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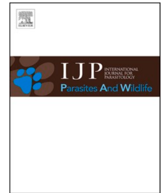

First report of cystic echinococcosis in rhinos: A fertile infection of *Echinococcus equinus* in a Southern white rhinoceros (*Ceratotherium simum simum*) of Kruger National Park, South Africa

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ABSTRACT

Despite being a parasitic disease known since ancient times, some epidemiological aspects of cystic echinococcosis (CE) remain unclear. Many studies describe its prevalence and genotyping in populations of domestic animals and livestock, but data regarding wildlife are often scarce and incomplete. The available literature suggests that CE has never been reported in African rhinos. Considering the fragile conservation status of these species due to continued poaching, this study tries to clarify some neglected epidemiological aspects. In February 2020, an adult female of the Southern white rhinoceros, *Ceratotherium simum simum* (Burchell, 1817), was killed by poachers. The subsequent necropsy performed by the state veterinary team revealed the presence of seven cysts within the pulmonary tissue (four cysts in the right medio-caudal lobe and three cysts in the left medio-caudal lobe) with a diameter of between 1.5 and 2.3 cm. Given the state of decomposition of the carcass, only two of these were suitable for microscopic examination. Specimens were examined under 10x and 40x microscopic magnification for the confirmation of fertility of the cysts, based on the presence of numerous protoscoleces in different stages of maturation. A histopathological examination was also performed to describe the relationship between parasite and host tissue reaction. Cyst samples were subjected to PCR. The primers successfully amplified the expected fragments of the cox-1 and the nad-1 gene from the isolated genomic DNA, revealing high sequence identity with published sequences of *Echinococcus equinus* Williams & Sweatman, 1963 isolate G4 and *E. equinus* isolate SLG5-G4.

1. Introduction

Echinococcosis is a cosmopolitan zoonosis caused by larval stages of cestodes belonging to the genus *Echinococcus* (Rudolphi, 1801) (Deplazes et al., 2017). Ever since its conception, the classification of species within the genus, based on morphological and biological characteristics, remained a taxonomic challenge (Romig et al., 2015; Laurimäe et al., 2018). More recently, the application of molecular genotyping, using mitochondrial DNA (mtDNA) sequences, has enabled scientists to identify 5 species formerly grouped under *Echinococcus granulosus* (Batsch, 1786) *sensu lato* (*s. l.*): *E. granulosus sensu stricto* (*s. s.*) (genotypes G1 and G3) (Busi et al., 2007; Kinkar et al., 2017), *E. equinus* Williams & Sweatman (G4), *E. ortleppi* Lopez-Neyra & Soler Planas, 1943 (G5) (Thompson and McManus, 2002), *E. canadensis* Webster & Cameron, 1961 (G6-7, G8 and G10) (Nakao et al., 2007, 2013; Laurimäe et al., 2018), and *E. felidis* Ortlepp (1937), the "lion strain" (Bowles et al.,

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1992; Bowles and McManus, 1993; Lavikainen et al., 2003). Despite considerable progress on the development of a stable taxonomy for *E. granulosus s. l.*, the species status of some of these remains controversial, in particular for *E. canadensis* in which a division into two species has been proposed, respectively G6/G7 and G8/G10 (Laurimäe et al., 2018). All five species within the complex of *E. granulosus s. l.* are endemic in sub-Saharan Africa: *E. granulosus s. s.*, *E. canadensis*, *E. ortleppi*, *E. equinus* and *E. felidis* (Hüttner et al., 2009); the species status of the latter three has rarely been contested (Romig et al., 2015; Lymbery, 2017; Laurimäe et al., 2018). *Echinococcus felidis*, a species endemic to African wildlife, was first described some 80 years ago in a lion, *Panthera leo* (Linnaeus, 1758), from the Northern Transvaal (presently Limpopo province), South Africa (Ortlepp, 1937).

The ability of *E. granulosus s. l.* to infect a wide range of hosts species and its great genetic variability contribute to the universal distribution of this parasite (Poglayen et al., 2017a). The life cycle is indirect and includes two hosts. The definitive host is always a carnivore, in which the adult form is located in the small intestine. Adults are small (2–7 mm in length) and the strobila is composed of few proglottids. Proglottids, when gravid, can contain about 500–800 eggs, which cannot be morphologically distinguished from other taeniid eggs.

The intermediate hosts can be herbivores, omnivores or even rodents. They become infected when ingesting eggs containing the so-called oncosphere larvae (Budke et al., 2002). The larvae then penetrate the lamina propria of the intestinal mucosa and are transported passively through the blood or lymph system to the liver, lungs, or other organs, where the oncosphere larvae develop into hydatid cysts (metacestode larvae). In cattle, higher prevalence of cysts was observed in the liver and lungs, with a probability of 60–80% and 10–20%, respectively, compared to other organs (Poglayen et al., 2017a).

Larval infection, also called hydatid disease or hydatidosis, is characterised by long term growth of metacestode cysts in the intermediate host (McManus and Thompson, 2003). Cysts consist of two parasite-derived layers: an inner nucleated germinal layer, and an outer acellular laminated layer surrounded by a host-derived fibrous capsule. Brood capsules and protoscoleces bud from the germinal layer (McManus and Thompson, 2003). The infectious capacity of a hydatid cyst is closely related to the vitality of the protoscoleces; therefore, they are classified as fertile or sterile (acephalocysts), based on the presence or absence of protoscoleces, respectively (Laurimäe et al., 2018). Development times of hydatid cysts are highly variable and depend on the animal species, the parasite species and the organ affected. On average, it is estimated that the growth of a cyst can vary in diameter from 1 to 5 cm per year (Taylor et al., 2010). From an epidemiological point of view, human and other primates represent “aberrant” intermediate hosts, because the transmission cycle of the parasite usually ends within these hosts. Despite this, CE is considered one of the five most frequently reported parasitic zoonoses and remains a public health problem globally (Sadjjadi, 2006).

Studies conducted in the Kruger National Park (KNP) have revealed that some species are particularly susceptible to *Echinococcus* infection. According to Young (1975/a, b), numerous cystic lesions have been found in the Burchell’s zebra, *Equus quagga burchellii* (Gray, 1824), with an estimated prevalence of 60% in the population included in the study. These lesions could likely have been caused by *E. equinus*, but definitive confirmation of species identification is not possible due to a lack of molecular and microscopic diagnostics in these studies. The morphological characteristics of *E. equinus* appear to be similar across the globe, which explains how it has easily adapted in different contexts and different host species (Kumaratilake et al., 1986).

As mentioned above, despite it being an ancient genus, the ecology and epidemiology of *Echinococcus* spp. remain largely unknown. A wildlife cycle of *E. equinus* has recently been identified, involving lions, black-backed jackals, *Canis mesomelas* Schreber, 1775 and Burchell’s zebras, in the Etosha National Park, Namibia (Wassermann et al., 2015). There is a possibility that more strains do exist in wildlife species due to the co-existence of wild/domestic animals and humans in sub-Saharan Africa. Further research is required to characterize the strains/species in both livestock and wildlife (Magambo et al., 2006). Reports of CE in wild intermediate hosts are very rare, in comparison with the studies carried out for domestic species (Poglayen et al., 2017b). This is ultimately attributable to sampling bias and the difficulty in necropsying free-ranging wild intermediate hosts with any frequency in ecosystems with large carnivores. It is particularly difficult to access fresh carcasses for thorough inspection of the organs in order to confirm the presence of parasitic cysts. Due to the illegal trade in their horns, an increase in rhino poaching in the KNP since 2012 has made it theoretically possible to carry out epidemiological investigations on carcasses of poached rhinos. Although several necropsies were performed during the last five years, no reports of lesions attributable to CE could be found in the literature. The use of improved microscopic techniques and molecular biology made it possible to, for the first time, confirm the presence of *E. equinus* (G4) in a Southern white rhinoceros, *Ceratotherium simum simum* (Burchell, 1817), originating from the KNP.

2. Materials & methods

2.1. Study area

Covering a surface area of 19 485 km2, the KNP is South Africa’s largest wildlife refuge and a critical biodiversity resource. It is situated in the lowveld of the northeastern Mpumalanga and Limpopo provinces of South Africa, bordering Mozambique in the east and touching on Zimbabwe in the north. It is an elongated park of about 2 million ha, extending roughly 320 km from north to south, with an average width of 65 km (Michel et al., 2006). KNP also forms part of the Great Limpopo Transfrontier Conservation Area which includes private reserves to the west of the KNP, Gonarezhou National Park in Zimbabwe, Limpopo National Park in Mozambique, and eventually also several communal conservation areas in between. Removal of fences with Mozambique started in 2003 and has allowed for livestock and wild animals from both sides to mix to an extent. The KNP supports 147 mammal species.

2.2. The Southern white rhinoceros: conservation status and biological traits

As of December 31, 2017, there were an estimated 18,064 white rhinos, including both subspecies, in the wild, the majority (99.3%) of them occurring in just five countries (South Africa, Namibia, Kenya, Botswana and Zimbabwe) (Emslie et al., 2019). The Southern white rhinoceros continues to be listed as Near Threatened by the IUCN (the International Union for Conservation of Nature), mainly as a result of poaching (Emslie, 2020).

White rhinos are bulk grazers, using their broad lips to pluck grass close to ground level. Short grass areas are favoured for most of the year, while during the late dry season stands of tall *Themeda triandra* are grazed. Drinking usually takes place only every 2–4 days during the late dry season, while mud wallowing is frequent during the summer (Owen-Smith, 1973). Rhinos are hindgut fermenters (Van Hoven et al., 1987), an aspect shared with zebras and other equids, exposing these animals to a similar risk profile regarding infection with *E. equinus*.

2.3. Sample origin

In February 2020, an adult female white rhino was shot in the late afternoon by poachers in an attempt to remove the two keratin horns attached to her skull. The poachers were unsuccessful in removing the horns because the gunshots alerted patrol rangers to the imminent presence of danger. Horn measurements, together with dental impressions, used to estimate the age of the female, suggested that she was approximately 20 years old (Hilmann-Smith et al., 1986).

Unfortunately, the state veterinary team was only able to perform the

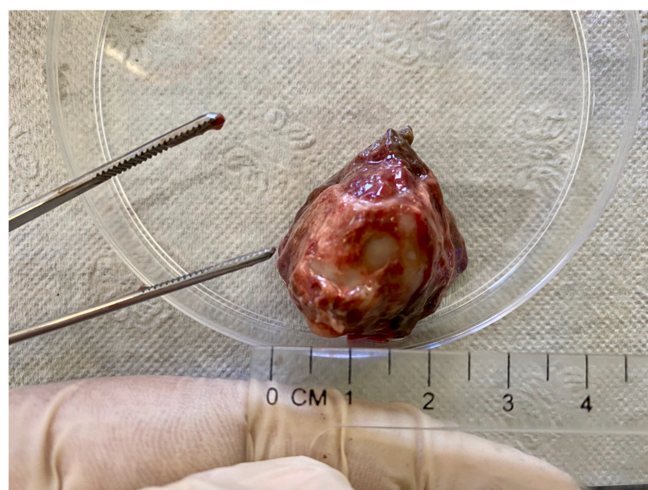

Fig. 1. Section of rhino lung containing hydatic cyst.

necropsy on the carcass the following day, with substantial damage done to the carcass by lions and hyaenas during the night. Despite this, the thick skin layer and close arrangement of the costal arches protected the anatomical structure of the thorax, and consequently, the main thoracic organs and partial abdominal organs remained intact. The animal had an older gunshot wound to the back of the withers (possibly from a prior failed poaching attempt), with the lesions suggesting a chronic inflammatory process. Inspection of the internal thoracic organs revealed the presence of seven cysts within the pulmonary tissue: four cysts in the right medio-caudal lobe and three cysts in the left medio-caudal lobe. The diameter of the main cysts ranged between 1.5–2.3cm (Fig. 1). The cysts presented with different degrees of autolysis, and from a total of seven cysts, microscopic tests could only be performed on two. Four other cysts had signs of severe decomposition, one with waxy/purulent material indicating a state of lysis prior to the death of the animal. The biological material extracted was divided and stored for further analysis.

2.4. Sample processing

An aliquot of cyst aspirate was subjected to direct microscopic investigation. For confirmation of diagnosis, fertility was assessed under a microscope at 10x and 40x magnifications, observing vitality and motility of protoscoleces as well as flame cell movements without staining (Varcasia et al., 2007). Protoscoleces removed from the wall of the hydatid cyst or taken from the cyst fluid were individually mounted and cleared in Hoyer's medium. Light pressure on the coverslip was used to spread the hooks, which were then measured in lateral view as illustrated by (Halajian et al. 2017). Hooks measured originated from six different protoscoleces, while the number of hooks was counted in five rostellar crowns. Tissues submitted for histopathology, comprising a section of cyst wall and lung tissue of the host, were fixed in 10% neutral buffered formalin, processed routinely through graded alcohols and embedded in paraffin wax. Histologic sections were prepared using haematoxylin and eosin stain (Fig. 2).

2.5. PCR analysis

Protoscoleces from the inner germinal layer of hydatid cysts were extracted and stored in 70% ethanol. Prior to DNA isolation, the samples were washed three times in 10 ml 1 X PBS to remove residual ethanol. The pellet was resuspended in 500 μl 1 X PBS and the mixture was transferred to a tube containing MagNa Lyser green beads (Roche Molecular Diagnostics). The sample was homogenized twice at 6800 rpm for 30 s followed by immediately cooling on ice. DNA was isolated using the digestion workflow of the MagMax Core Nucleic Acid Purification Kit (ThermoFisher Scientific) on the KingFisher™ Duo Prime

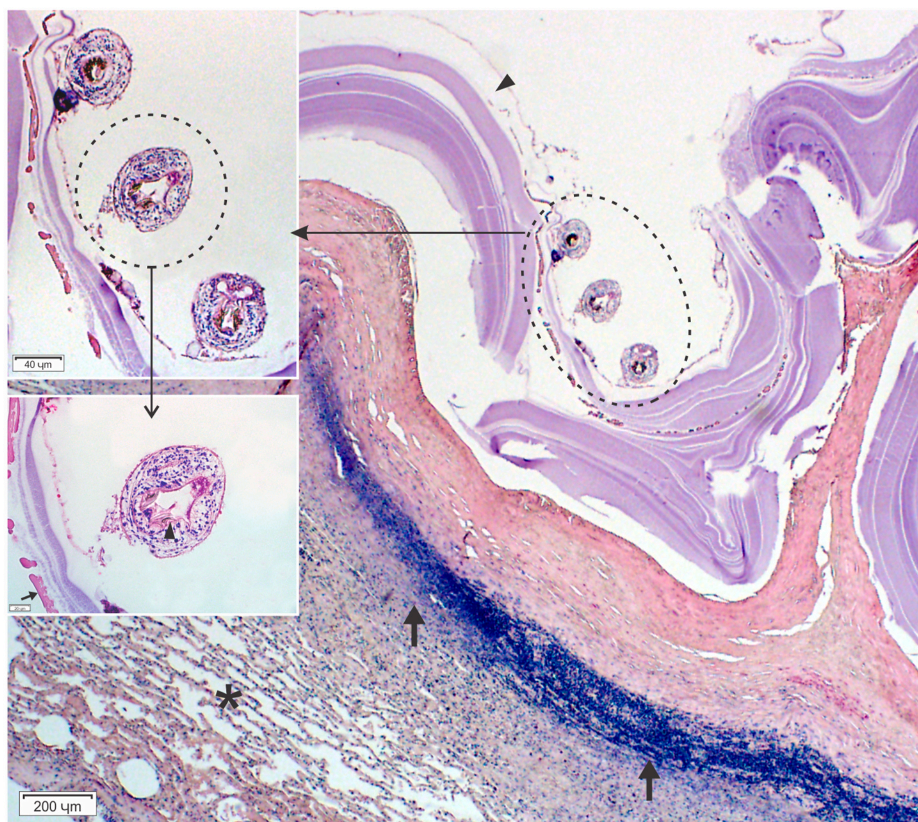

Fig. 2. Histopathological section of the lung (*). The tissue contains a partially septate cystic structure, surrounded by a thick layer of mature fibrous connective tissue infiltrated by lymphocytes and plasma cells mixed with smaller numbers of eosinophils and macrophages (arrows). Three small (100–125 μm in diametre) cestode protoscoleces are loosely attached to a laminated basophilic granular inner layer (arrowhead). Protoscoleces contain large hooklets in the rostellum (upper inset, and lower inset (arrowhead)). The inner layer of the cyst wall is lined with amorphous acellular eosinophilic material (arrow, lower inset).

Table 1
Oligonucleotide primers used in the amplification and direct sequencing of the cox-1 and nad-1 genes.

Primer	Sequence 5′-3′	Reference
JB3- cox F	TTTTTTGGGCATCCTGAGGTTTAT	Bowles et al. (1992)
JB4.5 -cox R	TAAAGAAAGAACATAATGAAAATG	
JB11_nad F	AGATTCGTAAGGGGCCTAATA	Bowles and McManus (1993)
JB12_nad R	ACCACTAACTAATTCACTTTC	

Purification System (ThermoFisher Scientific). Published primers designed to amplify regions of the mitochondrial cytochrome oxidase subunit I (cox-1) and NADH dehydrogenase 1 (nad-1) genes were used to analyze the isolated genomic DNA (Table 1). Reactions were carried out in a final volume of 25 μl, comprising GoTaq® Green master mix (Promega), 0.2 μM of each primer and 2.5 μl of genomic DNA. The cycling conditions were an initial denaturation of 3 min at 95 °C, followed by 30 cycles of 30 s at 95 °C, 1 min at 50 °C and 1 min at 72 °C, with a final extension of 7 min at 72 °C. The amplicons were visualized

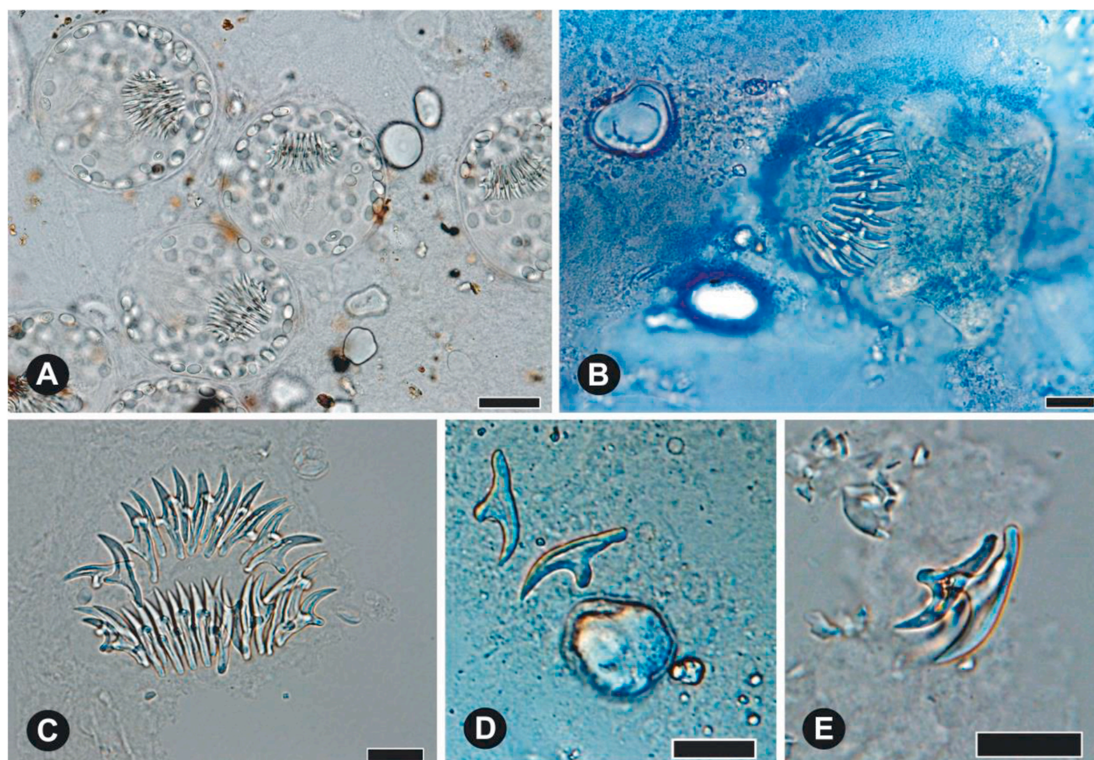

Fig. 3. Micrographs of metacestodes of *Echinococcus equinus* from *Ceratotherium simum simum*. **A.** Protoscoleces in cyst fluid (hydatid sand). **B.** Evaginated protoscolex showing suckers and rostellar hook crown. **C.** Rostellar hooks flattened under coverslip pressure. **D**, **E.** Pairs of hooks; small hook on the left, large hook on the right. Scale bars: 50 μm (**A**), 20 μm (**B-E**).

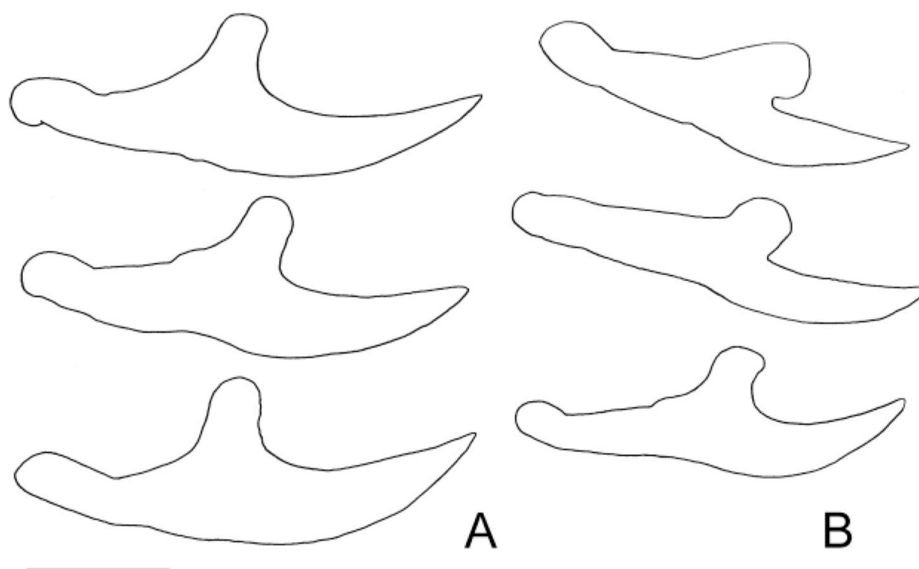

Fig. 4. Line drawings of large (**A**) and small rostellar hooks (**B**) of protoscoleces of *Echinococcus equinus* from *Ceratotherium simum simum*. Scale bar: 10 μm.

Table 2
Comparative measurements of large and small hooks of *Echinococcus equinus* metacestodes from *Ceratotherium simum simum* in the Kruger National Park, South Africa and equids in Europe, New Zealand and Namibia. The range in parentheses follows the mean ± S.D. Measurements of metacestodes from the rhino were taken according to Halajian et al. (2017, Fig. 2). L, large hooks; S, small hooks; TL, total length; TW, total width; AL, anterior length; PL, posterior length; GL, guard length; BL, blade length.

Host, hooks (no. measured)	TL	TW	AL	PL	GL	BL	Country	Reference
Rhino, L (n = 16)	30.9 ± 0.8 (29.2–32.1)	11.4 ± 1.2 (9.2–13.5)	16.2 ± 0.6 (14.7–17.2)	16.9 ± 1.4 (14.3–19.3)	6.1 ± 0.7 (5.0–7.4)	14.3 ± 0.9 (12.5–15.9)	South Africa	This study
Rhino, S (n = 15)	26.9 ± 1.1 (25.2–28.4)	8.7 ± 1.0 (6.4–10.7)	11.8 ± 0.6 (11.0–13.1)	16.7 ± 1.0 (15.3–18.6)	4.8 ± 0.6 (3.6–5.9)	10.4 ± 0.6 (9.4–11.7)	South Africa	This study
Zebra, L (nd)[a]	30.3 ± 0.6 (29.2–31.5)	–	–	–	–	14.9 ± 0.5 (14.0–16.0)	Namibia	Kumaratilake et al. (1986)
Zebra, S (nd)[a]	26.4 ± 1.6 (22.0–28.5)	–	–	–	–	10.3 ± 0.7 (9.0–12.0)	Namibia	Kumaratilake et al. (1986)
Donkey, L (nd)[a]	29.4 ± 0.9 (27.0–31.0)	–	–	–	–	14.3 ± 0.8 (12.5–15.5)	Switzerland	Kumaratilake et al. (1986)
Donkey, S (nd)[a]	25.9 ± 1.1 (23.0–28.0)	–	–	–	–	10.3 ± 0.8 (8.5–11.5)	Switzerland	Kumaratilake et al. (1986)
Horse, L (nd)[a]	31.1 ± 0.9 (30.0–33.0)	–	–	–	–	15.2 ± 0.4 (14.5–16.0)	New Zealand	Kumaratilake et al. (1986)
Horse, S (nd)[a]	28.2 ± 1.5 (26.0–31.0)	–	–	–	–	11.1 ± 0.6 (10.0–12.2)	New Zealand	Kumaratilake et al. (1986)
Horse, L (nd)[a]	30.6 ± 1.2 (28.0–32.0)	–	–	–	–	14.4 ± 0.6 (13.0–15.5)	Belgium	Kumaratilake et al. (1986)
Horse, S (nd)[a]	27.7 ± 0.8 (25.5–29.0)	–	–	–	–	9.5 ± 0.7 (8.0–10.5)	Belgium	Kumaratilake et al. (1986)
Horse, L (nd; cyst no. 5)[b]	29.0 (26–33)	10.8 (10–12)	–	–	–	14.8 (14–18)	England	Williams and Sweatman (1963)
Horse (nd; cyst no. 6)[b]	28.6 (28–30)	10.7 (10–12)	–	–	–	14.5 (14–15)	England	Williams and Sweatman (1963)

[a] The authors reported their specimens as '*Echinococcus granulosus* of equine origin'.
[b] Only the mean and range were reported by the authors; nd, not determined.

by 1.5% agarose gel electrophoresis, and positive PCR products were purified using the PCR purification protocol of the PureLink™ Quick Gel and PCR Purification combo kit (ThermoFisher Scientific). The samples were submitted to Inqaba Biotechnologies (South Africa) for direct sequencing and capillary gel electrophoresis. Sequences were assembled and edited using the CLC Main Workbench (CLC Bio version 20.0.4) and queried against previously reported cox-1 and nad-1 sequences from *Echinococcus* spp. using BLASTn under default algorithm parameters (NCBI BLAST).

3. Results

3.1. Microscopic analysis

Histologically, partially septate encapsulated parasitic cystic structures were present in the lung (Fig. 2). Protoscoleces were invaginated and ranged in diameter from 137 to 152 μm, with a mean of 146 μm (n = 11). Rostellar hooks were typically arranged in two rows and varied in number from 31 to 32, with a mean of 31.8 (n = 5). Protoscoleces and hooks are illustrated in Figs. 3 and 4, and measurements of large and small hooks are presented in Table 2. The blade was shorter than the handle in the small hooks (AL < PL; acronyms defined in Table 2). In the large hooks, the guard was located in the middle or slightly posterior in eight of the 16 measured hooks, whereas it was slightly anterior in the remaining eight hooks. Williams and Sweatman (1963) assigned metacestodes collected from horses in England to the newly erected subspecies *E. equinus*. The number of large and small rostellar hooks in 100 scoleces varied from 25 to 41, with a mean of 31.6 (Williams and Sweatman, 1963). The number of rostellar hooks as well as the total length, width and blade length of the large hooks obtained in the present study fall well within the range of the hooks of metacestodes of *E. equinus* as described by Williams and Sweatman (1963) and recorded by Kumaratilake et al. (1986) from horses, donkeys and zebras (Table 2). Based on the molecular evidence and observed morphological similarities (see below), we assign our specimens to *E. equinus*.

3.2. PCR analysis

The primers successfully amplified the expected fragments of the cox-1 gene (450 bp) and the nad-1 gene (500 bp) from the isolated genomic DNA. BLASTn analysis of the nad-1 gene showed 99% sequence identity to published *E. equinus* isolate G4, while the cox-1 gene had 100% sequence identity to the *E. equinus* isolate SLG5-G4.

4. Discussion

The present study conducted within the KNP, represents the first reported case of *E. granulosus s. l.* in African rhinos. Though most pathogens and parasites infect multiple hosts, parasite ecology studies in wildlife tend to focus on single parasite-host systems, and concentrate on commonly occurring host species, particularly when postmortem sampling is necessary for data acquisition. Rare or threatened animals, such as white rhinos remain poorly studied. However, one can infer that mega herbivores (over 1000 kg), due to their low predation risk as adults (Pringle, 2018), will represent poor intermediate host potential. It would therefore seem that from an epidemiological point of view white rhinos would represent aberrant or accidental hosts in the biological cycle of *E. granulosus s. l.*

Since 2008, white rhino populations have been severely impacted by illegal hunting (poaching) for their horn. In South Africa, the annual number of rhinos poached increased from 200 in 2009 to a peak of 1300 in 2015 (Emslie et al., 2018). Levels of poaching have differed between sites, with KNP, with the largest rhino population also having the greatest number of rhinos poached to date, representing an 8% loss per annum for the past 5 years (Emslie et al., 2018). In this scenario, interventions to counter the poaching threat, include managing for maximum productivity (Balfour et al., 2019). In threatened and stressed populations, impacts of parasites, may become more serious. The rhino in the present study was compromised by a festering gunshot wound and was found in poor body condition at the time of its death. Whether this made the parasite infection worse is unknown but is a factor that needs

to be considered. Poaching pressure may push white rhinos into less preferred habitats that may not only restrict access to optimal grazing but may also increase exposure to parasites and lead to higher parasite burdens.

This study describes a new host species description for *E. equinus*, and a new geographic confirmation of the parasite, within the KNP. Previous records of hydatic cysts in zebras in the KNP (,(Young, 1975a,b) lacked morphological descriptions and molecular strain information, but were presumably *E. equinus* (Young, 1975a,b). The morphological description of structures of the protoscoleces together with molecular data presented herein can serve as a comparative reference for future studies in other species. Previous studies in sub-Saharan Africa, have shown that lions often represent the definitive host in the life cycle of *E. equinus* (Wassermann et al., 2015). Completion of the life cycle in the present study would seem to be entirely dependent on wild/free-ranging hosts, since the game fence surrounding the KNP largely prevents contact between domestic and wild animals; it is, however, noteworthy, that the fence is incomplete in places or might at times be compromised. Thus, we cannot exclude that contacts with domestic animals in the surrounding areas of the park and those living in the neighboring Limpopo National Park in Mozambique may create a wildlife/livestock/human interface. Recent establishment of a Greater Limpopo Conservation Area has facilitated the movement of animals from adjacent parks. Importantly, in the Mozambican area, many rural communities, including their livestock and pets, live in close vicinity to the park. This certainly must be considered a factor when assessing epidemiological risks. Though the parasite life cycle may be relatively conserved in wild hosts, exposure to domestic animals and an increased wildlife-livestock-human interface may increase exposure of wildlife to novel and diverse strains of parasites. The results of our microscopic study of protoscoleces of *E. equinus* indicate their morphological similarity to samples from different parts of the world. This highlights that *E. equinus* has undergone very little change over time despite being isolated in different ecosystems for decades. A reflection must be made on the incredible adaptability that *E. granulosus s. l.* retains in different host species. Whatever its final host, we can say that very few species are completely resistant to this parasite.

5. Conclusion

Molecular tools are changing our understanding of shared parasites in multi-host systems. Environmental, host and parasite factors combine to determine which parasites are shared and which are of consequence to a given host. When managing for optimal productivity, especially in a rare or threatened species, conservation managers would be well-advised to consider parasite life cycles, as well as factors that may increase parasite prevalence, such as stress, overgrazing and stagnation, and find means to limit them.

Declaration of competing interest

All authors declare that they have no competing interests.

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