Detection in and circulation of Bluetongue virus among domestic ruminants in Madagascar

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Running Title: Bluetongue Virus in Madagascar

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34 **Highlights**

- 35 • Bluetongue virus serotype 2 (BTV-2) circulates in Madagascar.
- 36 • BTV is endemic and highly prevalent among cattle
- 37 • Yearly incidence among cattle suggests low pathogenicity of circulating strains

38

39

Abstract

So far, no published data was available concerning the circulation of Bluetongue virus (BTV) in Madagascar. During a survey on Rift Valley Fever, we were able to detect a virus belonging to BTV. Therefore, we conducted a study aiming at characterizing molecularly the BTV isolated and assess the importance of circulation of BTV in Madagascar. A total of 4,393 sera from ruminants selected randomly by stratification and sampled in 30 districts of Madagascar were tested for BTV. Moreover, 175 cattle were followed during 11 months. Phylogenetic analyses were performed from virus isolated from unfed pools of mosquitos. Overall, the estimated mean seroprevalence of infection at the national level was 95.9% (95% CI: [95.2-96.5]) in cattle and 83.7% (95% CI: [81.4-85.9]) in small ruminants. Estimation of incidence rate was 54 per 100 cattle-years assuming that the incidence rate is constant all year along. Phylogenetic analyses revealed that BTV detected belong to serotype 2. In conclusion, our results showed that BTV is endemic in Madagascar and highly prevalent among cattle. In our study we did not work on the vector involved in transmission of BTV in cattle. Thus, research should be conducted to better describe epidemiology of BTV in Madagascar including vectors and assess economic impact of the disease associated to BTV infections.

Keywords

Bluetongue virus, *Bovidae*, Madagascar.

Background

Bluetongue disease is a disease of ruminants caused by Bluetongue Virus (BTV). This arthropod-borne virus is transmitted primarily by biting midges belonging to the *Culicoides* genus (reviewed in Coetzee 2012) (Coetzee et al., 2012a; Coetzee et al., 2012b). BTV is circulating widely and its distribution pattern is related to the distribution of its vectors. BTV is believed to have expanded in recent years as a possible result of climate warming (Purse et al., 2005; Tabachnick, 2004). Areas like Northern and Western Europe, previously BTV-free were recently affected by the disease (Wilson and Mellor, 2009). Direct or indirect economic impacts were important in some areas of the world (Dungu et al., 2004a; MacLachlan, 2010; MacLachlan and Osburn, 2006; Tabachnick et al., 1996). BTV can cause severe disease in certain species of sheep, especially European fine wool and mutton breeds; however in endemic regions, local ruminants do not express clinical disease (Coetzee et al., 2012b). The clinical signs of BTV infection can vary between species. Sheep may have mainly fever, serous bloody nasal discharge, edema erosions and ulcers. Cattle and other ruminants may have ocular discharge, conjunctivitis, oral mucosal congestion, ulceration of muzzle, and teats (reviewed in MacLachlan, 2009) (MacLachlan et al., 2009). Previous studies showed that ruminants of African origin are apparently resistant to the infection (Fernandez-Pacheco et al., 2008; Mauroy et al., 2008). This resistance to BTV might explain why the disease was never described in Madagascar. In this study, we describe for the first time the detection of BTV serotype 2. Moreover, serosurvey amongst cattle revealed high seroprevalence of BTV infection and large distribution of the virus in Madagascar.

Methods

Specimen collection

A total of 12,785 adults mosquitoes were collected on April 2009 in Fianarantsoa and Ambalavao (Central South of Madagascar) during investigations due to Rift Valley Fever

86 outbreaks. Mosquitoes were pooled (23-30) by species and then stored at -80°C. A total of
87 390 pools containing monospecies of unfed female mosquitoes were grinded and supernatants
88 were collected for subsequent viral analysis.

89 To address seroprevalence of BTV infection, we used sera from cattle and small ruminants
90 collected in Madagascar in August 2008 during Rift valley fever outbreak. Sampling methods
91 were described in Jeanmaire *et al.*, 2011 (Jeanmaire et al., 2011). Briefly, Madagascar was
92 divided into 10 sampling areas representing the combination of two stratification factors, the
93 cattle density and the different ecozones. In these 10 sampling areas, 30 districts were
94 randomly sampled. In each district 33 to 200 animals were sampled. Only animals that were
95 born and lived in the same sampling area were considered.

96 For estimation of incidence, we included retrospectively sera collected from a cohort of cattle
97 from 5 and 3 villages located respectively in the Southwestern (Tulear II district) and
98 Northwestern (Mampikony district) regions of Madagascar (Figure 1A). These two districts
99 were chosen because of their agro-system differences. Tulear II is a semi-arid ecosystem and
100 Mampikony has an environment of a dry and deciduous forest. In these study sites, 20 to 30
101 cattle were sampled and followed up monthly from May 2010 to April 2011. In collaboration
102 with veterinary service and the farmers, cattle included in this cohort study were firstly
103 identified and marked using number hooked around their neck. Sera from these cattle were
104 collected monthly by a study veterinarian and were stored in -80°C until use.

105 **Viral isolation and detection**

106 Virus isolation was performed on mosquito AP61 cell lines and virus identification was firstly
107 performed by an indirect immunofluorescence assay (IFI) as previously described (Digoutte et
108 al., 1992; Ratovonjato et al., 2011). One pool containing supernatant of *Anopheles squamosus*
109 individuals tested positive for BTV and was subsequently analyzed (see viral detection and
110 analysis). (Ratovonjato et al., 2011).

Nucleic acid sample preparation, RT-PCR reactions and sequencing

Total RNA was extracted from 100 µL of infected culture cells using the QIAcube robot (Qiagen, Courtaboeuf, France) and the QIAamp Viral kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. Finally, the RNAs were eluted with 50 µL of ultrapure water. Reverse transcription (RT) and amplification (PCR) were performed using a commercial real-time RT-PCR kit (ADI-352, AES) according to the manufacturer's instructions. For the segment 2 sequencing, RT-PCR were performed using a single tube RT-PCR method and following the "One-step RT-PCR Kit" protocol (Qiagen, Courtaboeuf, France). Group-specific primer-pairs derived from the nucleotide sequence data of genome segment 2 of BTV available in GenBank were used (not described). Amplified RT-PCR products were sequenced directly, in both directions, using the primer-pairs (Eurofins MWG Operon, Ebersberg, Germany). Sequences were assembled by SeqMan (DNASTar programs, Lasergene) and compared to the homologous sequences available in GenBank. Sequence alignments were performed using MegAlign-Clustal V method (DNASTar software).

Serological Analysis

Serological tests were performed using LSIVet™ Ruminant Bluetongue Advanced II - Serum ELISA Kit (LSI, Lissieu, France). This kit which is based on the principle of a blocking ELISA, has been shown to detect specific antibodies directed against VP7 of all BTV serotypes without cross reactions against antibodies induced by Epizootic Hemorrhagic Disease Virus and has a high level of sensitivity and specificity (Niedbalski, 2011).

Data analysis

Statistical analysis was performed using R software version 3.1.2 (R Core Team (2014)). Data were described nationwide and on stratification by sampling zone. Mean seroprevalence of antibodies directed against BTV was estimated at the national level and by sampling zone with accompanying 95% confidence interval (CI). Student's t-test was used to compare mean

age of two sub population, Chi-square test was used to compare frequency between two or more sub population with $p=0.05$ as degree of signification. Odds ratio (OR) were calculates to quantify effects of age in seroprevalence. Survival analysis was used to assess incidence rate, and median time from birth to seroconversion for the cohort population of cattle. Event is defined as ELISA positive during cohort observation. Time to seroconversion is defined as time from birth to seroconversion. In this study the time point is defined as the end of the survey. Animal is right censored if at the time point it did not have IgG against BTV or if survey of cattle did not reach the time point. Animals were left-censored when time to seroconversion could not be estimated because they were seropositive for BTV (IgG) at the beginning of the survey.

Results

Virus detection and molecular analysis

Molecular analysis of virus isolated the supernatant of *Anopheles squamosus* individuals from Ambalavao inoculated in mosquito cells (AP61) revealed that genomic segment 2 (encoding the VP2 serotype-specific protein) of the isolate belonged to the serotype BTV-2 (Figure 2). The comparison with homologous sequences available in GenBank showed a nucleotide identity from 79.5 to 99% (data not shown).

Serological results

To assess the seroprevalence of BTV, a total of 4,393 samples (3,395 cattle and 998 small ruminants: sheep and goats) from 30 of the 114 districts of Madagascar were tested.

The cattle population had a range of age from 4 months to 19 years, with a median age at 5 years and a mean at 5.5 (123 missing data). Small ruminant had a range of age population from 6 months to 7 years, with a median age at 2 years and a mean at 2.4 (244 missing data). The sex ratio (M/F) was 1.37 (26 missing data) and 0.4 (4 missing data) in cattle and small ruminants respectively. Fifteen percent of cattle were sampled in slaughterhouses, 46.5% on

farms, and 6.2% in markets. Most of the small ruminants (97.7%) were sampled in farms. Overall, 95.9% (95% IC = [95.2-96.5]) of cattle sampled and 83.8% (95% IC = [81.4-85.9]) of small ruminants sampled showed detectable antibodies levels against BTV indicating past infections. Twenty-one (0.62%) cattle and 19 (1.9%) little ruminants presented doubtful results. Due to small amount of sera available, we were not able to re-test samples with doubtful results. In all sampling areas there were animals with antibodies against BTV with a seroprevalence in animals ranging from 85.2% (one district of zone 10) to 100% (Figure 1B). The seroprevalence of infection was significantly higher in males compared to females in cattle (Pearson's Chi-squared test $p=0.001$) but not in small ruminants (Pearson's Chi-squared test $p=0.075$). Prevalence of antibodies against BTV increased significantly with the age in cattle with an OR=1.24 (CI 95% [1.15-1.35], Pearson's Chi-squared test $p<0.0001$) but not in small ruminants (Pearson's Chi-squared test $p=0.69$). The value of OR showed more important risk depending on age category (Table 1). There were no significant differences in mean BTV-seroprevalence between animals sampled in farm, slaughterhouse or market.

To assess incidence-rate of BTV infection, a cohort of 182 cattle sampled from 2 regions of Madagascar was included. Age was missing for 7 animals that were then excluded from our statistical analysis. This cohort had a range of age from 7 to 12 months, with a median age at 8.7 months. Sex-ratio was 0.9 (2 missing data). From this cohort of 175 cattle, 77.7% (n=136) had already IgG against BTV; 39 cattle remained seronegative at the beginning of the survey. Mean age of IgG positive population was significantly higher to mean age of remaining at risk population (Student's t-Test $p=0.03$). Difference of seroprevalence was significantly more important in Northwestern region than Southern region (Pearson's Chi-squared test $p<10^{-3}$). No significant differences were observed between sexes concerning seropositivity of BTV in this cohort. During this longitudinal survey, 1 animal was lost before its seroconversion; and 19 seroconversions were observed. The median time to seroconversion was 9 months (95% CI:

[8-9.5]) (Figure 3). Estimation of incidence rate was 89.5 per 100 cattle-year assuming this incidence rate is constant.

Discussion

Our study confirms the circulation of BTV serotype 2 and the widespread distribution of this virus amongst domestic ruminant from Madagascar. The molecular analysis of genome segment 2 of the strain isolated in this study showed a high homology in nucleotides (99%) with the homologous gene from a strain isolated in 2009 in Reunion Island, 800 km to the East from Madagascar (Sailleau et al., 2012). These data suggest that the same strain has been circulating on both islands.

No clinical manifestations due to BTV infection were reported in Madagascar during the last decade except a suspicion of a BTV outbreak among cattle in the southern region of Madagascar in 1999-2000. Unfortunately, this outbreak was not confirmed by laboratory testing (Herbas J, personal communication). The high seroprevalence of BTV infection and the estimated incidence are in favor of a silent circulating of BTV in cattle of Madagascar as observed in other parts of Africa and in all of BTV enzootic-areas (Gibbs and Greiner, 1994; MacLachlan and Osburn, 2006). The lack of clinical manifestation may be due to this high seroprevalence. Indeed, Park *et al.*, have demonstrated that areas with intermediate seroprevalence have high number of cases reporting whereas low case reporting was associated with a high seroprevalence (Park et al., 2013). Only one unpublished study has evaluated seroprevalence of BTV infection in small ruminants of Madagascar (Rasamoelina-Andriamanivo, Dakar: UCAR, 2005). Indeed, this study showed a seroprevalence of 17.7% (n=92) amongst 520 sheep sampled in the district of Ambatondrazaka in 2005 (East part of Madagascar). In the same district, three years later, we found a seroprevalence of antibodies against BTV of 100% (95% CI, [89.6-100]) amongst cattle using the same assay. Although we cannot exclude a difference in terms of risk of infection between cattle and sheep, the

actual seroprevalence observed amongst both species and the clinically described BTV epidemic in 1999-2000, could be in favor of a hypothesis of a fairly recent introduction in Madagascar. Nevertheless, due to lack of historical data at the national level, we could not confirm our hypothesis.

Our results indicate that BTV infection increased significantly with age of ruminants demonstrating an enzootic circulation. We also demonstrate that annual incidence of the disease was 54 per 100 cattle-year. This incidence could be in favor of many episodes of circulation per year or a yearly circulation of BTV. Nevertheless, since BTV infection seems to be silent we could only address the question of seasonality and/or episodes of circulation with yearly virological surveillance.

Our study has some limitation. Indeed, BTV was detected from a pool of mosquitoes which is uncommon. No BTV wild strain was present in the laboratory at the time of viral isolation and detection of the Malagasy strain. Thus, a laboratory contamination at this level can be excluded. Some fresh blood from cattle was perhaps still present in the female mosquitoes constitutive of the BTV positive pool. Biting midges may have also contaminated mosquito's pools. Unfortunately, due to lack of specimen we could not look for DNA material from cattle or midges in the positive pool. Finally, incidence was estimated considering that rate of infection was constant all year around which might not be the case. A longer survey period that includes entomological and virological surveillance will be needed to address the question of seasonality or period of BTV transmission.

Conclusions

Our study reports the first BTV isolation in Madagascar. Madagascar is a BTV endemic-area and we can conclude that at least serotype 2 of BTV have circulated in Madagascar. Farming ruminants are largely traditional with local breeds. However importation of ameliorated breeds in dairy farms raises concern about the susceptibility of these animals to BTV

circulating in Madagascar. More studies about the incidence, the circulating serotypes, the vectors and clinical manifestations of BTV in Madagascar should be conducted at a larger scale to evaluate the associated risk for cattle. Due to high circulation of BTV in Madagascar, vaccine implementation can prevent infection amongst introduced breeding ruminants to avoid economic loss. Nevertheless, this implementation should be discussed due to the risk of recombination between vaccine and circulating strains (Dungu et al., 2004b).

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Competing interests

The authors declare no conflict of interest. None of the authors have financial or personal conflicts of interest related to this study. The corresponding author has full access to all data in the study and final responsibility for the decision to submit this publication.

Authors' contributions

JMH and SFA executed and coordinated the study, analyzed data and coordinated all process of writing of this work. CV, JMR, JPR and CS performed viral diagnosis and analysis. AAS provided reagents and help writing the paper. MLT and NE conducted vector survey and help

design the study. EC helped with serological analysis. SZ helped design the study and participated to the writing.

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Table 1. Relation between age group and seroprevalence of Bluetongue virus infection in cattle population, Madagascar, 2008 survey.

Figure 1. Seroprevalence of Bluetongue virus infection, Madagascar 2008-2009. **A.** Sampled area. Longitudinal study area: Tulear II in the Southern region (3 sites) and Mampikony in the Northern Region (2 sites). Sample district area: 30 districts sampled for the survey. **B.** Seroprevalence of BTV in sampled area. White areas represent districts where no sample was collected.

Figure 2. Phylogenetic tree based on the full-length segment 2 sequences from BTV-2 strains selected in GenBank (clustal analysis)

Figure 3. Estimation of survival parameters during longitudinal survey, Madagascar 2010-2011. Kaplan-Meier curve.

Table 1. Relation between age group and prevalence of Bluetongue virus infection in cattle population, Madagascar, 2008 survey.

Age group (years)	OR	p-value*	95% CI**
≤5***	1		
]5-10]	2.5	0.000	[1.6-3.9]
]10-20]	9.5	0.025	[1.3-68.4]

*p-value: Fisher’s exact test (univariate analysis), OR: univariate analysis

**95% CI: Confidence interval of OR

***≤5: reference category

Figure1A

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Figure1B

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Figure2

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Figure3

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