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Research Article

Molecular and Microbiological Characterization of *Brucella* Species Isolated in Sudan between 2005 - 2015

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Abstract

Brucellosis is considered one of the most important bacterial zoonosis worldwide. The disease in Sudan is caused by biovars of *Brucella abortus and B. melitensis* and affects most domestic livestock species (i.e. cattle, sheep and goats, camels and equines) and humans. little Information is known about circulating biovars and suitable molecular assays for diagnosis and characterization of *Brucella* spp. in this country. The aim of the current study was to characterize *Brucella* spp. obtained in this and previous studies using microbiological and molecular methods.

In the current study 20 *Brucella* isolates were microbiologically typed (biotyped) as *B. melitensis* (n = 3) bv 1, 2 and 3 and *B. abortus* (n = 17) bv 1 (n = 1), bv 3 (n = 1), bv 6 (n = 13). Two *Brucella abortus* isolates were doubtful with biotyping as either bv 6 or 1 due to inconclusive growth in thionin-added medium. The *Brucella* species-specific Bruce-ladder PCR confirmed the 17 *B. abortus* isolates along with the three *B. melitensis*. While the AMOS PCR identified all *Brucella melitensis* and only the two doubtful *Brucella abortus* as S19 vaccine strain (*B. abortus* bv 1). *B. abortus* bv 6 is the predominant cause of bovine brucellosis in this country. This knowledge and further molecular testing could contribute towards brucellosis control in Sudan.

Keywords: *Brucella abortus; B. melitensis; Sudan*

Abbreviations

AMOS: First Latter from Abortus, melitensis, ovis, and suis; bv: Biovar; bp: Base Pair; DNA: Deoxyribonucleic Acid; ITS: Interspacer; min: Minute; ml: Millimeter; µl: Micrometer; mM: Micro Mole; mTM: Modified Thayer Martin Medium; ng: Nanogram; n: Number; PCR: Polymerase Chain Reaction; sec: Second; µg: Microgram; xg: Relative centrifuge force measured in multiples of the standard acceleration due to gravity at the Earth's surface

Introduction

Brucellosis is an important bacterial zoonosis worldwide [1]. The disease in human is a flu-like febrile illness that can be mild, acute or chronic debilitating illness [2]. However; the disease in animals affects mainly the reproductive performance resulting in abortion, stillbirths, giving birth to unthrifty calves, reduction in milk yield, mastitis, endometritis, and placentitis and orchitis and epididymitis in male [3]. The disease is caused by members of the genus *Brucella* of which *B. abortus* and *B. melitensis* were the only (to the moment)

reported species responsible for animal brucellosis in Sudan. Bennet in 1943 [4] reported the first isolation of *B. abortus* from a dairy farm in Khartoum State. *B. melitensis* isolation reported in 1957 from Al Gazira region (Central Sudan) when cases of febrile illness observed among foreigners visiting the area [5]. Later, several studies were conducted mostly towards understanding the epidemiology of brucellosis with the majority of these studies applying serology [6-12] rather than isolation [13-17] with few researches using molecular tools [18-20]. Apart from the limitations associated with isolation of brucellae (i.e. slow growth with high contamination, intermittent shedding that confer most sampling to yield negative results… etc.), the use of molecular testing is rather not performed in most diagnostic facilities in Sudan. This may be due to lack of equipment, skilled personnel, adequate funds that hampering the capability of researchers to conduct comprehensive studies. Data on species-specific PCR tests for *Brucella* were few if not any. The aim of current study was to identify *Brucella* isolated in Sudan between 2005 - 2015 using microbiological methods and to apply *Brucella* specific PCR assays such as (ITS), AMOS and Bruce-Ladder PCRs to investigate suitability of these assays to be used for molecular identification of *Brucella* in Sudan.

Materials and Methods

Study area, samples collection, isolation and *Brucella* stains

This study conducted in Khartoum State included 127 dairy cattle farm distributed in six localities. Milk samples collected during 2014 - 2015 from brucellosis seropositive cows (n = 541) tested previously [6] (Table 1). Sampled animals were selected based on owner/farmers' consent and ethical approval (attached) obtained from the Ethical Committee of the Deanship of Scientific Research (University of Sudan of Science and Technology) prior to samples collection. 20 ml milk samples collected from each teat directly into a sterile 100 ml falcon tubes after discarding the first streams. Milk collection performed after cleaning and disinfecting the udder. The milk samples transported on ice to the laboratory were stored at 4°C until examined. Culture attempted on modified Thayer Martin (mTM) medium and processed as previously described [21,22]. Briefly, mTM inoculated with pellets and deposits of milk samples centrifuged at 3000 xg at 4°C for 15 minutes followed by incubation in 10% CO_2 atmosphere at 37°C and examined for growth of *Brucella*-like organisms after 3 - 7 days [23]. Suspect colonies were examined with Gram's and modified Ziel-Neelsen's staining methods. Acid fast small gram-negative coccobacilli from suspect colonies were further biotyped at the Animal and Plant Health Agency bacteriology laboratory in Surry, UK (Table 2). Biotyped *Brucella* strains (n = 7; Table 2 (SN 15-21)) from 2005 to 2015 of the Central Veterinary Research Laboratories (CVRL) collection in Sudan were included in this study along with *Brucella* reference strains (Table 2; SN 22-26).

Locality	Cattle population (adults)	No. of seropositive animals	Prevalence (%)*	Sample size	Brucella species ** and biovars (bv)
Karrari	8032	2217	27.6	58	*B. abortus* bv. 6 (n = 1)
Omdurman	7846	1495	19.1	79	-
Umbada	17019	3506	20.6	73	*B. abortus* bv 6 (n = 2)
Bahri	16188	4937	30.5	132	*B. abortus* bv 6 (n = 1); *B. abortus* S19 (n = 2)
(Shargalnile) East Nile	79777	18668	23.4	167	*B. abortus* bv 6 (n = 7)
Jabel Awolia	11764	4117	35	32	*B. abortus* bv 6 (n = 1)
Total	143688	39566		541	14 *B. abortus* isolates

Table 1a: *Information of samples collected for Brucella culturing from brucellosis rose Bengal test (RBT) seropositive animals in Khartoum state localities in Sudan.*

**Result summary obtained from [33].*

*** Brucella species isolated from milk in Sudan listed in Table 2; SN: 1-14 based on biotyping.*

Strain number (SN)	Strain lab ID	Geographic origin	Year	Specimen	Host	*Brucella* species and biovar[a]
1	333_2/14	Khartoum/SD	2014	Milk	Bovine	*B. abortus* bv 6
2	333_2/15	Khartoum/SD	2015	Milk	Bovine	*B. abortus* bv 6
3	188_4/14	Khartoum/SD	2014	Milk	Bovine	*B. abortus* bv 6
4	188_4/15	Khartoum/SD	2015	Milk	Bovine	*B. abortus* bv 6
5	1/M_15	Khartoum/SD	2015	Milk	Bovine	*B. abortus* bv 6
6	2/M_15	Khartoum/SD	2015	Milk	Bovine	*B. abortus* bv 6
7	6_7_15	Khartoum/SD	2015	Milk	Bovine	*B. abortus* bv 6
8	6_8_15	Khartoum/SD	2015	Milk	Bovine	*B. abortus* bv 6
9	Braig715	Khartoum/SD	2015	Milk	Bovine	*B. abortus* bv 6
10	BtBraig715	Khartoum/SD	2015	Milk	Bovine	*B. abortus* bv 6
11	Soba9_15	Khartoum/SD	2015	Milk	Bovine	*B. abortus* bv 6
12	B_Soba 915	Khartoum/SD	2015	Milk	Bovine	*B. abortus* bv 6
13	Sarah1114	Khartoum/SD	2014	Milk	Bovine	*B. abortus* bv 6
14	Sarah1115	Khartoum/SD	2015	Milk	Bovine	*B. abortus* bv 6
15[b]	SO_M1_08	Khartoum/SD	2008	LN	Camel	*B. melitensis* bv 2
16[b]	SO_M3_05	Darfur/SD	2005	LN	Camel	*B. melitensis* bv 1
17[b]	SO_M2_05	Khartoum/SD	2005	Placenta	Bovine	*B. melitensis* bv 3
18[b]	SO_BA1_05	Darfur/SD	2005	LN	Camel	*B. abortus* bv 1
19[b]	SO_BA6_06	Gazira/SD	2006	Milk	Bovine	*B. abortus* bv 6
20[b]	SO_BA3_09	Khartoum/SD	2009	Milk	Caprine	*B. abortus* bv 3
21[b]	BMH_14	Gadarif/SD	2014	Blood	Human	*B. melitensis* bv 1
22[c]	REF86/8/59	England		Milk	Bovine	*B. abortus* bv 2

Table 1b: *Brucella strains and information used in the current study.*

BCCN: Brucella culture collection, Nouzilly, France; SD: Sudan.

[a]: Identification based on biotyping

[b]: Isolates obtained from the CVRL-Sudan collection.

[c]: Reference strains.

LN: Lymph Node.

Strain number (SN)	Geographic origin	Specimen	Year	Host	*Brucella* species and biovar[#]
1	Khartoum/SD	Milk	2014	Bovine	*B. abortus* bv.6
2	Khartoum/SD	Milk	2015	Bovine	*B. abortus* bv.6
3	Khartoum/SD	Milk	2014	Bovine	*B. abortus* bv.6
4	Khartoum/SD	Milk	2015	Bovine	*B. abortus* bv.6
5	Khartoum	Milk	2015	Bovine	*B. abortus* bv.6
6	Khartoum	Milk	2015	Bovine	*B. abortus* bv.6
7	Khartoum	Milk	2015	Bovine	*B. abortus* bv.6
8	Khartoum	Milk	2015	Bovine	*B. abortus* bv.6
9	Khartoum	Milk	2015	Bovine	*B. abortus* bv.6
10	Khartoum	Milk	2015	Bovine	*B. abortus* bv.6
11	Khartoum	Milk	2015	Bovine	*B. abortus* S19

12	Khartoum	Milk	2015	Bovine	*B. abortus S19*
13	Khartoum	Milk	2014	Bovine	*B. abortus* bv. 6
14	Khartoum	Milk	2015	Bovine	*B. abortus* bv.6
15*	Khartoum	L. Node	2008	Camel	*B. melitensis* bv.2
16*	Darfur	L. Node	2005	Camel	*B. melitensis* bv.1
17*	Khartoum	Placenta	2005	Bovine	*B. melitensis* bv.3
18*	Darfur	L Node	2005	Camel	*B. abortus* bv.1
19*	Gazira	Milk	2006	Bovine	*B. abortus* bv. 6
20*	Khartoum	Milk	2009	Caprine	*B. abortus* bv. 3
21*	Gadarif	Blood	2014	Human	*B. melitensis* bv.1
22**	USA			Dog	*B. canis* (RM6/66)
23**	Australia			Ovine	*B. ovis*
24**	England	Milk		Bovine	*B. abortus* bv.2 (BCCN R5; REF86/8/59)
25**	USA	Milk		Bovine	*B. abortus* S19 vaccine strain
26**					*B. melitensis* rev1 vaccine strains

Table 2: *Brucella strains and information used in the current study.*

BCCN: Brucella culture collection, Nouzilly; SD: Sudan.

 # Identification based on biotyping; AMOS and Bruce-ladder PCR assays.

**: Isolates obtained from the CVRL-Sudan collection;*

***: Reference strains.*

DNA extraction and PCR assays and criteria for analysis

Genomic DNA was extracted from all *Brucella* strains (n = 21) from Sudan (Table 2). Extraction was done using the High Pure PCR Template Preparation Kit (Roche Diagnostics), following the manufacturer's instructions. Extracted DNA was quantified and store at -20°C until tested. Whole DNA amplification was performed for samples with low DNA concentration (SN. 8 and 21) using the GenomiPhi V2 DNA Amplification Kit following to the manufacturer's instruction (GE Health Care, USA, http://www3.gehealthcare.com/en).

Brucella specific 16S-23S rDNA interspacer PCR

The *Brucella* specific 16S-23S rDNA interspacer region was amplified with primers ITS66: ACA TAG ATC GCA GGC CAG TCA and ITS279: AGA TAC CGA CGC AAA CGC TAC as described by Keid., *et al* [24]. The PCR reaction in 15 µl composed of 1x MyTaq mix (Bioline), 0.4 mM of each primer and 10 ng template DNA. PCR conditions consisted of an initial denaturation at 95°C for 2 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing 62°C for 30 seconds, extension at 72°C for 30 seconds and final extension at 72°C for 5 minutes. *Brucella* reference strains (i.e. *Brucella canis*, *B. ovis*, *B. abortus* bv 2, *B. abortus* S19 vaccine and *B. melitensis* Rev1 vaccine (Table 2)) were included as controls. Amplification was performed on a GeneAmp-PCR System 2700 thermal cycler (Applied Biosystems) and PCR products were separated by gel electrophoresis on a 2% agarose gel stained with ethidium bromide (0.5 µg/ml, Invitrogen). The DNA bands were visualized under UV-illuminator camera (Bio-Rad) and photographed.

AMOS and Bruce-ladder PCR

The AMOS-PCR was performed as described previously [25-27]. The PCR mixture contained 1X MyTaq mix (Bioline), a combination of five *Brucella* species specific forward primers (0.2 µM each) and reverse IS711 (1 µM), 10 ng DNA in 15 µl reaction. The PCR conditions

consisted of an initial denaturation at 95°C for 30 minutes followed by 35 cycles of denaturation at 95°C for 1 minute, annealing 60°C for 2 minutes and extension at 72°C for 2 minutes. AMOS PCR is capable of identifying *B. abortus* bv 1,2 and 4, *B. melitensis* (all biovars), *B. ovis* and *B. suis* bv 1 and *Brucella* vaccine strains.

Bruce-ladder PCR was performed in a 15 µl PCR reactions composed of 1X MyTaq mix (Bioline), 0.4 mM of each primer (8 primer pairs) and 10 ng template DNA. PCR conditions consisted of initial denaturation at 95°C for 3 minutes, followed by 25 cycles at 95°C for 35 secs, 64°C for 45 seconds and 72°C for 3 minutes and a final extension at 72°C for 5 minutes on a GeneAmp-PCR System 2700 thermal cycler (Applied Biosystems). *Brucella* reference DNA and water as positive and negative controls were respectively included for both PCR assays. PCR products were separated by gel electrophoresis on a 2% agarose gel stained with ethidium bromide (0.5 µg/ml, Invitrogen), and DNA bands visualized under UV-illuminator camera (Bio-Rad) and photographed. This test can differentiate in a single step all of the classical *Brucella* species, including those found in marine mammals and the S19, RB51, and Rev.1 vaccine strains [28,29].

Results

Brucella strains from Sudan

Brucella strains in this (n = 14; SN1-14) study were all shown to be non-motile, gram-negative, oxidase positive acid-fast small rods. Likewise, on biotyping these strains showed identical traits and characterized as *B. abortus* bv 6 except SN 11-12 which were doubtful as either bv1 or 6 due to inconclusive growth on media plates impregnated with thionin dye (Table 2). Three *Brucella abortus* bv 1, 6 and 3 (SN 18-20) along with four *B. melitensis* bv 1, 2 and 3 "n = 2" (SN 15-17 and 21) were identified from isolates in CVRL collection.

Brucella specific 16S-23S rDNA interspacer PCR

All 21 isolates from Sudan were confirmed as *Brucella* using the *Brucella* specific 16S-23S rDNA interspacer region PCR as the 214 bp product was amplified (Figure 1). *Brucella* strain SN 8 did not amplify initially but the DNA template was increase with the GenomPhi kit (GE Health Care, USA, http://www3.gehealthcare.com/en) where after it produced PCR products (Figure 2).

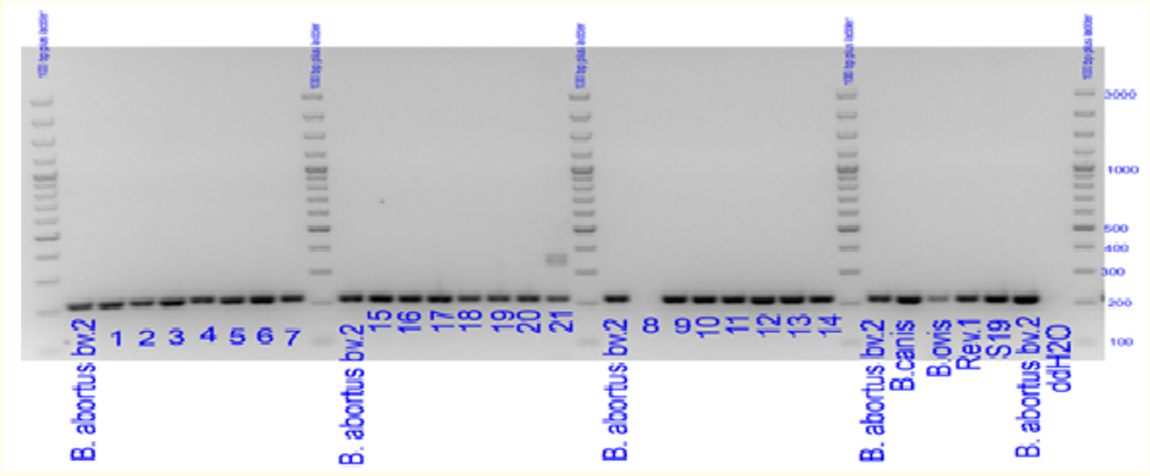

Figure 1: *Brucella specific 16S-23S rDNA interspacer PCR profile. Lanes M contain the Gene Ruler 100bp plus DNA marker; lanes B. abortus bv.2 (BCCN R5; REF86/8/59); lane B. canis (RM6/66); B. ovis (REF63/290); Rev.1 (B. melitensis Rev.1vaccine stain); S19 (B. abortus S19 vaccine strain); lane ddH2O (negative control), lanes 1-7, 8-14, 15-21 (SN) contains DNA from Brucella isolates from Sudan shown in table 2.*

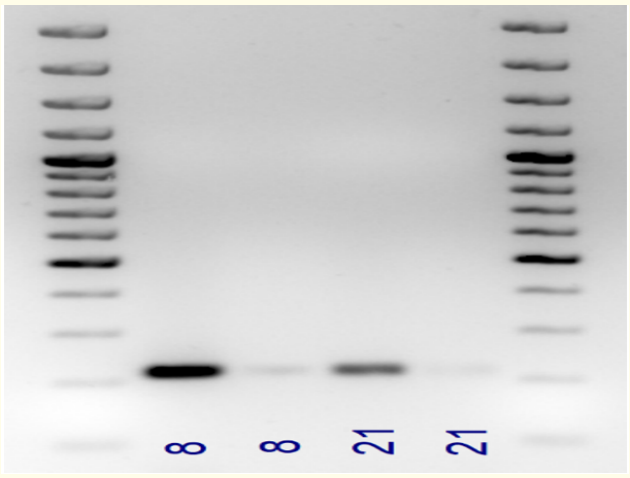

Figure 2: *16S-23S rDNA PCR Profile. Data shown: lanes 1 and 6 consisted of 100 bp plus DNA marker (Invitrogen); lanes 2-3 consisted of duplicated DNA from sample SN 8; lanes 4-5 consisted of duplicated DNA from sample SN 21.*

AMOS and Bruce-ladder PCR

Using the AMOS-PCR assay two samples SN 11-12 (Table 2) were identified as *B. abortus* S19 vaccine strain as the eri locus was not amplified in these samples, and three samples SN 15-17 (Table 2) were identified as *B. melitensis*. AMOS PCR did not amplify the other *Brucella* samples (Figure 3).

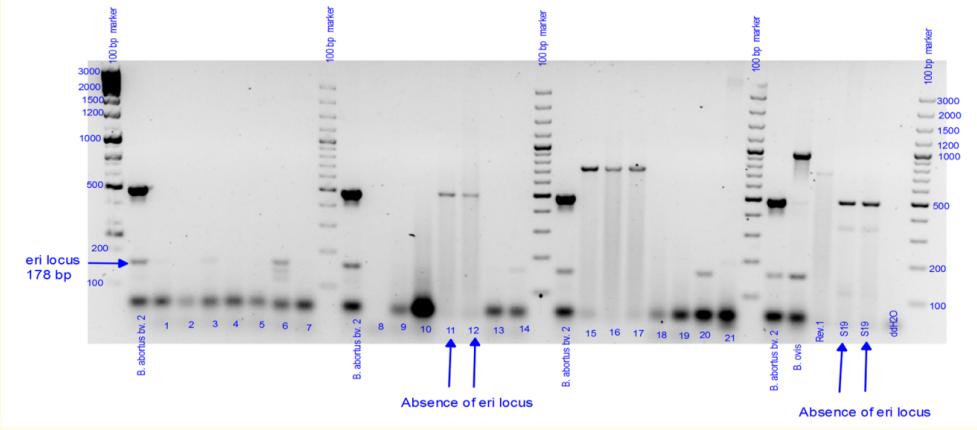

Figure 3: *AMOS PCR profile. Lanes 100 bp plus ladder contain the Gene Ruler 100 bp plus DNA size marker; lanes B. abortus bv 2 (REF REF86/8/59), lane B. canis (RM6/66); Rev.1 vaccine (B. melitensis Rev. 1 vaccine stain), S19 (B. abortus S19 vaccine strain); lane ddH2O (negative control); lanes 1-7, 8-14, 15-21 (SN) contain DNA from Brucella isolates from Sudan shown in table 2.*

Out of the 21 isolates the Bruce-ladder PCR assay confirmed 16 as *B. abortus* and three as *B. melitensis* (Figure 4). Samples SN 8 and 21 did not amplify and therefore could not be characterized using this *Brucella* species-specific PCR assays.

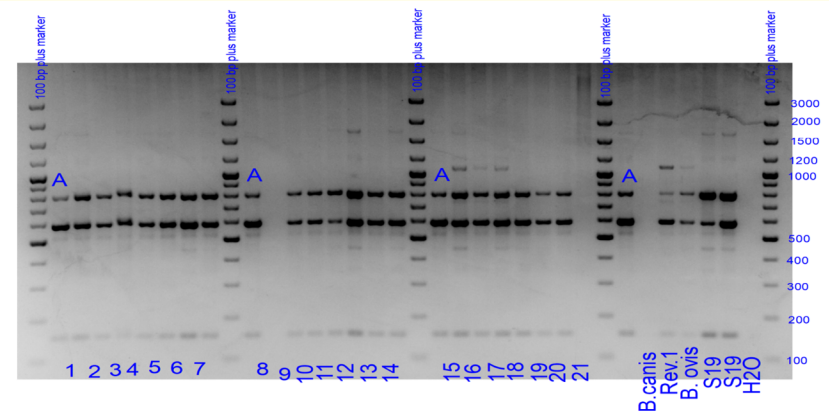

Figure 4: *Bruce-ladder PCR profile. Lanes 100 bp ladder contains the Gene Ruler 100 bp plus DNA size marker; Lanes A consisted of B. abortus bv2 (REF REF86/8/59, BCCN R5) reference strain, lane B. canis consisted of B. canis (RM6/66) reference strain, lane B. ovis consisted of B. ovis () reference strain, lane S19 (B. abortus S19 vaccine strain); lane ddH2O (as positive and negative controls); lanes 1-7, 8-14, 15-21 consisted of DNA from Brucella isolates from Sudan shown in table 2.*

Discussion

Although culture and biotyping remain the gold standard methods for diagnosis and identification of brucellosis, these methods are time-consuming and difficult to interpret [22,23]. *Brucella* species-specific PCR assays like AMOS and Bruce-ladder could be useful for identification of *Brucella* spp. in resource-limited countries like Sudan. These assays allow for rapid speciation and can identify and differentiate most of *Brucella* spp. and biovars. In this study *B. abortus* isolates SN 11 and 12 biotyping results were doubtful (either *B. abortus* bv 1 or 6) were identified with the AMOS PCR assay as *B. abortus* S19 vaccine strain as demonstrated by the absence of the eri locus that exist in other *Brucella* strains (Figure 3). However; AMOS could not identify *B. abortus* bv 6 and 3 which were confirmed in Sudan by biotyping and Bruce-ladder PCR (Table 2; Figure 3 and 4). This indicates that Bruce-ladder, beside biotyping, are more suitable for characterization of *Brucella* spp. in Sudan. In addition, the modified AMOS-ERY PCR, which is capable of differentiating *B. abortus* biovars 5, 6 and 9 and the new subgroup 3b of biovar 3 as well as other *Brucella* spp. [31], is could also be suitable option for Sudan. The farm owners from where *B. abortus* S19 vaccine strains were isolated, confessed that beside calves, they vaccinated adult cows when neighbouring farms experienced abortion storms believed to have been due to brucellosis. This could be true since the live vaccine strains can be isolated from milk and abortion material from vaccinated animals [32,33] and transmission to humans is not uncommon. Interestingly, human infection with *B. abortus* S19 was reported earlier in Sudan among farm workers who used to consume raw milk and have no real awareness of brucellosis transmission [18].

According to our and previous studies findings we recommend a national-wide survey to identify all existing *Brucella* spp. using more cheaper and rapid PCR assays like AMOS - ERY and Bruce-Ladder, along with determining the source of infection. People working in close proximity with animals should be educated about the risk of infection.

Conclusion

Brucella abortus bv 6 is the predominant biovar circulating in Sudan, but it cannot be detected by AMOS PCR; therefore, Bruce-ladder AMOS -ERY PCR assays are better options. The use of *B. abortus* live vaccines in cattle and the correction of vaccination protocol should receive more attention. Moreover, the community should be educated about the effect of brucellosis and its routes of transmission. We present this knowledge and believe it can be used towards control of brucellosis in Sudan.

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Ethical Consideration

A permission to collect bovine milk samples for this study issued in 2011 by the ethical committee of the Deanship of Scientific Research, Sudan University of Science and Technology (SUST/DSR/IEC/EA/2011). The samples' collection did not induce pain and all samples collected during routine milking after obtaining oral consent from the owners.

Conflict of Interest

The authors have declare that there is no competing interests exist.

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