Phenotypic and genotypic characterization of *Brucella* strains isolated from autochthonous livestock reveals the dominance of *B. abortus* biovar 3a in Nigeria

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**A B S T R A C T**

Brucellosis is a worldwide widespread zoonosis caused by bacteria of the genus *Brucella*. Control of this disease in a given area requires an understanding of the *Brucella* species circulating in livestock and humans. However, because of the difficulties intrinsic to *Brucella* isolation and typing, such data are scarce for resource-poor areas. The paucity of bacteriological data and the consequent imperfect epidemiological picture are particularly critical for Sahelian and Sub-Saharan African countries. Here, we report on the characterization of 34 isolates collected between 1976 and 2012 from cattle, sheep and horses in Nigeria. All isolates were identified as *Brucella abortus* by Bruce-ladder PCR and assigned to biovar 3 by conventional typing. Further analysis by enhanced AMOS-ERY PCR showed that all of them belonged to the 3a sub-biovar, and MLVA analysis grouped them in a cluster clearly distinct from that formed by European *B. abortus* biovar 3b strains. Nevertheless, MLVA detected heterogeneity within the Nigerian biovar 3a strains. The close genetic profiles of the isolates from cattle, sheep and horses, suggest that, at least in some parts of Nigeria, biovar 3a circulates among animal species that are not the preferential hosts of *B. abortus*. Consistent with previous genetic analyses of 7 strains from Ivory Coast, Gambia and Togo, the analysis of these 34 Nigerian strains supports the hypothesis that the *B. abortus* biovar 3a lineage is dominant in West African countries.

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1. Introduction

Bacteria of the *Brucella* genus are the aetiological agents of brucellosis, a zoonotic disease of worldwide distribution that affects a broad range of mammals. Presently, the genus includes eleven nominal species (http://www.bacterio.net/-allnamesac.html), among which *Brucella melitensis*, *Brucella abortus* and *Brucella suis* are divided into 3, 7 and 5 different biovars, respectively, based on dye and phage sensitivity, metabolic and agglutination tests (Alton et al., 1988). *B. melitensis* and *B. abortus* preferentially infect small ruminants and cattle, respectively, causing abortions and infertility (Corbel et al., 2006) and these animals are the most common source of human brucellosis, a debilitating chronic condition which untreated can lead to permanent sequelae (Zinsstag et al., 2011). Although *B. melitensis* and *B. abortus* and some other species of the genus have a preferential host, cross infections are significant in...
mixed husbandry systems and at the domestic-wildlife interface (Corbel et al., 2006; Verger et al., 1989). Thus, knowledge of the circulating Brucella species is important both to understand the epidemiology and to control the disease.

Brucellosis is considered a major problem in many areas of Asia and Africa (Grace et al., 2012; McDermott et al., 2013). In Nigeria (the most populous country in Africa with about 20.5 million cattle, 23.1 million sheep and 28.1 million goats) consistent serological evidence shows its importance since British colonial times (Ducrotoy et al., 2014). According to the scarce bacteriological studies conducted, B. abortus biovar 1 is the main cause of cattle brucellosis (41 strains out of 45 isolates reported; Table S1), which is in contrast with the reported dominance of biovar 3 in surrounding countries (Bankole et al., 2010; Dean et al., 2014; Sanogo et al., 2013a). Since knowledge of the circulating Brucella species and biovars is of great practical importance, the objective of this work was to characterize previously reported (Ocholi et al., 2004) and new strains isolated by the National Veterinary Research Institute in Vom, Northern Nigeria, between 1976 and 2012. To this end, we used conventional typing plus MLVA-16 (Multiple-locus variable-number tandem-repeat analysis) (Le Fleche et al., 2006) and AMOS-ERY PCR (Ocampo-Sosa et al., 2005), two molecular methods allowing refined analyses.

### 2. Materials and methods

#### 2.1. Samples, bacteriological procedures and primary isolation

Samples consisting of aborted foetuses and placentae, milk from cattle, sheep and horses, hygroma fluid and vaginal swabs from recently aborting animals or animals that had history of abortion were collected from Plateau, Adamawa, Taraba, Bauchi, Kaduna, Kano, Borno, Sokoto, Nassarawa and Kogi states of Nigeria between 1976 and 2012 (Table 1 and Fig. S1).

Primary isolation of Brucella was achieved by culturing the samples on Farrell’s medium supplemented with 5% horse serum (Alton et al., 1988). The inoculated plates were incubated at 37 °C aerobically and in an atmosphere of 5–10% CO₂, and examined after 3–7 days. Suspicious colonies morphologically consistent with Brucella were sub-cultured onto serum dextrose agar from which subsequent colonies were examined by Gram and Stemp’s modified Ziehl–Neelsen stain, and by agglutination with anti-Brucella antibody. They were also tested for catalase production and the cytochrome C oxidase test was performed (Alton et al., 1988). Thirty-four isolates identified as Brucella spp. were lyophilized and stored at -20 °C.

#### 2.2. Phenotypic typing

The freeze-dried strains were rehydrated with sterile phosphate buffered saline (150 mM NaCl, 7.3 mM KH₂PO₄, 11.5 mM K₂HPO₄, pH 6.8) and cultured on blood agar base (BAB no. 2, Oxoid, UK) supplemented with 5% sterile bovine serum (PAN Biotech, No. 0402-P101305, USA) for 48–72 h at 37 °C in a 5–10% CO₂ atmosphere. After assessing for dissociation by the crystal violet exclusion test and absence of contamination, colonies were sub-cultured on the same medium for 24 h under the same conditions and submitted to conventional biotyping (Alton et al., 1988): oxidase and urease tests, CO₂ requirement, agglutination with monospecific anti-A and anti-M sera, lysis with phages Tb, Wb, Lz1 and R/C and sensitivity to thionin (10 μg/mL and 20 μg/mL), basic fuchsin (20 μg/mL) and safranin (100 μg/mL). B. melitensis 16 M, B. abortus 519, B. suis 1330 and B. ovis 63/290 reference strains (originally supplied by Dr. J.M. Verger, Laboratoire de Pathologie Infectieuse et Immunologie-Nouzilly, France- and kept freeze-dried at CITA-Spain) were used as controls.

#### 2.3. Molecular typing

Genomic DNA was extracted from pure Brucella cultures using a commercial microbial DNA extraction kit (QIAamp DNA, QIAGEN, Hamburg, Germany). The Bruce-ladder multiplex PCR (8 target genes) (Garcia-Yoldi et al., 2007) was first performed to identify the Brucella species. The AMOS-ERY PCR which discriminates B. abortus (biovars 1–2 and 4), B. melitensis (biovars 1–3), B. ovis and B. suis (only biovar 1) (Ocampo-Sosa et al., 2005) then enhanced the identification of the B. abortus biovar 3 subtypes (3a or 3b). Moreover, MLVA-16, the current optimal strategy for genotyping Brucella strains, was undertaken (Al-Dahouk et al., 2007). The 16 markers were classified into two panels: stable panel 1 with eight minisatellite loci (Bruce06, Bruce08, Bruce11, Bruce12, Bruce42, Bruce43, Bruce45, Bruce55) and hypervariable panel 2 with eight microsatellite loci: 3 loci (Bruce18, Bruce19 and Bruce21) for panel 2A and 5 loci (Bruce04, Bruce07, Bruce09, Bruce16 and Bruce30) for panel 2B. Fragment sizes were converted to repeat unit (U) numbers and analyzed as a character data set, using BioNumerics v7.1 (Applied Maths, Belgium). The cluster analysis was performed using the UPGMA (Unweighted Pair Group Method Algorithm) with an Euclidean distance or a minimum spanning tree (MST) with distance matrices for categorical data.

The MVLA-16 patterns obtained with the 34 Nigerian strains were compared to the reference strain B. abortus biovar 3 Tulya and to some biovar 3 field strains reported in other Sub-Saharan countries (10 from Kenya, 3 from Togo and one from Sudan) from...