Epizootic ulcerative syndrome – First report of evidence from South Africa's largest and premier conservation area, the Kruger National Park

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ABSTRACT

This study reports on the first evidence of genomic material of the causative agent for epizootic ulcerative syndrome (EUS), Aphanomyces invadans, from fish in the Limpopo River system and the Kruger National Park, South Africa. Fourteen fish species were collected from various depressions in the floodplains of the Limpopo and Luvuvhu Rivers in the Makuleke Wetlands during 2015 and 2017. A single individual of Clarias gariepinus was found to have a suspected epizootic ulcerative syndrome (EUS) lesion. Samples were collected and evidence of A. invadans DNA in the samples was found through PCR and amplicon sequencing. The spread of EUS into this premier conservation area is of concern as it could potentially spread across borders and into other naïve river systems with important conservation statuses.

1. Introduction

The Kruger National Park (KNP) is the flagship conservation park of South Africa. The northern region of the park is characterised by the floodplains of the Limpopo and Luvuvhu Rivers and is known as the Makuleke Wetlands, a Ramsar wetland of international importance (Malherbe et al., 2017; Malherbe, 2018). These floodplains have numerous perennial and ephemeral depressions (approximately 30) that play a functional role as water source for mammals and habitat for unique aquatic biota.

The occurrence of Aphanomyces invadans, the causative agent of epizootic ulcerative syndrome (EUS) in various freshwater fish species, was first identified in southern Africa in 2007 from Botswana and then in 2008 from Zambia (Andrew et al., 2008). Since then, approximately 93 fish species from these two countries have been shown to be susceptible to EUS (see Huchzermeyer et al., 2018). These outbreaks have mostly been documented from floodplain systems, where the disease appears annually after flooding events. In South Africa, EUS has only been confirmed from the Palmiet and Eerste Rivers in the Western Cape (Huchzermeyer and Van der Waal, 2012). However, observational data of EUS in the North West Province (upper reaches of Limpopo River) was provided by Huchzermeyer et al. (2018). Here we present the first evidence of genomic material of A. invadans (EUS) in the Limpopo River system in South Africa.

2. Materials and methods

Fish were collected for an assessment of the fish population of the Makuleke Wetlands and not as a targeted survey for EUS (Malherbe et al., 2017; Malherbe, 2018). Fish were sampled using electroshockers, (Smith & Root LR-24), a small fyke net (10 mm mesh, 25 m), a seine net (10 mm mesh, 10 m long), and cast nets at ten different floodplain depressions within the Makuleke Wetlands in April 2015 (late summer high flow), September 2015 (early spring low flow) and May 2017 (late summer high flow) (Fig. 1). Fish were visually assessed for external lesions and abnormalities before being released. If any abnormal lesions were visible, fish were humanely killed using approved methods (NWU Ethics no. NWU-00095-12-A4). Briefly, fish where stunned using blunt force trauma which was immediately followed by pithing of the spinal cord and brain stem. Fish with lesions were photographed and the lesions were surgically removed and preserved in 96% ethanol for PCR analysis for genomic material of A. invadans.

Initial or preliminary detection of Aphanomyces invadans DNA in the fish tissue was achieved through the amplification of a 234 bp fragment of the internal transcribed spacer (ITS) gene region using the A. invadans specific primers recommended by the OIE (OIE, 2019) namely, forward primer Ainvad-2F (5′-TCA TTG TGA GTG AAA CGG TG-3′) and reverse primer Ainvad-ITSR1 (5′-GGC TAA GGT TTC AGT AGT TAG-3′) of Vandersea et al. (2006). Duplicate sets of DNA isolated from the
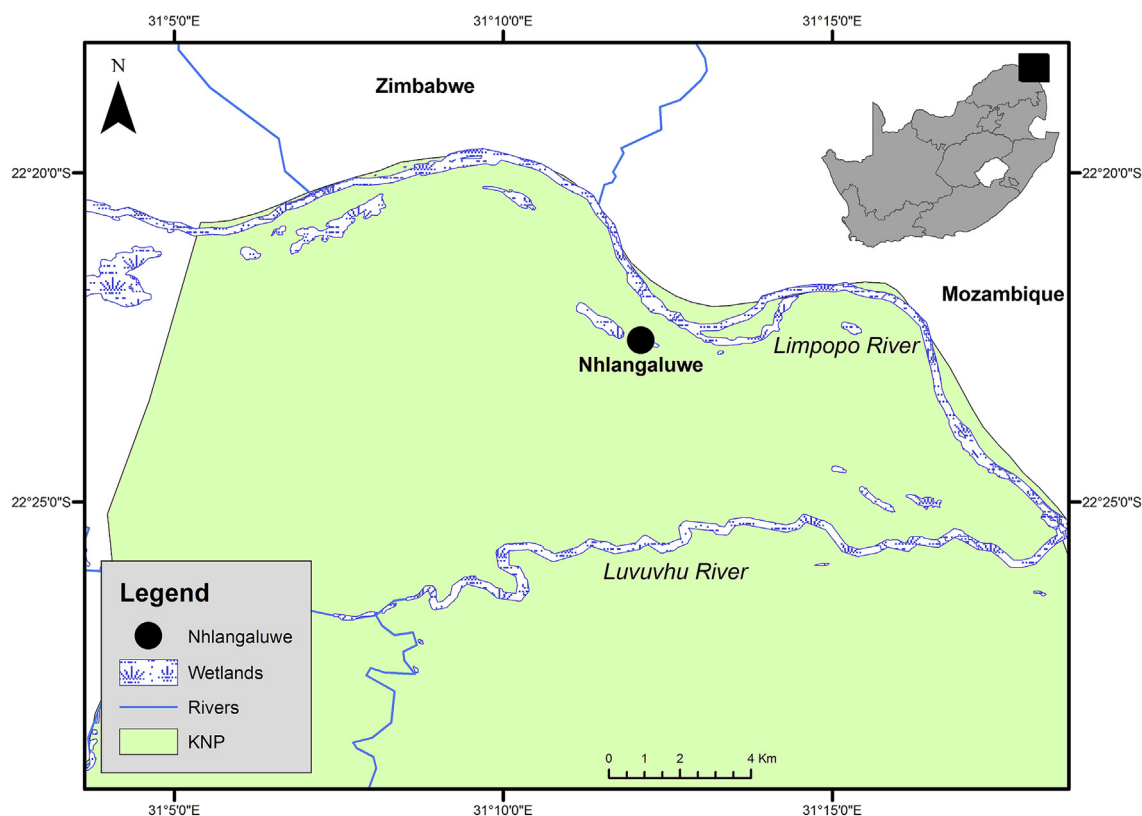

Fig. 1. Sampling location from which evidence of genomic material of *Aphanomyces invadans* was obtained in *Clarias gariepinus* from Nhlangaluwe Pan on the Limpopo River in the Kruger National Park, South Africa.

Table 1
Fish species, numbers and mean lengths of fishes sampled in the various depressions within the Makuleke Wetlands during 2015 and 2017.

Fish Name	April 2015		Sept 2015		May 2017	
	N	Size (mm)	N	Size (mm)[a]	N	Size (mm)
Glossogobius giuris	76	37				
Mesobola brevianalis	5	48				
Brycinus imberi	1	70			1	6
Synodontis zambezensis	1	80				
Clarias gariepinus	17	240.7	17		8	249.7
Infected *C. gariepinus*					**1**	**250**
Schilbe intermedius	2	175				
Oreochromis mossambicus adults	20	180.8	16		6	192.5
Oreochromis mossambicus juveniles	280	40				
Oreochromis niloticus	33	10	9		9	159.4
Tilapia sparrmanii	1	140				
Coptodon rendalli	6	15			1	
Labeo rosae	9	10			5	128.3
Enteromius toppini	6	4			1	6
Enteromius paludinosus	3	8	25			
Enteromius afrohamiltoni	144	66				

[a] Fish were not measured during this survey.

tissue sample and the reference samples (following the method of Greeff et al., 2012) were subjected to PCR (Labnet Multigene™ Thermal Cycler (Labnet International, Inc.)). Known *A. invadans* DNA as well as infected and non-infected reference tissues were included in the analysis to confirm the sensitivity and specificity of the assay. Additionally, non-template controls were included in the assay to ensure that the reagents were not contaminated. The reaction mixtures (25 μL) were prepared using 2 μL of supernatant, PCR-Master Mix (Kapa Biosystems; Cat# KK1006) and 400 nM of each primer. Amplification consisted of an initial denaturation of 2 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 45 s at 56 °C and 2.5 min at 72 °C, with a final extension of 5 min at 72 °C. The PCR products were run on a 0.8% agarose gel electrophoresis to verify analysis specificity and the fragment size.

Further evidence of *A. invadans* DNA in the fish tissue was provided through amplicon sequencing of a 550bp partial fragment of the ITS nuclear rRNA gene region using the primer pairs ITS11 (5′-GCC-GAA-GTT-TCG-CAA-GAA-AC-3′) and ITS23 (5′-CGT-ATA-GAC-ACA-AGC-ACA-CCA-3′) (Phadee, 2004; OIE, 2019). Amplification was conducted using the Labnet Multigene Thermal Cycler (Labnet International, Inc.) and consisted of an initial denaturation of 5 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at 56 °C and 1 min at 72 °C, with a final extension of 5 min at 72 °C. The amplified PCR products (10 μL) were analyzed by 0.8% agarose gel electrophoresis to verify reaction specificity and fragment size (550 bp) before being purified using a PCR purification kit (Roche). The purified PCR products were sequenced using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) and ABI3730xl Genetic Analyzer (Applied Biosystems) according to the sequencer manufacturer's instructions. Both forward and reverse primers (ITS11 and ITS23) were used for cycle sequencing. Each sequence was edited and assembled using CLC Main Workbench version 6.9. Homology searches were carried out using the BLASTN algorithm (Altschul et al., 1990) provided by the Internet service of the National Centre for Biotechnology Information (http://www.ncbi.blast.nlm.nih.gov/BLAST/).

3. Results and discussion

A total of fourteen fish species were collected during all the surveys (Table 1). These species represent approximately 37% of the fish species diversity reported from the Limpopo and Luvuvhu Rivers (Malherbe et al., 2017). No abnormal skin lesions were evident on any of the fish species collected in 2015. However, during the 2017 survey a

Fig. 2. Photographs of a lesion suspicious of epizootic ulcerative syndrome (EUS) on *Clarias gariepinus* from the Makuleke Wetlands.

single sharptooth catfish, *Clarias gariepinus* (Burchell, 1822) with a suspected EUS lesion (Fig. 2) was collected from the Nhlangaluwe Pan (S 22° 22′ 33.25″ E 31° 12′ 06.88″; Fig. 1) in the Limpopo River floodplain. The 2017 sampling trip was at the end of the summer high flow season with water levels already receding from the floodplains. Potentially, the single infected *C. gariepinus* could have been a remaining individual from a larger unrecorded outbreak during the preceding flooding period.

The suspected lesion was a large bleeding, ulcerative lesion extending from below the lateral line to above the pelvic fin with an approximate diameter of 3–4 cm. Genomic DNA was successfully isolated from the fish and control tissue samples and subjected to PCR. The fish tissue samples as well as the positive control samples all produced positive results in both PCR reactions producing bands of expected molecular weight, approximately 234 bp and 550 bp, respectively Phadee et al., (2004); Vandersea et al. (2006). No amplification was evident in any of the samples included as negative controls or non-template controls. A 480 bp nucleotide sequence was determined from the fish tissue sample and was deposited in the GenBank data base as accession number MN453589. A BLAST search of the GenBank Database showed 100% similarity between the gene sequences derived from our isolate and all other *A. invadans* ITS sequences in the GenBank database. These provide further support to the PCR findings in this study and evidence for the presence of *Aphanomyces invadans* DNA in the *Clarias gariepinus* tissue sample collected in 2017 from the Limpopo River and the KNP. Diagnoses based on PCR of tissue extracts and sequencing and analysis of PCR products are both diagnostic methods recommended by the OIE (OIE, 2019) as confirmatory diagnostic methods for reasons of utility and diagnostic specificity and sensitivity. Consequently, this data partly satisfies the OIE conditions for a confirmed case (OIE, 2019). Ideally, confirmatory diagnostic information from two independent diagnostic methods that have been validated for use in both healthy and clinically affected animals would be required for a confirmed case. A widely used secondary method would generally be the observation of mycotic granulomas by histopathology in the tissue (OIE, 2019). This method was not possible due to the necessary tissue fixatives not being available during the field work. This report of EUS in the Limpopo system, approximately 30 km from Mozambique

makes it a possibility that it could be reported from Mozambique in the future. Currently, EUS has not been reported from Mozambique (OIE-WAHIS, 2019) even though it has been extensively found in the Zambesi River system in Zambia and Zimbabwe (Huchzermeyer and Van der Waal, 2012). As the confluence of the Limpopo and Luvhuvhu Rivers is in the Makeluke Wetlands, the likelihood that EUS will be found in the Luvuvhu River is also high.

There are approximately 38 fish species within the Limpopo and Luvhuvhu rivers (46 species within the entire KNP) (Skelton, 2001), with many of these species listed by the OIE as being susceptible to EUS. In order to ensure that EUS is not spread accidently through monitoring or research practices, strict biosecurity protocols should be in place to prevent the spread into other KNP rivers. As the KNP rivers are already impacted by anthropogenic activities (Gerber et al., 2015), the risk of clinical infection is increased due to compromised host immunity and broad host range currently reported for this disease.

The extent of the impact of EUS was not assessed during this study due to the small sample size (n = 32) and low apparent prevalence (3.1%) but it is recommended that future targeted surveillance be undertaken to determine the occurrence of EUS in the Limpopo River (South Africa), throughout other rivers in the KNP as well as in Mozambique. It is recommended that extensive sampling of fish in the Limpopo River is undertaken during the highest risk periods to determine the occurrence of EUS throughout the catchment.

Further research is needed to understand the epidemiology of EUS in South African fish populations, especially regarding its distribution, transmission, and environmental conditions enabling survival. This information will then lead to the implementation of biosecurity practices to prevent or reduce the rate of spread of this disease in Southern Africa.

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Declaration of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2019.08.007.

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