



## Application of Bruce-Ladder Multiplex PCR for Identification of *Brucella abortus* Isolated from Cattle in Kachia Grazing Reserve and Jos Plateau

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### SUMMARY

A study was carried out to isolate *Brucella* strains from cattle in Kachia Grazing Reserve (KGR) and some communities on Jos Plateau and to carry out phenotypic and molecular characterization of the isolates along with other isolates obtained from field submissions and those in the archive of National Veterinary Research Institute (NVRI), Vom. A total of 63 vaginal swabs, 36 milk samples, and 2 hygroma fluids were collected from KGR while 70 vaginal swabs, 50 milk samples and 2 hygroma fluids were collected on the Jos Plateau for *Brucella* isolation. They were cultured for *Brucella* isolation according to standard *Brucella* isolation protocol. Three *Brucella abortus* strains were isolated from KGR while 4 *Brucella abortus* strains were isolated from the Jos Plateau respectively. Eight isolates from field submissions and 5 from the archive were collected and resuscitated. Comprehensive characterization of the isolates in this study revealed that they were all *Brucella abortus*. Similarly, characterization of archived isolates and those from field submissions showed that they were *Brucella abortus*. The classical biotyping of all the isolates revealed that they were *Brucella abortus* biotype 3. Molecular characterization of all the isolates by Bruce-ladder multiplex Polymerase chain reaction (PCR) showed bands consistent with *Brucella abortus*. This is the first molecular characterization of *Brucella* isolates from Nigeria using the Bruce-ladder multiplex PCR and the first study that established that *Brucella abortus* biotype 3 is the predominant *Brucella* strain in Nigeria. The study established the endemicity of brucellosis due to *Brucella abortus*, in the two study areas. These findings have great veterinary and public health implications. There is therefore an urgent need for the institution and implementation of brucellosis control measures in these areas.

Keywords : *Brucella abortus*, isolation, biotype 3, bruce-ladder, multiplex, PCR

## INTRODUCTION

Brucellosis is caused by slow-growing, small, gram negative, cocobacilli, facultative intracellular bacteria belonging to the genus *Brucella*. Members of the genus represent some of the world's major zoonotic pathogens responsible for enormous economic losses and considerable human morbidity (Pappas *et al.*, 2006). Six species were earlier recognized which include *Brucella abortus* (cattle), *Brucella melitensis* (goats and sheep), *Brucella suis* (pigs), *Brucella ovis* (sheep), *Brucella canis* (dog), *Brucella neotomae* (desert wood rat) (Osterman and Moriyon, 2006). Much later, two species were reported from marine mammals namely, *Brucella ceti* (porpoises and dolphins) and *Brucella pennipediae* (seals) (Foster *et al.*, 2007). *Brucella microti* was later isolated from the common vole (*Microtus arvalis*) and *Brucella inopinata* from human breast implant (Scholz *et al.*, 2010; Nymo *et al.*, 2011).

Brucellosis remains a major bacterial zoonotic disease of global importance (Cutler *et al.*, 2005). The main symptoms in female animals are abortion (especially at the second half of gestation), premature or full term birth of weak or dead offspring, retained placenta and metritis, reduced milk production and orchitis in males (Acha and Szyfres, 2003). While *Brucella* is sometimes cited as a re-emerging pathogen, often reflecting socioeconomic changes in particular locations, the organism has been recognised as a major scourge of mankind since its first isolation from humans (Bruce, 1887) cited by Alton *et al.* (1988). The prevalence of brucellosis is highest in the Mediterranean region, the Middle East and Asia (Pappas *et al.*, 2006). It is also regarded as a major problem among ruminants in sub-Saharan Africa and its epidemiology and impact has been reported in several countries ((Mcdermott and Arimi, 2002; Bronsvort *et al.*, 2009).

The most common symptoms of brucellosis in man include undulant fever, night sweat with

peculiar odour, chills and weakness (Zinsstag *et al.*, 2011). Other symptoms are malaise, insomnia, anorexia, headache, arthralgia, constipation, sexual impotence, nervousness and depression (Acha and Szyfres, 2003).

Key to understanding of brucellosis epidemiology is identification of circulating *Brucella* species (and biovars). This is because antibodies are not species specific. There are various reports on cultural isolation of *Brucella* organisms from Nigerian livestock over the years. Based on their cultural morphology, biotyping and biochemical characteristics, *Brucella* species may be subdivided into biotypes (Alton *et al.*, 1988).

Although, isolation of the organism remains the most reliable and confirmatory diagnosis of brucellosis (OIE, 2009; FAO, 2003), it is usually laborious, time consuming and sometimes very hazardous (Lopez-Goni *et al.*, 2008; OIE, 2009).

The advent of molecular methods, especially polymerase chain reaction (PCR) technique has enhanced disease diagnostic capacities to a point that identification of causative agents is less obscure (Ron-Roman *et al.*, 2012). One of these methods is a multiplex conventional PCR called Bruce-Ladder (Garcia-Yoldi *et al.*, 2006; Lopez-Goni *et al.*, 2008). The Bruce-ladder multiplex PCR is robust, performs very well and can differentiate all known *Brucella* species and the vaccine strains and is as sensitive as classical culture-based techniques (Lopez-Goni *et al.*, 2008). The method is capable of accurately typing *Brucella* based on specific identification of *Brucella* nucleotide sequences associated with the genus and the species (Garcia-Yoldi *et al.*, 2006).

The Kachia Grazing reserve and the Jos Plateau are home to semi-sedentary and nomadic Fulani herdsmen that practice extensive animal husbandry with free movement of the animals within and outside their locations. This life style could encourage the spread of brucellosis among cattle and herdsmen in the areas. The aim of this study

was to isolate *Brucella* organisms from cattle in the two study areas and carry out classical biotyping and molecular characterization of the isolates using Bruce-ladder multiplex PCR.

## MATERIALS AND METHODS

### Study Areas

#### Kachia Grazing Reserve

Kachia Grazing reserve (KGR) in Kaduna state was established in 1968 but developmental work did not commence until 1970. It is located between Latitude 10.10112-10.29477<sup>0</sup>N and Longitude 8.00708-8.15154<sup>0</sup>E. It is a wide span of flat lowland area covering about 33, 411 hectares covering three LGAs in the present Kaduna state, namely Kachia, Kajuru and Zangon Kataf. The livestock population in the Grazing Reserve was made up of 41,234 cattle, 10,161 sheep, 4, 828 goats. Record of previous brucellosis prevalence in the area is lacking but a prevalence of 8% has been reported for Kaduna State (Ocholi, 1990).

#### Jos Plateau

The Jos Plateau is located within the Guinea Savannah zone of Nigeria. It is the highest landmass in Nigeria, comprising high plains of about 1,300m above sea level and a number of granite-hill ranges of over 1,900m. The *Jos Plateau* study area comprises villages in Bokkos, Mangu and Pankshin LGAs of Plateau State. It comprises 10 selected villages with cattle population totalling 10,264.

#### Sampling Design

A purposive sampling technique was used. Samples were collected only from cattle with history of abortion, repeat breeding and those with hygroma.

#### Sample Collection and Handling

Fifty-five (55) vaginal swabs, 70 milk samples, 2 hygroma fluids and 1 placenta were collected from cattle in selected herds in KGR while 63 vaginal swabs, 36 milk samples and 2 hygroma fluids were collected from cattle on the Jos Plateau. Milk samples and hygroma fluids

were collected into sterile 10 ml sample tubes and 10 ml syringes respectively, all containing tryptone broth (transport medium) and properly labelled. Vaginal swabs were collected using cotton swabs sticks in which 2ml of tryptone broth was added by the manufacturers. The samples were transported on ice in cold boxes to the Brucellosis Research Laboratory at the National Veterinary Research Institute, Vom for culture.

#### Culturing of samples and biochemical tests

All samples for isolation were cultured immediately on arrival to the laboratory on serum dextrose agar (SDA) or trypticase soy agar (TSA) (*Brucella* selective media) for isolation of *Brucella* organisms as described by Alton *et al.* (1988). The inoculated plates were incubated at 37<sup>0</sup>C for 3-7 days in the presence of 5-10% CO<sub>2</sub> atmosphere.

Isolates that appeared typical of *Brucella* organisms under the microscope were further subjected to classical biotyping tests including; growth in presence of CO<sub>2</sub>, urease test, oxidase test, agglutination with monospecific antisera A and M, sensitivity to *Brucella* phages (Wb, Tb, Tz, R/C), sensitivity to thionin and basic fuchsin dyes as described by Alton *et al.* (1988).

Isolates were then lyophilized and stored at -20<sup>0</sup>C for further work.

#### *Staphylococcus aureus* Cowan. 1 test

Each of the 32 lyophilized vials were reconstituted with 1ml of Phosphate buffered saline (PBS) using a 1000 $\mu$ l microtitre pipette and allowed to stand without shaking for 10 minutes. PBS was used because the isolates were freeze-dried with lactoalbumin as a constituent. A loopful of the reconstituted bacterial suspension was aseptically plated on Trypticase soy agar (TSA) and incubated according to Alton *et al.* (1988).

This test was used to confirm whether or not the isolates were *Brucella*. The *Staphylococcus* Cowan 1 strain had been sensitized with *Brucella* antiserum and therefore readily produced agglutination when it was emulsified

with a colony of *Brucella* organisms. The presence of agglutination confirmed that the isolates were *Brucella*. *Brucella melitensis* 16M strain was used as control. Sixteen isolates were confirmed by this test as *Brucella*.

### Classical Biotyping

All the sixteen *Brucella* isolates were subjected to classical biotyping tests including; growth in presence of CO<sub>2</sub>, urease test, oxidase test, hydrogen sulphide production, agglutination with monospecific antisera A and M, sensitivity to *Brucella* phages (Wb, Tb, Tz, R/C), sensitivity to thionin and basic fuchsin dyes as described by Alton *et al.* (1988).

### Growth in presence of CO<sub>2</sub>

Some *Brucella* species grow both in air and in atmosphere containing 5-10% CO<sub>2</sub> while others grow only on CO<sub>2</sub> supplemented atmosphere. A loopful of *Brucella* suspension was sub-cultured onto serum dextrose agar plates in duplicates. One set was incubated in anaerobic jar containing carbon dioxide generating pack while the other set was incubated in air within the incubator. Growth on plates containing CO<sub>2</sub> and absence of growth in plates in air indicates positive CO<sub>2</sub> requirement.

### Hydrogen Sulphide Production

The *Brucella* colonies to be tested were inoculated onto a serum-dextrose agar slope and a strip of lead acetate paper is placed in the tube such that it does not touch the inoculated media in the tube. It was incubated at 37<sup>o</sup>c and observed daily for 24-48 hours. The lead acetate paper is blackened at its tip down its length when hydrogen sulphide is produced. The colour of the lead acetate paper remains unchanged if hydrogen sulphide is not produced.

### Urease Test

A slope of Christensen's medium with addition of 20% urea solution was inoculated with a loopful of *Brucella* cultures. The bottle was incubated at 37<sup>o</sup>c and observed for colour

change. When the medium changes to purple-pink, it shows urease activity and it indicates positive reaction. Almost all *Brucella abortus* strains have urease activity. *B. suis* produces the fastest reaction.

### Agglutination with positive and negative *Brucella* sera.

A loopful of *Brucella* suspension was added to a drop of both positive and negative *Brucella* sera on a clean glass slide. They were mixed using wire loop, rocked and observed for agglutination. *Brucella* organisms will produce agglutination with *Brucella* positive control serum but absent with the negative control serum.

### Agglutination with Monospecific Antisera A and M

A drop of diluted anti-A and anti-B sera were placed on a clean glass slide. A loopful of *Brucella* culture was emulsified with each of the sera, rocked gently for one minute and observed for agglutination. *B. abortus* will agglutinate with monospecific antisera A while *B. melitensis* will agglutinate with antisera M. This test was carried out along with reference strains of *Brucella abortus* (S19) and *Brucella melitensis* (16M) as controls.

### Sensitivity to thionin and basic fuchsin dyes

Three sets of 300ml trypticase soy media or blood agar base were prepared. 3ml, 6ml and 12ml of thionin respectively was added to the three media to give three different concentrations of 10, 20 and 40 percent of thionin. The plates were labelled with the different concentrations.

Two sets of 300ml media was also prepared for the addition of 3ml and 6ml basic fuchsin dye to obtain two different media with 10 and 20 percent of basic fuchsin. The plates were labelled with the different concentrations.

All the dye plates (three sets for thionin and two sets for basic fuchsin) were marked for inoculating 4 different *Brucella* isolates. They were arranged from the highest concentrated to the lowest concentrated plates for each dye so

that inoculation was started from the lowest to the highest concentration. A suspension of each of the *Brucella* isolates to be inoculated was made by suspending a loopful of a 24-hour culture in 0.5ml of saline in a sterile tube and covered with its sterile cover. Following the numbers on the Plates and using a sterile cotton swab soaked in the bacterial suspension, a horizontal line was streaked across the media using the same side of the swab for the same type of dye but changing to another side for a different dye. This was done for each of the isolate. The five different control strains (*Brucella abortus* S19, *Brucella melitensis* 16M, *Brucella suis* 1330, *Brucella ovis* (Bow) and *Brucella melitensis* Rev.1) were also inoculated in similar manner. The plates were incubated at 37°C in atmosphere of 5-10% CO<sub>2</sub> and another set of plates of the same dyes, concentrations, same number of *Brucella* isolates and controls were inoculated and incubated at the same conditions but in the absence of CO<sub>2</sub>.

Absence of growth on any of the dye media at any concentration is considered a negative reaction while the presence of growth is regarded as a positive reaction.

#### **Sensitivity to *Brucella* phages (Wb, Tb, Iz, R/C)**

The *Brucella* isolates to be tested were grown on media along with the control strains for 24 hours. Care was taken to ensure all suspensions had uniform turbidity. Just for dyes plates, the plates were labelled to be inoculated with four samples while that of the controls were labelled for five strains. The plates prepared from Trypticase soy agar (TSA) containing 1% yeast extract and 5% sterile newborn calf serum must be well dried in the incubator. They were inoculated with the *Brucella* strains and then the controls strains in similar pattern as in the case of the dyes but in this case only changing the swabs after inoculating each isolate. There were five sets of plates and each of the set has four plates. The first four sets of plates were labelled with four *Brucella* isolates

so that one set of plates had all the 16 isolates. The 16 isolates were replicated in the other three sets. The last set was labelled with the control strains and the plates were inoculated such that there were five controls per plate. One plate from the control was transferred to each of the four sets of plates already inoculated with the isolates. This gave rise to four sets with five plates each. Each set of plates was assigned to one phage as the samples were to be tested with four phages namely Tb, Wb, Iz and R/C. Using a Pasteur pipette, 1 drop of routine test dilution (RTD) of the phage was carefully applied on each streaked line in alternating pattern to avoid coalescing of the drops. This was done for all the five plates per phage. Other sets of plates were also inoculated similarly for incubation without CO<sub>2</sub>. The plates were left to stand for the phages to fully absorb into the media before they were packed and incubated at 37°C both in 5-10% CO<sub>2</sub> and in absence of CO<sub>2</sub> and observed after three days.

The plates were then removed from the incubator and observed for presence or absence of lysis on the streaked areas where phages were applied. A zone of lysis on the streaked areas where phages were applied means positive result while absence means negative result. *B. melitensis*, *B. canis* and *B. ovis* are negative (no lysis) by all phages at both dilutions, *B. abortus* is positive (lysis) by all phages at both dilutions while *B. suis* and *B. neotomae* are negative at RTD but positive at 10000 x RTD.

#### **Bruce-Ladder Multiplex PCR**

##### **Extraction of genomic DNA**

Genomic DNA was extracted from pure *Brucella* cultures using a standard commercial microbial DNA extraction kit (QIAamp DNA Mini Kit, Homburg, Germany) protocol. Briefly, fresh *Brucella* cells were resuspended in 0.5 ml of sterile TE buffer (10nM Tris-HCL, 1mM EDTA, pH, 8.0). The suspension was heated at 80°C for 15 minutes and then incubated at 37°C for 1 hour with 0.5% sodium

dodecyl sulphate (SDS) and Proteinase K (200 mg/ml). Cell wall debris, denatured proteins and polysaccharides were then removed by precipitation with 5M NaCl and CTAB-NaCl solution and incubated at 65°C for 10 minutes. DNA was then extracted by a standard protocol with phenol-chloroform-isoamyl alcohol, precipitated with isopropanol, washed with

ethanol. The DNA pellet was re-dissolved in 50-100µl of sterile distilled water for PCR.

### Preparation of primer cocktail and Master Mix

The primers presented in table 1 were from earlier studies (Lopez-Goni *et al.*, 2008), purchased from Sigma Aldrich (<https://www.sigmaaldrich.com>) that currently belongs to Merck KGaA, Darmstadt, Germany.

**Table I: Oligonucleotides used in Bruce-ladder multiplex PCR and their sequences**

Primer	Sequence (5'-3')	Amplicon (bp)	DNATarget	Source of genetic difference
BMEI0998f BMEI0997r	ATC-CTA-TTG-CCC-CGA-TAA-GG GCT-TCG-CAT-TTT-CAC-TGT-AGC	1682	Glycosyltransferase wboA	IS711 insertion in BMEI0998 in <i>B. abortus</i> RB51, and deletion of 15,079 bp in BMEI0993-BMEI1012 in <i>B. ovis</i>
BMEI0535f BMEI0536r	GCG-CAT-TCT-TCG-GTT-ATG-AA CGC-AGG-CGA-AAA-CAG-CTA-TAA	450 (1320) <sup>b</sup>	Immunodominant antigen, gene <i>bp26</i>	IS711 insertion in BMEI0535-BMEI0536 in <i>Brucella</i> strains isolated from marine mammals
BMEII0843f BMEII0844r	TTT-ACA-CAG-GCA-ATC-CAG-CA GCG-TCC-AGT-TGT-TGT-TGA-TG	1071	Outer membrane protein, gene <i>omp31</i>	deletion of 25,061 bp in BMEII0826-BMEII0850 in <i>B. abortus</i>
BMEI1436f BMEI1435r	ACG-CAG-ACG-ACC-TTC-GGT-AT TTT-ATC-CAT-CGC-CCT-GTC-AC	794	Polysaccharide deacetylase	deletion of 976 bp in BMEI1435 in <i>B. canis</i>
BMEII0428f BMEII0428r	GCC-GCT-ATT-ATG-TGG-ACT-GG AAT-GAC-TTC-ACG-GTC-GTT-CG	587	Erythritol catabolism, gene <i>eryC</i> (Derythrose-1-phosphate dehydrogenase)	deletion of 702 bp in BMEII0427-BMEII0428 in <i>B. abortus</i> S19
BR0953f BR0953r	GGA-ACA-CTA-CGC-CAC-CTT-GT GAT-GGA-GCA-AAC-GCT-GAA-G	272	ABC transporter binding protein	deletion of 2653 bp in BR0951-BR0955 in <i>B. melitensis</i> and <i>B. abortus</i>
BMEI0752f BMEI0752r	CAG-GCA-AAC-CCT-CAG-AAG-C GAT-GTG-GTA-ACG-CAC-ACC-AA	218	Ribosomal protein S12, gene <i>rpsL</i>	point mutation in BMEI0752 in <i>B. melitensis</i> Rev.1
BMEII0987f BMEII0987r	CGC-AGA-CAG-TGA-CCA-TCA-AA GTA-TTC-AGC-CCC-CGT-TAC-CT	152	Transcriptional regulator, CRP family	deletion of 2,203 bp in BMEII0986-BMEII0988 in <i>B. neotomae</i>

- Designations are based on the *B. melitensis* (BME) or *B. suis* (BR) genome sequences. f: forward; r: reverse.
- Due to a DNA insertion in the *bp26* gene, the amplicon size in *Brucella* strains isolated from marine mammals is 1320 bp

0.5µl of the 16 primers (8 pairs) was aliquoted into a pre-labelled 1.5ml thin walled microfuge tube. A total of 8 µl of primer cocktail was required for each isolate. This was prepared for 25 isolates, giving a total volume of 200 µl of primer cocktail which was briefly vortexed to ensure homogeneity.

Preparation of master mix was carried out in laminar flow hood. The hood was thoroughly disinfected with methylated spirit. All pipettes, tips, tubes, PCR water needed for this work were left opened in the hood and sterilized by exposure to UV light for 10 minutes. Reaction reagents were pipetted into a sterile 1.5ml microfuge tube in the order below (figure1).

<b>Reagents</b>	<b>Final conc.</b>	<b>1rxn</b>	<b>x 25rxn</b>
PCR buffer 10 X	1x	2.5µl	62.5
dNTPs Mix (2mM)	400mM each one	5.0µl	125.0
MgCl <sub>2</sub> (50mM)	3.0mM	1.5µl	37.5
Bruce-ladder eight primer cocktail (12.5)	6.25pmol each one	7.6µl	190.0
H <sub>2</sub> O (PCR grade)	-	7.1µl	177.5
DNA Polymerase	1.5U	0.3µl	7.5
<b>Total Volume</b>		<b>24.0µl</b>	<b>600.0µl</b>

**Figure 1: Reagents and quantities used for master mix**

The mix was vortexed briefly to ensure adequate mixing of reagents and to avoid bubbling.

24 µl was pipetted into 25 pre-labelled 0.2 ml thin-walled microfuge tubes. 1.0 µl of template DNA was added to the 24 µl PCR reaction mixture, giving a total volume of 25 µl for each sample. All the samples were set for DNA amplification and were taken to be programmed in the thermocycler.

#### **PCR Cycling Conditions.**

PCR reaction mixture was placed in a thermocycler and set for amplification. The initial denaturation temperature was 95 °C for 7 minutes, template denaturation was 95 °C for 35 seconds and primer annealing at 64 °C for 45 seconds. This was followed by primer extension at 72 °C for 3 minutes, final extension at 72 °C for 6 minutes for a total of 25 cycles and cooled at 4°C (Garcia-Yoldi *et al.*, 2006; Lopez-Goni *et al.*, 2008).

#### **Gel Electrophoresis and Analysis**

After PCR amplification, 2 µl of PCR product and 8 µl of bromophenol blue (loading buffer) were loaded into wells in 1.5% agarose gel in TBE in a cuvette flooded with TBE slightly covering the gel. The loading of the PCR products in well in the agarose gel must correspond with the arrangement or numbering of the samples and always starting from the second well.

100-bp DNA ladder or 1kb plus DNA ladder (Invitrogen LTD) ([www.invitrogen.com](http://www.invitrogen.com)) was used as molecular marker. This was loaded in the first well of the gel.

Sterile ultrapure water was used as negative control and was loaded in the last well of the gel.

The gel was stained with 7% SYBR Safe DNA Gel Stain ([www.lifetechnologies.com](http://www.lifetechnologies.com)). The cuvette was covered with the electrophoresis equipment, and the electrodes applied accordingly. The electrophoresis equipment was set to run at 130V for 50 minutes. The gel was then visualised under UV light in the computerized gel documentation equipment.

The bands were observed; the pictures were downloaded on to the computer, analyzed and saved appropriately.

## RESULTS

Three *Brucella abortus* isolates were obtained from Kachia Grazing Reserve while four *Brucella abortus* were isolated from Jos Plateau. The isolates grew after 3 days incubation at 37°C in the presence of 10% CO<sub>2</sub> and without CO<sub>2</sub> respectively. The colonies were tiny, smooth, with pale honey colour on transmitted light and bluish in reflected light. They were all Gram negative, cocobacilli, arranged mostly in singles but rarely in pairs and in groups. They produced hydrogen sulphide (H<sub>2</sub>S), they were urease positive, oxidase positive, positive with positive *Brucella* sera but negative with negative *Brucella* sera. They produced agglutination with *Brucella* monospecific antisera A but

negative with *Brucella* monospecific antisera M. They produced agglutination in *Staphylococcus aureus* Cowan 1 co-agglutination test. They were all lysed by Weybridge (Wb), Tbilisi (Tb), Izadnagar (Iz), smooth *Brucella* phages at routine test dilution (RTD) but were not lysed by rough culture phage (R/C).

All the *Brucella abortus* isolates grew on agar plates containing 10µg/ml concentration of thionin dye but did not grow on media containing 20µg/ml and 40µg/ml concentrations of thionin. They grew on media containing 20µg/ml and 40µg/ml basic Fuchsin dye. However, *Brucella abortus* S19 which is biotype 1 did not grow on agar plates containing thionin dye at 10µg/ml. All the *Brucella abortus* isolates were therefore confirmed as *Brucella abortus* biotype 3 (Tables II and III).

**Table II: Biotyping of *Brucella abortus* isolates from field submissions and archive based on their Growth on various concentrations of Thionin and Basic Fuchsin dyes.**

Isolates / Ref. Strains	Growth on Thionin dye			Growth on basic Fuchsin dye		<i>Brucella</i> spp / biovar
	10µg/ml	20µg/ml	40µg/ml	10µg/ml	20µg/ml	
S19*	-	-	-	+	+	
1330*	+	+	+	-		
16M*	+	+	-	+	+	
Ovis63/290*	+	+	-	-	-	
Rev.1*	+	+	-	-	-	
MBL**	+	-	-	+	+	<i>B.abortus</i> 3
HZ16***	+	-	-	+	+	<i>B.abortus</i> 3
MKD**	+	-	-	+	+	<i>B.abortus</i> 3
VSM5***	+	-	-	+	+	<i>B.abortus</i> 3
HK15***	+	-	-	+	+	<i>B.abortus</i> 3
VSM1***	+	-	-	+	+	<i>B.abortus</i> 3
HM14***	+	-	-	+	+	<i>B.abortus</i> 3
HP19***	+	-	-	+	+	<i>B.abortus</i> 3
HO18***	+	-	-	+	+	<i>B.abortus</i> 3
JOS**	+	-	-	+	+	<i>B.abortus</i> 3
HG13***	+	-	-	+	+	<i>B.abortus</i> 3

Key: \* *Brucella* reference strains (Controls)\*\* Archived isolates\*\*\*Field submissions isolates

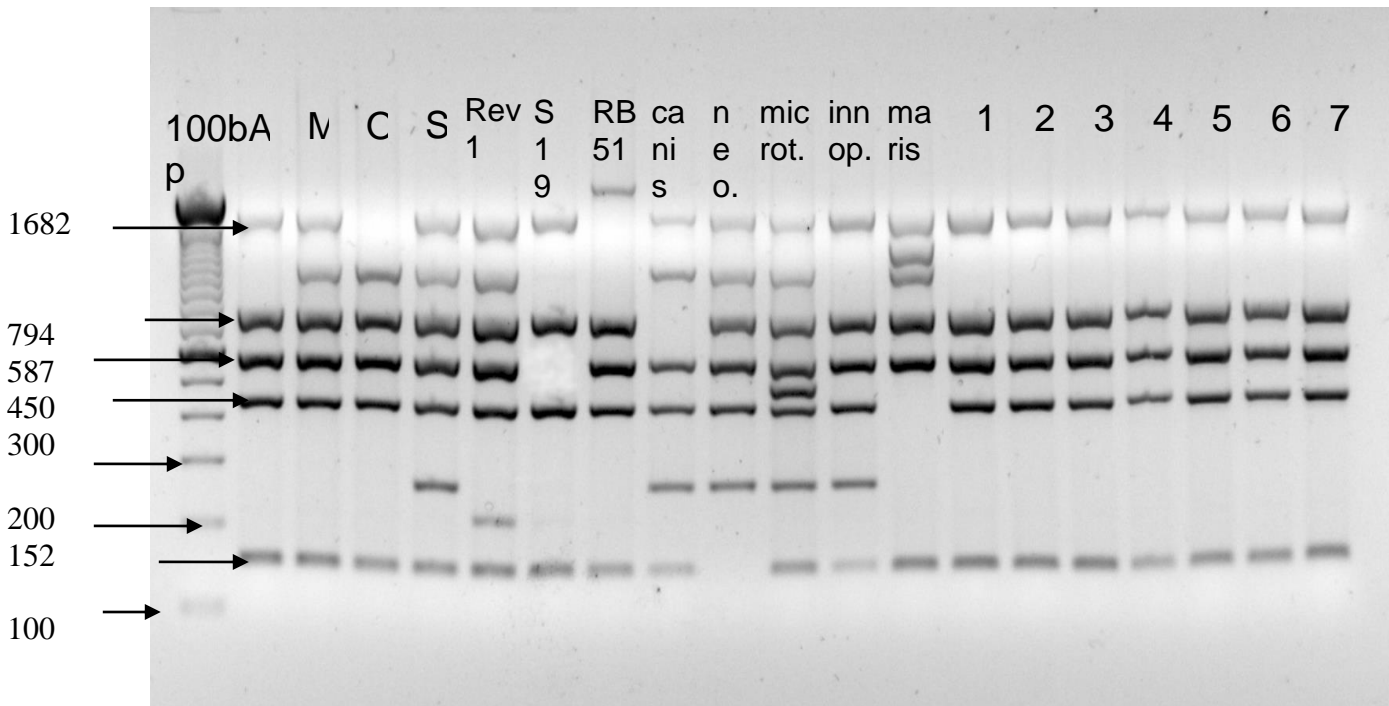


**Table III: Biotyping of *Brucella abortus* isolates from this study based on their Growth on agar plates containing various concentrations of thionin and basic fuchsin dyes**

Isolates / Ref. Strains	Growth on Thionin dye			Growth on basic Fuchsin dye		<i>Brucella</i> Species and Biovars
	10µg/ml	20µg/ml	40µg/ml	10µg/ml	20µg/ml	
S19*	-	-	-	+	+	
1330*	+	+	+	-		
16M*	+	+	-	+	+	
Ovis63/290*	+	+	-	-	-	
Rev.1*	+	+	-	-	-	
KGR1**	+	-	-	+	+	<i>B.abortus</i> 3
KGR2**	+	-	-	+	+	<i>B.abortus</i> 3
KGR3**	+	-	-	+	+	<i>B.abortus</i> 3
PLM1**	+	-	-	+	+	<i>B.abortus</i> 3
PLB2**	+	-	-	+	+	<i>B.abortus</i> 3

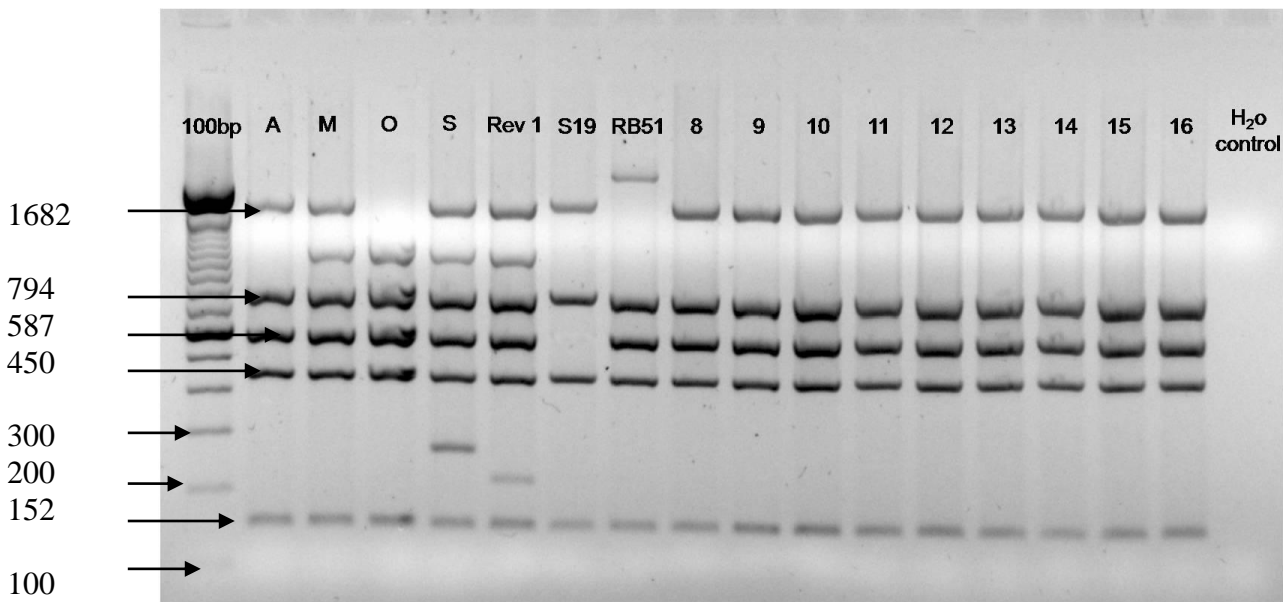
Key: \* *Brucella* reference strains (controls)  
 The results of Bruce-ladder multiplex PCR for all the *Brucella* isolates obtained in this study, field submissions and those from the archive are presented (Figures 2 and 3).  
 The amplified amplicons of the isolates were compared with those of the reference *Brucella* strains. The Bruce-ladder multiplex PCR from DNA from the sixteen *Brucella abortus*

\*\* *Brucella* isolates from this study amplified five fragment sizes of 1,682bp, 794bp, 587bp, 450bp and 152bp which were consistent with amplicons amplified by DNA of *Brucella abortus* S19 reference strain used as control. All the 16 *Brucella abortus* isolates were therefore confirmed as *Brucella abortus* by the Bruce-ladder multiplex PCR (figures 2 and 3).



Molecular marker (100bp); lane 1, *B. abortus* 2308; lane 2, *B. melitensis* 16M; lane 3, *B. ovis*; lane 4, *B. suis* 1330; lane 5, *B. melitensis* Rev1 vaccine; lane 6, *B. abortus* S19 vaccine; lane 7, *B. abortus* RB51 vaccine; lane 8, *B. canis*; lane 9, *B. neotomae*; lane 10, *B. microti*; lane 11, *B. inopinata*; lane 12, *B. ceti*; lane 13 (1), MBL; lane 14(2), KGR2; lane 15(3), PLM1; lane 16(4), PLB2; lane 17(5), KGR2; lane 18(6), KGR1, lane 19(7), HZ16.

**Figure 2: Gel photograph of Bruce-ladder multiplex PCR for *Brucella* isolates 1-7 and the 12 *Brucella* reference control strains**



Molecular marker (100bp); lane 1, *B. abortus* 2308; lane 2, *B. melitensis* 16M; lane 3, *B. ovis*; lane 4, *B. suis* 1330; lane 5, *B. melitensis* Rev1 vaccine, lane 6, *B. abortus* S19 vaccine; lane 7, *B. abortus* RB51 vaccine; lanes 8(8), MKD; lane 9(9), VSM5; lane 10(10), HK15; lane 11(11), VSM1; lane 12(12), HM14; lane 13(13), HP19; lane 14(14), HO18; lane 15(15), Jos; lane 16(16), HG13; lane 17, H<sub>2</sub>O (negative control).

**Figure 3: Gel photograph of Bruce-ladder multiplex PCR for *Brucella* isolates 8-16 and 7 *Brucella* reference control strains**

## DISCUSSION

Since brucellosis was first reported in Nigeria in 1927 (Banerjee and Bhatta, 1970), to the best of my knowledge, only five bacteriological studies have been undertaken in cattle in various locations and *Brucella abortus* isolated (Ducrotoy et al., 2014). Other previous studies (Ishaya et al., 2012; Bertu et al., 2015a) reported successful isolation of brucella organisms in multispecies livestock farm and in cattle with bilateral hygroma respectively. All the isolates were *Brucella abortus* biotype 1. There is concern that brucellosis could emerge as a result of the settling of previously migratory herds or from increased contacts between infected nomadic herds and susceptible intensive commercial or settled semi-intensive herds (Ducrotoy et al., 2014). This concern has been confirmed by the successful isolation of *Brucella abortus* in both KGR and on the Jos Plateau, indicating that brucellosis is endemic in the study areas. This is very significant and has far reaching

implications as the infected cows may spread the infection to other cattle within the herds as well as sheep and goats which are usually reared along with cattle as the organisms are shed on pasture. Secondly, infection could also be spread from infected herds to non-infected herds through contact and through loaning of heifers and bulls commonly practiced by herdsmen. Thirdly, infection could be spread to non infected herds by dogs which are scavengers and usually carry aborted fetuses and placentae from one area to another and in the process contaminating the pastures on which animals graze with *Brucella* organisms. Further spread of brucellosis from both KGR and Jos Plateau to other animals could also occur during migration as the cattle move along their various routes to distant areas in search of greener pasture.

The isolation of *Brucella abortus* from milk in both KGR and Jos Plateau is of great public health significance because *Brucella* organisms are usually shed through the milk of infected cows which is freely consumed raw or as

fermented milk. The most common means of transmission of brucellosis from animals to humans is through the consumption of unpasteurized or raw milk and milk products (Kang'ethe *et al.*, 2000, Capasso, 2002).

The isolation of *Brucella abortus* from the vaginal swab is also of great public health significance. This is because herdsmen are in the habit of using their bare hands to assist cows to ensure successful parturition in cases of dystocia or to pull out placenta in cases of retained placenta. These habits and practices expose them to high risk of contracting brucellosis through direct contact.

There was higher rate of isolation of *Brucella* organisms from hygroma fluids in this study compared to other samples. A possible explanation to this is that hygroma fluid is comparatively less contaminated than vaginal swabs, milk or aborted tissues which may easily become contaminated prior to or during sample collection. This finding is consistent with reports of previous investigators in Nigeria where a majority of isolates were from hygroma fluid and only a few from other samples such as milk, vaginal swabs, aborted foetuses and placentae (Eze, 1978; Bale and Kumi-Diaka, 1981; Ocholi *et al.*, 2004b). Successful isolation of *Brucella abortus* from specimens examined in this study confirmed its presence in the study areas and this agrees with reports of previous investigators that bovine brucellosis in Nigeria is generally caused by *Brucella abortus* (Eze, 1978; Bale and Kumi-Diaka, 1981; Ocholi *et al.*, 2004a, b; Ocholi *et al.*, 2005). The findings have provided additional data on *Brucella abortus* isolation in Nigeria. The fact that *Brucella abortus* was isolated from field samples submitted from various States confirms the widespread distribution of brucellosis due to *Brucella abortus* in the country. Archived isolates also show consistency with those in this study, indicating that the prevailing species of *Brucella* in Nigerian cattle over the years has been *Brucella abortus*.

Based on classical biotyping, the isolates were all identified as *Brucella abortus* biotype 3.

This finding differs from those of previous investigators (Eze, 1978; Ocholi *et al.*, 2004a, b; Ocholi *et al.*, 2005) in which *Brucella abortus* biotype 1 was reported as the prevailing biotype in Nigeria. This may be due to improved biotyping expertise as well as use of newly developed *Brucella* selective media and characterization techniques. Differences between biotypes 1 and 3 are not very apparent and can only be distinguished based on their sensitivity to thionin dye (Banai and Corbel, 2010). While *Brucella abortus* S19 Biotype 1 did not grow in presence of thionin at all three concentrations of 10µg/ml, 20µg/ml and 40µg/ml, all the sixteen isolates tested in this study grew in the presence of thionin only at 10µg/ml which is a typical behaviour of *Brucella abortus* biotype 3. This confirmed all the isolates as *Brucella abortus* biotype 3. All the isolates from the archive which were earlier reported as being biotype 1, have been confirmed as biotype 3. This finding is consistent with the report from other West African and other African countries where biotype 3 was found to be the prevailing biotype circulating. Such studies were from Senegal (Verger *et al.*, 1979), Togo (Verger *et al.*, 1982), Niger (Akakpo *et al.*, 1986), The Gambia (Bankole *et al.*, 2010) and Ivory Coast (Sanogo *et al.*, 2013). Similar trends were reported in Central Africa (Domenech *et al.*, 1983), Kenya (Muendo *et al.*, 2012).

This study may be the first to report the use of Bruce-ladder multiplex PCR for the characterization of *Brucella* isolates from Nigeria. The Bruce-ladder multiplex PCR successfully identified all the sixteen phenotypically characterized *Brucella abortus* isolates to species level as *Brucella abortus*. Although this PCR cannot differentiate biovars from the same species, it is species specific and can identify and differentiate all the *Brucella* species and vaccine strains in the same test in one reaction (Garcia-Yoldi *et al.*, 2006; Lopez-Goni *et al.*, 2008). To our knowledge, this is the first molecular technique used to identify *Brucella* to species level in Nigeria. Further

genotypic and phylogenetic aspect of this study has been published (Bertu *et al.*, 2015b).

These findings have established the endemicity of *Brucella* infection due to *Brucella abortus* biotype 3 in KGR and on the Jos Plateau. The study provides useful data for the establishment of appropriate brucellosis intervention and control measures in the study areas

## CONCLUSION

The successful isolation of *Brucella* in KGR and Jos Plateau shows that brucellosis is prevalent in the areas. *Brucella abortus* is the prevailing *Brucella* species. All the species from the study areas were *Brucella abortus* biotype 3. Molecular characterization by Bruce-ladder Multiplex PCR of all the isolates from the study areas showed that they were *Brucella abortus*. The classical biotyping of *Brucella* isolates from the archive in NVRI revealed that they were *Brucella abortus*, they were all biotype 3. The archived isolates were also genetically identified as *Brucella abortus* by Bruce-ladder multiplex PCR.

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