



SHORT COMMUNICATION

Molecular Typing of *Brucella abortus* Strains Isolated from Cattle in Different Districts of Pakistan Based on Bruce-Ladder-PCR and MLVA-16 Assays

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ARTICLE HISTORY (18-410)

Received: October 21, 2018
Revised: November 25, 2018
Accepted: November 26, 2018
Published online: January 24, 2019

Key words:

Bovine
Brucellosis
Genotyping
Punjab

ABSTRACT

Bovine brucellosis is endemic in Pakistan. But little is known about the prevalent *Brucella* species and biovars. We have performed molecular typing of 17 *Brucella* isolates from cattle by Bruce-Ladder-PCR and Multiple-Locus Variable number tandem repeat Analysis (MLVA)-16 genotyping. These *Brucella* isolates were recovered from aborted fetuses, milk samples and vaginal swabs from recently aborted cattle in different districts in Pakistan. All isolates were confirmed by conventional culture methods and PCR. All strains had identical MLVA-16 profiles. Results of this study concluded that the same genotype of *B. abortus* is circulating in different districts of Pakistan which considered as the main risk of spread of this infection in Pakistan. This data will be helpful to develop prevention and control strategies for human and bovine brucellosis in Pakistan.

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To Cite This Article: Ali S, Akhter S, Khan I, Ahmed H, Maalik A, Neubauer H, Melzer F, El-Adawy HH, 2019. Molecular typing of *Brucella abortus* isolated from cattle in different districts of Pakistan based on Bruce-ladder-PCR and MLVA-16 assays. Pak Vet J. <http://dx.doi.org/10.29261/pakvetj/2019.014>

INTRODUCTION

Brucellosis is a major zoonotic disease of humans, livestock and wildlife worldwide. This disease is responsible for significant economic losses in terms of morbidity and mortalities in farm animals (Ali *et al.*, 2015; Ali *et al.*, 2016; Ali *et al.*, 2018). Currently, the genus *Brucella* (*B.*) has 12 species, which are differentiated into various biovars/biotypes (Scholz and Vergnaud, 2013). The division of *Brucella* species was commonly done on the basis of their host preferences and pathogenicity. In humans and small ruminants *B. melitensis* is identified as the main causative agent of brucellosis. *B. abortus* and *B. suis* cause bovine and swine brucellosis, respectively. But cross species infections are reported for various *Brucella* species for environments where different livestock species share the same resources (Weiner *et al.*, 2011). Various techniques are used for the identification and characterization of *Brucella* isolates from human and animal origin. Historically, identification and typing of *Brucella* isolates requires culture and physical and biochemical tests.

However, PCR based techniques are safe, quick and easy to perform. Moreover, genotyping using PCR helps us to determine phylogenetic and geographic association of zoonotic agents like *B. abortus*. Although, brucellosis is endemic in Pakistani cattle's no information is available about genetic diversity of prevalent *Brucella spp.* The aim of the present study was to use Bruce-ladder and MLVA-16 genotyping assays for confirmation and to identify genetic variations in *B. abortus* isolates from cattle of different districts of Pakistan.

MATERIALS AND METHODS

Isolation of *Brucella* isolates: *Brucella abortus* isolates were isolated from serologically positive milk samples, vaginal swabs and aborted fetuses collected from cattle having a recent abortion history in different regions of the Potohar Plateau, Pakistan (Table 1). Modified Farrell's serum dextrose agar was used for isolation of *Brucella*. Suspected colonies were identified according to standard procedures by modified Ziehl-Neelsen staining and biochemically (Alton *et al.*, 1988).

Extraction of DNA: Extraction of DNA from *Brucella* colonies was done using the High Pure PCR Template Preparation Kit (Roche Diagnostics®, Mannheim, Germany) and stored at -20°C for further analysis.

PCR-Bruce Ladder and MLVA-16 Assays: Genotyping of *Brucella* isolates was performed using Bruce-ladder - PCR assays and MLVA-16 assays. The Bruce-ladder PCR was performed using eight pairs of primers for a multiplex PCR assay and PCR products were analyzed by gel electrophoresis (Garcia-Yoldi *et al.*, 2006). MLVA-16 assays were performed using 16 pairs of primers and data were analyzed as described earlier (Le-Fleche *et al.*, 2006).

RESULTS AND DISCUSSION

All 17 *Brucella* isolates were phenotyped as *B. abortus* biovar 1. These isolates were isolated from aborted fetuses (n=6), vaginal swabs (n=6) and milk samples (n=5). The isolates were confirmed by Bruce-Ladder PCR as *B. abortus* as shown in Fig. 1. Bovine brucellosis caused by *B. abortus* is an important zoonotic disease with a great economic impact. In Pakistan no control or eradication programs are in place and no specialized laboratories are available to perform sophisticated phenotyping of *Brucella* isolates. PCR based methods can reduce the risk of laboratory infection and are useful tools in epidemiology to identify links. These techniques have also the potential to be implemented in diagnostic laboratories in Pakistan. Commonly, *B. abortus* is prevalent in case of brucellosis in bovines, *B. melitensis* and *B. suis* have also been identified as the causative agents in regions where bovines have close contact to goats and sheep or pigs, respectively (Liu *et al.*, 2012; Fretin *et al.*, 2013). Cohabitation of bovines and small

ruminants is common in Pakistan. Under these conditions, the use of multiplex PCR is very important for differentiation of *Brucella* at species level. A new Bruce-ladder multiplex PCR assay was introduced for diagnosis of *B. abortus* (biovar 1, 2 or 4) and all biovars of *B. melitensis*, *B. suis* and *B. ovis* biovar 1 and to differentiate between vaccine strains S99 and RB51 of *B. abortus* (Lopez-Goni *et al.*, 2011). In the present study, all isolates could be confirmed genetically as *B. abortus* based on Bruce-Ladder PCR, which is in accordance to previous findings from other countries i.e. India and Tanzania (Nagalingam *et al.*, 2012; Mathew *et al.*, 2015). *Brucella abortus* isolates have been isolated from Pakistani cattle and buffaloes and phenotyped previously (Ali *et al.*, 2014).

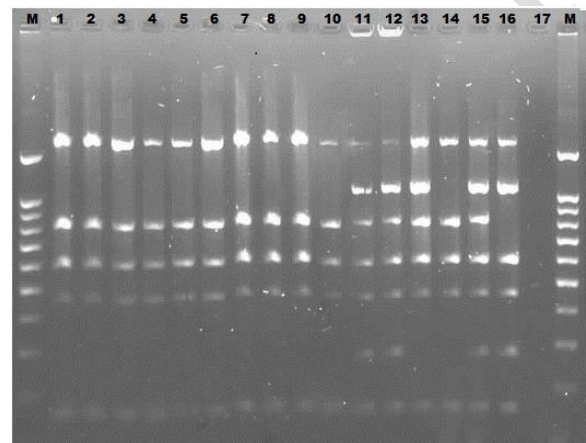


Fig. 1: Bruce-Ladder PCR results for *Brucella* isolates from Pakistan. Line M=100-bp mark; Line 1-10= DNA of *B. abortus* isolates from Pakistan; Line 11-12 and 15= *B. suis*; line 13=*B. melitensis* 16 M as positive control; Line 14=*B. abortus* BA544 as positive control; Line 16= *B. canis*; Line 17 negative control.

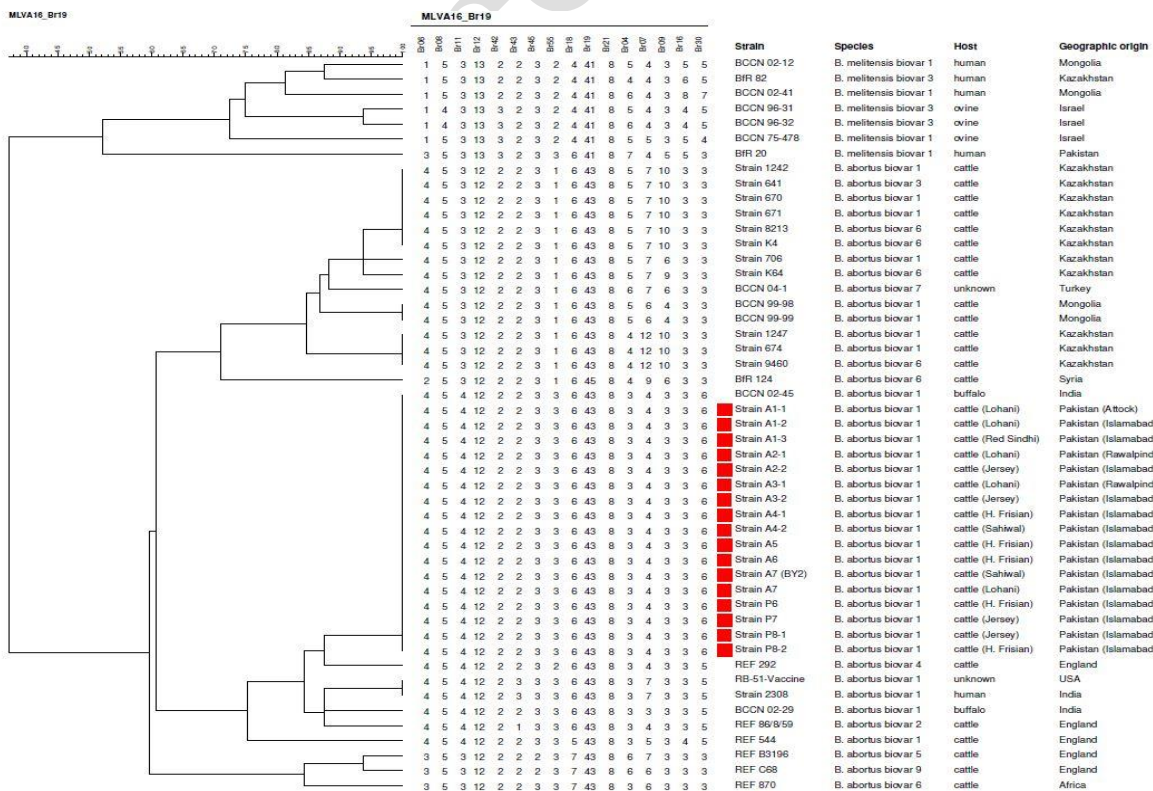


Fig. 2: Dendrogram showing relationships between the 17 *B. abortus* biovar I isolates from cattle in Pakistan and other *Brucella* spp. isolated from neighboring countries.

Table 1: Source of *Brucella abortus* isolates from cattle in Pakistan

Isolate No.	Sample source	District	Animal breed
1	AF	Attock	Lohani
2	AF	Rawalpindi	Lohani
3	AF	Rawalpindi	Lohani
4	VS	ICT	H. Frisian
5	VS	ICT	H. Frisian
6	VS	ICT	H. Frisian
7	VS	ICT	Lohani
8	VS	ICT	Lohani
9	M	ICT	H. Frisian
10	M	ICT	H. Frisian
11	VS	ICT	Jersey
12	AF	ICT	Jersey
13	AF	ICT	Red Sindhi
14	AF	ICT	Jersey
15	M	ICT	Jersey
16	M	ICT	Sahiwal
17	M	ICT	Sahiwal

AF aborted fetus; M milk; VS vaginal swab; ICT Islamabad Capital Territory.

In the present study, the phylogenetic relationship of 17 *B. abortus* biovar1 isolates was assessed with MLVA-16. All had the same genotype (Fig. 2). This genotype was also previously isolated from a buffalo in India (Fig. 2). On the basis of genetically similarity of 17 *B. abortus* biovar 1 isolates of Pakistan with Indian isolates. It could be assumed that the source of brucellosis infection in Pakistani cattle is the same as previously reported in India. The phylogenetic relationship of local isolates of *B. abortus* was investigated successfully using MLVA-16 genotyping which is a prove of the effectiveness of this genotyping method as a useful tool to trace back infection in the setting of Pakistan. The genotype found was different to that of *B. abortus* and *B. melitensis* isolates from different countries (<http://microbesgenotyping.i2bc.paris-saclay.fr/>) (Fig. 2). MLVA genotyping was used to characterize *Brucella* spp. isolated from animals and humans in China and South Korea (Jiang *et al.*, 2011; Kang *et al.*, 2011) and various genotypes were found in each country. However, in the present study, only one single genotype of *B. abortus* (biovar1) was identified as the causative agent of brucellosis in cattle pointing to the fact that a single introduction event or single source led to the spread of brucellosis in the study area. Possible reason for the lack of genetic diversity found may be use of an inadequate vaccine strain, import of an infected exotic or cross breed cattle being the index case or sampling bias (e.g. animals belonging to the same production unit). Next generation sequencing (NGS) has higher resolution power than MLVA and might be helpful in this setting.

Conclusions: This study provides the first evidence that only one genotype of *B. abortus* is circulating in Pakistani cattle of the Potohar district. Topographically the study area is different from others parts of Pakistan and our findings might be only a local phenomenon. A detailed study including various sampling areas within the country

with representative numbers of *Brucella* isolates is now mandatory.

Funding: We gratefully acknowledge the financial support by the “Higher Education Commission, Pakistan.

Acknowledgements: The authors thank Dr. Gernot Schmoock at Friedrich-Loeffler-Institut, Jena, Germany for his help in MLVA phylogenetic tree formulation. The authors thank Federal Foreign Office, Germany “German Biosecurity Programme” for their support to achieve this work.

Authors contribution: SA, ShA and IK conceived and designed the study. SA and IK performed the study and analyzed the data. AM, SA, FM, HN, HE and HA wrote the article. SA, ShA, IK, HA, FM, HN, HE and AM proofread the manuscript and approved final version.

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