Application of MLVA-15 genotyping for typing of *Brucella abortus* isolates from India

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Introduction

- Brucellosis is considered as the most wide spread zoonosis in the world and a True zoonosis
- Genus - *Brucella* - Gram negative bacteria
- Bovine brucellosis - *B. abortus*
  - most widespread form in Cattle
  - economic impact on livestock industry due to abortions, stillbirths, weight loss, decreased milk production, hazard to human health
  - barriers to international trade of animals and their products (Corbel, 2006)
- Control and Eradication of infection - continuous surveillance and epidemiological trace-back

Trace-back Analysis

Strain-specific identification is essential to know the source of infection

Requires a suitable typing technique for identification at sub-species level

Molecular typing of *Brucella*

Outer membrane protein typing	Species-specific polymorphisms
Insertion sequence based typing	Limited subspecies level diversity
Pulsed field gel electrophoresis	-do-
Amplified fragment length polymorphism	-do-
Random Amplified Polymorphic DNA analysis	Sensitive to environmental conditions
Repetitive sequences based typing approaches (ERIC-PCR, REP-PCR, BOX A1R-PCR, (GTG)5-PCR, *etc*.)	Sensitive to environmental conditions
Multilocus sequence-typing (MLST)	Global epidemiology of bacteria
Multilocus Variable-Number Tandem-Repeat Analysis (MLVA)	Local epidemiology and epidemiological trace-back in outbreak cases

Multilocus Variable-Number Tandem-Repeat Analysis (MLVA)

- Tandemly repeated sequences observed throughout the prokaryotic and eukaryotic genomes in thousands of copies

  (Bennett, 2000; van Belkum *et al*., 1999)

- Minisatellites : repeat unit sizes of 9 bp or greater
  Microsatellites : repeat unit sizes of up to 8 bp

  (Vergnaud and Pourcel. 2006)

- Combinations of minisatellite and microsatellite repeats in MLVA proven highly discriminatory in subtyping of monomorphic bacterial species, like *Brucella*

  (Bricker et al., 2003; Whatmore et al., 2006; Le Fleche et al., 2006)

- High-speed molecular clocks (van Belkum, 1999).

MLVA contd….

- ✓ PCR-based
- ✓ Multiple alleles can be present at a single locus
- ✓ Size differences could be easily resolved by electrophoresis (Lindstedt , 2005; Vergnaud and Pourcel , 2006)
- ✓ When multiple loci are analyzed, the resulting fingerprint can be highly discriminatory or even unique.
- ✓ Use of multiple loci avoids dangers of incorrect conclusions being drawn from single loci
- ✓ Data can be easily stored and compared between laboratories leading to the development of International databases accessible via the Internet.

MATERIALS & METHODS

- **Field isolates of *B. abortus*- 13**
- **Reference strains- 4**

Sl. No.	Reference Strains	Species-biovar	Acc. No.
1	544	*B. abortus bv 1*	ATCC 23448/NCTC10093
2	S19	*B. Abortus*	NCTC8038
3	S99	*B. abortus*	
4	1119-R	*B. abortus*	

- ***B. melitensis* 16M- Standard strain**

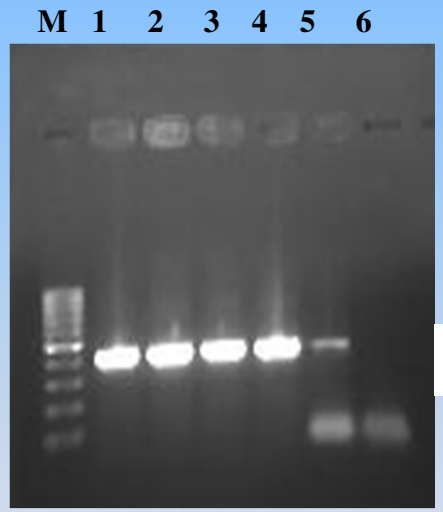
Field Isolates Of *Brucella abortus* Used For The Present Study

S. N.	Sample No./ Strain	Species-biovar	Host	Occupation	Source of isolation	Year of isolation	Place
1	12/02_VPH	*Brucella abortus*				2002	
2	M06_VPH	*Brucella abortus*				2006	
3	07/VPH	*Brucella abortus*	Bovine		Aborted fetus	2006	Kolkata
4	19/VPH	*Brucella abortus*	Bovine			2007	West Bengal
5	21/VPH	*Brucella abortus*	Bovine			2007	West Bengal
6	22/VPH	*Brucella abortus*	Bovine			2007	West Bengal
7	47a(8)/VPH	*Brucella abortus*	Cattle			2008	Bangalore
8	11/08_VPH	*Brucella abortus*				2008	
9	60/VPH	*Brucella abortus*	Cattle		Aborted materials	2009	CADRAD
10	61/VPH	*Brucella abortus*	Cattle		Aborted materials	2009	CADRAD
11	75/VPH	*Brucella abortus*	Bovine		Fetus	2010	Mizoram
12	76/VPH	*Brucella abortus*	Man	Butcher	Blood	2010	Maharashtra
13	BAB_VPH	*Brucella abortus*					

All the isolates were checked for purity & biochemical characteristics along with genus specific PCR (16S - 23S r-RNA spacer gene) before use in the study

	Name	Forward Primer (5'-3')	Reverse Primer (5'-3')	References
Genus specific PCR	16S-23S r-RNA spacer genes	AAC ATA GAT CGC AGG CCA GTC AGC	TGC CAA TAT CCG TCT CAA GAC CAA	Kumar (2007)

Reaction mixture		Cycle condition	
10X PCR buffer	2.5 µl	Initial denaturation	94°C for 5 min
dNTP	2.5 µl(2mM each)	Denaturation	94°C for 45 s
MgCl2	2.5 µl(2.5 mM)	**Annealing**	**55°C for 45 s**
Forward primer	1 µl (10 pmol/µl)	Extension	72°C for 1 min
Reverse primer	1 µl(10 pmol/µl)	Final extension	72°C for5 min
Taq DNA polymerase	0.2 µl (1 unit)	Cycles	30
Genomic DNA	3 µl	**Amplicon size: 410 bp**	
NFW	12.8 µl		
Total Volume	25 µl		



Oligonucleotide Primers used for MLVA Typing

	Name	Forward Primer (5'-3')	Reverse Primer (5'-3')	References
MLVA-15	**Panel 1**	**Minisatellite Loci**		Le Fleche *et al.* (2006)
	Bruce 6	ATG GGA TGT GGT AGG GTA ATC G	GCG TGA CAA TCG ACT TTT TGT C	
	Bruce 8	ATT ATT CGC AGG CTC GTG ATT C	ACA GAA GGT TTT CCA GCT CGT C	
	Bruce 11	CTG TTG ATC TGA CCT TGC AAC C	CCA GAC AAC AAC CTA CGT CCT G	
	Bruce 12	CGG TAA ATC AAT TGT CCC ATG A	GCC CAA GTT CAA CAG GAG TTT C	
	Bruce 42	CAT CGC CTC AAC TAT ACC GTC A	ACC GCA AAA TTT ACG CAT CG	
	Bruce 43	TCT CAA GCC CGA TAT GGA GAA T	TAT TTT CCG CCT GCC CAT AAA C	
	Bruce 45	ATC CTT GCC TCT CCC TAC CAG	CGG GTA AAT ATC AAT GGC TTG G	
	Bruce 55	TCA GGC TGT TTC GTC ATG TCT T	AAT CTG GCG TTC GAG TTG TTC T	
	Panel 2	**Microsatellite Loci**		
	Bruce 4	CTG ACG AAG GGA AGG CAA TAA G	CGA TCT GGA GAT TAT CGG GAA G	
	Bruce 7	GCT GAC GGG GAA GAA CAT CTA T	ACC CTT TTT CAG TCA AGG CAA A	
	Bruce 9	GCG GAT TCG TTC TTC AGT TAT C	GGG AGT ATG TTT TGG TTG TAC ATA G	
	Bruce 16	ACG GGA GTT TTT GTT GCT CAA T	GGC CAT GTT TCC GTT GAT TTA T	
	Bruce 18	TAT GTT AGG GCA ATA GGG CAG T	GAT GGT TGA GAG CAT TGT GAA G	
	Bruce 21	CTC ATG CGC AAC CAA AAC A	GAT TCG TGG TCG ATA ATC TCA TT	
	Bruce 30	TGA CCG CAA AAC CAT ATC CTT C	TATGTGCAGAGCTTCATGTTCG	

MLVA-15 Genotyping

PCR reaction mix for 15 VNTR loci

Sr. No.	Bruce	NFDW	Glycerol (10%)	10x PCR Buffer	2 mM dNTP Mix	MgCl2 (25mM)	Taq DNA Polymerase (5U/µl)	Forward Primer (10pm)	Reverse Primer (10pm)	Genomic DNA	Total Reaction Volume
1	**06**	8.8 µl	2.5µl	2.5 µl (Final conc. of 1X)	1.5 µl (Final conc. of 120µM of each)	1.5 µl (1.5mM)	0.2 µl (Final conc. 1U)	1 µl (Final conc. of 10pM)	1 µl (Final conc. of 10pM)	4 µl	25 µl
2	**08**	13.3 µl									
3	**11**	13.3 µl									
4	**12**	13.3 µl									
5	**42**	8.8 µl	2.5µl								
6	**43**	13.3 µl									
7	**45**	13.3 µl									
8	**55**	13.3 µl									
9	**04**	13.3 µl									
10	**07**	13.3 µl									
11	**09**	13.3 µl									
12	**16**	13.3 µl									
13	**18**	13.3 µl									
14	**21**	13.3 µl									
15	**30**	13.3 µl									

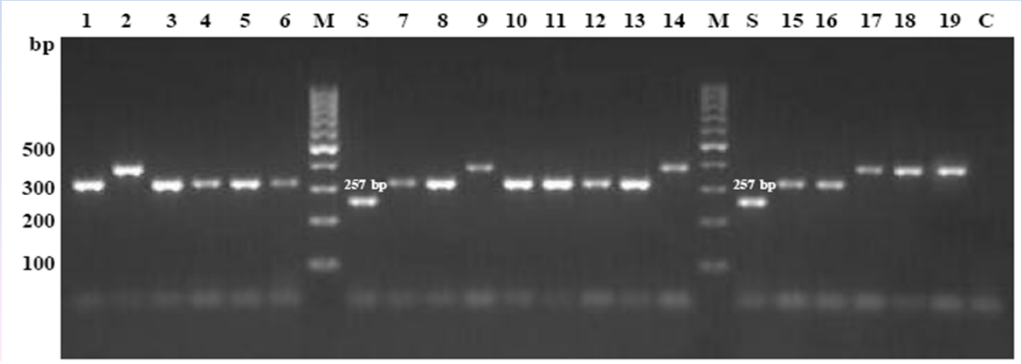
Amplification conditions for the 15 VNTR loci

Sr. No.	Bruce	STEP1 Initial Denaturation	STEP2 Denaturation	STEP3 Annealing	STEP4 Extension	STEP5 Repeat (Steps 2 to 4)	Final Extension	Hold
1	06	96°C for 5 min	96°C for 30 sec	62°C for 30 sec	70°C for 1 min	30 cycles	70°C for 5 min	4°C for Infinite
2	08			60°C for 30 sec				
3	11							
4	12							
5	42							
6	43							
7	45			61°C for 30 sec				
8	55							
9	04			55°C for 30 sec		34 cycles		
10	07			61°C for 30 sec		30 cycles		
11	09			55°C for 30 sec		34 cycles		
12	16							
13	18							
14	21			60°C for 30 sec		30 cycles		
15	30			55°C for 30 sec		34 cycles		

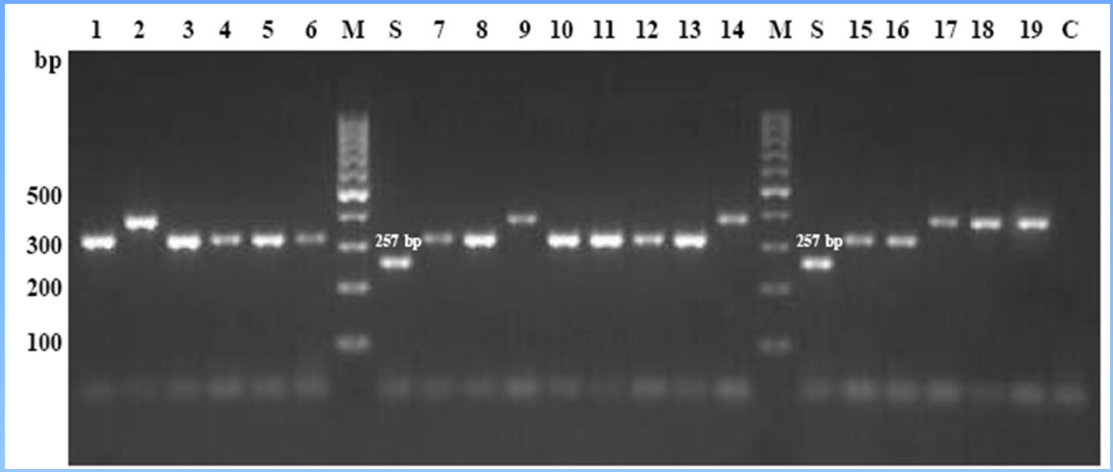
Analysis of PCR Products

Done by Agarose Gel Electrophoresis

- **Minisatellites (Panel 1 Loci)**
  - ✓ 2% (w/v) Agarose gel
  - ✓ 100bp and 50bp DNA ladder
  - ✓ constant 80V for 3 to 4 h depending on the particular VNTR loci.
- **Microsatellites (Panel 2 Loci)**
  - ➢ 4% (w/v) High Resolution Agarose gel
  - ➢ 20bp DNA ladder
  - ➢ constant 80V for 6 h



Amplicon size determination and calculation of number of repeat at particular loci



- Comparing with standard strain's amplicon size (*B. melitensis* 16M, ATCC 23456)
- 100bp DNA ladder
- 50bp DNA ladder
- 20bp DNA ladder
- Respective number of repeat at particular loci with the help of VNTR allelic Table

locus \ unit number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	28
Panel 1 (agarose2%)																										
bruce06-BRU1322_134bp_408bp_3u	140	274	**408**	542																						
bruce08-BRU1134_18bp_348bp_4u		312	330	**348**	366	384																				
bruce11-BRU211_63bp_257bp_2u		**257**	320	383		509		635	698			887		1013	1076											
bruce12-BRU73_15bp_392bp_13u							302	317	332	347	362	377	**392**	407	422	437	452									
bruce42-BRU424_125bp_539bp_4u	164	289	414	**539**	664	789	914																			
bruce43-BRU379_12bp_182bp_2u	170	**182**	194																							
bruce45-BRU233_18bp_151bp_3u		133	**151**	169	187																					
bruce55-BRU2066_40bp_273bp_3u	193	233	**273**	313	353	393	433			*553																
Panel 2A (agarose3%)																										
bruce18-BRU339_8bp_146bp_5u			130	138	**146**	154	162	170	178	*186																
bruce19-Bru324_6bp_163bp_18u				79	85	91												**163**	169	175	181	187	193		205	
bruce21-BRU329_8bp_148bp_6u					140	**148**	156	164	172																	
Panel 2B (agarose3%)																										
bruce04-BRU1543_8bp_152bp_2u	144	**152**	160	168	176	184	192	200	208	216	224	232	240	248	256	264	272	280	288	296	304	312	320			360
bruce07-BRU1250_8bp_158bp_5u		134	142	150	**158**	166	174	182	190	198	206	214	222	230		246										
bruce09-BRU588_8bp_156bp_7u			124	132	140	148	156	**164***	172	180	188	196	204	212	220	228	236	244	252	260	268	276	284	292		
bruce16-BRU548_8bp_152bp_3u		144	**152**	160	168	176	184	192	200	208	216	224	*232	240	248	**254		**270								
bruce30-BRU1505_8bp_151bp_6u		119	127	135	143	**151**	159	167	175	183	191	199														

* not expected size in available 16M reference strain

Le Flèche et al. 2006 version 3.3 (last modified june 2nd 2009)

** Alleles observed in B. microti isolates*

*** Alleles observed in B. ceti isolates*

Repeat number present at each locus in standard strain (*Brucella melitensis* 16M, ATCC 23456)

Sl. No.	Locus	VNTR Name	PCR product size(bp)	Tandem repeats (bp)	Unit size
	Panel 1				
1	Bruce06	BRU1322_134bp_408bp_3u	408	134	3
2	Bruce08	BRU1134_18bp_348bp_4u	348	18	4
3	Bruce11	BRU211_63bp_257bp_2u	257	63	2
4	Bruce12	BRU73_15bp_392bp_13u	392	15	13
5	Bruce42	BRU424_125bp_539bp_4u	539	125	4
6	Bruce43	BRU379_12bp_182bp_2u	182	12	2
7	Bruce45	BRU233_18bp_151bp_3u	151	18	3
8	Bruce55	BRU2066_40bp_273bp_3u	273	40	3
	Panel 2				
9	Bruce04	BRU1543_8bp_152bp_2u	152	8	2
10	Bruce07	BRU1250_8bp_158bp_5u	158	8	5
11	Bruce09	BRU588_8bp_156bp_7u	156	8	7
12	Bruce16	BRU548_8bp_152bp_3u	152	8	3
13	Bruce18	BRU339_8bp_146bp_5u	146	8	5
14	Bruce21	BRU329_8bp_148bp_6u	148	8	6
15	Bruce30	BRU1505_8bp_151bp_6u	151	8	6

Analysis of MLVA-15 genotyping

- START (Sequence Type Analysis and Recombinational Test) software, version 1.0.5 (Jolley *et al.*, 2001)
  - Allelic profile frequencies
  - Allele frequencies
  - Phylogenetic tree by UPGMA method
- Hunter-Gaston diversity index (HGDI)
  - Measure of the variability of the TRs copy number at each locus.
  - V-DICE (VNTR diversity and confidence extractor) (http://www.hpa.org.uk)

RESULTS

Number of tandem repeats determined at each locus for *B. abortus* strains

S.N.	*B. abortus* strains	6	8	11	12	42	43	45	55	4	7	9	16	18	21	30
1	12/02_VPH	4	5	4	12	2	2	3	3	4	4	3	3	6	8	6
2	M06_VPH	4	5	4	12	2	2	3	3	3	5	3	3	7	8	6
3	07/VPH	4	5	4	12	2	2	3	3	3	4	3	3	6	8	8
4	19/VPH	4	5	4	12	2	2	3	3	3	4	3	3	6	8	7
5	21/VPH	4	5	4	12	2	2	3	3	3	4	3	3	6	8	7
6	22/VPH	4	5	4	12	2	2	3	3	3	4	3	3	7	8	7
7	47a(8)/VPH	4	5	4	13	2	2	3	3	3	4	3	3	7	8	5
8	11/08_VPH	4	5	4	12	2	2	3	3	3	4	3	3	6	8	6
9	60/VPH	4	5	4	12	2	2	3	3	3	4	3	3	6	8	6
10	61/VPH	4	5	4	12	2	2	3	3	3	4	3	3	6	8	6
11	75/VPH	4	5	4	12	2	2	3	3	3	4	3	3	6	8	5
12	76/VPH	4	5	4	12	2	2	3	3	3	5	3	3	6	8	5
13	BAB_VPH	4	5	4	12	2	2	3	3	3	6	3	3	6	8	5
14	Ref 544	4	5	4	12	2	2	3	3	3	5	3	4	6	8	5
15	Ref S 19	4	5	4	12	2	2	3	3	3	6	3	3	6	8	5
16	Ref S99	4	5	4	12	2	2	3	3	3	5	3	4	6	8	6
17	Ref 1119-R	4	5	4	12	2	2	3	3	3	6	4	3	6	8	5

Allelic profile frequencies of *B. abortus*

13 different profile(s) in dataset (displayed in descending order of frequency)

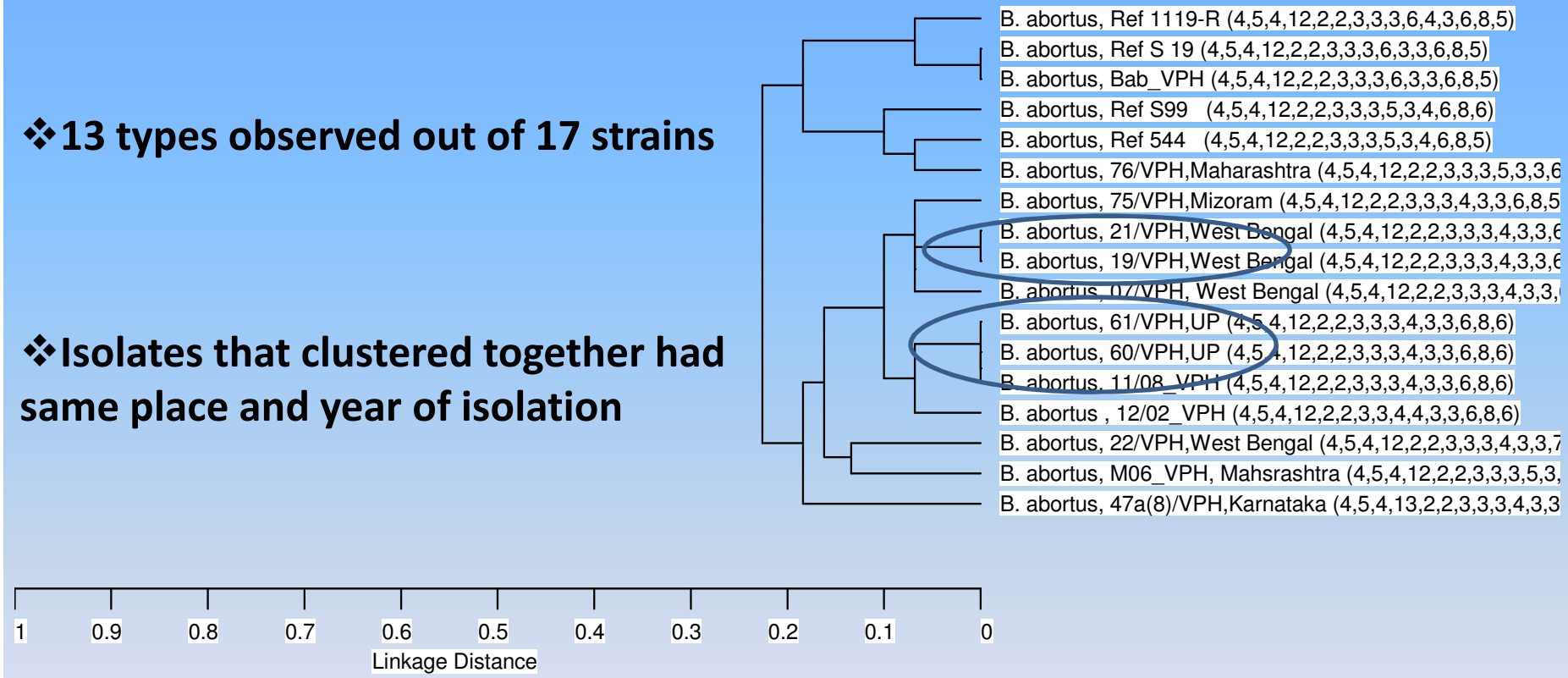
Genotype	Profile (Bruce 06, 08, 11, 12, 42, 43, 45, 55, 04, 07, 09, 16, 18, 21, 30)	Frequency	% of dataset
A1	4, 5, 4, 12, 2, 2, 3, 3, 3, 4, 3, 3, 6, 8, 6	3	17.65
A2	4, 5, 4, 12, 2, 2, 3, 3, 3, 4, 3, 3, 6, 8, 7	2	11.76
A3	4, 5, 4, 12, 2, 2, 3, 3, 3, 6, 3, 3, 6, 8, 5	2	11.76
A4	4, 5, 4, 12, 2, 2, 3, 3, 4, 4, 3, 3, 6, 8, 6	1	5.88
A5	4, 5, 4, 12, 2, 2, 3, 3, 3, 5, 3, 3, 7, 8, 6	1	5.88
A6	4, 5, 4, 12, 2, 2, 3, 3, 3, 4, 3, 3, 6, 8, 8	1	5.88
A7	4, 5, 4, 12, 2, 2, 3, 3, 3, 4, 3, 3, 7, 8, 7	1	5.88
A8	4, 5, 4, 13, 2, 2, 3, 3, 3, 4, 3, 3, 7, 8, 5	1	5.88
A9	4, 5, 4, 12, 2, 2, 3, 3, 3, 4, 3, 3, 6, 8, 5	1	5.88
A10	4, 5, 4, 12, 2, 2, 3, 3, 3, 5, 3, 3, 6, 8, 5	1	5.88
A11	4, 5, 4, 12, 2, 2, 3, 3, 3, 5, 3, 4, 6, 8, 5	1	5.88
A12	4, 5, 4, 12, 2, 2, 3, 3, 3, 5, 3, 4, 6, 8, 6	1	5.88
A13	4, 5, 4, 12, 2, 2, 3, 3, 3, 6, 4, 3, 6, 8, 5	1	5.88

Allelic frequencies and Allelic Diversity of *B. abortus*

Allele	*6*	*8*	*11*	*12*	*42*	*43*	*45*	*55*	*4*	*7*	*9*	*16*	*18*	*21*	*30*
1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	17	17	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	17	17	16	-	16	15	-	-	-
4	17	-	17	-	-	-	-	-	1	10	1	2	-	-	-
5	-	17	-	-	-	-	-	-	-	4	-	-	-	-	7
6	-	-	-	-	-	-	-	-	-	3	-	-	14	-	6
7	-	-	-	-	-	-	-	-	-	-	-	-	3	-	3
8	-	-	-	-	-	-	-	-	-	-	-	-	-	17	1
9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12	-	-	-	16	-	-	-	-	-	-	-	-	-	-	-
13	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-
Total	1	1	1	2	1	1	1	1	2	3	2	2	2	1	4
Allelic Diversity	0.000	0.000	0.000	0.118	0.000	0.000	0.000	0.000	0.118	0.603	0.118	0.221	0.309	0.000	0.713

Phylogenetic tree constructed from MLVA-15 fingerprinting of *B.abortus* strains using UPGMA

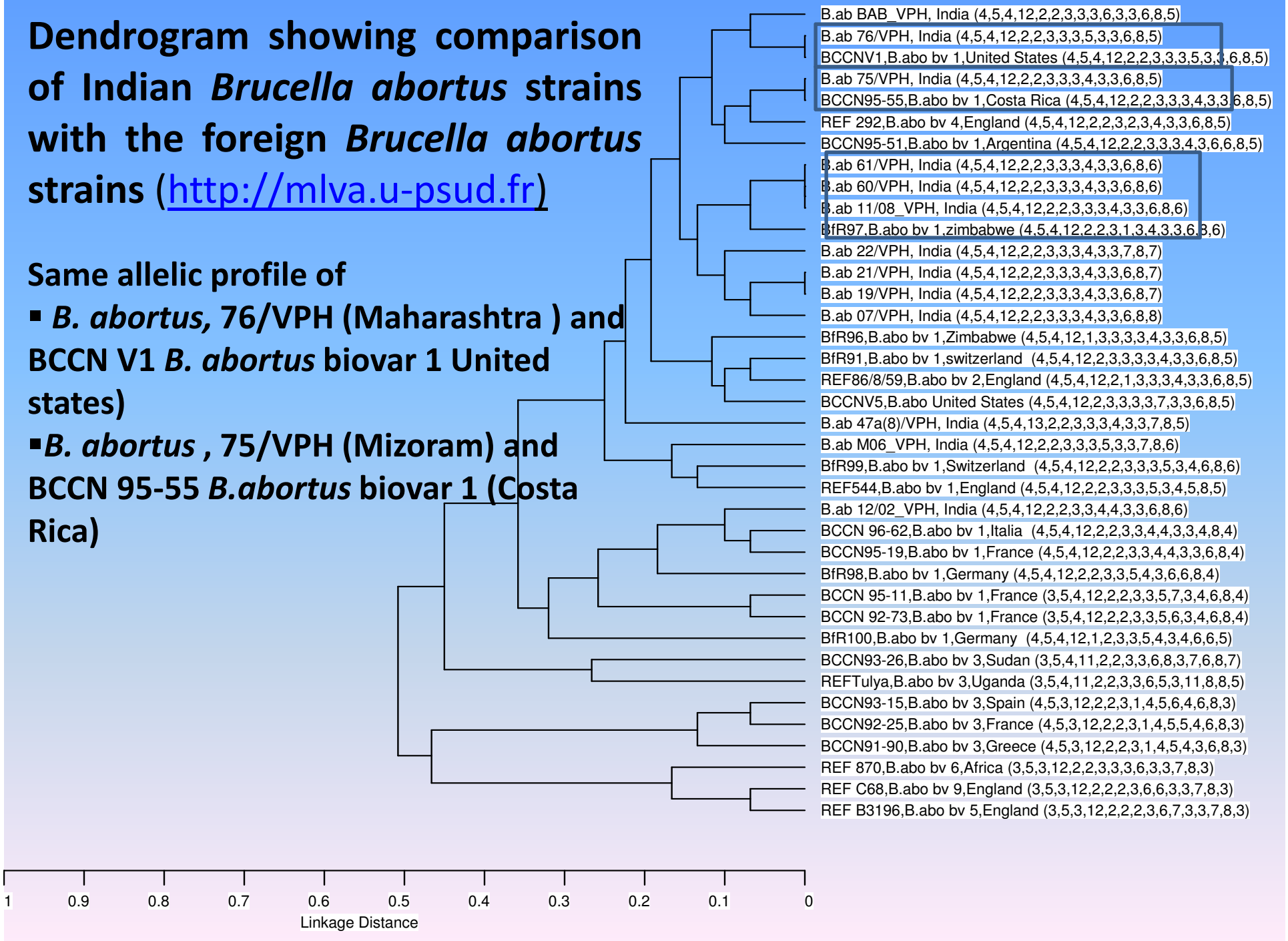
- 13 types observed out of 17 strains
- Isolates that clustered together had same place and year of isolation



Dendrogram showing comparison of Indian *Brucella abortus* strains with the foreign *Brucella abortus* strains (http://mlva.u-psud.fr)

Same allelic profile of

- ***B. abortus,* 76/VPH (Maharashtra) and BCCN V1 *B. abortus* biovar 1 United states)**
- ***B. abortus* , 75/VPH (Mizoram) and BCCN 95-55 *B.abortus* biovar 1 (Costa Rica)**



SUMMARY AND CONCLUSION

- **17 *Brucella abortus* strains (13 field isolates & 4 reference strains) were used in MLVA-15 genotyping**
- **MLVA-15 genotyping clearly discriminated Indian field isolates of *Brucella abortus* producing 13 genotypes**
- **The isolates that grouped together were of same source**

So, MLVA-15 genotyping sufficiently discriminated Indian field isolates of *Brucella abortus*.

Specific grouping was also observed according to epidemiological data/source of infection


THANKS