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Antibiotic resistance profiles of oral flora in hippopotami (*Hippopotamus amphibius*): implications for treatment of human bite wound infections

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Abstract

Background The common hippopotamus (*Hippopotamus amphibius*) is found in aquatic environments throughout sub-Saharan Africa and is known to cause attacks on humans living or working close to water bodies. Victims surviving an attack often suffer from the consequences of severe wound infections caused by the animal's sharp canine teeth.

Objective Isolation of normal flora bacteria from the oral cavity of common hippopotami (*Hippopotamus amphibious*) followed by antibiotic susceptibility testing to aid in the identification of a targeted antibiotic treatment regimen for hippopotamus attack victims.

Methods Oral swabs were collected from 34 free-ranging hippopotami in three reserves within the Greater Kruger National Park Complex in South Africa and cultured for aerobic and anaerobic bacteria. Antibiotic susceptibility testing was conducted using the disc diffusion method (Kirby-Bauer method) and a panel of 16 antibiotic drugs representing 10 antibiotic categories.

Results Culturing of 50 oral swab samples from 34 hippopotami yielded 188 aerobic isolates belonging to 30 bacterial genera and 41 bacterial species (Gram-negative: 70.7%; Gram-positive: 29.3%) and 16 obligate anaerobic isolates from two genera. Three bacterial species, namely *Aeromonas hydrophila, Aeromonas sobria* and *Shewanella putrefaciens* accounted for 52% of the aerobic isolates. The anaerobic isolates were identified as *Prevotella melaninogenica* and *Clostridium spp*.

Antimicrobial susceptibility testing was performed for 112 aerobic isolates (Gram-negative: 93 (83%); Gram-positive: 19 (17%)) representing all isolated bacterial species. High levels of antibiotic resistance were observed among the Gram-negative species especially to most beta-lactam antibiotics (50.5% to 80.7%). Multidrug resistance was detected in 22.6% of Gram-negative isolates and in 24.1% of all isolates.

Conclusions This study provides the first investigation of the oral flora bacteria of the common hippopotamus. Among the 32 mostly aerobic bacterial genera the most abundant bacterial species were *A. hydrophila*, *A. sobria* and *S. putrefaciens*. They are typical inhabitants of the aquatic habitat of the hippopotamus and of zoonotic importance as opportunistic human pathogens. The antibiotic susceptibility profiles demonstrated that quinolones,

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aminoglycosides, and tetracyclines were highly efficacious against these bacterial species which otherwise showed moderate to high levels of resistance to the traditional bite wound treatment with amoxicillin/clavulanate and 1st and 2nd generation cephalosporins.

Keywords *Aeromonas hydrophila*, *Aeromonas sobria*, Antibiotic resistance, Bite wound infection, *Hippopotamus amphibius*, Human-wildlife conflict, Public health, *Shewanella putrefaciens*

Background

The common hippopotamus (*Hippopotamus amphibius*), taxonomic group *Artiodactyls*, is a semi-aquatic mammal living in wetlands, rivers and lakes with a geographical distribution limited to sub-Saharan Africa and known to aggressively defend its territory and its young if threatened. All *Artiodactyls* are herbivores, but the hippopotamus is an exception. It is not a ruminant and has a three-chamber stomach. Individuals may be roaming far in the dark, usually covering considerable distances. Humans living close to water bodies are at the highest risk of encountering hippopotami when these animals leave the water at dusk to graze and at dawn when they return to the water from their sleeping place on land. Hippopotami spend most of the day in the water for shelter and may attack humans in the water or boats if they feel threatened [1].

The common hippopotamus has the largest mouth of all terrestrial mammals and can open more than 150˚ [2]. The lower canine teeth project about 30 cm from the gum and are kept razor sharp by grinding against the upper canine [2]. In a hippopotamus attack, the canine teeth cause deep lacerations and damage skin and subcutaneous tissue, as well as muscular tissue with a high risk of microbial contamination [3]. Hippopotami have a biting force of 2 000 PSI, in contrast to the 1000 PSI of spotted hyena [4], which can lead to fatal or severe crushing injuries during attacks on humans followed by tissue devitalization [3]. Near-amputation of extremities has also been reported [5]. For this reason, medical professionals have expressed their support to triage hippopotamus inflicted injuries as major trauma rather than a mammalian bite [6].

In the Mpumalanga Province (MP) the annual hippopotamus aerial surveys for the Crocodile River (Montrose to Kruger National Park boundary, 92.6 km) dating back from 1992 to the present year indicated a population increase of 245% (70 individuals in 1992 and 242 in 2024). Similar drastic increases of the hippopotamus populations in tributaries of other rivers have been noted. Hippopotami roam in and out of the KNP protected area into private land and adjacent human settlements close to rivers, which can be classified as Human-Wildlife Conflict (HWC) animals [7]. The increased hippopotamus population numbers can be directly related to increased HWC hippopotamus complaints (42 to 61 annually for the past 5 years) to MTPA. These complaints consisted mainly of crop damages to citrus, macadamia orchards, and sugar cane fields as well as threats to human lives. Owing to the ever-increasing number of security fences on farms, hippopotami are often trapped in these corridors resulting in additional HWCs. During the high rainfall season and the flooding of river macro-channels hippopotami migrate to safety out of the river mainstems into secondary river channels with pools and dams. These spatial movement patterns linked to intraspecific competition result in hippopotami-occupying areas where they are in direct conflict with humans, often resulting in injuries and death to humans. Unfortunately, not all hippopotamus attacks on humans are reported, and the exact number of hippopotamus attacks on humans per year is unknown [8, 9]. A study modelling the hippo-human conflict incidences in Kenya over a 12-year period reported a mean rate of 54 incidences per year, whereby this increased to several hundred during severe droughts [8]. Haddara et al. (2020) estimated that up to 74 people may have died during a 2-year period after a hippopotamus attack before they could reach a hospital in Burundi [6]. In the MP attacks appear to be on the increase, with four cases reported for the first half of 2024 and four patients admitted by one private hospital in MP alone in 2024 (A Schoeman, personal communication). A study by Dunham et al. (2010) described HWC in Mozambique over a period of 27 months. Among the 265 people killed and 82 people injured by wildlife. hippopotami were responsible for 4.5% and 12%, respectively [10]. Hippopotamus–human conflict has also been reported by rural communities in Nigeria, Zimbabwe and Tanzania [11, 12].

Wild animal attacks including fatal injuries in humans appear to involve more often crocodiles, lions and elephants than hippopotami but quantitative data are scarce [9]. In contrast to crocodile and lion attacks, hippopotami pose a double threat as they may attack humans on land and in water. In particular, people living near water are exposed to attacks when they encounter grazing or sleeping animals on land or in the water while bathing, washing clothes or fishing [13]. Regarding the latter, there was a reported increase in hippopotamus attacks in Kenya during the COVID-19 pandemic, illustrating their

impact on livelihoods [14]. In many other cases, crop-raiding hippopotami may attack people who attempt to chase them off the land [11, 12, 15]. Most of these incidents are underreported or appear "hidden" in studies investigating HWC in general [8, 13, 15].

More often, media releases cover hippopotamus attacks on tourists, fishermen, game rangers, small scale farmers and illegal migrants in South Africa (iSimangaliso News Flash 21 October 2022; News24; News24, 10 December 2021, News24, 13 August 2023; News24, 22 November 2023), Zimbabwe (News24, 4 December 2018; 13 October 2017) and Kenya (News24, 12 August 2018). These and other reports illustrate that the likelihood of death after a hippopotamus attack is high but moreover, victims who survive an attack usually sustain severe injuries and trauma caused by the mega-herbivore's long and razor-sharp canine teeth [6].

Hippopotamus wounds in humans typically present with long bone fractures, deep lacerations, tendon or joint damage, crushing injuries, organ damage, and chest or abdomen penetration [3, 16, 17]. The complications of these injuries can include chronic osteomyelitis, possibly necessitating limb amputation, laparotomy, and other specialized surgical interventions [5, 6]. While surgical site infections remain the most common complication in animal bite wounds in general [18], the prevalence of deep wound infections in hippopotamus bite wounds in humans has reached 37.4% [6] and may lead to sepsis [3].

Due to their amphibious lifestyle, the oral cavities of hippopotami harbour aquatic aerobic and anaerobic bacteria and may differ from those encountered in bite wounds inflicted by domestic animals; hence standard antibiotic treatment regimens tailored for these species could be ineffective in hippopotamus inflicted bite wound infections of the inherent antibiotic resistance of the causative bacteria.

This study aimed to culture and identify the recoverable aerobic and anaerobic bacterial species present on the oral mucosa of the hippopotamus and to determine the antibiotic susceptibility profiles of all identified strains. The findings can assist health care professionals in the development of informed and improved antibiotic treatment regimens for victims of hippopotamus attacks.

Materials and methods

Study area and study animals

Between May and September 2021, samples were collected from 34 healthy hippopotami at four different sites within the southern Greater Kruger National Park Complex in the MP. The individual reserves included the Kruger National Park (KNP) ($n=7$), a private game reserve ($n=8$) and a provincial game reserve ($n=19$) and included a river and three dams. Twenty-seven animals were sampled during translocation for population management purposes whereas seven animals were sampled after humane culling (preferably of adult males) due to overpopulation.

Sample collection

Single oral samples were collected from 18 hippopotami, and two separate samples were collected from the other 16 hippopotami. In both cases, Copan regular rayon swabs (Transystem) with Amies agar gel (commercial semi-solid transport medium suitable to collect and preserve aerobic and anaerobic organisms in clinical specimens, particularly those from throat and wound swabs for bacterial culture) were used to swab the sublingual area and the dorsal tooth pocket in the maxillary buccal mucosa where the crown of the mandibular canine extends into. All the samples were stored for between four and eight weeks at 4 °C until analysis.

Culture and species identification

A total of 50 swab samples were processed in a BSL 2 laboratory by plating each bacterial swab onto two Columbia blood agar plates with 5% horse blood (ThermoFisher Scientific, Johannesburg, South Africa) (CBA), and one MacConkey agar plate w/o salt (ThermoFisher Scientific, Johannesburg, South Africa) (McC). Inoculations were performed in a biosafety class 2 cabinet. One inoculated CBA plate was incubated with 5%-CO_2, the other was incubated in a Bactron anaerobic chamber and the McC plate was incubated in normal air. All the plates were incubated at 37 °C. After 24 h the different bacterial colonies of each individual hippopotamus sample were subcultured on new blood agar and McC plates for purification. The original aerobic plates were reincubated to monitor for additional bacterial growth as follows: aerobic plates for 72 h; anaerobic plates for 5 days. The subcultures on CBA and McC were incubated for 24 h at 37 °C. The anaerobic plates were examined after 3 days of incubation. Suspect colonies were subcultured onto two CBA plates. One was incubated anaerobically, and the other was incubated in normal air. Isolates that did not grow in normal air were identified further. Conventional bacteriology was used to identify the bacterial species isolated from the samples. Primary identification included Gram staining and catalase, oxidase and spot indole tests. Secondary identification was based on the following API test kits: API 10S for non-fastidious Gram-negative rods, API 20NE for non-fastidious non-enteric Gram-negative rods and API 32A for anaerobic bacteria (bioMérieux, Lyons, France).

Antibiotic susceptibility testing

Drug sensitivity profiles were determined using disc diffusion susceptibility testing (Kirby-Bauer method) [19]. A

bacterial colony was picked with a dry sterile swab and emulsified in a 0.5% saline solution. The turbidity of the bacterial suspension was adjusted to that of a 0.5 MacFarland standard. A new sterile dry swab was used to streak the bacteria on Mueller–Hinton agar plates (MH) in three different directions. Standardized, impregnated antibiotic discs (Oxoid, Basingstoke, UK) were placed on the agar surface. A panel of 16 antibiotics representing 10 antibiotic categories was included in the test. The following antibiotic categories were selected: β-lactam antibiotics represented by penicillins (penicillin G: 10 IU, ampicillin: 10 µg, amoxicillin/clavulanate: 30 µg) and cephalosporin (ceftiofur: 30 µg and cephalotin: 30 µg), fluoroquinolone (enrofloxacin: 5 µg), polymyxin (colistin: 10 µg), lincosamide (clindamycin: 2 µg), tetracycline (tetracycline: 30 µg, doxycycline: 5 µg), amphenicol (florfenicol: 30 µg), aminoglycoside (gentamycin: 10 µg), sulphonamides and trimethoprim (sulfonamides: 300 µg, sulfamethoxazole-trimethoprim: 25 µg), macrolide (tilmicosin: 15 µg) antibiotics and phosphonic acids (fosfomycin: 200 µg).

The inoculated MH plates were placed in an incubator at 37 °C in normal air for 24 h. For *Streptococcus* species, MH agar with 5% sheep blood was used and the plate was incubated for 20–24 h in a CO_2 atmosphere. The zones of inhibition were measured after 24 h using a sliding calliper. The measurements were recorded in millimetres and interpreted using the CLSI 2020 standard. The measurements were compared to zone sizes for resistant, intermediate and sensitive, published in the CLSI guideline for each antibiotic disk [19]. Each isolate was classified as resistant, intermediate or sensitive to each antibiotic according to the zone of no growth. Multi-drug resistance (MDR) was defined as resistance or intermediate susceptibility to at least one antibiotic from three different antibiotic classes, whereby only antibiotic resistance to drugs with expected susceptibility was taken into consideration.

Data analysis

Quantitative data referring to the bacterial genera and isolates per genus and the antibiotic susceptibility test results were entered into a Microsoft Excel spreadsheet. The data set was analyzed using descriptive statistics in Excel to characterize the set of bacterial isolates with regard to species composition (proportions) and antibiotic resistance. Antibiotic resistance levels were depicted for an individual antibiotic if it was the sole representative of its antibiotic category. When more than one antibiotic drug per category was tested, the highest resistance value for the category was depicted (Fig. 1). For β-lactam antibiotics, resistance values were reported for penicillin,

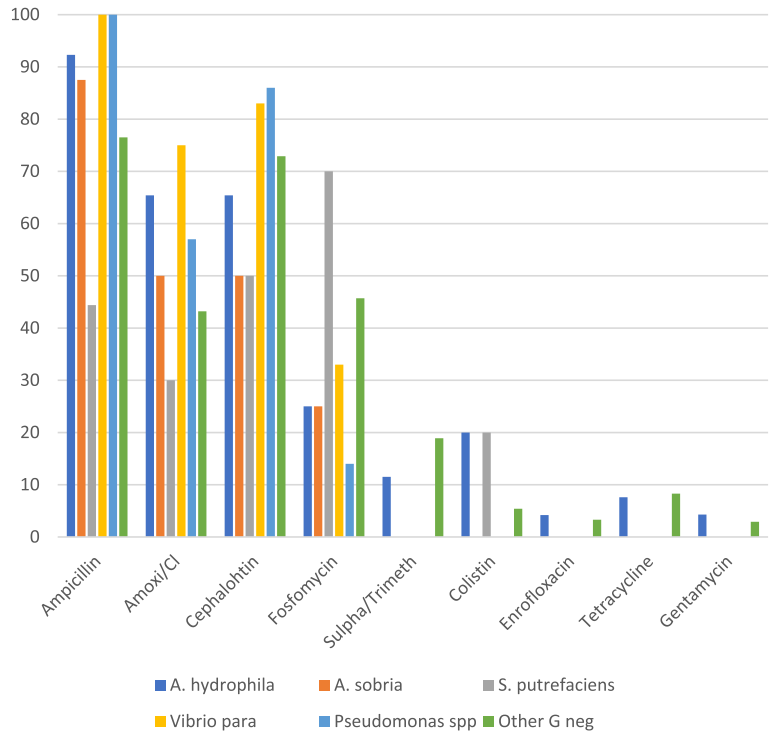

Fig. 1 Antibiotic resistance in Gram-negative isolates, including the most frequently isolated bacterial species (MFS) (*n* = ,44), *Vibrio parahaemolyticus* (*n* = 12), and *Pseudomonas spp.* (*n* = 7)

ampicillin, amoxicillin/clavulanate and cephalosporins as applicable. Bacterial isolates were considered non-susceptible if they tested resistant or intermediate to an antibiotic drug.

Results

Bacterial culture

A total of 188 aerobic and 16 anaerobic isolates were recorded, consisting of 30 (94%) aerobic and two (6%) obligate anaerobic bacterial genera, respectively. The distribution of recovered bacterial genera across the study animals in the three reserves is outlined in Table 1. The majority of aerobic isolates (78%) were detected in hippopotami from all three geographical sites; 4% were detected at two sites and 18% at one of the three sites. Among the obligate anaerobic species, *Prevotella melaninogenica (P. melaninogenica)* was isolated from all three sites whereas *Clostridium spp* was only found at one site (private nature reserve).

The majority of the 41 aerobic bacterial species isolated were Gram-negative (70.7%) and 29.3% of the isolates were Gram-positive (Supplementary Table 1). The

Table 1 Bacterial genera isolated from the oral flora of 34 hippopotami in three nature reserves in MP, South Africa

Genus	Gram stain	No. isolates	% of total	Private NR	Prov NR	KNP
Aeromonas	G-	72	35.3	15	48	9
Shewanella	G-	26	12.7	1	23	2
Vibrio	G-	16	7.8	0	7	9
Escherichia	G-	11	5.4	7	2	2
Pseudomonas	G-	8	3.9	0	6	2
Acinetobacter	G-	6	2.9	3	2	1
Enterococcus	G+	5	2.4	3	2	0
Streptococcus	G+	5	2.4	4	1	0
Enterobacter	G+	4	1.9	0	4	0
Bacillus	G+	3	1.5	1	0	2
Hafnia	G-	3	1.5	3	0	0
Micrococcus	G+	3	1.5	0	0	3
Pantoea	G-	3	1.5	2	0	1
Ralstonia	G-	3	1.5	0	1	2
Actinomyces	G+	2	1.0	0	1	1
Brevundimonas	G-	2	1.0	1	0	1
Rhodococcus	G+	2	1.0	0	2	0
Sphingomonas	G-	2	1.0	0	2	0
Arcobacter	G-	1	0.5	0	1	0
Citrobacter	G-	1	0.5	1	0	0
Comamonas	G-	1	0.5	0	1	0
Corynebacterium	G-	1	0.5	0	1	0
Dietza	G+	1	0.5	1	0	0
Klebsiella	G-	1	0.5	1	0	0
Lactobacillus	G+	1	0.5	0	1	0
Lactococcus	G+	1	0.5	0	1	0
Listeria	G+	1	0.5	1	0	0
Mannheimia	G-	1	0.5	0	1	0
Pasteurella	G-	1	0.5	1	0	0
Proteus	G-	1	0.5	0	1	0
Sub-total: aerobic	**30**	**188**	**92.2**	**45**	**108**	**35**
Prevotella	G-	14	6.9	5	6	3
Clostridium	G+	2	1.0	0	2	0
Sub-total: anaerobic	2	16	7.8	5	8	3
Total	**32**	**204**	**100**	**50**	**116**	**38**

NR Nature reserve, *Prov* Provincial, *KNP* Kruger National Park

bacterial species represented by at least 10% of the total isolates were classified as *Most Frequently Isolated Species* (MFS). Three aerobic species were classified as MFS, namely *Aeromonas hydrophila (A. hydrophila)* at 24.5%, *Shewanella putrefaciens (S. putrefaciens)* at 12.7% and *Aeromonas sobria (A. sobria)* at 10.8% and accounting for 52.1% of the aerobic isolates and 48% of all the isolates. The two isolated anaerobic bacterial genera were *P. melaninogenica* and *Clostridium spp.* (Table 1).

Antimicrobial susceptibility testing

Among the 188 aerobic isolates, 112 isolates representing all the isolated genera and species were subjected to antibiotic susceptibility testing representing all the isolated bacterial species with between one and 26 isolates per species. In total, 93 (83%) Gram-negative isolates and 19 (17%) Gram-positive isolates were profiled (Table 2). A total of 44 MFS isolates were included in the antibiograms as follows: *A. hydrophila (n = 26), A. sobria* (*n* = 8), *and S. putrefaciens* (*n* = 10).

Antibiotic resistance was observed for all categories of antibiotics employed as depicted in Table 2. Most Gram-negative bacteria are intrinsically resistant to penicillin G, macrolides and lincosamides; whereas most Gram-positive bacteria are intrinsically resistant to polymyxins [20]. High levels of resistance were detected for most beta-lactam antibiotics especially in the *Aeromonas spp.* (50.5% to 80.7%) (Fig. 1), except for ceftiofur (10% to 19.2%), a veterinary 3rd generation cephalosporin (3GC) (Table 2). The five-fold difference in the number of Gram-negative isolates which were resistant to cephalotin, a 1st generation cephalosporin, compared to ceftiofur is noteworthy (Table 2). The resistance of Gram-negative isolates to sulphonamides, amphenicols and phosphonic antibiotics was moderate while it was very low for tetracyclines (< 8%), gentamycin (< 5%) and fluoroquinolone (enrofloxacin) (< 5%) (Fig. 1).

Among the 19 Gram-positive isolates (belonging to 15 species) tested, the highest levels of antibiotic resistance were recorded for clindamycin (46.2%), tilmicosin (40%), gentamycin (33.3%) and fosfomycin (33.3%). Intermediate susceptibility to β-lactam antibiotics was mostly observed in Gram-negative bacteria (Table 2) but was negligible to other antibiotic drugs. Full sensitivity to enrofloxacin and doxycycline was observed in Gram-positive isolates and *A. sobria* and *S. putrefaciens* (Table 2).

MDR was detected among the Gram-negative (22.6%) and the Gram-positive (31.6%) isolates (Table 2). Overall, one *A.hydrophila* isolate and one *Pantoea spp* isolate were resistant to the entire panel of 16 antibiotics (Supplementary Table 1).

Discussion

Few studies report on the pathogen(s) responsible for infections due to hippopotamus bites, although the risk of infection is well-recognized [3, 6]. In this study we investigated the cultivable bacteria in the sublingual and gingival flora of 34 free-ranging hippopotami and their antibiotic susceptibility profiles. The animals lived in dams and a river in three different conservation areas within the Greater Kruger National Park of South Africa. *Aeromonas spp.* and *S. putrefaciens* constituted 48% of all isolates but only 6% of all bacterial genera and 7% of the species recovered. *A. hydrophila* and *S. putrefaciens* were isolated from animals in all three and *A. sobria* was isolated from two of the three reserves. These findings suggest that these species are common oral flora organisms of hippopotami. The diversity of mostly aerobic bacterial

Table 2 Antibiotic susceptibility profiles for 112 aerobic bacterial isolates recovered from 34 hippopotami

Antibiotic class	Antibiotic drug	Bacterial isolates (resistant/intermediate[a]/total tested) (%)						
		A. hydrophila (n=26)	*A. sobria* (n=8)	*S. putrifaciens* (n=10)	Other G- isolates (n=49)	Total G- isolates (n=93)	Total G+ isolates (n=19)	Total (n=112)
	Penicillin G			Intrinsic resistance			4/0/15 (26.7)	4/0/15 (26.7)
	Ampicillin	23/0/26 (88.5)	7/0/8 (87.5)	4/0/9 (44.4)	37/1/45 (84.8)	71/1/88 (81.8)	3/3/15 (40.0)	74/4/103 (75.7)
Beta-lactams	Amoxi/Clav	16/5/26 (80.8)	4/3/8 (87.5)	3/1/10 (40.0)	24//8/49 (65.3)	47/17/93 (68.8)	2/0/19 (10.5)	49/17/112 (58.9)
	Cephalothin	15/3/26 (69.2)	4/1/8 (62.5)	5/1/10 (60.0)	36/2/49 (77.6)	60/7/93 (72.0)	3/0/18 (16.7)	63/7/111 (63.0)
	Ceftiofur	1/4/26 (19.2)	0/1/8 (12.5)	1/0/10 (10.0)	9/11/49 (40.8)	11/16/93 (29.0)	4/4/18 (44.4)	15/20/111 (31.5)
Lincosamides	Clindamycin			Intrinsic resistance			5/13 (46.2)	5/13 (46.2)
Polymyxins	Colistin	3/25 (12.0)	0/8 (0.0)	2/10 (20.0)	2/48 (4.2)	7/91 (7.7)	Intrinsic resistance	7/91 (7.7)
Fluoroquinolones	Enrofloxacine	1/24 (4.2)	0/8 (0.0)	0/9 (0.0)	1/42 (2.4)	2/83 (2.4)	0/9 (0.0)	2/92 (2.2)
Phosphonics	Fosfomycin	5/24 (20.8)	2/8 (25.0)	7/10 (70.0)	20/47 (42.6)	34/89 (38.2)	3/9 (33.3)	37/98 (37.8)
Amphenicols	Florfenicol	2/23 (8.7)	0/7 (0.0)	1/10 (10.0)	9/38 (23.7)	12/78 (15.4)	2/15 (13.2)	14/93 (15.0)
Aminoglycoside	Gentamycin	1/23 (4.3)	0/8 (0.0)	0/10 (0.0)	1/45 (2.2)	2/86 (2.3)	1/3 (33.3)	3/89 (3.4)
Sulphonamides	Sulphonamide	2/26 (7.7)	1/8 (12.5)	4/10 (40.0)	5/49 (10.2)	12/93 (12.9)	6/19 (31.6)	18/112 (16.1)
	Sulpha/Trimethoprim	1/26 (3.8)	0/8 (0.0)	0/10 (0.0)	7/49 (14.3)	8/93 (8.6)	2/19 (10.5)	10/112 (8.9)
Tetracyclines	Tetracycline	2/26 (7.7)	0/8 (0.0)	0/10 (0.0)	3/49 (6.1)	5/92 (5.4)	1/19 (5.3)	6/111 (5.4)
	Doxycycline	2/26 (7.7)	0/8 (0.0)	0/10 (0.0)	3/46 (6.5)	5/90 (5.6)	0/17 (0.0)	5/107 (4.7)
Macrolides	Tilmicosin			Intrinsic resistance			2/5 (40.0)	2/5 (40.0)
Drug non-susceptibility		9/44 (20.5)			12/49 (24.5)	21/93 (22.6)	6/19 (31.6)	27/112 (24.1)

[a] No. of intermediate isolates are only shown for beta-lactam antibiotics. For these, the % reflects the resistant and intermediate isolates as a fraction of the total number tested

isolates can be attributed to the amphibious lifestyle of hippopotami as the composition of the oral flora is largely determined by their aquatic environment. In comparison, the oral flora bacteria isolated from dogs and dog bite wound infections represent both aerobic and anaerobic bacteria in similar numbers and most commonly include *Actinomyces spp., Pasteurella spp., Staphylococcus spp, Streptococcus spp., Prevotella spp., Bacteroides spp. Porphyromonas spp*. [21, 22].

Hippopotami share their aquatic environment with the Nile crocodile (*Crocodylus niloticus*) and crocodilian bites are more commonly reported in the scientific literature. Previous reports of infection associated with crocodile bites in humans have confirmed the presence *Aeromonas* species [23] and an Australian series reported the presence of *Citrobacter koseri*, *Proteus vulgaris*, *Bacillus cereus*, *Pseudomonas aeruginosa* and *Enterococcus* species [24]. In their case series of *Aeromonas* infections associated with the aquatic environment in southern Africa, all cases were treated with ceftazidime and an aminoglycoside. In contrast, *Shewanella sp.* has not typically been associated with aquatic infections following animal bites, although the organism is recognized to survive in aquatic environments [25]. *Shewanella sp.* has also been reported following stingray attacks, in association with some of the pathogens we identified from hippopotami, but also with marine pathogens such as *Mycobacterium marinum* and *Photobacterium damselae* [26]. Most patients in these series received a third-generation cephalosporin or a penicillin/penicillinase inhibitor combination, with a selected few receiving ciprofloxacin [26, 27].

Based on available data on bite wounds in humans which are primarily caused by dogs, followed by cats and humans [28], wound infections occur in 10% – 20% of cases with a higher risk of infection in deep wounds, those associated with severe tissue destruction, joints, and bones and wounds older than 8 days [21, 29, 30]. There is consensus among authors that bite wound infections are derived primarily from the oral flora of the biting animal and in fewer cases from the skin of the victim and the environment. For this reason, prophylactic administration of antibiotics is based on the pathogen profile established for dog, cat and human bite wounds, with the drug of choice being amoxicillin/clavulanate [29]. In patients allergic to penicillins, cephalosporins are usually used [31]. In the absence of wildlife species-specific guidelines this or a similar broad-spectrum penicillin treatment must be adopted for victims of hippopotamus bites [5]. Based on the findings in this study, an antibiotic regimen based on amoxicillin/clavulanate and 1st and 2nd generation cephalosporins is not effective against the bacteria found in the oral cavity of hippopotami. This was demonstrated by 80.7% and 50.5% of the Gram-negative isolates being resistant to ampicillin and amoxicillin/clavulanate, respectively. For *A. hydrophila* isolates the resistance rates were even slightly higher (Fig. 1). Considering the heterogeneity of the antibiotic sensitivity profiles of the MFS, varying and variable susceptibilities to antibiotic classes other than tilmicosin and clindamycin were observed (Supplementary Table 1). Whether this is an indication of possible acquired antimicrobial resistance in the aquatic bacteria due to greater environmental exposure to human than to veterinary antibiotic drugs is unknown. For cephalosporins, similar levels were detected for cephalothin, while ceftiofur, a 3GC registered only for veterinary use, demonstrated a considerably higher efficacy towards Gram-negative isolates. Ceftazidime with an aminoglycoside has notably been used to treat an *Aeromonas* infection associated with aquatic exposure to a crocodile bite successfully in a similar environment in Southern Africa [23]. A recent literature review examining stingray injuries highlighted susceptibility of both *Aeromonas* and *Shewanella* species to third generation cephalosporins, aminoglycosides and fluoroquinolones [26].

Gram-positive aerobic bacterial species were isolated in low numbers and presented only moderate to low or even full susceptibility (enrofloxacin and doxycycline) apart from the intrinsic resistance to colistin (Table 2). Unlike in dogs [18], anaerobic bacteria were rarely isolated in this study and were limited to two genera, which may be attributed to the amount of time hippopotami spend at least partially submerged in water.

Multi-drug resistance (MDR) is generally defined as non-susceptibility to at least one agent in three or more antimicrobial categories, whereby non-susceptibility includes both resistant and intermediate strains [32]. The definition is unclear as to whether MDR should strictly encompass acquired resistance or include intrinsic resistance. In this study, the former definition was applied and revealed that 24.1% of all the isolates tested, showed multi drug resistance to drugs expected to be effective. When intrinsic resistance was included, the MDR rate increased from 21.4% to 38.4%. We argue that both rates are important to note in the context of this study for the following reasons. The absence of treatment guidelines for hippopotamus bite wounds and the resource constraints in rural clinical settings in South Africa where most hippopotamus bite wound patients seek medical care may lead to the choice of non-optimum or available, cheapest antibiotics, resulting in a poor prognosis for the treatment of such infections. This is further emphasized by the finding that *A. hydrophila* which yielded the highest number of isolates showed the highest resistance to broad spectrum penicillins (Table 2) and one of the *A.*

hydrophila strains was resistant to the entire spectrum of antibiotics employed (Supplementary Table 1).

The overall susceptibility profiles of all bacterial strains to the antibiotic panel were established semi-quantitatively (Kirby-Bauer method). Although it did not afford us the enhanced resolution of susceptibility profiling by minimum inhibitory concentrations for the bacteria identified, it demonstrated a clear pattern. Most aerobic strains tested (>90%) including the three MFS strains, were sensitive to enrofloxacin, gentamycin, tetracycline and doxycycline, followed by a slightly reduced sensitivity to florfenicol, colistin and sulfamethoxazole/trimethoprim (Supplementary Table 1). To the contrary, penicillin, amoxicillin/clavulanate and macrolide antibiotics showed a high level of resistance observed in this and other studies [33–35]. Environmental *Aeromonas spp.* are known to be intrinsically resistant to penicillin and some other drugs and may even be a risk in the transfer of resistance to clinically significant bacteria in nosocomial infections [34].

For the successful treatment of hippopotamus and other wild animal bite wounds it is still important to prepare wound cultures for the exact speciation of the causative agents, especially for wounds older than eight hours and those patients who do not respond to initial antibiotic treatment [29]. This is supported by our own work, which possibly points towards acquired resistance to some of the antibiotics tested, and the potential for *Shewanella sp.* to develop resistance to quinolones [36] further supports wound culture, particularly if a patient is not responding to empiric therapy. Given the extent and severity of hippopotamus bites, any antimicrobial therapy should be associated with extensive surgical debridement of the bite wound, which may include orthopaedic repair or amputation, depending on the site and extent of the bite [3, 5, 6, 17].

We acknowledge that the composition of the hippopotamus oral flora most likely harbours a much higher microbial diversity than could be detected with culture-based methods and a more comprehensive view of hippopotamus oral microbiome would be obtained through the use of metagenomic sequencing. Nevertheless, and although the hippopotamus oral flora might differ slightly between aquatic environments and between seasons, the ubiquitous presence of the environmental bacteria *Aeromonas spp.* and *S. putrefaciens* is typical for all types of water bodies especially those in moderate and warm climates. *Shewanella putrefaciens* has been reported as an opportunistic human pathogen following occupational or recreational exposure, causing wound infections, bacteraemia and osteomyelitis, amongst other conditions, in many countries including in southern Africa [37, 38]. *Aeromonas hydrophila* and *A. sobria* are recognized human enteric pathogens mostly derived from fish and causes of bacteraemia and soft tissue infections following trauma including necrotizing fasciitis which can lead to necrosis and gangrene, with a case fatality rate of 50% [39]. In the context of hippopotamus-human conflict it is therefore critical to consider all three MFS as zoonotic pathogens.

Our study had a number of limitations. As the work was done in a veterinary laboratory, the antimicrobials tested were primarily veterinary antimicrobials and we didn't test for resistance to the 3GCs that are specific for human use, particularly ceftazidime versus ceftriaxone, which have slightly different spectra of activity (Aarestrup), thus our findings were extrapolated in the case of humans suffering hippopotamus bites. We also did not screen our isolates for carbapenem susceptibility or extended spectrum beta-lactamase production, or characterize the resistance genes at a molecular level, so cannot categorically state whether resistance mechanisms were acquired or arose de novo, and whether the resistance we observed was transmitted between the organisms we isolated. Lastly, although there are now newer, more rapid and more reliable methods to identify pathogenic bacteria, these were not available in our laboratory, and we thus relied on API to confirm the identity.

In conclusion, this study provides the first investigation of the oral flora bacteria of the common hippopotamus which has revealed 32, mostly aerobic, bacterial genera. The 204 bacterial isolates largely consisted of strains of *Aeromonas spp.* and *S. putrefaciens* which are mostly typical inhabitants of the aquatic habitat of hippopotami, and opportunistic human pathogens. The antibiotic susceptibility profiles of the 10 antibiotic categories demonstrated that quinolones, aminoglycosides, and tetracyclines were highly efficacious against these bacterial species which otherwise showed moderate to high levels of resistance to the traditional bite wound treatment with amoxicillin/clavulanate and 1st and 2nd generation cephalosporins.

The findings of this study can hopefully promote follow-up investigations in collaboration with medical practitioners to include clinical cases of human bite wound infections.

Abbreviations

3GC	Third generation cephalosporin
A. hydrophila	*Aeromonas hydrophila*
A. sobria	*Aeromonas sobria*
CBA	Columbia blood agar
HWC	Human-Wildlife Conflict
KNP	Kruger National Park
McC	MacConkey agar
MDR	Multi-drug resistance
MFS	Most frequently isolated species
MP	Mpumalanga Province
MTPA	Mpumalanga Tourism and Parks Agency
S. putrefaciens	*Shewanella putrefaciens*

Supplementary Information

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Supplementary Material 1.

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Authors' contributions
ALM conceived the project idea and designed the project, analyzed the laboratory data, wrote the manuscript and prepared tables and the figure. FR conducted the collection of field samples and contributed information to the manuscript. ME was an undergraduate veterinary student at the time of the project and participated in the bacterial isolations in the laboratory. JW assisted with project coordination; AJ supervised all the laboratory analyses, interpreted the results and reviewed the manuscript.

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Data availability
One dataset was generated and analyzed during the current study.

Declarations

Ethics approval and consent to participate
This study was approved by the University of Pretoria's Research Ethics and Animal Ethics Committees (approval number REC050-21) and the National Department of Agriculture, Land Reform and Rural Development in terms of the Animal Diseases Act. No human data were collected or used. The welfare of animals was ensured by the use of compliant procedures by a registered wildlife veterinarian during all capture events.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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