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Evaluation of EryC as Molecular Marker for Differential Identification of *Brucella abortus* from Vaccine Strain S 19

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ABSTRACT

Background: To prevent the losses caused by brucellosis, development of diagnostic tools for differentiating vaccinated cattle from infected one is priority in the eradication of the disease. The present study was undertaken for differential identification of *Brucella abortus* from vaccine strain S19 by EryC PCR.

Methods: In this study, 1145 clinical samples of unvaccinated cattle and buffaloes (200 blood samples, 710 sera, 190 vaginal swabs, 20 abomasal contents of foetus, 25 foetal tissues) and 146 blood samples of vaccinated animals were collected from dairy farms in and around Mumbai and Pune region. These samples were processed for isolation of *Brucella* organisms and further characterized by EryC PCR.

Result: The EryC PCR yielded the amplicon of 1257 bp in *B. abortus* 544, *B. melitensis* Rev1, all field isolates, 67 vaginal swabs, 23 foetal tissue samples, 80 (40%) blood samples of unvaccinated animals and 21 blood samples of vaccinated animals. Only two out of the 146 blood samples from S19 vaccinated animals showed amplicon of 555 bp. The EryC PCR developed during the present study was found useful in differentiating wild type of *Brucella* strains from *B. abortus* S19 and also found effective when tried on reference strains and field samples.

Key words: *Brucella abortus*, *B. abortus* 544, EryC PCR, Vaccine strain S19.

INTRODUCTION

Brucellosis is a widespread, economically devastating and highly infectious zoonotic disease of animals prevalent throughout the world (Dawood, 2008). The disease causes serious economic losses to the livestock industry in terms of loss of calves, sterility, infertility, reduction or complete loss of milk yield after the abortion (Chahotal *et al.* 2003). Brucellosis continues to be of great health significance and economic importance in many countries.

Brucellosis can be eradicated from animals by quarantine of infected herds, vaccination, test and culling policy and regular surveillance. Use of attenuated live *Brucella abortus* strain S19 vaccine has been introduced in several states in the country for controlling the disease. One of the major drawbacks of this vaccine is the development of postvaccinal antibody response that interferes with the interpretation of serodiagnostic tests in eradication programmes (Nicoletti, 1980). 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 differentiate between *B. abortus* S19 from virulent *Brucella* strains, the studies have recently been focused on the *eryC* gene encoding D-erythrulose 1-phosphate dehydrogenase, which is involved in the erythritol metabolism in virulent *B. abortus* strains. However, there is a deletion of 702 bp sequence in *ery*C gene of *B. abortus* S19 (Sangari *et al.*, 1994; Patil *et al.*, 2014). This deletion of 702 bp sequence of *ery*C gene in *B. abortus* S19 strain thus provides a method to distinguish animals infected with virulent *B. abortus* strain from *B. abortus* S19 vaccinated animals by PCR amplification of this genomic region using an appropriate pair of primers (Eoh *et al.*, 2010).

The present study was planned with a view to develop a tool to differentiate *B. abortus* S19 vaccinated cattle from those infected with wild strain of *B. abortus* using EryC PCR assay.

MATERIALS AND METHODS

The permission was taken from Institutional Biosafety Committee for conducting research as per letter ref No.BVC/Dean/VPH/IBSC/221/2016 dated 02/07/2016. Reference strains of *Brucella i.e Brucella abortus* 544, *Brucella melitensis* Rev 1 and *Brucella abortus* S-19 were purchased from Division of Biological Standardization, IVRI, Izatnagar, U.P. Along with that, two isolates received from J.J. Hospital

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Mumbai and three isolates received from BLDE University, Bijapur are included in the study. A total 1145 clinical samples derived from unvaccinated cattle and buffaloes including (200) blood samples, (710) sera, (190) vaginal swabs and (20) abomasal contents of foetus and (25) foetal tissues were collected in Tryptose broth from dairy farms in and around Mumbai and Pune. Also 146 blood samples were collected from vaccinated animals from Mumbai and Pune region. The sera samples were screened by RBPT. The vaginal swabs, abomasal content and foetal tissues were processed for isolation of *Brucella* by standard methods. The isolates suspected to be of *Brucella* were subjected biochemical tests for identification (OIE, 2009). The vaginal swabs, abomasal content and foetal tissue samples were used for isolation of *Brucella* organisms and further subjected to PCR. The blood samples, vaginal swabs, abomasal content and foetal tissue samples were also used for direct DNA extraction and further characterized by EryC PCR assay. The extraction of genomic DNA of *B. abortus* from the blood samples, bacterial cultures, *Brucella* reference strains and Foetal tissue, abomasal contents was carried out by Leal-Klevezas *et al*., (1995), Romero *et al.* (1995a) and O'Leary *et al.,* (2006) and Leal-Klevezas *et al.,* (1995) with slight modifications, respectively.

The nucleotide sequence of eryC gene of *B. abortus* was retrieved from NCBI GenBank and multiple sequence alignment of eryc gene of *B. abortus* S 19 and virulent *B. abortus* 544 was carried using Clustal W tool. The designing of primers for amplification of the eryC gene was undertaken using bioinformatics tools available online using the Primer Express Software Version 2.0.at ABI. The newly designed oligonucleotide primer sequences were synthesized and supplied by Urofins India Pvt. Ltd. The details of primer sequences designed is EryC-F (5′-CATGACACGCGGCA TATAAC-3′) and EryC-R (5′- GACCTCCAGCTTACCCATGA-3′). The reaction mixtures were prepared using 200 ng/µl genomic DNA, 2.50 µl 10X PCR buffer, 2.0 µl 25 mM $MgCl_2$, 0.50 µl 10 mM dNTPs, 1 µl (10 picomols) of primers each, 0.2 µl 5U Taq polymerase. The reaction mixtures prepared as above were subjected to cyclic conditions of Initial denaturation at 94°C for 3 min; 35 cycles of denaturation at 94°C for 3 min, annealing at 60°C for 45 Sec, extension at 72°C for 1 min and final extension at 72°C for 6 min. In order to assess the ability of EryC PCR assay to detect *Brucella* spp., blood samples spiked with different concentrations of *Brucella abortus* 544 and S19 were subjected to EryC PCR assay. EryC PCR assay was also applied on all the field isolates and directly on clinical specimens like blood samples (unvaccinated and vaccinated animals), vaginal swabs, aborted foetus tissues and abomasal contents.

RESULTS AND DISCUSSION

Serological detection of brucellosis

A total of 710 bovine sera samples were processed for detection of *Brucella* antibodies using RBPT. Of these, 200 samples were found positive by RBPT with an overall serological prevalence of 28.16%.

The seroprevalence of brucellosis in animals in India has been studied by several workers in India. Handa *et al.* (1998) reported prevalence of 6.6% in Delhi; Thakur and Thapliyal (2002) observed prevalence of 4.97% in Uttaranchal, Kumar and Nanu (2005) reported prevalence of 2.45% in Kerala. Similarly, Rana *et al.* (1985) reported a prevalence of 27.7% and Kumar *et al*. (1997) found prevalence of 28.57% in Delhi. As far as the studies conducted in Maharashtra are concerned, Aher (2010) studied the seroprevalence of brucellosis in bovines using RBPT and recorded prevalence of 64.81% in Pune and 16.16% prevalence in Thane. Differences in the serological test sensitivity, infection stage, duration and design of study and variations within infected flocks may be the possible explanation for these variations among different studies (Al-Talafhah *et al*., 2003).

Isolation

A total of 26 (11.06%) *brucella* isolates were recovered from 235 clinical samples. Five human isolates are also included in the study. All the isolates exhibited morphology and staining characteristics typical of *Brucella* spp. *i.e*. they were Gram negative coccobacilli, showed acid-fastness in MZN staining and appeared red coloured. The colonies of organisms isolated on BAM appeared round, glistening and smooth; those on MacConkey agar were non lactose fermenting and on blood agar the colonies were non haemolytic. The isolates recovered were further confirmed as members of *Brucella* spp. employing different biochemical tests. All isolates produced oxidase, catalase, urease, reduced nitrates while none produced indole.

From 235 clinical samples processed, 26 isolates were recovered including 4 from foetal tissues, 6 from abomasal content and 16 from vaginal swabs. Highest proportions of (30%) isolates were recovered from abomasal contents of aborted fetuses followed by 16% isolates from foetal tissues. The isolation rate was relatively low (8.42%) from vaginal swabs.

Several workers in India have made an attempt towards isolation of *B. abortus* with varying rates of isolation. Jeyaprakash *et al.,* (1999) recorded isolation of *B. abortus* in 15 per cent cases from vaginal swabs employing tryptose agar while Das *et al.,* (1990) using *Brucella* selective medium recorded an isolation rate of 38.18 per cent in cows and 14.28 per cent in buffaloes. Isolation rates are much higher during first two weeks of symptoms (80 - 90-% in acute form and 30-70% in chronic form) (Al Dahouk *et al.,* 2003). Relatively low isolation rate (11.06%) recorded in the present study is in agreement with previous report of 6.4% (Celebi and Otlu, 2011; Kala *et al.,* 2018). Slow growing and fastidious nature of the pathogen could be another explanation for a relatively lower isolation rate (Seleem *et al*., 2010).

Differentiation of *Brucella abortus* S 19 and wild strains by EryC PCR

In order to assess the ability of EryC PCR assay to detect *Brucella* spp., blood samples spiked with different concentrations of *Brucella abortus* 544 and S19 were subjected to EryC PCR assay. The results are presented in Table 1. The results showed that no amplification was generated at a concentration of 4×10^3 organisms per ml of blood while specific amplification product of 555 bp and 1257 bp was generated at the concentrations of 2×10^4 and 3×10^4 organisms per ml of blood (Plate 1). In blood samples spiked with *B. abortus* 544 a product of 1257 bp was generated while in those spiked with *B. abortus* S19 a product of 555 bp was generated. In samples spiked with both *i.e*. *B. abortus* 544 and *B. abortus* S19 two bands one each of 1257 bp and 555 bp was generated. The results clearly suggested that the EryC PCR assay developed during the present work is effective in differentiating between *B. abortus* virulent strains and vaccine strain S19 and could be used for differentiation of vaccinated and infected animals.

All the isolates and reference strains were also simultaneously subjected to EryC PCR. *Brucella abortus* S 19 revealed amplicon of 555 bp while *B. abortus* 544 and *B. melitensis* Rev1 showed amplicon of 1257 bp. All the 31 isolates showed amplification product of 1257 bp (Plate 2).

This EryC PCR was also applied directly on clinical samples *i.e* blood samples (146) of vaccinated animals. Twenty one blood samples showed amplification product of 1257 bp while two blood samples showed amplicon of 555 bp. The results of EryC PCR assay are presented in Table 2. Thus *B. abortus* S19 specific amplification product of 555 bp was noticed in only two out of the total 146 vaccinated animals. The detection rate of *B. abortus* S19 from vaccinated animals was relatively low by EryC PCR which could possibly be

Table 1: Detection of *Brucella* from spiked blood samples by EryC PCR assay.

Organism (s) spiked	Concentration of organisms	Amplification product of 1257 bp	Amplification product of 555 bp
B. abortus 544	4×10^3	-	-
	2×10^4	+	-
	3×10^4	+	-
B. abortus S19	4×10^3	-	-
	2×10^4	-	+
	3×10^4	-	+
B. abortus 544 + *B. abortus* S19	4×10^3	-	-
	2×10^4	+	+
	3×10^4	+	+

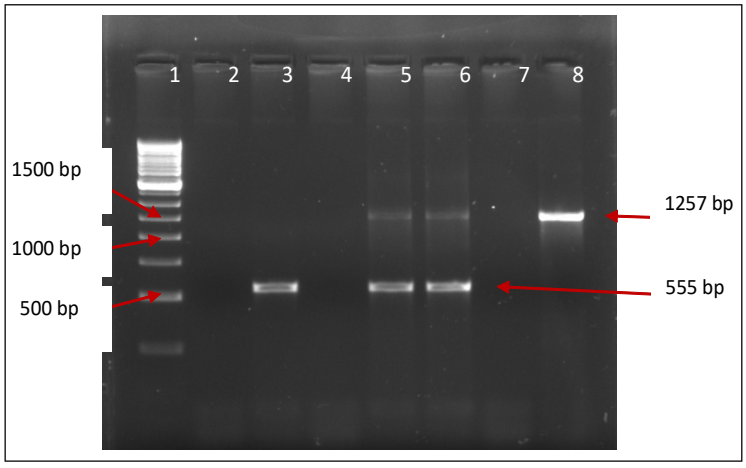

Plate 1: Detection of *B. abortus* S19 and 544 in spiked blood samples by EryC PCR.
Lane 1- 250 bp Ladder, 2 and 3 -S19, 4, 5 and 6- S19 and 544, 7 and 8- 544.
Concentration of S19 in 2 and 3- (4×10^3 and 2×10^4).
Concentration of S19 and 544 in 4, 5 and 6- (4×10^3, 2×10^4 and 3×10^4).
Concentration of 544 in 7 and 8- (4×10^3 and 2×10^4).

Table 2: Direct detection of *Brucella* spp. from clinical samples by EryC PCR.

Type of clinical sample	No. of samples processed	No of samples positive for 1257 bp product	No of samples positive for 555 bp product
a) Whole blood samples from unvaccinated animals	200	80	00
b) Whole blood samples from vaccinated animals	146	21	02
Vaginal swab	190	67	00
Abomasal content and Foetal tissue	45	23	00
Total	581	191	02

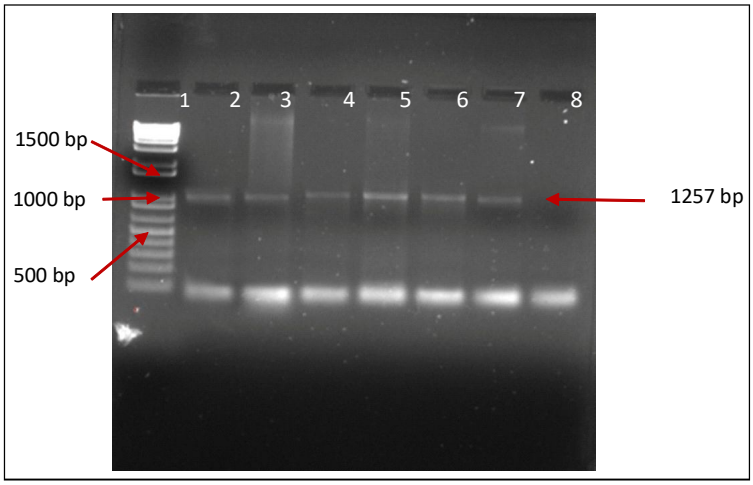

Plate 3: Identification of vaginal swabs, Foetal tissue and Abomasal content by EryC PCR assay. Lane 1 - 1 Kb Ladder, 2 and 3- Vaginal swabs, 4 and 5- Foetal tissue, 6 and 7- Abomasal content, 8-Negative Control.

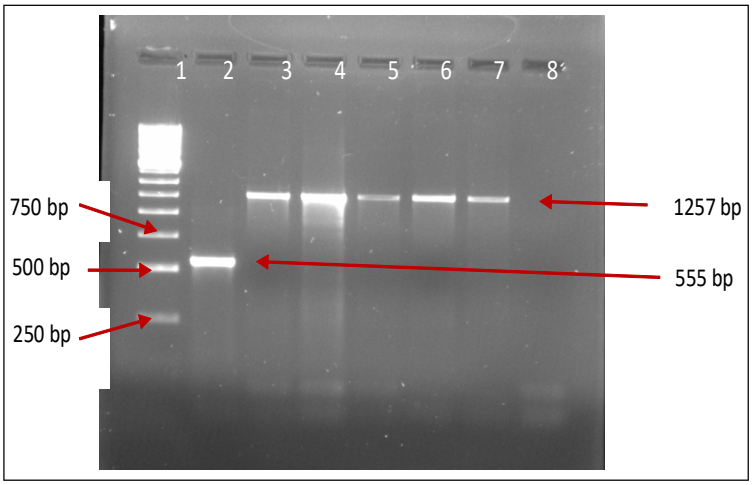

Plate 2: Identification of field isolates of *Brucella* spp. by EryC PCR. Lane B-250 bp Ladder, 2-S19, 4- *B. abortus* 544, 3 and 5-7- Isolates, 8- Negative control.

attributed to the stage of collection of blood after vaccination. In the present investigation, the blood samples were collected from vaccinated animals randomly without following any specific timeline between collection of blood and vaccination. Among the other clinical samples tested including 200 blood samples of unvaccinated animals, 190 vaginal swabs and 45 abomasal contents and foetal tissues, none was found positive for *B. abortus* S19 specific amplification product of 555 bp (Plate 3).

Perusal of literature on development of PCR assay for differentiation of *B. abortus* S19 from wild type of *Brucella* strains indicates that several workers have attempted development of assay targeting EryC gene (Tuba *et al*., 2012, Chavarria *et al*., 2006, Sangari *et al*., 1994). The results of EryC PCR regarding its efficacy in differentiating the vaccinated and infected animals are in agreement with the findings of Patil *et al.* (2014).

The EryC PCR assay developed during the present study proved effective in differential identification of animals infected with virulent *Brucella* strains from those vaccinated with *B. abortus* S19.

CONCLUSION

In countries where vaccination program is implemented to eradicate *Brucellosis*, it is important to have a tool that can differentiate animals infected with wild strain from those vaccinated with S19 strain. The reliable differentiation of vaccine strains from field isolates is an important element in brucellosis control programs. The conventional methods cannot meet this requirement however *B. abortus* S19 vaccine strain can be readily differentiated from field strains by EryC PCR. The EryC PCR assay developed during the present investigation thus could be an effective tool in

differentiation of S19 vaccine strain from wild type of strains of *Brucella* spp.

Conflict of interest: None.

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