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EryC PCR assay as DIVA tool for Bovine Brucellosis

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ABSTRACT: The present study focuses on differential identification of vaccinated and infected animals of brucellosis (DIVA) by EryC PCR assay. Clinical specimen (400) comprising of 200 (162 blood, 27 milk, 10 vaginal swab and one tissue) samples from vaccinated and 200 (51 blood and 149 milk) samples from unvaccinated cattle. These samples were collected from four different regions of Maharashtra. The RBPT recorded overall prevalence of 39.9% (85/213) with 50.98% (26/51) in unvaccinated animals and 36.41% (59/162) in vaccinated animals. According to MRT results, the overall percent positivity observed was 58.5% (103/176) with vaccinated animals showing a 74.07% positivity (20/27) and unvaccinated animals showing 55.70% positivity (83/149).
A sum of 199 clinical samples (85 blood, 103 milk, 10 vaginal swab and one tissue sample) subjected to differentiate vaccine strain and wild strains of *B. abortus*,by targeting deletion of 702bp. The EryC PCR assay generated amplicon in 57 clinical samples i.e. 28.64% (19 from vaccinated and 38 from unvaccinated animals) of them found positive by EryC PCR. 16 samples from vaccinated animals amplified amplicon of 1257bp and only three samples showed amplicon of 555bp. Eight samples from unvaccinated animals amplified amplicon of 1257bp, 30 milk samples from unvaccinated animals generated amplicon of 555bp suggesting infection is due to vaccine strain.

KEYWORDS: *B. abortus* S 19, Milk Ring Test, Rose Bengal Plate Test, EryC PCR, DIVA

INTRODUCTION

The population of bovine in India and its contribution to national economy is of great importance, but infectious diseases are being constant threat to it. Brucellosis is one among them.The brucellosis is an infectious re- emerging, neglected bacterial zoonosis of global importance. *Brucella* spp. belongs to a genus of the family *Brucellaceae*, order Rhizobiales, class Alphaproteobacteria and phylum Proteobacteria (Glowacka *et al*., 2018). The causative agent is gram negative coccobacilli, non- capsulating, non – motile, non - sporulating which infects wide host range. Genus *Brucella* has nine recognized species, seven of them that affect terrestrial animals are: *B. abortus* (bovine), *B. melitensis* (caprine), *B. suis* (swine), *B. ovis* (ovine), *B. canis* (canine), *B. neotomae* (wood rats), and *B. microti* (mice) and two that affect marine mammals are: *B. ceti* and *B. pinnipedialis*(Seleem*et al*., 2010). Brucellosis in humans is caused by *B. abortus, B. melitensis, B. canis,* and *B. suis* (Berhanu *et al.,* 2020).
To prevent brucellosis in bovine population, the first effective *Brucella* vaccine was developed by using *Brucella abortus* strain 19, a laboratory derived strain attenuated by an unknown process during subculture. One of the major drawbacks of this vaccine is the development of post-vaccinal antibody response that interferes with the interpretation of serological -diagnostic tests in eradication programmes (Nicoletti *et al*.,1981). This vaccine induces fair immune response against *B. abortus*, but at the expense of persistent serological responses (Schuring *et al*., 2002). To overcome this problem, attention has been focused on the development of diagnostic tools for differentiating vaccinated cattle from infected cattle (Nielsen *et al*.,1989).
In order to develop a tool to distinguish between of *B. abortus* S19 from virulent *Brucella* strains, the studies have recently been focused on the EryC gene which encodes D-erythrulose 1-phosphate dehydrogenase, which is involved in the erythritol metabolism in virulent *B. abortus* strains. However, there is a deletion of 702bp sequence in EryC gene of *B. abortus* S19 (Sangari and Aguero *et al*, 1994;Patil *et al*., 2014).The *B. abortus* S19 vaccine strain is unable to use erythritol because the EryC gene is truncated (Sperry and Robertson *et al*., 1975). Therefore, EryC may constitute a *B. abortus* target antigen for diagnostic tests, as it would distinguish the individuals infected with virulent *B. abortus* strain from *B. abortus* S19 vaccinated individuals (Eoh *et al*., 2010). The EryC gene was evaluated as a molecular marker for differential identification of *Brucella abortus* from vaccine strain S 19 (Thorat *et al*., 2022).

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Considering all these aspects the present study was planned to evaluation of EryC PCR assay for differential identification of wild strains of *Brucella abortus* from vaccine strain S 19.

MATERIAL AND METHODS

Reference strains - The standard strains i.e*Brucellaabortus* S19 and *Brucella abortus* 544 were obtained from the Division of Biological Standardization, Indian Veterinary Research Institute (IVRI), Izatnagar, Uttar Pradesh and maintained in the Department of Microbiology, Mumbai Veterinary College, Mumbai for the present study.

Clinical specimen - A total of 400 comprising of 200 (162 blood, 27 milk, 10 vaginal swab and one tissue) samples from vaccinated and 200 (51 blood and 149 milk) samples from unvaccinated cattle. These samples were collected from four different regions of Maharashtra namely Pune, Ahilyanagar, Akola and Mumbai.

Processing of specimen - The sera samples were screened by Rose Bengal Plate Test and milk samples by Milk Ring Test. The extraction of genomic DNA of *B. abortus* from the blood and milk was done by Leal-Klevezas*et al*., (1995) and Romero and Lopez-Goni (1999) respectively. The extraction of genomic DNA of *B. abortus* from the reference strains *Brucella abortus* S 19, *Brucella abortus* 544, vaginal swabs and tissue samples, the protocol described by Romero *et al*. (1995a) was followed with slight modifications.

EryC PCR assay - EryC primers were used for differentiation of vaccinated and infected animals of brucellosis. These primers were supplied by Eurofins India Pvt. Ltd. The details of primers given in the table 1.

Table 1. Oligonucleotide primers

Sr. No.	Name of the PCR	PCR Primer Sequence	
1.	EryC PCR	EryC (F)	5' CATGACACGCGGCATATAAC 3'
		EryC (R)	5' GACCTCCAGCTTACCCATGA 3'

The reaction mixture was set in a final volume of 25µl consisting of nuclease free water (NFW)16.8 µl, 10× PCR buffer with $MgCl_2$ 4.5 µl, dNTPs mix (10 mM) 0.5 µl, Primers EryC F (10 pM/ µl) 1 µl and EryC R (10 pM/ µl) 1 µl, template DNA 1 µl (200 ng) and Taq DNA polymerase (5 U /µl) 0.2 µl.
The PCR reactions were conducted in a thermal cycler (Master Cycler Nexus gradient, Eppendorf) with initial denaturation 94°C for 3 min followed by 35 Cycles of denaturation at 94°C for 3 minutes, annealing at 60°C for 45 seconds, extension at 72°C for 1 minutes. The final extension was carried out at 72ºC for 5 minutes. The amplified products of EryC PCR assay were analyzed using Agarose Gel Electrophoresis (AGE) in 1.5% gel stained with ethidium bromide. The products were visualized using the gel documentation system (Gel Doc EZ Imager, Bio-Rad).

RESULTS AND DISCUSSION

Serological detection of brucellosis - A total of 162 sera from vaccinated animals and 51 sera from unvaccinated animals were processed for detection of *Brucella* antibodies by RBPT. From these 213 sera samples, 85 showed the presence of *Brucella* antibodies showing overall prevalence of 39.9%(Table 2). The collected data showed a prevalence of *Brucella* antibodies is more in unvaccinated cattle, i.e. 50.98% in comparison with vaccinated cattle, i.e. 36.41%. The collected data indicates that Pune had the highest percent positivity (57%), followed by Ahilyanagar (55.8%), while Mumbai recorded the lowest (11.3%).

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Table 2. Detection of *Brucella* antibodies in cattle sera by RBPT

Sr. No.	Region	No. of samples from vaccinated animals	No. of positive samples	No. of samples from unvaccinated animals	No. of positive samples	Region-wise Percent positivity
01.	Pune	89	50	11	07	57%
02.	Ahilyanagar	01	-	33	19	55.8%
03.	Mumbai	72	09	07	-	11.3%
		162	59	51	26	39.9%

The similar results were noted by Londhe *et al*. (2011)where, overall positivity was 40.40% (40/99). The incidence reported from cattle was 37.14%. The author analysed a sum of 99 blood sera from Mumbai, Thane, Satara, Kolhapur and Ahmednagar. A highest percent positivity was noted in Satara district i.e. 70% and the lowest was noted in Ahmednagar i.e. 26.31%.

Detection of *Brucella* antibodies from milk samples - A total of 176 raw milk samples, 27 from vaccinated animals and 149 from unvaccinated animals were collected from four regions of Maharashtra and subjected to milk ring test (MRT) for detection of *Brucella* antibodies. From these 176 raw milk samples, 103 were found to be positive for MRT, suggesting the presence of *Brucella* antibodies with an overall prevalence of 58.5%Table 3. Pune region showed highest percent positivity (71.8%) followed by Ahilyanagar region (63.0%), and Akola region (57.8%) and the lowest percent positivity observed in the Mumbai region (36.3%). The collected data showed prevalence of *Brucella* antibodies through MRT is less in unvaccinated cattle i.e. 55.70% in comparison with vaccinated cattle i.e. 74.07%.

Table 3. Results of the analysis of cattle milk samples by MRT

Sr. No.	Region	No. of samples from vaccinated animals	No. of positive samples	No. of samples from unvaccinated animals	No. of positive samples	Percent positivity
01.	Pune	26	19	06	04	71.8%
02.	Ahilyanagar	01	01	72	45	63.0%
03.	Akola	-	-	38	22	57.8%
04.	Mumbai	-	-	33	12	36.3%
		27	20	149	83	58.5%

The similar results were obtained by Lindahl *et al*. (2018) who analysed 81 raw milk samples in Haryana, India for detection of *Brucella* antibodies bymilk ring test. Out of 81 samples, 45 found to be positive suggesting 55.6% of seroprevalence.

EryC PCR Assay – In EryC PCR assay, *B. abortus* 544, *B. abortus* S 19 and 199 clinical samples were analysed. *B. abortus* 544 and*B. abortus* S 19 amplified amplicon of 555bp and 1257bp respectively (Fig. 1). Then the EryC PCR assay generated amplicon in 57 clinical samples i.e. 28.64% (19 from vaccinated and 38 from unvaccinated animals) of them found positive by EryC PCR. 16 samples from vaccinated animals amplified amplicon of 1257bp and only three samples showed amplicon of 555bp.

Out of 38 samples of unvaccinated animals, eight samples comprising of two samples (one milk sample and one blood sample) from Pune region, four (one blood and three milk samples) from Ahilyanagar region, two (two milk samples) from Akola region generated amplicon of 1257bp suggesting infection is due to wild strain. In addition, two milk samples from Pune region, 25 milk samples from Ahilyanagar region, two milk samples from Akola region and one milk sample from Mumbai region generated amplicon of 555bp indicating the infection is due to vaccine strain S19.

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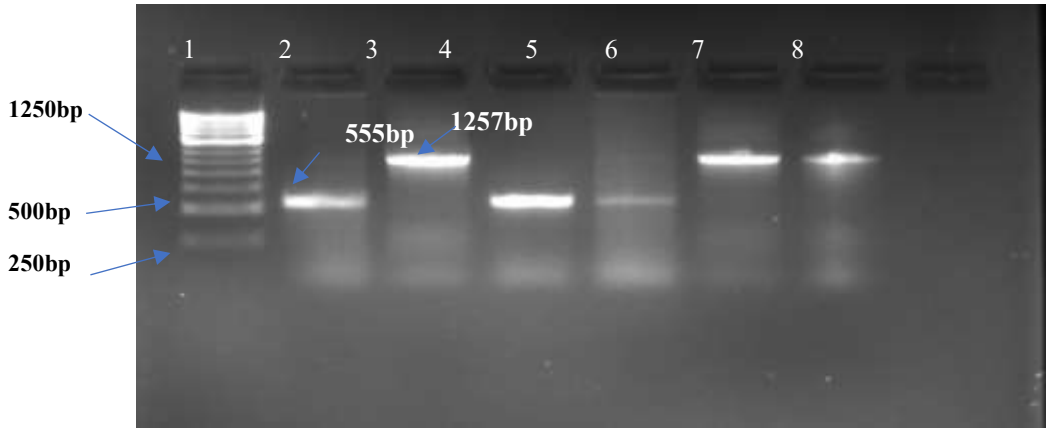

Fig. 1. Lane 1 – Ladder 250bp, Lane 2 – Positive control - *B. abortus* S19, Lane 3 – Positive Control - *B. abortus* 544, Lane 4 – Tissue Sample, Lane 5 – Milk Sample, Lane 6 – Milk sample, Lane 7 – Blood Sample, Lane 8 – Negative control

The prime goal of this investigation was to apply EryC PCR assay on clinical samples to differentiate wild strains of *Brucella abortus* from vaccine strain S19. The previous study hasstated that EryC PCR is reliable, sensitive,exhibits excellent linearity, and shows high PCR efficiency (Patil *et al*., 2014). Eoh *et al*. (2010) concluded that *Brucella abortus* EryC would be a potential reagent fordiagnosis of bovine brucellosis as a single protein antigen.

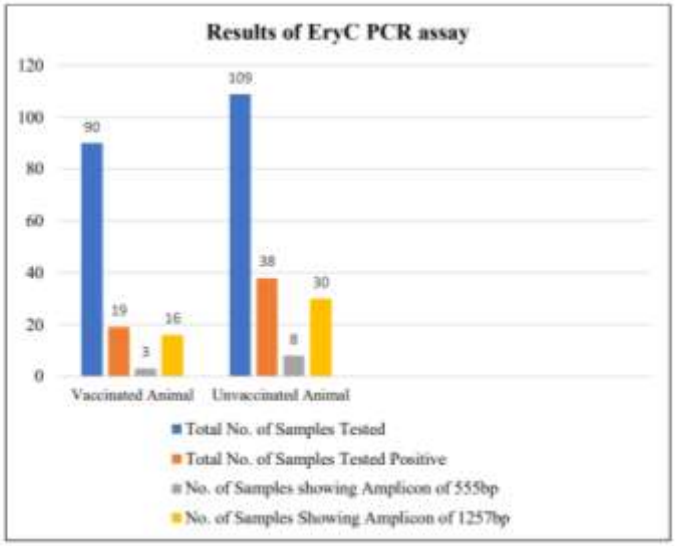

Fig. 2 – Results of EryC PCR assay

The findings of the present study differentiate wild strains of *B. aborus* from vaccine strain S19. The similar findings were reported by Sangari *et al.* (1994), Chavarria *et al.* (2006), Patil *et al.* (2014) and Thorat *et al.* (2022). Sangari *et al.* (1994) observed that vaccine strain differs from other *B. abortus* strains in its sensitivity to erythritol and carried out PCR by using primers for detection of 702bp deletion in the vaccine strain.Chavarria *et al.* (2006) standardized PCR assay, which allowed identification of strain S19 from other species, a 1063bp DNA fragment was amplified from nine strains while the other two were identified as vaccine strain S19 by amplification of 361bp DNA fragment. The difference of 702bp was obtained. In addition to this, Patil *et al.* (2014)designed a TaqMan-based 5'nuclease- real-time PCR for molecular diagnosis by targeting the 702bp deleted sequence of EryC gene from *B. abortus* S19and found that the assay was 100 per cent specific. Thorat *et al.* (2022) also, observed the difference of 702bp between wild strains of *B. abortus* and in vaccine strain S19. The similar findings were noted in the present investigation where *B. abortus* 544 amplified the amplicon of 1257bp and *B. abortus*S 19 generated amplicon of 555bp, where the deletion of 702bp was noted.

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19 positive samples were found among vaccinated animals where 16 samples generated amplicon of 1257bp suggesting the infection is due to wild strain of *B. abortus.Brucella abortus* vaccine efficacy studies conducted for cattle and demonstrated only 70% protection against wild strains of *B. abortus* (Nicolettiet *al*., 1990). Three samples generated amplicon of 555bp suggesting infection is due to vaccine strain. Similar findings were noted by Thorat *et al*. (2022) where two samples from vaccinated animal generated amplicon of 555bp.

Out of 38 samples positive from unvaccinated animals, 30 milk samples amplified a 555bp amplicon suggesting infection is due to vaccine strain S19 and eight samples amplified a 1257bp amplicon suggesting infection is due to wild strain. The similar findings were observed by Montovaniet *al*. (2020) where the vaccine strain S19 was detected in unvaccinated calves. The capacity of the vaccine strain to be transmitted between the population can explain this finding. Pachecoet *al*. (2012) studied the excretion of *Brucella abortus* vaccine B19 during a reproductive cycle of dairy cattle. They found excretion of vaccine strain in urine is much higher than milk samples up to 9 years of age, which means the organism was viable and multiplying in the urogenital system and kidneys in addition to recognised sites like lymph nodes, spleen and mammary gland. The author also concluded that the vaccinated population can be potential renal carriers. This finding may link to the present findings of the research, suggesting persistent excretion of vaccine strain, contaminating the environment and infecting unvaccinated animals through it. Contamination of bedding material with aborted material, placenta from vaccinated animal may also serve as a reason for infection to unvaccinated cattle when kept together.

CONCLUSION

The overall presence of *Brucella* antibodies in blood sera and milk were found to be 39.9% and 58.5% through rose bengal plate test (RBPT) and milk ring test (MRT) respectively The EryC PCR was employed to differentiate vaccine strain and wild strains of *B. abortus*,by targeting deletion of 702bp. The vaccine strain S19 developed amplicon of 555bp and an amplicon of 1257bp was generated by *Brucella abortus* 544. The EryC PCR assay can be utilised to distinguish between naturally infected and vaccinated population and found to be efficient for screening of cattle population for brucellosis.

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