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Original Article

Genotyping of Indian antigenic, vaccine, and field *Brucella* spp. using multilocus sequence typing

Rajeswari Shome[1], Natesan Krithiga[1], Padmashree B Shankaranarayana[1,2], Sankarasubramanian Jegadesan[6], Vishnu Udayakumar S[6], Bibek Ranjan Shome[1], Girin Kumar Saikia[3], Narendra Kumar Sharma[4], Harshad Chauhan[5], Bharat Singh Chandel[5] , Rajendhran Jeyaprakash[6], Habibur Rahman[1]

[1] Bacteriology Lab, ICAR - National Institute of Veterinary Epidemiology and Disease Informatics (ICAR - NIVEDI) (Formerly PD_ADMAS),Yelahanka, Bengaluru, Karnataka, India
[2] Department of Biochemistry, Jain University, Bengaluru, Karnataka, India
[3] Department of Microbiology, College of Veterinary Sciences, Assam Agricultural University, Khanapara, Guwahati, Assam, India
[4] Department of Veterinary Microbiology, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab, India
[5] Department of Animal Biotechnology, College of Veterinary Science and Animal Husbandry, Sardarkrushinagar Dantiwada Agricultural University, Sardarkrushinagar, Gujarat, India
[6] Department of Genetics, School of Biological Sciences, Madurai Kamaraj University, Madurai, Tamil Nadu, India

Abstract

Introduction: Brucellosis is one of the most important zoonotic diseases that affects multiple livestock species and causes great economic losses. The highly conserved genomes of *Brucella*, with > 90% homology among species, makes it important to study the genetic diversity circulating in the country.

Methodology: A total of 26 *Brucella* spp. (4 reference strains and 22 field isolates) and 1 *B. melitensis* draft genome sequence from India (*B. melitensis* Bm IND1) were included for sequence typing. The field isolates were identified by biochemical tests and confirmed by both conventional and quantitative polymerase chain reaction (qPCR) targeting *bcsp* 31Brucella genus-specific marker. *Brucella* speciation and biotyping was done by Bruce ladder, probe qPCR, and AMOS PCRs, respectively, and genotyping was done by multilocus sequence typing (MLST).

Results: The MLST typing of 27 *Brucella* spp. revealed five distinct sequence types (STs); the *B. abortus* S99 reference strain and 21 *B. abortus* field isolates belonged to ST1. On the other hand, the vaccine strain *B. abortus* S19 was genotyped as ST5. Similarly, *B. melitensis* 16M reference strain and one *B. melitensis* field isolate were grouped into ST7. Another *B. melitensis* field isolate belonged to ST8 (draft genome sequence from India), and only *B. suis* 1330 reference strain was found to be ST14.

Conclusion: The sequences revealed genetic similarity of the Indian strains to the global reference and field strains. The study highlights the usefulness of MLST for typing of field isolates and validation of reference strains used for diagnosis and vaccination against brucellosis.

Key words: *Brucella*; AMOS PCR; Bruce PCR; Indian isolates; MLST; qPCR.

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Introduction

Ever since the discovery of *Brucella* causative agent more than a century ago, brucellosis remains major problem for animals and mankind [1]. Cross-border epizootic [2] and regional outbreak incidences [3] observed in last few decades and the continuous changing trend in its epidemiological distribution across the globe led *Brucella* to be recognized as a re-emerging pathogen [1]. It is also credited as a biological warfare agent due to its highly infectious nature and airborne transmission [4]. The disease has been successfully eradicated in the cattle population of only a handful of developed countries [5], but it is still highly prevalent and often regionally neglected in many parts of the world, including the Indian subcontinent [6,7].

India has been recognized to be among the regions with the highest prevalence of brucellosis in the world [8], with annual economic losses to the tune of US $58.8 million. *B. abortus* S99 antigen extract-based diagnostics for surveillance and *B. abortus* S19 vaccination were recently implemented in the country for control of brucellosis in the bovine population. Knowledge about the prevalent strains in a given geographical area is a component of major importance

in surveillance systems in order to define control strategies. However, the strain typing of existing and new isolates within the genus *Brucella* is a great challenge because members of the genus have highly conserved genomes with more than 90% DNA-DNA hybridization homology [9], have almost identical 16S rRNA [10], and share more than 98% sequence identity [11,12].

Multilocus sequence typing (MLST) has been recently demonstrated as useful tool [13] for genotyping and for phylogenetic and global epidemiology studies of genetically conserved pathogens like *Brucella* [14,15]. Using the MLST technique, nine new sequence types, which were quite different from those observed in other countries, were defined in China [16]. Similarly, genetic differences of novel strains of *Brucella inopinata* were also recently documented [14]. To date, genotying of field *Brucella* isolates of India and comparing these to global isolates had not yet been done. Comparative analysis between the prevalent *Brucella* strains in India and those found in other regions is still not possible because there is no genotyping information about *Brucella* isolated in India. Therefore, this study was undertaken to type a limited number of Indian field isolates from domestic ruminants along with reference strains used for vaccine and diagnostics for preliminary study.

Methodology

Isolation and biochemical characterization of Brucella spp.

The isolations were carried out using samples that included placenta, fetal stomach contents, fetal membrane, fetal heart blood, and vaginal discharge from recently aborted animals, collected by a convenient sampling approach in Karnataka, Assam, Gujarat, and Punjab. All the aborted animals included were serologically screened for brucellosis using the Rose Bengal plate test (RBPT) (Institute of Animal Health & Veterinary Biologicals (IAH &VB), Bengaluru, India) [17]. Similarly, samples were screened by protein G based indirect enzyme-linked immunosorbent assay (iELISA) (ICAR-NIVEDI, Bengaluru, India) using smooth lipopolysaccharide antigen from standard strain *B. abortus* S99. The cut-off values established for diagnosis were decided after thorough screening and validation of the assay [18]. Any sample of percent positivity value below 55%, between 55% and 65%, and more than 65% were considered, negative, moderate positive, and strong positive, respectively. Isolations were carried out in *Brucella* selective broth (Condaa Pronadisa Laboratories, Conda, S.A. Madrid, Spain,) containing antibiotic supplements (nystatin, bacitracin, polymyxin-B, cycloheximide, nalidixic acid) in a laboratory that had a class II biosafety cabinet facility. Sample inoculated tubes were incubated with and without 10% CO_2 at 37°C for 72 hours. A loopful of broth culture from both sets were streaked onto *Brucella* selective agar with supplements and incubated with and without 10% CO_2 at 37°C until the appearance of growth or up to one week. Purified colonies recovered from the processed clinical samples along with four reference strains (*B. abortus* S99, *B. melitensis* 16M, *B. suis* 1330, and vaccine strain *B. abortus* S19) procured from the National Culture Repository, Indian Veterinary Research Institute, Izatnagar, India, were identified by Gram staining, CO_2 requirement, H_2S production, catalase, urease, oxidase tests, and inhibition of growth by basic fuchsin and thionin dyes [17]. All the isolates were grown to stationary phase at 37°C in tryptic soy broth (TSB) or tryptic soy agar (TSA) (Becton Dickinson, Franklin Lakes, USA) and preserved without extensive laboratory passage.

Table 1. Oligonucleotide sequences used for the amplification and sequencing of nine genetic loci.

No.	Locus	Putative function	Forward primer (5'–3')	Reverse primer (5'–3')	Product size (bp)
1.	*gap*	Glyceraldehydes 3-phosphate dehydrogenase	YGCCAAGCGCGTCATCGT	GCGGYTGGAGAAGCCCCA	589
2.	*aro*A	3-phosphoshikimate 1-carboxyvinyltransferase	GACCATCGACGTGCCGGG	YCATCAKGCCCATGAATTC	565
3.	*glk*	Glucokinase	TATGGAAMAGATCGGCGG	GGGCCTTGTCCTCGAAGG	475
4.	*dna*K	Chaperone protein	CGTCTGGTCGAATATCTGG	GCGTTTCAATGCCGAGCGA	470
5.	*gyr*B	DNA gyrase B subunit	ATGATTTCATCCGATCAGGT	CTGTGCCGTTGCATTGTC	469
6.	*trp*E	Anthranilate synthase	GCGCGCMTGGTATGGCG	CKCSCCGCCATAGGCTTC	486
7.	*cob*Q	Cobyric acid synthase	GCGGGTTTCAAATGCTTGGA	GGCGTCAATCATGCCAGC	422
8.	*omp*25	25 kDa outer-membrane protein	ATGCGCACTCTTAAGTCTC	GCCSAGGATGTTGTCCGT	490
9.	*int-hyp*	Upstream and extreme 5' of hypothetical protein (BruAb1_1395)	CAACTACTCTGTTGACCCGA	GCAGCATCATAGCGACGGA	430

Table 2. *Brucella* isolates and their geographical region of origin.

No.	Strain No.	Place of isolation	Animal species	Age (yrs)	Stage of pregnancy	Animal tissue used for isolation	Species identified
1	ADMAS- G1	Karnataka	Goat	2	3rd month of pregnancy	Aborted placenta of goat	*B. melitensis*
2	ADMAS- C1	Karnataka	Cow	6	7th month of pregnancy	Vaginal discharge (collected 10 days after abortion)	*B. abortus*
3	ADMAS- C2	Karnataka	Cow	6	6th month of pregnancy	Vaginal discharge (collected 10 days after abortion)	*B. abortus*
4	ADMAS- C3	Karnataka	Cow	6	9th month of pregnancy	Vaginal discharge (collected 8 days after abortion)	*B. abortus*
5	ADMAS- C4	Karnataka	Cow	6	8th month of pregnancy	Vaginal discharge (collected 12 days after abortion)	*B. abortus*
6	ADMAS- C5	Karnataka	Cow	5	7th month of pregnancy	Vaginal discharge (collected 13 days after abortion)	*B. abortus*
7	BrAs-01	Assam	Cow	6	6th month of pregnancy	Uterine discharge (collected after abortion)	*B. abortus*
8	BrAs-02	Assam	Cow	5	6th month of pregnancy	Uterine discharge (collected after abortion)	*B. abortus*
9	SKN1	Gujarat	Cow	6	7th month of pregnancy	Placenta	*B. abortus*
10.	SKN2	Gujarat	Cow	7	6th month of pregnancy	Placenta	*B. abortus*
11	SKN12	Gujarat	Buffalo	7	8th month of pregnancy	Vaginal discharge	*B. abortus*
12	SKN13	Gujarat	Buffalo	7	8th month of pregnancy	Fetal heart blood	*B. abortus*
13	SKN14	Gujarat	Buffalo	7	9th month of pregnancy	Placenta	*B. abortus*
14	SKN15	Gujarat	Buffalo	7	7th month of pregnancy	Placenta	*B. abortus*
15	SKN16	Gujarat	Buffalo	7	8th month of pregnancy	Placenta	*B. abortus*
16	SKN17	Gujarat	Buffalo	7	6th month of pregnancy	Placenta	*B. abortus*
17	SKN18	Gujarat	Buffalo	7	6th month of pregnancy	Placenta	*B. abortus*
18	SKN19	Gujarat	Cow	7	8th month of pregnancy	Placenta	*B. abortus*
19	LMN1	Punjab	Pig	4	3rd month of pregnancy	Aborted fetus	*B. abortus*
20	LMN2	Punjab	Cow	6	7th month of pregnancy	Placenta	*B. abortus*
21	LMN3	Punjab	Cow	4	7th month of pregnancy	Placenta	*B. abortus*
22	LMN3	Punjab	Cow	7	6th month of pregnancy	Placenta	*B. abortus*
23	*B. abortus* S99						
24	*B. abortus* S19						
25	*B. melitensis* 16M				Reference strains		
26	*B. suis* 1330						

Molecular characterization of cultures

Cultures were grown on TSA at 37°C for 48 hours, inactivated for 2 hours at 80°C, and DNA was extracted with the QIAamp DNA mini kit (Qiagen, Dusseldorf, Germany). For *Brucella* genus-specific detection, sequences were amplified by genus-specific primers [19]. Species were identified as *B. abortus*, *B. melitensis*, and *B. suis* by multiplex Bruce ladder polymerase chain reaction (PCR) [20]; biovar typing was done by modified *abortus-melitensis-ovis-suis* (AMOS) PCR (primers by Eurofins Genomics Pvt. Ltd, Bengaluru, India) [21]. Similarly, the isolates were subjected to both genus and speciation real-time quantitative polymerase chain reaction (qPCR) assays that are routinely carried out in the laboratory [22,23].

MLST typing and sequence analysis

Genotyping was done for 22 *Brucella* field isolates and four reference strains by amplifying nine distinct genetic loci (seven housekeeping and one each outer membrane protein and intergenic fragment) using primers (Table 1) [24]. The PCR-amplified products were purified by a QIAquick PCR purification kit (Qiagen) and sequenced at M/S Eurofins Genomics India, (Bangalore, India) using an ABI 3730 sequencer. The sequences were edited using Chromas Lite 2.01 software (http://www.tech-nelysium.com.au/chromas_lite.html). MLST sequences (accession numbers AM694191 through AM695630) of the strains were downloaded from the GenBank database. Distinct alleles at each of the loci were given a numerical designation according to the sequence of defined alleles. The unique allelic pattern over all loci was identified as a sequence type (ST). The sequences of the nine loci were concatenated to produce a sequence of 4,396 bp for each genotype. Genotype relatedness analysis along with global isolates obtained

Table 3. Phenotypic and biochemical profile of Indian field *Brucella* spp. along with four reference strains.

No.	Strain No.	Gram staining	CO_2 requirement	H_2S production	Indole production	Motility *	Catalase	Oxidase	Urease	Growth in thionine 1:50,000	Growth in basic fuchsine 1:50,000	Species identified
1	ADMAS- G1	-	-	-	-	-	+	+	+	+	+	*B. melitensis*
2	ADMAS- C1	-	+	+	-	-	+	+	+	-	+	*B. abortus*
3	ADMAS- C2	-	+	+	-	-	+	+	+	-	+	*B. abortus*
4	ADMAS- C3	-	+	+	-	-	+	+	+	-	+	*B. abortus*
5	ADMAS- C4	-	+	+	-	-	+	+	+	-	+	*B. abortus*
6	ADMAS- C5	-	+	+	-	-	+	+	+	-	+	*B. abortus*
7	BrAs-01	-	+	+	-	-	+	+	+	-	+	*B. abortus*
8	BrAs-02	-	+	+	-	-	+	+	+	-	+	*B. abortus*
9	SKN1	-	+	+	-	-	+	+	+	-	+	*B. abortus*
10	SKN2	-	+	+	-	-	+	+	+	-	+	*B. abortus*
11	SKN12	-	+	+	-	-	+	+	+	-	+	*B. abortus*
12	SKN13	-	+	+	-	-	+	+	+	-	+	*B. abortus*
13	SKN14	-	+	+	-	-	+	+	+	-	+	*B. abortus*
14	SKN15	-	+	+	-	-	+	+	+	-	+	*B. abortus*
15	SKN16	-	+	+	-	-	+	+	+	-	+	*B. abortus*
16	SKN17	-	+	+	-	-	+	+	+	-	+	*B. abortus*
17	SKN18	-	+	+	-	-	+	+	+	-	+	*B. abortus*
18	SKN19	-	+	+	-	-	+	+	+	-	+	*B. abortus*
19	LMN1	-	+	+	-	-	+	+	+	-	+	*B. abortus*
20	LMN2	-	+	+	-	-	+	+	+	-	+	*B. abortus*
21	LMN3	-	+	+	-	-	+	+	+	-	+	*B. abortus*
22	LMN4	-	+	+	-	-	+	+	+	-	+	*B. abortus*
23	*B. abortus* S99	-	+	+	-	-	+	+	+	-	+	*B. abortus*
24	*B. abortus* S19	-	+	+	-	-	+	+	+	-	+	*B. abortus*
25	*B. melitensis* 16M	-	-	-	-	-	+	+	+	+	+	*B. melitensis*
26	*B. suis* 1330	-	-	+	-	-	+	+	+	+	-	*B. suis*

* Motility status: + for motile, - for non-motile.

in the database was carried out using MEGA 6.06 software (http://www.megasoftware.net/mega.php).

Results

All the aborted animals' serum samples were positive by RBPT and protein G based iELISA. From these samples, 22 *Brucella* isolates were recovered; 21 were identified as *B. abortus* and 1 as *B. melitensis.* These isolates were from different geographical regions of the country: four *B. abortus* isolates from Punjab and ten from Gujarat, two from Assam and five from Karnataka, and one isolate of *B. melitensis* from Karnataka (Tables 2 and 3) . In the PCR, all the isolates showed 223 bp amplification specific for *Brucella* genus (Figure 1) and species-specific amplification products by multiplex Bruce ladder PCR (Figure 2). In AMOS PCR, a 498 bp amplicon was observed in all the *B. abortus* isolates, confirming their biovars to be either of I, II, or IV biovar; 731 bp in *B. melitensis* strains and a 285 bp product in *B. suis* biovar I (Figure 3). MLST typing revealed five distinct STs; the *B. abortus* S99 reference strain (antigenic strain) and 21 *B. abortus* field isolates belonged to ST1, whereas *B. abortus* S19 (vaccine strain) was found to be ST5. Similarly, the *B. melitensis* 16M reference strain and one *B. melitensis* field isolate (*B. melitensis* ADMAS-G1) were clustered into ST7. Recently, a draft genome sequence of *B. melitensis* Bm IND1 field isolate was reported to be ST8, and *B. suis* 1330 reference strain was recognized as ST14 (Table 4). The sequence divergence by neighbor-joining dendrogram showing the genetic relatedness among the five STs of the 27 Indian isolates (including four reference strains) and nine STs of the 24 global isolates is represented in Figures 4 and 5. Genotypic relatedness analysis classified the STs into different groups. Cluster C1 is divided into 2 clades. Clade 1 comprises *B. neotomae* and *B. inopinata* in two close subclades, indicating the close genetic resemblance between these species. Similarly, clade 2 of cluster C1 is divided into many sub-clades comprising different *Brucella* spp. – *B. abortus, B. melitensis*, *B. ovis*, *B. suis B. microti*, *B. ceti*, *B. pinnipedialis*, and *B. canis* – under different clades and subclades. This cluster is the largest group, comprising eight different *Brucella* spp. holding five different STs of Indian isolates together with eight different STs of global isolates.

Table 4. Allele profile and sequence type of the *Brucella* species

No.	Species/strain No.	*gap*	*aroA*	*glk*	*dnaK*	*gyrB*	*trpE*	*cobQ*	*Omp25*	*Int-hyp*	ST
1	ADMAS -G1	3	5	3	2	1	5	2	10	2	7
2	ADMAS -C1	2	1	1	2	1	3	1	1	1	1
3	ADMAS -C2	2	1	1	2	1	3	1	1	1	1
4	ADMAS- C3	2	1	1	2	1	3	1	1	1	1
5	ADMAS- C4	2	1	1	2	1	3	1	1	1	1
6	ADMAS- C5	2	1	1	2	1	3	1	1	1	1
7	BrAs-01	2	1	1	2	1	3	1	1	1	1
8	BrAs-02	2	1	1	2	1	3	1	1	1	1
9	SKN1	2	1	1	2	1	3	1	1	1	1
10	SKN2	2	1	1	2	1	3	1	1	1	1
11	SKN12	2	1	1	2	1	3	1	1	1	1
12	SKN13	2	1	1	2	1	3	1	1	1	1
13	SKN14	2	1	1	2	1	3	1	1	1	1
14	SKN15	2	1	1	2	1	3	1	1	1	1
15	SKN16	2	1	1	2	1	3	1	1	1	1
16	SKN17	2	1	1	2	1	3	1	1	1	1
17	SKN18	2	1	1	2	1	3	1	1	1	1
18	SKN19	2	1	1	2	1	3	1	1	1	1
19	LMN1	2	1	1	2	1	3	1	1	1	1
20	LMN2	2	1	1	2	1	3	1	1	1	1
21	LMN3	2	1	1	2	1	3	1	1	1	1
22	LMN4	2	1	1	2	1	3	1	1	1	1
23	*B. abortus*S99	2	1	1	2	1	3	1	1	1	1
24	*B. abortus*S19	2	1	1	2	1	4	1	1	1	5
25	*B. melitensis*16M	3	5	3	2	1	5	2	10	2	7
26	*B. suis* 1330	1	6	4	1	4	3	5	2	1	14

ST: sequence type.

Discussion

Brucellosis, a persistent bacterial disease of livestock and humans, causes huge economic losses and significant morbidity [25]. Accurate identification of the prevailing strains of *Brucella* in a given geographical area is central to epidemiological surveillance and public health decisions. Since 1947, there were studies pertaining to isolation of *Brucella* spp. in many regions of the country. Isolation of both *B. abortus* and *B. melitensis* from horses [26] and *B. abortus* bv. I and III [27], bv IV [28], and bv. I [29] from cattle has been reported. Similarly, *B. melitensis* from cattle [30], bv. I of *B. melitensis* from sheep and goats [31], *B. suis* bv. II from a cow [32], and *B. suis* bv. I and II to V from aborted sows have been reported [33,34]. All these studies attempted to identify *Brucella* species and biovars by conventional methods such as analysis of an array of phenotypic characteristics and serological and phage typing. These conventional methods, though time consuming, biohazardous to laboratory personnel, and difficult to differentiate between vaccine and wild strains, have been used worldwide for isolation and typing over the years. With the advent of molecular tools, the conventional typing methods have slowly been replaced by highly discriminatory sequence-based typing. In India, apart from recently reported draft genome sequences of few *Brucella* isolates [35,36], there is no documentation of genetic diversity studies of *Brucella* isolates.

In the study, 13, 7, and 1 *B. abortus* isolates recovered from cattle, buffalo, and pigs, respectively, and 1 *B. melitensis* from goats were identified and confirmed by biochemical methods and PCR. It has been reported that *B. abortus* is the most prevailing species in bovines in the country [37]. It is noteworthy that isolation of *B. abortus* from pigs clearly indicates the possibility of transmission among the livestock species in a mixed farming system. Though *Brucella* isolates were recovered from distant geographical regions within the country, most of them had similar sequence types. The current study highlights the genetic similarity among Indian isolates and with other global isolates. It has been observed that ST1 of *B. abortus* was predominant among the limited number of sequenced strains. In further studies, sequencing large numbers of field isolates will provide the most prevalent sequence types in the country.

Among reference strains, *B. abortus* S99, which is being used for diagnostic antigen preparations in various organizations in the country, belonged to ST1 and was phylogenetically related to the global designate reference stain (*B. abortus* 9-941). Similarly, *B. abortus* S19, used for calfhood vaccine preparation is in the ST5 group, has also been found to be genetically related to the other global vaccine strains such as *B. abortus* S19, 2308, and 13334. The other two reference strains, *B. melitensis* 16M and *B. suis*, belonged to ST7 and ST14, respectively. *B. melitensis* 16M is being used for preparation of plain agglutination antigen for diagnosis of *B. melitensis* infection in small ruminants and humans in the country. *B. suis*, on the other hand, is of limited diagnostic and vaccine importance. The phenotypic and genetic typing of reference strains used for vaccine and diagnosis is important, as the Indian Government's Department of Animal Husbandry, Dairying, and Fisheries (DADF) has initiated a nationwide brucellosis control program. In the program, *B. abortus* S19 calfhood vaccination for female cattle and buffalo calves and surveillance by various diagnostic tests using antigen extracts from *B. abortus* S99 are recommended. Hence, evaluation of vaccine and diagnostic reference strains is justified. The other two reference strains, *B. melitensis* 16M and *B. suis*, belonged to ST7 and ST14, respectively. *B. melitensis* 16M is being used for preparation of plain agglutination antigen for diagnosis of *B. melitensis* infection in small ruminants and humans in the country, whereas *B. suis* is of limited diagnostic and vaccine importance.

From a public health perspective, data on prevailing strains will be useful in the investigation of pyrexia of unknown origin studies in humans [38], disease outbreaks in domestic ruminants, and to appreciate the appropriateness of antigen/vaccination strains used in the region. This information is also useful to differentiate between naturally occurring outbreaks and bioterrorism. Moreover, the MLST method can also provides a framework for typing any new or emerging *Brucella* strains. .

Conclusions

The present study is the preliminary work carried as part of the Department of Biotechnology (DBT) - Network project on brucellosis. This small-scale validation of the sequence typing method is significant because in the future, large numbers of isolates can be sequenced and genotyped in a similar order. Also, this study underlines the genetic relatedness of the *B. abortus* S19 vaccine and *B. abortus* S99 antigenic strains.

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Corresponding author

Rajeswari Shome
National Institute of Veterinary Epidemiology and Disease Informatics (NIVEDI) (Formerly PD_ADMAS),
Yelahanka, Bengaluru, Karnataka, India
Phone: +91 80 23093110/23093111
Fax: +91 80 23093222
Email: rajeswarishome@gmail.com

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