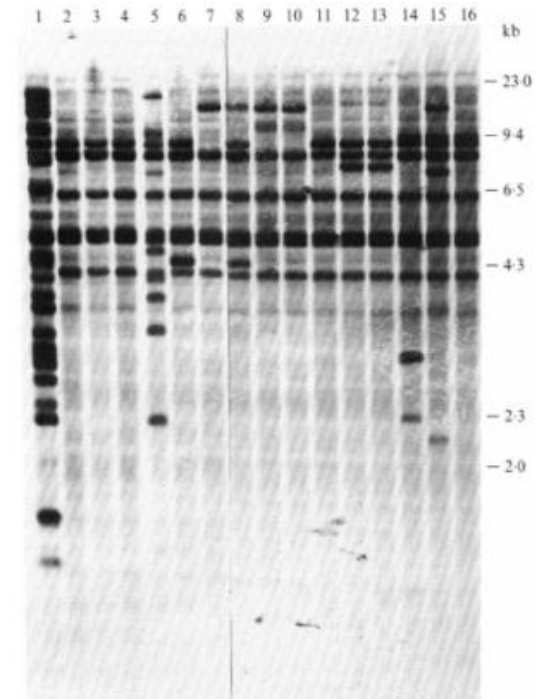
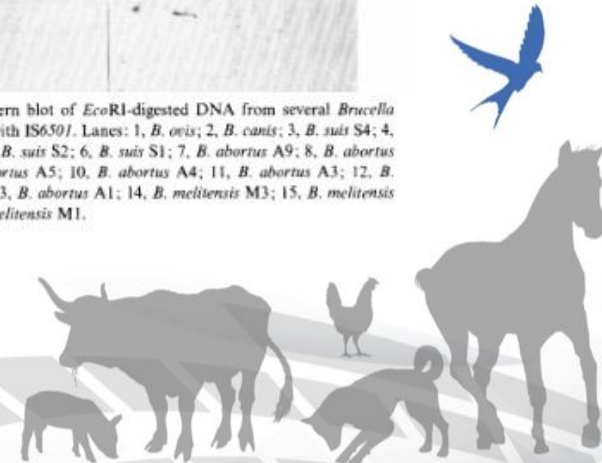


Fig. 5. Southern blot of *EcoRI*-digested DNA from several *Brucella* spp. probed with IS6501. Lanes: 1, *B. ovis*; 2, *B. canis*; 3, *B. suis* S4; 4, *B. suis* S3; 5, *B. suis* S2; 6, *B. suis* S1; 7, *B. abortus* A9; 8, *B. abortus* A6; 9, *B. abortus* A5; 10, *B. abortus* A4; 11, *B. abortus* A3; 12, *B. abortus* A2; 13, *B. abortus* A1; 14, *B. melitensis* M3; 15, *B. melitensis* M2; 16, *B. melitensis* M1.



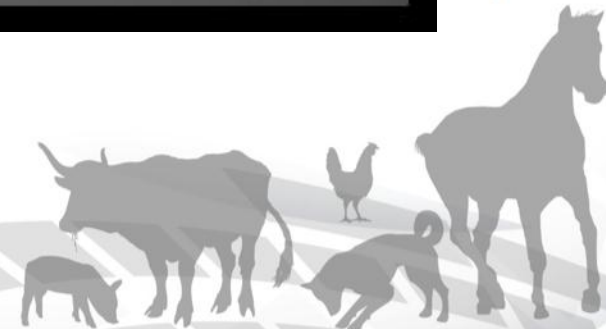
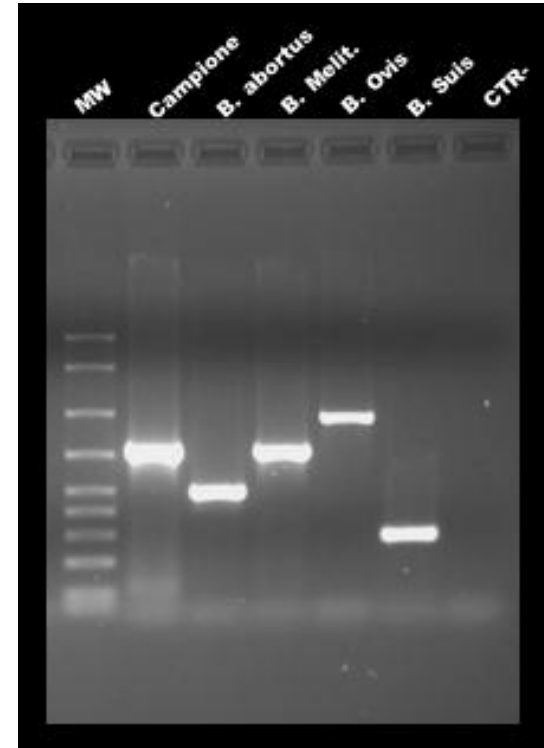
Brucella typing



PCR typing:

Amos PCR (*abortus, melitensis, ovis, suis*) Possibilità di identificare ceppi vaccinali S19 RB51

Bricker, B.J., Halling, S.M., (1994). Differentiation of *Brucella abortus* bv. 1, 2, and 4, *Brucella melitensis* s, *Brucella ovis*, and *Brucella suis* bv. 1 by PCR. J. Clin. Microbiol. 32, 2660–2666.



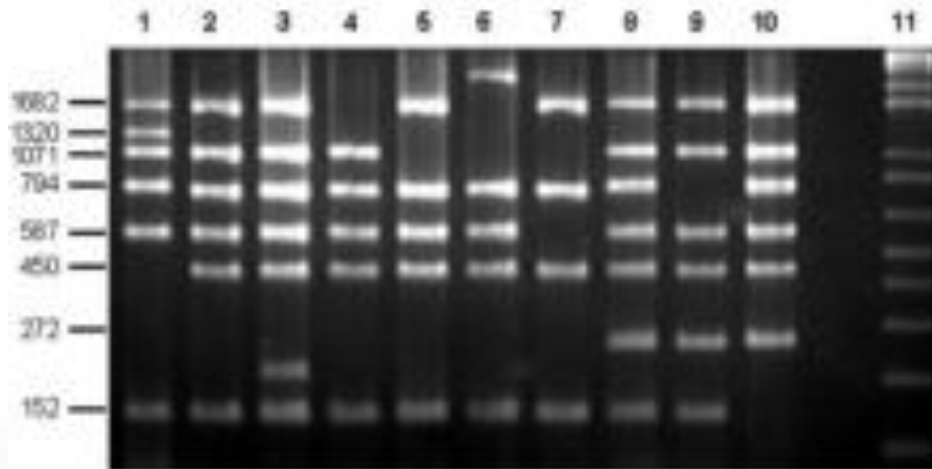
Brucella typing



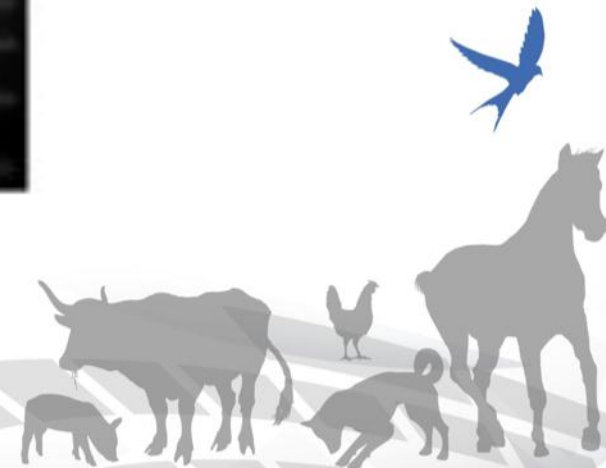
PCR typing:

Bruce ladder PCR

Identifica le 6 specie classiche, *Brucelle* marine e ceppi vaccinali S19 RB51



García-Yoldi, D. *et al.* (2006). Multiplex PCR assay for the identification and differentiation of all *Brucella* species and the vaccine strains *Brucella abortus* S19 and RB51 and *Brucella melitensis* Rev1. *Clinical Chemistry*, 52(4), 779-781.



Brucella typing

PCR typing:

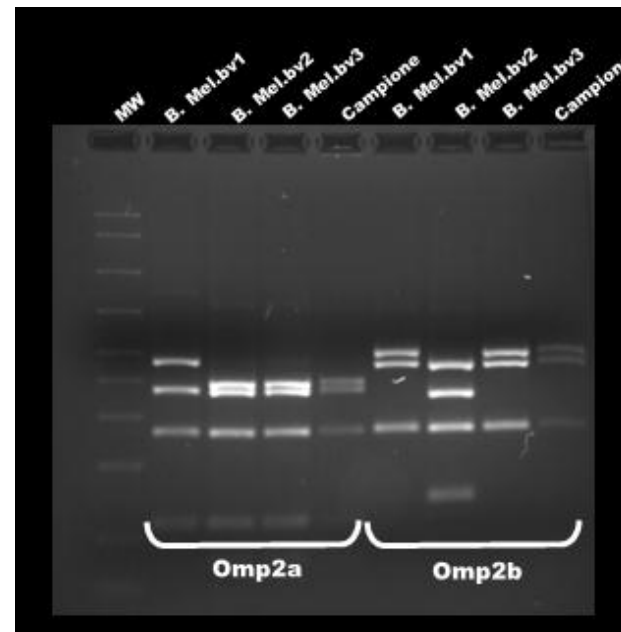
Restriction fragment length polymorphism (RFLP)

Su geni polimorfici

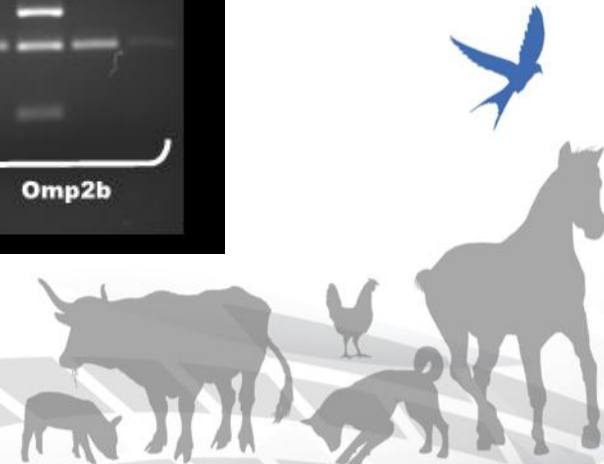
omp2

omp25

omp31



Cloekaert, A., Verger, J.M., Grayon, M., Grepinet, O., 1995. Restriction site polymorphism of the genes encoding the major 25 kDa and 36 kDa outer-membrane proteins of Brucella. Microbiology 141, 2111–2121.





Brucella typing

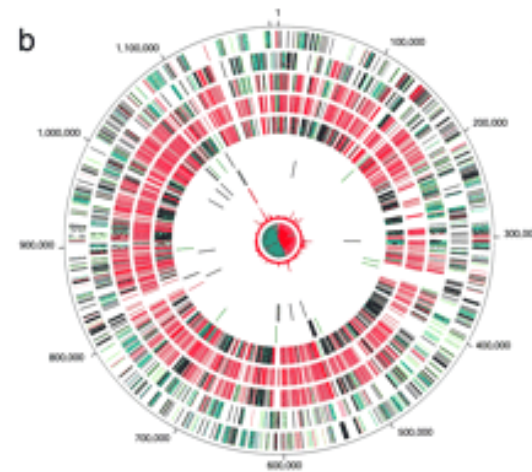
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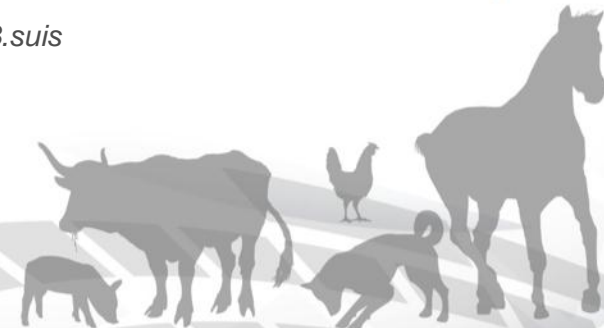
Genoma

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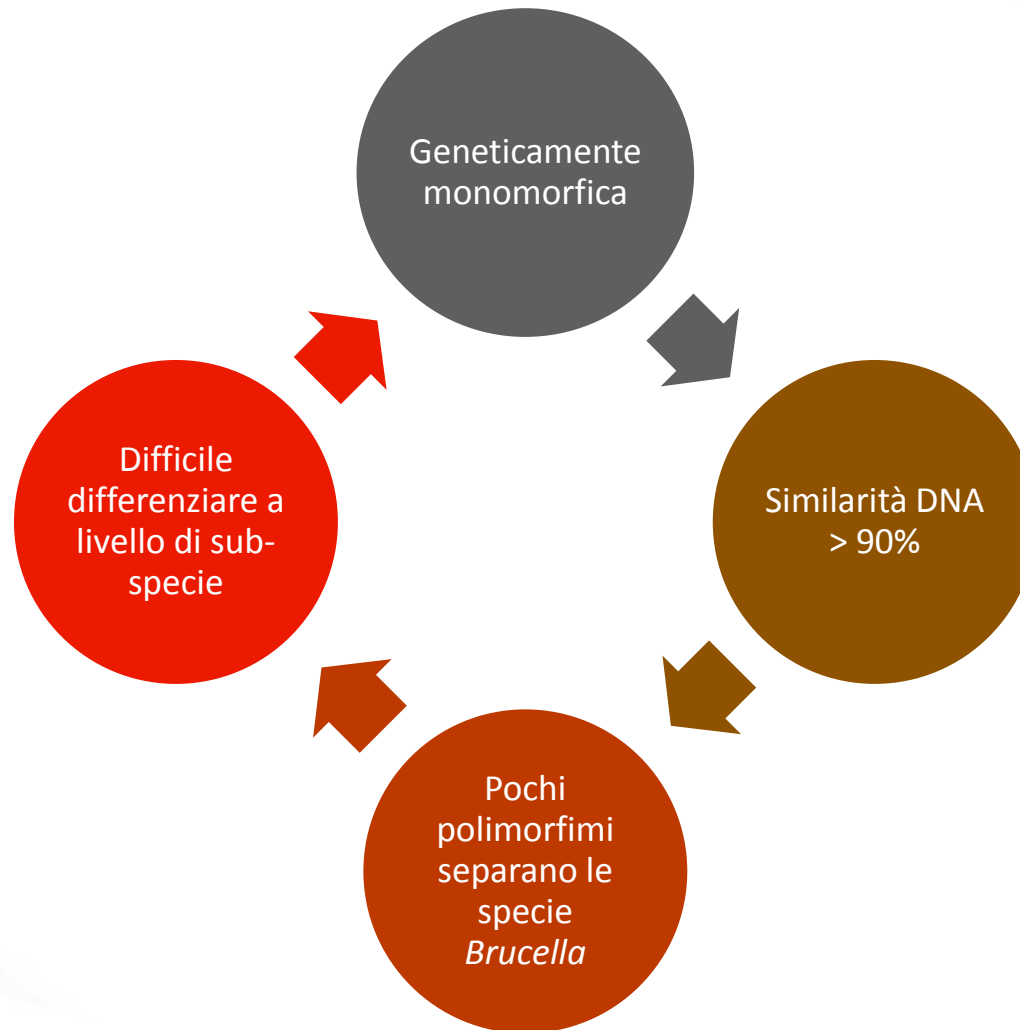
- ✓ Due cromosomi (3,5 Mb)
- ✓ Similarità DNA > 90%



B. suis



Brucella typing



Approccio di tipizzazione su intero genoma



Brucella typing

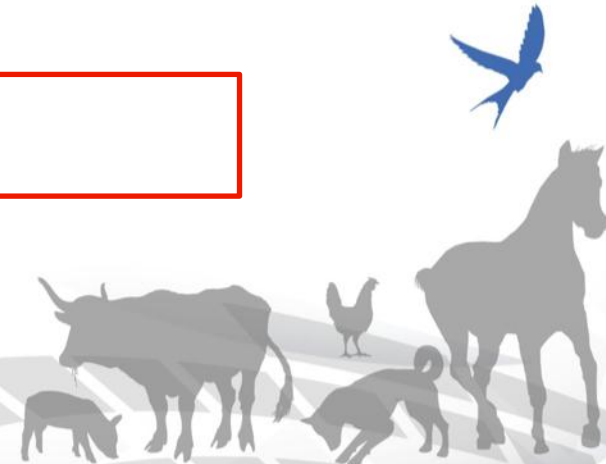


Metodi su base genomica:

MLST

MLVA

WGS



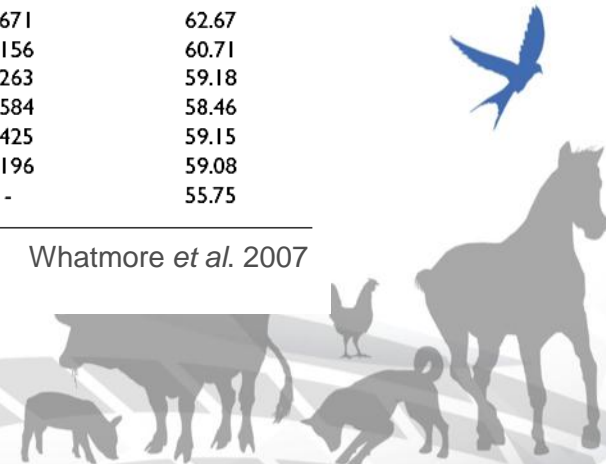
- Caratterizza loci genici multipli
- Sequenze nucleotidiche di geni (450-500 bp)
- Per ogni sequenza si definisce un numero allelico
- La combinazione di più alleli determina il ST

Table 2: Analysis of the nine loci examined in the *Brucella* strains sampled.

Locus	Alleles	Polymorphic sites (%)	d_N	d_S	d_N/d_S	Mean % GC
<i>gap</i>	6	7 (1.19)	0.0023	0.0105	0.217	58.33
<i>araA</i>	7	7 (1.24)	0.0032	0.0058	0.559	61.68
<i>glk</i>	10	11 (2.32)	0.0051	0.0031	1.671	62.67
<i>dnaK</i>	7	6 (1.28)	0.0016	0.0103	0.156	60.71
<i>gyrB</i>	6	5 (1.07)	0.0027	0.0102	0.263	59.18
<i>trpE</i>	6	5 (1.03)	0.0033	0.0056	0.584	58.46
<i>cobQ</i>	7	8 (1.90)	0.0055	0.0129	0.425	59.15
<i>omp25</i>	10	12 (2.45)	0.0032	0.0161	0.196	59.08
<i>int-hyp</i>	5	6 (1.40)	-	-	-	55.75

d_N = mean non-synonymous substitutions per non-synonymous site
 d_S = mean synonymous substitutions per synonymous site

Whatmore *et al.* 2007



Isolato

DNA

PCR

sequenza

DATI

Ricerca nel database

MLST
analisi

Indagini epidemiologiche per singoli focolai

Andamento globale e locale

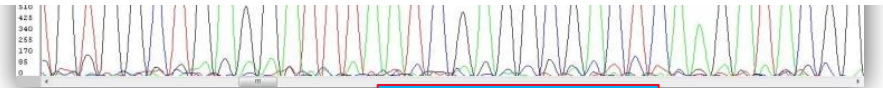
Dinamiche di popolazione

Analisi evolutive

Ceppi atipici



	111 111111111111 111111 22222 22223 33333333 33333333333 344444		
	12345 6677001 12223345556 667899 11344 67780 01333344 44677788888 900123		
	396223 4949495 62394503572 451928 66679 99954 7324824 992134812467 81695		
	1739310 7621854 54774230789 548598 69716 36888 27834755 89755508545 224406		
	gap aroA glk dnaK gyrB trpE cobQ omp25 int-hyp		
	SSSSNNN NSNNNSS NNNNNSSNNN NNSSS NSNS NSNNS SNNSSNN SSSNNNSNSN		
<i>B. abortus</i> ST1	CTCCGG GCGACG ATCGAGCGGA AGGCA CGGCG CGTGA GCAGCGG GCTCTGCCCG GGGTA	11	Biovars 1, 2, and 4
<i>B. abortus</i> ST2G.....	5	Biovars 3, 5, 7, and 9
<i>B. abortus</i> ST3T.....G.....	1	Biovar 7
<i>B. abortus</i> ST4G.....A.....	1	Biovar 6 reference only
<i>B. abortus</i> ST5A.....A.....	2	Vaccine strains only
<i>B. abortus</i> ST6	T..T...A...G..G..A..G.....G..T.....G..T.....	1	Biovar 3 reference only
<i>B. melitensis</i> ST7	..T.T..C...G...GT...G...G.....C.T.G..T...TC...T...T..CT	4	Biovars 1 and 2
<i>B. melitensis</i> ST8	..T.T..C...GT...G...G.....C.G..T...TC...T...T..CT	10	Biovars 1, 2, and 3
<i>B. melitensis</i> ST9	..T.T..C...GT...G...G.....C.G..T...TC...T...T..CT	1	Biovar 3 reference
<i>B. melitensis</i> ST10	..T.T..C...GT...G...G.....C.T.G..T...TC...T...T..CT	1	Biovar 1
<i>B. melitensis</i> ST11	..T.T..C...GT...G...G.....C.G..T...TC...T...T..CT	3	Biovars 1, 2, and 3
<i>B. melitensis</i> ST12	..T.T..C...GT...G...G.....C.T.G..T...TC...T...T..CT	2	Biovar 1
<i>B. ovis</i> ST13	.C.....C...T..G.....AAG.....TGA..A..C..A...A.....	11	All <i>B. ovis</i>
<i>B. suis</i> ST14	.C.....C..G..A..G.....G.....T..A..T.....TG..A..C.....	11	Biovar 1 only
<i>B. suis</i> ST15	.C.....C.....GG.....G.....T.....T.....TG.....A.....	26	Biovar 2 only
<i>B. suis</i> ST16	.C.....C.....GG.....G.....T.....T.....TG.....A.....	4	Biovar 2 only
<i>B. suis</i> ST17	.C.....C..G..A..G.....G.....T..AAT.....TG..A..C.....	7	Biovars 3 and 4
<i>B. suis</i> ST18	.C.....C..G..A..G.....G.....T..AAT.....TG..A..C.....	1	Biovar 4 only
<i>B. suis</i> ST19	.C.....C.....G.....G.....A.....TG.....A.....C.....	1	Biovar 5 only
<i>B. canis</i> ST20	.C.....C..G..A..G.....G.....T..AAT.....TG.....A.....	5	
<i>B. canis</i> ST21	.C.....C..G..A..G.....G.....T..AAT.....TG.....A.....	3	
<i>B. neotomae</i> ST22	.C.....C.....G.....G.....G.....G.....TG.....A.....	30	All <i>B. neotomae</i>
Marine <i>Brucella</i> ST23	.C.....CT.....G..A...G..A.....A.....A..TG..A..C.....	3	Predominantly porpoises
Marine <i>Brucella</i> ST24	.C.....C.....G..A...G..A.....A.....A..TG..A..C.....	3	Predominantly seals
Marine <i>Brucella</i> ST25	.C.....C.....G..A...G..A.....A.....A..TG..A..C.....	12	Predominantly seals
Marine <i>Brucella</i> ST26	.C.....C.....G.....G.....G.....A.....TG..AA..C.....	9	Dolphins
Marine <i>Brucella</i> ST27	.C.....C.....G.....G.....G.....A.....TG..AA..C.....	2	Human/dolphin



G
T
G
A
T
G
T
A
C



Variable Number Tandem Repeats

sono sequenze di DNA che possono variare da 2 fino a 100 nucleotidi organizzate in domini ripetuti

ATTCGATTTCGATTTCG
ATTCG è ripetuta 3 volte

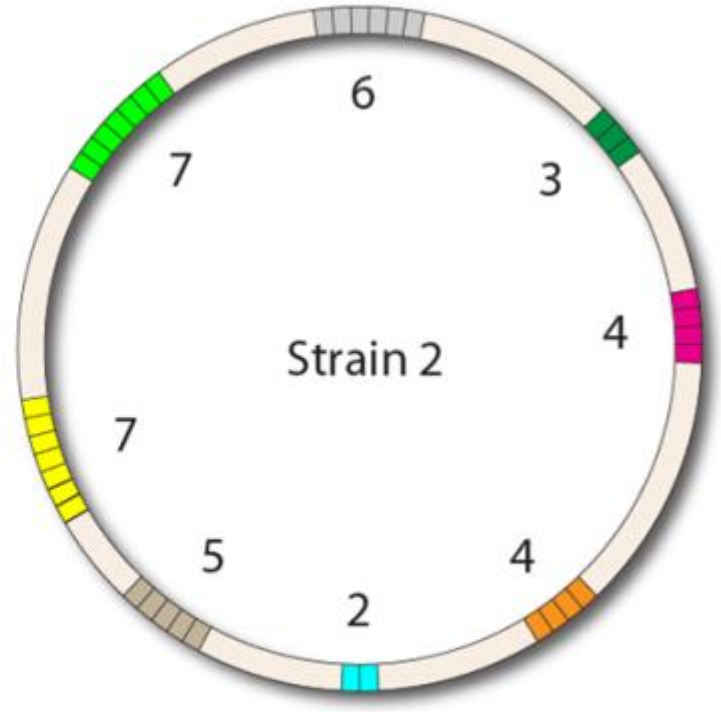
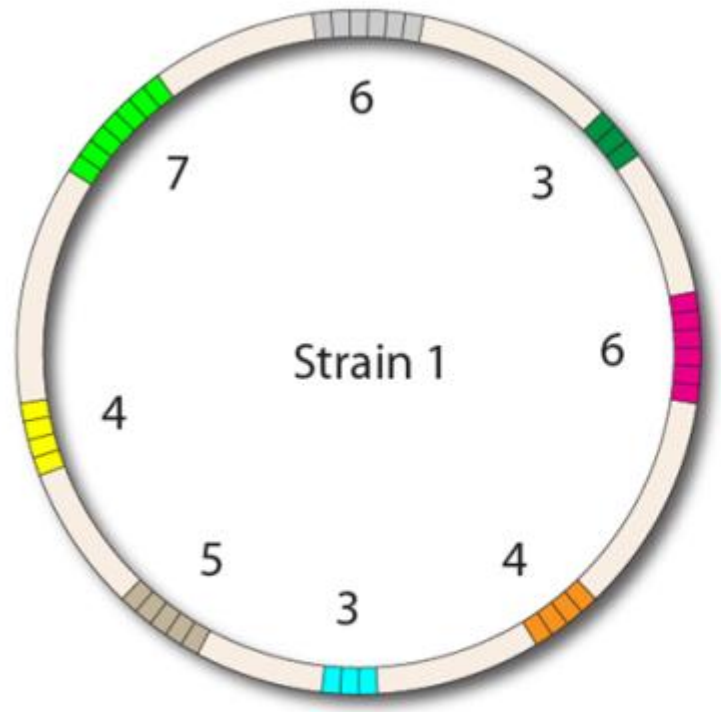




MLVA

Multiple locus VNTR (variable number of tandem repeats) analysis

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MLVA profile of strain 1: 6-3-6-4-3-5-4-7

MLVA profile of strain 2: 6-3-4-4-2-5-7-7





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MLVA

Multiple locus VNTR (variable number of tandem repeats) analysis

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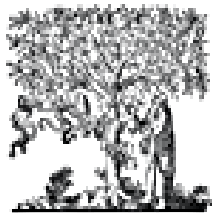
Flusso di lavoro:

- ✓ Amplificazione fino a 16 marker molecolari in regioni ipervariabili (tandem repeats)
- ✓ Lettura grandezza allele
- ✓ Conversione grandezza allele in unità di ripetizione
- ✓ Database (MLVA-NET for *Brucella*: <http://mlva.u-psud.fr/brucella/>)

Le Flèche *et al.*, 2006; Al Dahouk *et al.*, 2007



Analisi delle grandezze alleliche:



ELSEVIER

Journal of Microbiological Methods

Volume 92, Issue 2, 15 February 2013, Pages 103–107



Note

MLVA-16 loci panel on *Brucella spp.* using multiplex PCR and multicolor capillary electrophoresis

Giuliano Garofolo  ·  · , Massimo Ancora, Elisabetta Di Giannatale

Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise "G. Caporale" — National and OIE Reference Laboratory for Brucellosis, Italy



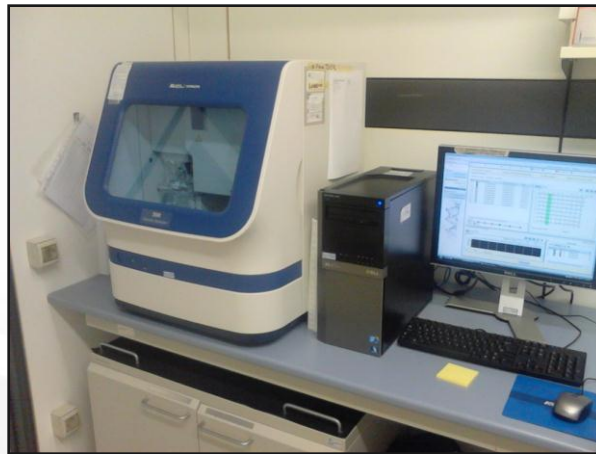


MLVA

Multiple locus VNTR (variable number of tandem repeats) analysis

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Flusso di lavoro:





MLVA

Multiple locus VNTR (variable number of tandem repeats) analysis

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Global
epidemiology

Outbreak
investigation





MLVA

Multiple locus VNTR (variable number of tandem repeats) analysis

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Variabilità genomica:

214 ceppi di *B.abortus* e *B.melitensis*

Tipizzazione convenzionale: *B.abortus* bv 1,3,6;

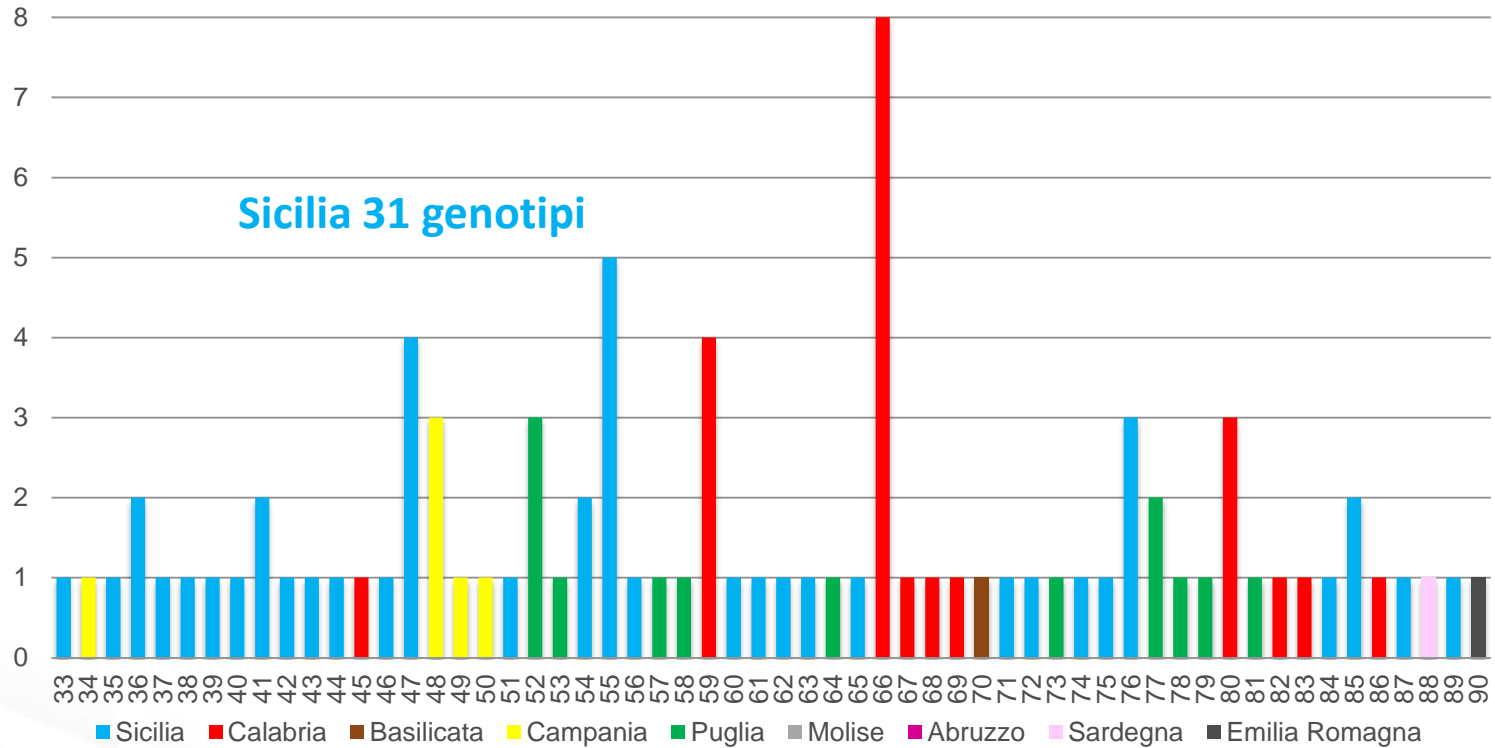
MLVA-16: 90 genotipi
B.abortus RB51
B.melitensis bv 1,3

B.melitensis: 58 genotipi *B.abortus*: 32 genotipi



MLVA-16 *B. melitensis*

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Sicilia 31 genotipi

Spiccata clusterizzazione geografica

58 genotipi con 45 di questi rappresentati da unico ceppo

43 ceppi identificati in 13 genotipi con indice di clusterizzazione del 50%



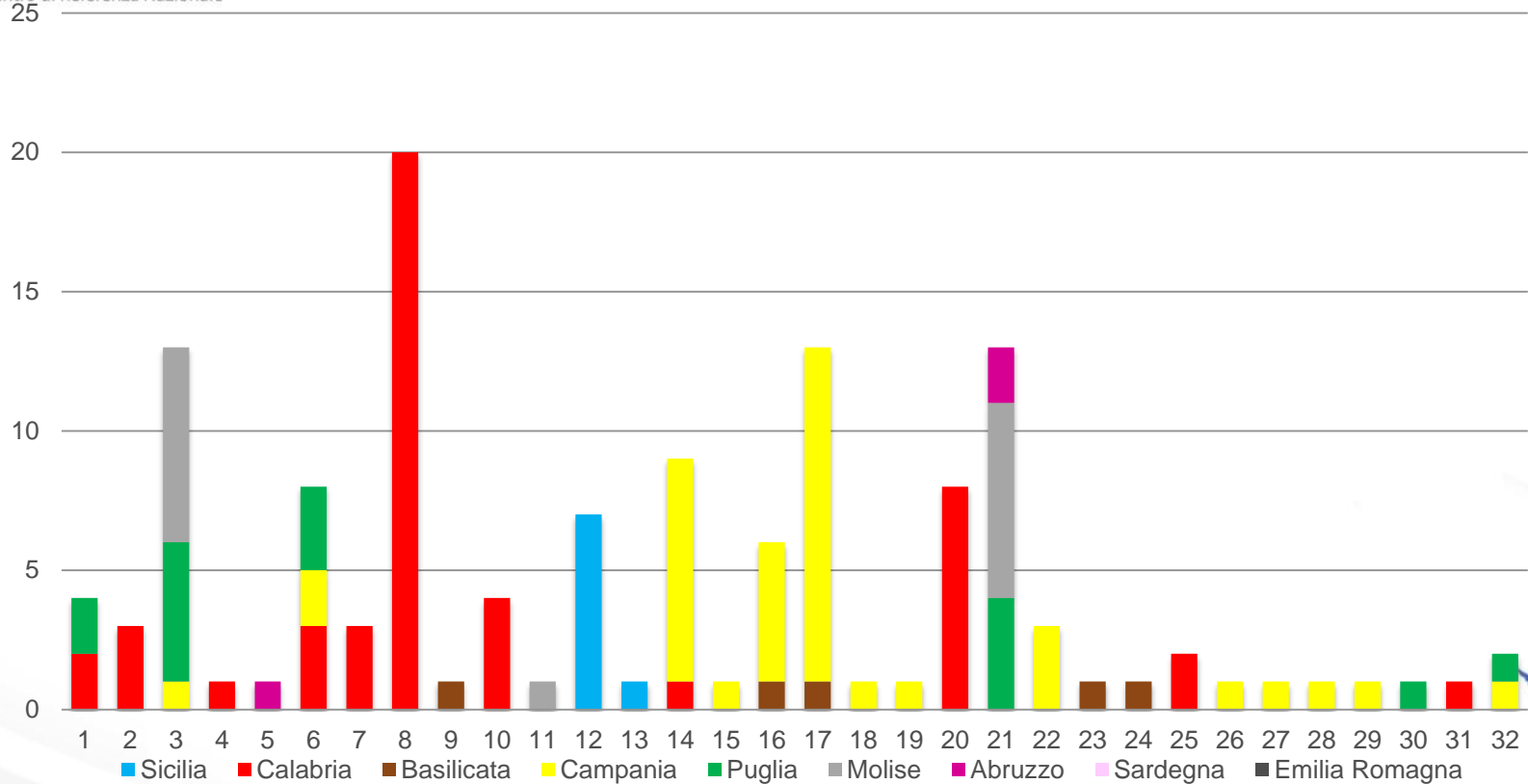


MLVA

Multiple locus VNTR (variable number of tandem repeats) analysis

MLVA -16 *B. abortus*

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Genotipi geograficamente misti

32 genotipi, 16 dei quali rappresentati da un ceppo unico

120 ceppi identificati da 17 genotipi con indice di clusterizzazione del 89,3%



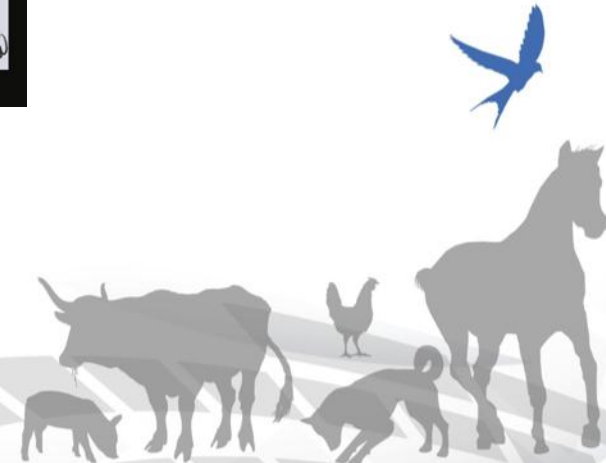


Qual è il metodo di discriminazione a più alta risoluzione?



Next Generation Sequencing (NGS)

Flusso di lavoro: wet/dry

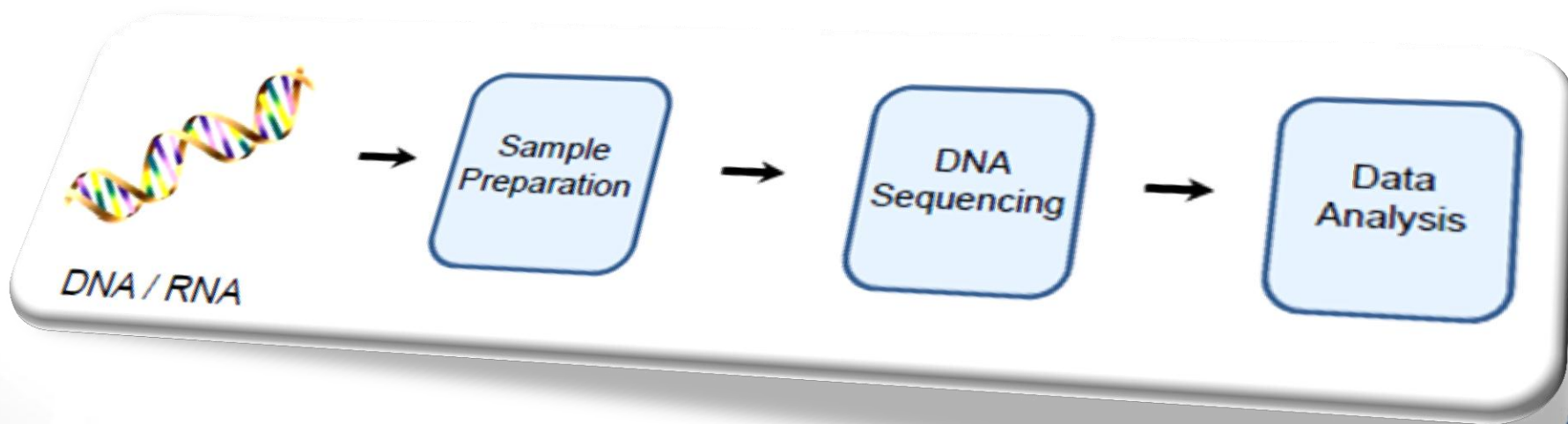


Next Generation Sequencing (NGS)



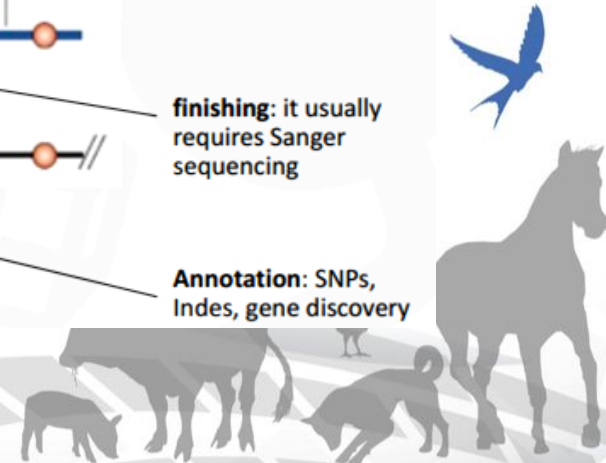
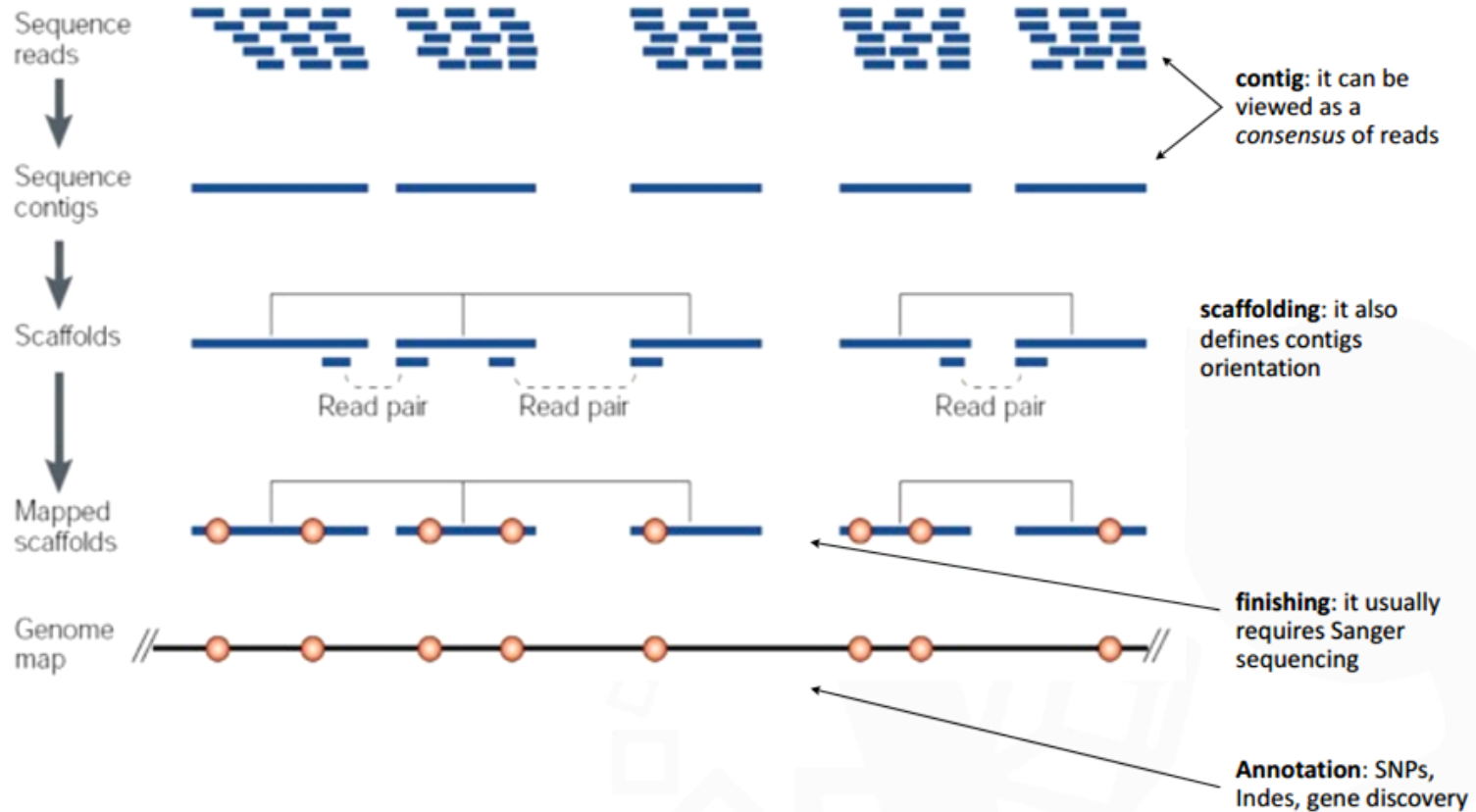
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 Flusso di lavoro:



Next Generation Sequencing (NGS)

Flusso di lavoro (dry):





Work in progress...

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Filogenesi MLVA-11 loci



Clusters clonali

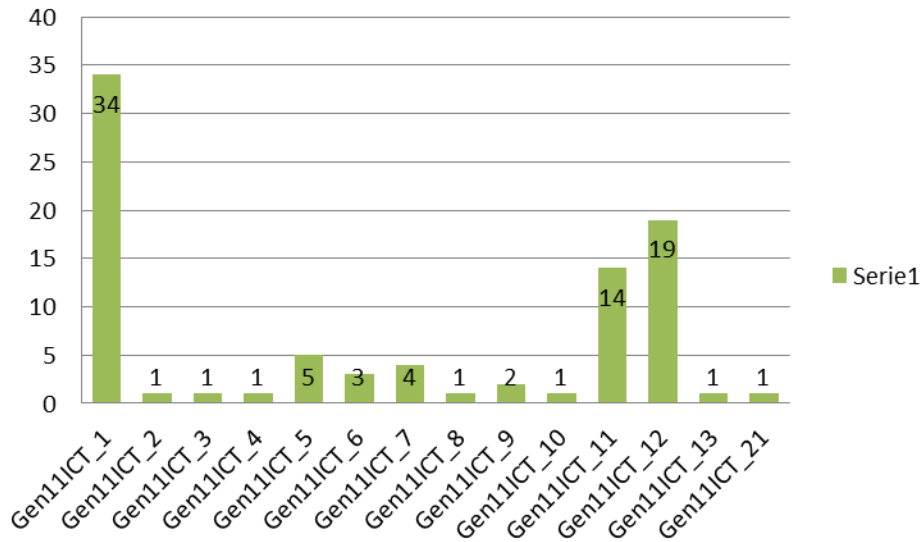
NGS



Genotipizzazione

PCR Real-Time



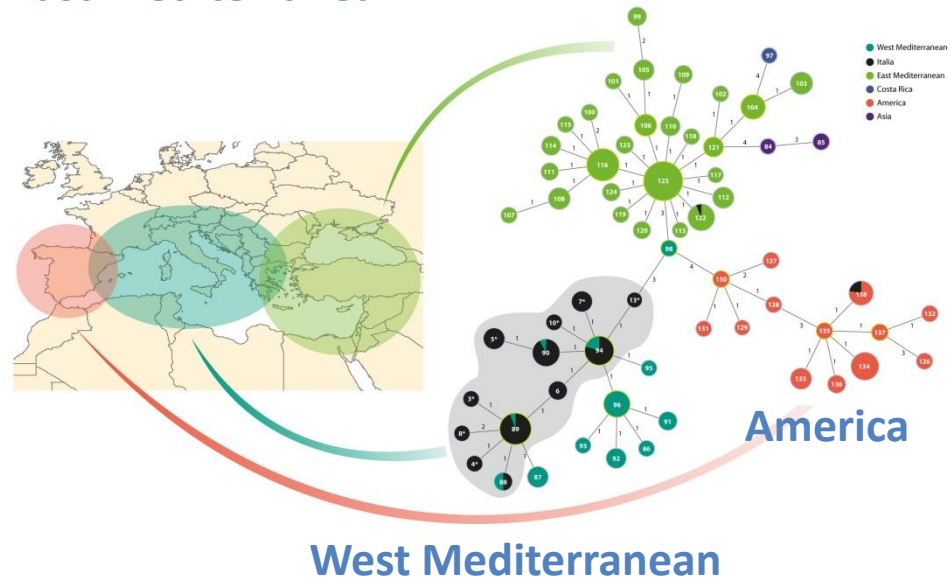


**12 MLVA-11 genotypes 98 % strains
Belong to West Mediterranean**

1 MLVA-11 genotype East Mediterranean

1 MLVA-11 genotype America

East Mediterranean



Relazioni filogenetiche: algoritmo goeBurst vs 700 profili database MLVA-net for Brucella





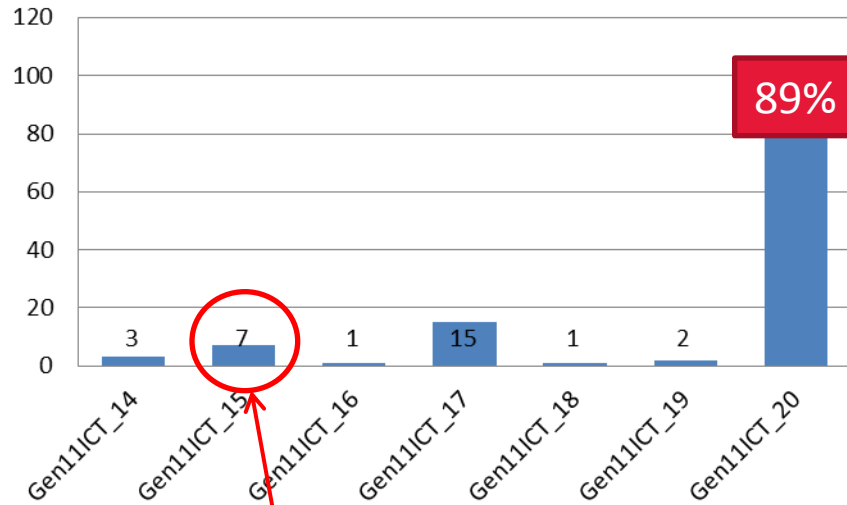
MLVA

Multiple locus VNTR (variable number of tandem repeats) analysis

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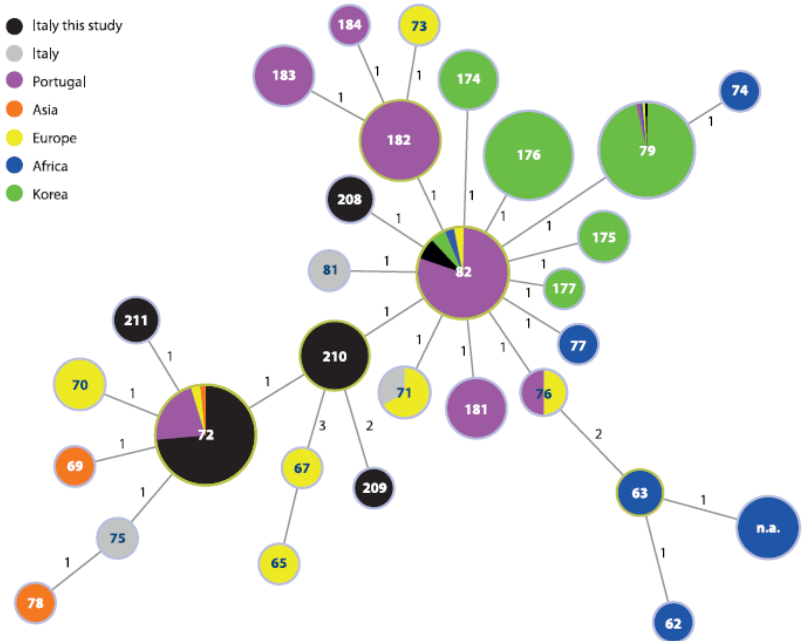
Filogenesi:

B. abortus



Biovar. 1

- Italy this study
- Italy
- Portugal
- Asia
- Europe
- Africa
- Korea



Relazioni filogenetiche: algoritmo goeBurst vs 700 profili database MLVA-net for Brucella





MLVA

Multiple locus VNTR (variable number of tandem repeats) analysis

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Arricchimento database internazionale (MLVA-NET for *Brucella*: <http://mlva.u-psud.fr/brucella/>)

Infection, Genetics and Evolution 19 (2013) 59–70



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journal homepage: www.elsevier.com/locate/meegid



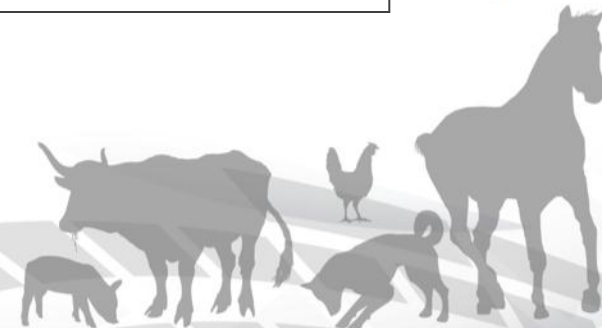
Investigating genetic diversity of *Brucella abortus* and *Brucella melitensis* in Italy with MLVA-16



Giuliano Garofolo^{a,*}, Elisabetta Di Giannatale^a, Fabrizio De Massis^a, Katuscia Zilli^a, Massimo Ancora^a, Cesare Cammà^a, Paolo Calistri^a, Jeffrey T. Foster^b

^aIstituto Zooprofilattico Sperimentale dell'Abruzzo e Molise "G. Caporale", National and OIE Reference Laboratory for Brucellosis, Via Campo Boario, 64100 Teramo, Italy

^bCenter for Microbial Genetics & Genomics, Northern Arizona University, Flagstaff, AZ 86011-4073, USA





MLVA

Genotipizzazione di *Brucella* in Italia

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Filogenesi:

214 ceppi

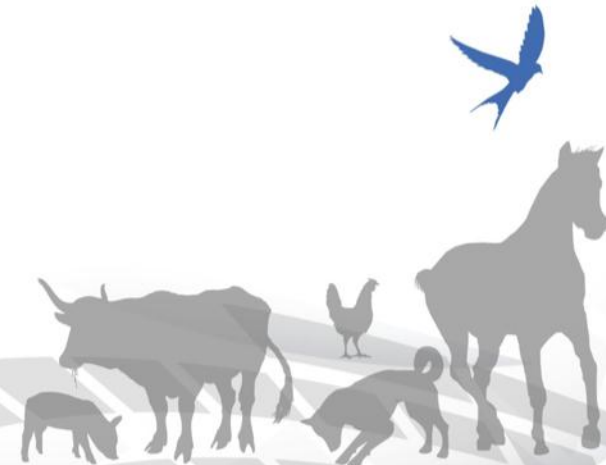


B.abortus: 7 genotipi

B.melitensis: 12 genotipi



NGS

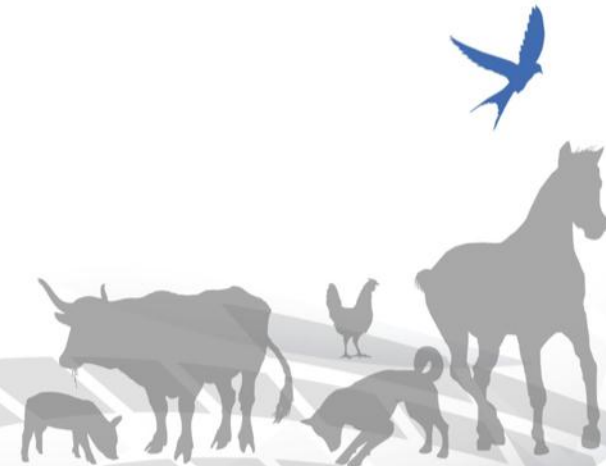




Next.....

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- **Sviluppo di un nuovo metodo di tipizzazione molecolare**
- **Canonical SNPs + pannello minimo di VNTRs**
- **WGS – fast clustering**





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