Aeromonas and *Plesiomonas* species from scarlet ibis (*Eudocimus ruber*) and their environment: monitoring antimicrobial susceptibility and virulence

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Abstract The present study aimed at evaluating the role of captive scarlet ibises (*Eudocimus ruber*) and their environment as reservoirs of *Aeromonas* spp. and *Plesiomonas* spp., and analyzing the in vitro antimicrobial susceptibility and virulence of the recovered bacterial isolates. Thus, non-lactose and weak-lactose fermenting, oxidase positive Gram-negative bacilli were recovered from cloacal samples (n = 30) of scarlet ibises kept in a conservational facility and from water samples (n = 30) from their environment. Then, the antimicrobial susceptibility, hemolytic activity and biofilm production of the recovered *Aeromonas* spp. and *Plesiomonas shigelloides* strains were

assessed. In addition, the virulence-associated genes of *Aeromonas* spp. were detected. Ten *Aeromonas veronii* bv. *sobria*, 2 *Aeromonas hydrophila* complex and 10 *P. shigelloides* were recovered. Intermediate susceptibility to piperacillin-tazobactam and cefepime was observed in 2 *Aeromonas* spp. and 1 *P. shigelloides*, respectively, and resistance to gentamicin was observed in 4 *P. shigelloides*. The automated susceptibility analysis revealed resistance to piperacillin-tazobactam and meropenem among *Aeromonas* spp. and intermediate susceptibility to gentamicin among *P. shigelloides*. All *Aeromonas* isolates presented hemolytic activity, while 3 *P. shigelloides* were non-

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hemolytic. All *Aeromonas* spp. and 3/10 *P. shigelloides* were biofilm-producers, at 28 °C, while 10 *Aeromonas* spp. and 6/10 *P. shigelloides* produced biofilms, at 37 °C. The most prevalent virulence genes of *Aeromonas* spp. were *asa1* and *ascV*. Scarlet ibises and their environment harbour potentially pathogenic bacteria, thus requiring monitoring and measures to prevent contamination of humans and other animals.

Keywords *Eudocimus ruber* · Scarlet ibis · *Aeromonas* sp. *Plesiomonas shigelloides* · Antimicrobial susceptibility · Virulence factor

Introduction

The scarlet ibis (*Eudocimus ruber*) is a tactile foraging, colonial wading bird that breeds in coastal regions and mangroves. Studies on this species are scarce and mainly focus on their behavioral and reproductive habits, with a lack of data on the health of these animals. Considering the importance of birds as hosts and carriers of potentially pathogenic bacteria, studying the bacterial microbiota of clinically healthy wild birds is important to understand the epidemiology of bacterial diseases that can affect not only birds themselves but also other animals and humans (Kim et al. 2015).

Among the bacteria with zoonotic importance, *Aeromonas* spp. and *Plesiomonas shigelloides* are notable, and are found in a wide variety of habitats. These microorganisms have been described as components of the microbiota of ectothermic animals and waterfowl and are reported as important animal pathogens, especially for fish (Janda and Abbott 2010; Kim et al. 2015; Janda et al. 2016). In humans, *Aeromonas* spp. and *P. shigelloides* are important waterborne and foodborne pathogens, causing acute diarrhea, or causing extraintestinal infections affecting several internal organs, as well as skin and soft tissues. These infections are more commonly described in food handlers and aquaculture professionals (Janda and Abbott 2010; Parker and Shaw 2011; Janda et al. 2016).

The pathogenesis of *Aeromonas* infections is yet to be clearly understood, but several virulence factors have been proposed, such as hemolysins, enterotoxin, and type III secretion systems, which have been

detected in strains recovered from animals and water sources (Castelo-Branco et al. 2015). On the other hand, virulence factors of *P. shigelloides* have not been fully elucidated, however the presence of plasmids, cytolysins, hemolysins, enterotoxins, including cholera-like toxins, has been reported (Janda et al. 2016). In addition, the production of biofilms by members of these bacterial genera is associated with their survival and maintenance in the environment and the animal host, hence, this ability is considered an important virulence trait (Igbinosa 2014; Dobiasova et al. 2014). Moreover, it is worth emphasizing that biofilm growth is associated with seafood colonization, which increases the risk of foodborne infections with these pathogens (Mizan et al. 2015).

Therefore, the present study aimed at evaluating the role of captive scarlet ibises (*E. ruber*) and their environment as reservoirs of *Aeromonas* spp. and *Plesiomonas* spp., and analyzing the in vitro antimicrobial susceptibility and virulence of the recovered bacterial isolates.

Materials and methods

Ethics

This project was approved by the Authorization and Information System for Biodiversity (SISBIO) of the Chico Mendes Institute for Conservation of Biodiversity (ICMBio, Brazil), under the license No. 45009-1. It was also approved by the Ethics Committee for the Use of Animals (CEUA) of the State University of Ceará, under the protocol number 4797437/2014.

Animal handling

For the isolation of microorganisms, cloacal samples were collected from scarlet ibis (*E. ruber*) kept in captivity at Parque Mangal das Garças, located in Belém, State of Pará, Brazil (S: 1°27'51.9" and W: 48°30'19.9"). All animals were clinically evaluated and their body score was assessed, classifying them as obese, normal or thin (Ritchie et al. 1994). Animals that were clinically ill and/or those that were obese or thin were not sampled for this research. Three sample collections were carried out with an interval of 1 month between them. At each collection point, ten animals (30 animals in total) were randomly captured

with pole nets to collect biological material. These animals were kept in three enclosures. In the enclosure of Aningas (Sector 1), a closed and collective enclosure, scarlet ibises cohabit with other species and were fed with fish and specific extruded bird ration for ibises and flamingoes. On the island of scarlet ibises (Sector 2), scarlet ibises live in semi-freedom along with other bird species and they receive only extruded feed. In the extra sector (Sector 3), where the surplus scarlet ibises of the Park intended for exchange with other zoos were kept, the birds receive only extruded feed. All three enclosures had a water tank/ponds and a proper ambiance to provide well-being and stimulate reproduction and nesting. All animals were daily assisted by a veterinarian and a biologist.

Sample collection of biological material

For each collection, cloacal samples ($n = 10$) were collected with sterile cotton swabs, which were inserted and rotated 180 degrees twice (Silva et al. 2004). Afterwards, the swabs were placed in Cary Blair transport medium (Silva et al. 2004). Water samples ($n = 10$) were collected from ten different points of two artificial lakes that surround the island of scarlet ibises. These lakes receive water from the Guamá River. For this purpose, sterile Falcon tubes were used, dipped rapidly from 15 to 30 cm below the water surface. The tubes were immediately sealed with plastic film around the lid, and kept refrigerated until processing (Castelo-Branco et al. 2015).

Sample processing

Samples were processed at the Laboratory of Emerging and Re-emerging Pathogens (LAPERÉ) of the Federal University of Ceará and were kept under refrigeration until processing, within 24 h after collection. Cloacal swabs were transferred to tubes with 1 mL of sterile saline (0.9 % NaCl) and homogenized with a vortex mixer for 3 min and left to decant for 30 min at room temperature (Filiú et al. 2002). Water samples were processed in duplicate, distributing 2500 μ L in each tube. Then, these aliquots were centrifuged at 3000 rpm for 20 min. After centrifugation, 2000 μ L of supernatant from each tube were discarded and the remaining volume of the tubes was mixed and sterile saline (0.9 % NaCl) was added to reach a final volume of 2000 μ L. The solution was homogenized with a

vortex mixer and left to settle out for 30 min at room temperature (Medeiros 2008). Finally, 10 μ L-aliquots of the supernatant from each cloacal and water sample were seeded in duplicate on MacConkey agar and thiosulphate citrate bile and sucrose (TCBS) agar. The plates were incubated at 35 °C for 24–48 h.

Bacterial isolation and identification

The colonies grown on the agar plates were counted and non-lactose and weak-lactose fermenting colonies on MacConkey medium were selected for screening, as well as the colonies grown on TCBS medium. First, a Gram stain of the selected colonies was performed to ensure that they were Gram-negative bacilli. Subsequently, they were tested for cytochrome oxidase production, ability to ferment and oxidize glucose, gas production from D-glucose, indole production and ability to grow on TCBS agar (Winn et al. 2006). *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 were used as quality control to validate the biochemical tests. The screening was performed based on established identification keys, following specific methodology for *Aeromonas* spp. and *P. shigelloides* (Winn et al. 2006). After the biochemical screening, the strains were subjected to automated biochemical identification, using the Vitek 2 system (bioMérieux), with Gram-negative identification cards. In these analyses, reference ATCC strains were also included, as recommended by the manufacturer, as well as previously identified *Aeromonas* and *Plesiomonas* strains. Only results with a probability level above 95 % were considered reliable (Castelo-Branco et al. 2015).

In vitro antimicrobial susceptibility testing

These analyses were performed through broth microdilution and automated methods (Vitek 2TM), for comparative purposes. The antimicrobial drugs used in broth microdilution susceptibility testing were selected according to the document M45-A2 (CLSI 2010). The minimum inhibitory concentrations (MIC) of piperacillin–tazobactam, cefepime, ceftazidime, meropenem, gentamicin and ciprofloxacin (Sigma Chemical Corporation, USA) against strains of *Aeromonas* spp. and *P. shigelloides* were determined through broth microdilution (document M07-A9, CLSI 2012). These assays were performed in

Mueller–Hinton broth (Difco, USA) with a final bacterial inoculum of 5×10^5 UFC/mL (CLSI 2010). Drug-free and bacterium-free wells were included as growth and sterility controls, respectively. The plates were incubated at 35 °C for 18–20 h (CLSI 2012). MICs were considered to be the lowest drug concentration capable of inhibiting 100 % of bacterial growth and the isolates were classified as susceptible (S), intermediate (I) and resistant (R), according to M45-A2 document (CLSI 2010). *E. coli* ATCC 25922 and *E. coli* ATCC 35218 were included in each test, as quality control (CLSI 2010).

For the automated analyses, the antimicrobial susceptibility card N-239 (bioMérieux, France) was used and all procedures were carried out according to the manufacturer's recommendations (Castelo-Branco et al. 2015). *E. coli* ATCC 25922 and *E. coli* ATCC 35218 were included as quality control (CLSI 2010).

Hemolytic activity of *Aeromonas* spp. and *P. shigelloides*

The hemolytic activity of the 12 *Aeromonas* spp. and 10 *P. shigelloides* isolates was evaluated, based on the methodology described by Yano et al. (2015). For such, bacterial colonies were suspended in 2 mL of sterile saline, until reaching a turbidity of 6 on McFarland scale. Afterwards, 10 µL of each bacterial inoculum were seeded on 5 % sheep blood agar and the plates were incubated at 37 °C for 48 h. Isolates were classified as β-hemolytic, when complete hemolysis was observed, characterized by the presence of clear halos surrounding the bacterial colonies; α-hemolytic, when partial hemolysis was observed, characterized by the presence of greenish halos surrounding the colonies, and non-hemolytic, when hemolysis was not observed. One strain of *Streptococcus pyogenes* and one *Streptococcus sanguinis* were included as controls for complete and partial hemolysis, respectively.

Biofilm production by *Aeromonas* spp. and *P. shigelloides*

The biofilm production of *Aeromonas* spp. and *P. shigelloides* was evaluated at two different temperatures (28 and 37 °C). For this, the strains were subcultured on brain heart infusion agar (BHI) and incubated at 37 °C for 24 h. Then, a bacterial

suspension was prepared in saline solution, reaching turbidity equivalent to 6 on McFarland scale. In parallel, 96-well flat-bottomed polystyrene microtiter plates were prepared by adding 175 µL of BHI broth supplemented with 1 % glucose (Bandeira et al. 2013). Afterwards, 25 µL of the bacterial suspension were added to each well, and the plates were incubated at 28 or 37 °C, for 48 h (Bandeira et al. 2013; Castelo-Branco et al. 2015). Three wells were used as negative growth control. Assays were carried out in triplicate at two different moments. After incubation, the supernatant was carefully aspirated from the wells, followed by three washes with PBS-Tween. Then, the wells were washed with 100 µL of 100 % methanol. After drying, 100 µL of a 0.3 % crystal violet solution were added. After 20 min, the crystal violet was removed, and the wells were washed twice with 200 µL of sterile distilled water. Then, 150 µL of 33 % acetic acid solution was added to the stained wells and left for 30 s. The acetic acid was, then, transferred to another 96-well polystyrene plate, which was read through spectrophotometry at a wavelength of 540 nm. The optical density values obtained at 540 nm (OD₅₄₀ nm) were corrected by subtracting the OD₅₄₀ nm obtained for the negative control (containing only RPMI 1640 broth). The cutoff point (OD_c) for biofilm production was defined as three standard deviations above the mean OD₅₄₀ nm obtained for the negative control. At the end, all the tested strains were classified according to Stepanovic et al. (2000), as non-biofilm producers (OD₅₄₀ nm ≤ OD_c), weak producers (OD_c < OD₅₄₀ nm ≤ 2 × OD_c), moderate producers (2 × OD_c < OD₅₄₀ nm ≤ 4 × OD_c) and strong producers (4 × OD_c < OD₅₄₀ nm).

For the microscopic analyses of *Aeromonas* spp. and *P. shigelloides* biofilms, two strains of *Aeromonas* spp. (1 *A. veronii* *sobria* and 1 *A. hydrophila* complex, both biofilm producers from water) and two *P. shigelloides* (both biofilm producers from water) were initially grown in a 24-well polystyrene plate containing a thermanox coverslide at the bottom of the well. Afterwards, the same methodology described for biofilm formation was used, adjusting the total volume of culture medium to 1000 µL. After 48 h of incubation, biofilms were evaluated through confocal and fluorescence microscopy. For the confocal microscopic analysis, the methodology described by Castelo-Branco et al. (2015) was used. The thermanox coverslides were transferred to glass-bottomed Petri

dishes and 200 µL of the Live/Dead (Invitrogen™) fluorescent dye was added. Afterwards, the coverslips were evaluated under a Confocal Microscope Nikon C2, at 488 nm for the detection of the SYTO9 fluorescent dye, which identifies live bacterial cells, and at 561 nm for the detection of the propidium iodide, which identifies dead bacterial cells. Z-stack images were obtained from five fields for each of the analyzed strains. Software COMSTAT was used for quantitative analysis of the image stacks produced by CLSM (Heydorn et al. 2000). The evaluated parameters were: total biomass, biomass average thickness, total average thickness, roughness coefficient and surface-volume ratio, whereas thresholding was calculated automatically by Otsu's method.

Detection of virulence genes of *Aeromonas* spp. encoding for type III secretion system, hemolysin and enterotoxin

DNA extraction was performed using a Wizard Genomic DNA Purification Kit (Promega, USA), according to the manufacturer's instructions. The concentration and purity of extracted products were measured, using Qubit® (Invitrogen, Carlsbad, USA). The detection of virulence factors was performed through conventional PCR using primers designed for *Aeromonas* spp. In order to detect the genes encoding enterotoxin (*act*), haemolysin (*asa1*), and type III secretion system (*ascV*) in strains of *Aeromonas* spp., the following primers were used: (5'–3') *act_F*-AGAAGGTGACCACCAAGAACA and *act_R*-AATGACATCGGCCTTGAATC (Kingombe et al. 1999), *asa1_F*-TAAAGGGAAATAATGACGGCG and *asa1_R*-GGCTGTAGGTATCGGTTTTTCG (Wang et al. 2003), and *ascV_F*-CTCGAACTGGAAGAGCAGAATG and *ascV_R*-GAACATCTGGCTCTCCTTCTC GATG (Martino et al. 2011). PCR amplification was performed in a final volume of 25 µL, as described by Castelo-Branco et al. (2015). The PCR conditions were performed according to Martino et al. (2011), with an initial denaturation of 2 min at 94 °C followed by 35 cycles of 20 s at 94 °C, 30 s at 56 °C for the genes *asa1* and *act* and 60 °C for *ascV* gene, 50 s at 72 °C and a final extension at 72 °C for 7 min.

PCR products were visualized on 1.8 % agarose gel, through an UV transilluminator. The specific products of the genes *act* (enterotoxin), *asa1*

(hemolysin) and *ascV* (type III secretion system) were 232, 249 and 577 bp, respectively. A molecular weight marker of 100 bp was included in order to analyze amplicon size. As positive control, a strain of *A. hydrophila* whose genes have been previously detected was included, and as negative control, PCR Mix without DNA sample was used (Castelo-Branco et al. 2015).

Statistical analysis

Mann–Whitney test was used for the comparison of the antimicrobial MIC values against *Aeromonas* spp. and *P. shigelloides* strains. Concerning biofilm production, the crystal violet absorbance values obtained for *Aeromonas* spp. and *P. shigelloides* incubated at both tested temperatures were compared through Student's T test. This same test was used to compare the biofilm production by *Aeromonas* spp. and *P. shigelloides*. Finally, Pearson's Chi square test and Fisher's exact test were applied to compare the categorical data between species and the presence of virulence genes. P-values lower than 5 % indicate significant conclusion.

Results

Overall, 22 non-lactose or weak-lactose fermenting, oxidase positive Gram-negative bacilli were recovered: 12 *Aeromonas* spp. (2 *Aeromonas hydrophila* complex and 10 *Aeromonas veronii* bv. *sobria*) from water and 10 *P. shigelloides* from cloacal swabs (n = 7) and water samples (n = 3) (Table 1). The highest number of isolates was obtained from water samples (15/22), followed by cloacal samples (7/22). *Aeromonas* spp. were only recovered from water samples, while *P. shigelloides* was more frequently recovered from cloacal samples (P = 0.0031) than water.

All *Aeromonas* spp. isolates were susceptible to ceftazidime, cefepime, meropenem, ciprofloxacin and gentamicin. Two strains of *A. veronii* bv. *sobria* presented intermediate susceptibility to piperacillin–tazobactam (MIC: 32/4 µg/mL; Table 2). Concerning *P. shigelloides*, all strains were susceptible to ceftazidime, meropenem, ciprofloxacin and piperacillin–tazobactam, but four strains were resistant to gentamicin (MIC: 16 µg/mL), and two had intermediate

Table 1 Isolation and identification of *Aeromonas* spp. and *P. shigelloides* from scarlet ibis and water

Isolation					
Collection	Origin and site (n)	Gram negative non/weak-lactose fermenting	Oxidase producer	Screening for <i>Aeromonas</i> and <i>Plesiomonas</i>	Identification (n)
1st	Cloaca (n = 10)	22	9	4	<i>P. shigelloides</i> (4)
	Water (n = 10)	13	8	7	<i>A. veronii</i> bv. <i>sobria</i> (6) <i>A. hydrophila</i> complex (1)
2nd	Cloaca (n = 10)	50	20	3	<i>P. shigelloides</i> (3)
	Water (n = 10)	25	5	4	<i>A. veronii</i> bv. <i>sobria</i> (2) <i>P. shigelloides</i> (2)
3rd	Cloaca (n = 10)	22	2	0	–
	Water (n = 10)	21	15	4	<i>A. veronii</i> bv. <i>sobria</i> (2) <i>A. hydrophila</i> complex (1) <i>P. shigelloides</i> (1)
Total					22

(–) No isolated strains

susceptibility to this drug (MIC: 8 µg/mL). Furthermore, it was observed that a gentamicin resistant strain from water showed intermediate susceptibility to cefepime (MIC: 16 µg/mL; Table 2). Gentamicin showed higher MIC values against *P. shigelloides* than *Aeromonas* spp. ($P = 0.001$), whereas piperacillin–tazobactam ($P = 0.002$), ceftazidime ($P = 0.0188$) and meropenem ($P = 0.0072$) showed higher MIC values against *Aeromonas* spp. than *P. shigelloides*.

The automated susceptibility analysis presented an overall good agreement level with the broth microdilution method, but some disagreements were observed. Vitek 2, for instance, demonstrated five and six *Aeromonas* spp. strains resistant to piperacillin–tazobactam (MIC: ≥ 128) and meropenem (MIC: ≥ 16), respectively, and three *P. shigelloides* strains with intermediate susceptibility to gentamicin (Table 2).

Concerning the hemolytic activity, all *Aeromonas* isolates were positive, with 7/12 β -hemolytic and 5/12 α -hemolytic isolates. As for *P. shigelloides*, 7/10 were α -hemolytic, 3/10 were non-hemolytic and no β -hemolytic isolates were observed.

Regarding biofilm production, when it was analyzed at 28 °C, all isolates of *Aeromonas* spp. were positive for the production of biofilms (5 weak and 7 moderate biofilm-producers), while only three *P. shigelloides* isolates produced biofilms (3 weak producers). When the

assays were performed at 37 °C, 2 isolates of *A. veronii* bv. *sobria* were non-biofilm producers and the remaining isolates were weak producers, while 6/10 *P. shigelloides* were biofilm-producers (5 weak and 1 moderate biofilm producer). The production of biofilm by *Aeromonas* spp. at 28 °C was statistically higher ($P < 0.05$) than that at 37 °C, while the opposite was observed for *P. shigelloides*, i.e., biofilm production was enhanced at the higher temperature ($P < 0.05$). When comparing both bacterial genera, *Aeromonas* spp. presented a statistically higher biofilm ($P < 0.05$) production than *P. shigelloides* at 28 °C, but not at 37 °C.

The analyses of the biofilm structures, through confocal microscopy, revealed that *Aeromonas* spp. biofilms presented a total biomass of $10.1 \pm 3.12 \mu\text{m}^3/\mu\text{m}^2$; a biomass average thickness of $21.54 \pm 3.7 \mu\text{m}$, a total average thickness of $19.94 \pm 5.03 \mu\text{m}$; a roughness coefficient of 0.34 ± 0.28 and surface-volume ratio of $1.59 \pm 0.19 \mu\text{m}^2/\mu\text{m}^3$ (Fig. 1a). As for *P. shigelloides*, biofilms presented a biomass of $9.16 \pm 5.29 \mu\text{m}^3/\mu\text{m}^2$; a biomass average thickness of $22.97 \pm 5.59 \mu\text{m}$, a total average thickness of $20.07 \pm 8.16 \mu\text{m}$; a roughness coefficient of 0.43 ± 0.32 and surface-volume ratio of $1.72 \pm 0.35 \mu\text{m}^2/\mu\text{m}^3$ (Fig. 1b).

The detection of a single virulence gene was observed in three strains of *A. veronii* bv. *sobria* (1/12 *act*, 1/12 *asaI* and 1/12 *ascV*), while the occurrence of more than one gene was observed in eight strains (6

Table 2 In vitro antimicrobial susceptibility of *Aeromonas* spp. and *P. shigelloides* using broth microdilution and Vitek 2 system

MIC		<i>A. veronii</i> bv. <i>sobria</i> (n = 10)				<i>A. hydrophila</i> complex (n = 2)		<i>P. shigelloides</i> (n = 10)		<i>Aeromonas</i> spp. (n = 12)			
Drugs	Profile	Broth microdilution (μg/mL)		Vitek 2	Broth microdilution (μg/mL)		Vitek 2	Broth microdilution (μg/mL)		Geometric mean	MIC ₅₀	MIC ₉₀	MIC ₅₀
PTZ	S	0.5/4–8/4 (8)	ND (1)	≤4 (1)	2/4–8/4 (2)	≤4 (1)	–	0.03125/4–2/4 (10)	≤4 (10)	3.0/4	1.5/4	32/4	0.0625/4
	I	32/4 (2)	–	–	–	–	–	–	–	–	–	–	–
	R	–	≥128 (4)	≥128 (1)	–	–	–	–	–	–	–	–	–
CAZ	S	0.0625–0.25 (10)	≤1 (10)	≤1 (2)	0.25–1 (2)	≤1 (2)	–	0.015625–4 (10)	≤1–8 (10)	0.15	0.1250	0.7750	0.01563
	I	–	–	–	–	–	–	–	–	–	–	–	–
	R	–	–	–	–	–	–	–	–	–	–	–	–
FEP	S	0.0625–1 (10)	≤1 (10)	≤1 (2)	0.0625–0.125 (2)	≤1 (2)	–	0.00390625–4 (9)	≤1–8 (10)	0.09	0.0625	0.7750	0.01172
	I	–	–	–	–	–	–	16 (1)	–	–	–	–	–
	R	–	–	–	–	–	–	–	–	–	–	–	–
MEM	S	0.0078125–0.5 (10)	≤0.25 (6)	–	0.25–1 (2)	–	–	0.015625 (10)	≤0.25 (10)	0.09	0.09375	0.85	0.01563
	I	–	–	–	–	–	–	–	–	–	–	–	–
	R	–	≥16 (4)	≥16 (2)	–	–	–	–	–	–	–	–	–
CIP	S	0.00098–0.0078 (10)	≤0.25 (10)	≤0.25–0.5 (2)	0.00390625–1 (2)	≤0.25–0.5 (2)	–	0.00195–0.0078 (10)	≤0.25–0.5 (10)	0.004	0.002930	0.7023	0.001953
	I	–	–	–	–	–	–	–	–	–	–	–	–
	R	–	–	–	–	–	–	–	–	–	–	–	–
GEN	S	1–2 (10)	1–2 (10)	2–4 (2)	2–4 (2)	2–4 (2)	–	4 (4)	2–4 (7)	1.6	2	3.4	8
	I	–	–	–	–	–	–	8 (2)	8 (3)	–	–	–	–
	R	–	–	–	–	–	–	16 (4)	–	–	–	–	–

(–) no strain, ND no data in the system, PTZ piperacillin-tazobactam, CAZ ceftazidime, FEP cefepime, MEM Meropenem, CIP ciprofloxacin, GEN gentamicin, S susceptible, I intermediate, R resistant, Geometric mean, MIC₅₀, MIC₉₀ were calculated for the MIC values obtained through broth microdilution

Fig. 1 Representative images of Confocal Laser Scanning Analysis of mature biofilms of *Aeromonas* spp. (**a**) and *P. shigelloides* (**b**). Note the more homogenous distribution of

Aeromonas cells and the decreased thickness of biofilm structure, when compared to that of *P. shigelloides*. Magnification of $\times 400$, images generated with 488 nm laser (SYTO9)

A. veronii bv. *sobria*, 2 *A. hydrophila* complex). The *ascV* gene (type III secretion system) was the most detected virulence gene in *Aeromonas* spp. ($P = 0.0391$), which was observed in nine strains, while the *asal* gene (hemolysin) was detected in eight strains and the *act* gene (enterotoxin) was detected in three strains of *Aeromonas* spp. Regarding the detection of more than one virulence gene per strain, the most frequent combination was *ascV* and *asal* ($P = 0.0107$), detected in six *A. veronii* bv. *sobria*. Only one strain of *A. hydrophila* complex simultaneously showed the three virulence genes, and one strain of *A. veronii* bv. *sobria* did not show any of the tested genes.

Discussion

Members of the genera *Aeromonas* and *Plesiomonas* have been described as components of the microbiota of ectothermic animals and aquatic birds and are considered potentially pathogenic for these animals and humans, causing waterborne diseases or infections by traumatic inoculation (Janda and Abbott 2010; Kim et al. 2015, Janda et al. 2016). These observations

reinforce the importance of monitoring different aquatic environments for the presence of these microorganisms, their antimicrobial susceptibility and pathogenic potential. In this study, *Aeromonas* spp. were isolated from water samples, while *P. shigelloides* was isolated from water samples and cloacal swabs obtained from scarlet ibis. These results demonstrate that scarlet ibis can act as reservoirs of this pathogen, possibly because of their feeding habits, i.e., fish and crustaceans. To our knowledge, this is the first time these waterborne pathogens were recovered from captive scarlet ibises and their habitat, emphasizing that conservational facilities should be constantly monitored for the presence of these pathogens, in order to promote human, animal and environmental health.

The identification of the recovered isolates was based on conventional biochemical screening, for genus identification, followed by biochemical identification through the system Vitek 2, which contains *Aeromonas* species and *P. shigelloides* in its database. All of the results presented high probability of success (above 95 %), and reference ATCC strains were included in order to validate the identification cards. In a previous study, the identification of *Aeromonas*

with Vitek 2 presented accuracy above 80 %, when compared to the molecular identification (Lamy et al. 2010). Even though molecular techniques have been applied, this biochemical method is routinely and widely used in clinical laboratories and it has also been used in other researches (Igbinosa 2014; Tang et al. 2014). However, phenotypic methods are mostly not sufficient to identify the species within the different *Aeromonas* species complex (Janda and Abbot 2010), therefore, they were not specified in the present research. As for *Plesiomonas*, a recent review discussed that *P. shigelloides* shows remarkably little strain-to-strain variation in its biochemical profile, thus, this species is easily identified through conventional tests (Janda et al. 2016).

Water is the major source of contamination with *Aeromonas* spp. or *P. shigelloides* and an important vehicle for the spread of these microorganisms (Kim et al. 2015). Water sources and aquatic ecosystems also contribute for the environmental dissemination of antimicrobial drugs that are indiscriminately used in farming practices, especially, aquaculture, contributing for the emergence of resistant bacterial strains, which are a public health problem worldwide (Wellington et al. 2013). In this study, intermediate susceptibility to piperacillin–tazobactam among strains of *A. veronii* bv. *sobria* and resistance to gentamicin and intermediate susceptibility to cefepime among strains of *P. shigelloides* were observed. Intermediate susceptibility indicates that resistance mechanisms have been acquired and resistant bacterial strains may soon emerge. The antimicrobial susceptibility was also assessed through the automated method Vitek 2 and disagreements between both methods were observed. However, broth microdilution is the gold standard method for antimicrobial susceptibility testing.

Phenotypic virulence traits of *Aeromonas* spp. and *P. shigelloides* were also assessed and, overall, *Aeromonas* spp. produced more virulence factors than *P. shigelloides*, as observed by the higher hemolytic activity and biofilm-forming ability of the former. Considering these bacterial genera, especially *Aeromonas* spp., are colonizers of aquatic environments and ectothermic animals and capable of causing infections in endothermic animals, two incubation temperatures were tested for biofilm production, i.e. 28 and 37 °C. *Aeromonas* spp. produced more biofilm than *P. shigelloides*, especially at 28 °C. The

enhanced biofilm formation by *Aeromonas* spp. at this temperature suggests the importance of this feature for bacterial survival and maintenance in the environment and within ectothermic hosts (Scoaris et al. 2008), contributing for the bacterial colonization of seafood, hence, the occurrence of foodborne infections (Mizan et al. 2015).

The confocal biofilm analyses showed that *Aeromonas* biofilms were flatter and more homogeneously organized than those of *P. shigelloides*, with bacterial cells more closely arranged to each other. In addition, *P. shigelloides* biofilms presented a higher surface-volume ratio and a larger external surface than those of *Aeromonas* spp. These structural characteristics, associated with the crystal violet assay, suggest that the biofilms of *Aeromonas* spp. are more closely distributed, with more packed cells and less pores and voids within the biofilm, than those of *P. shigelloides*, as shown by the higher biomass values and lower biomass average thickness (Heydorn et al. 2000; Schaber et al. 2007). It is important to highlight that to our knowledge this is the first systematic characterization of the biofilm-forming ability of *P. shigelloides* and the first confocal structural analysis of *Aeromonas* and *Plesiomonas* biofilms.

Moreover, virulence genes of *Aeromonas* spp. were also investigated, and the *ascV* (type III secretion system) and *asal* (hemolysin) genes were the most frequently detected genes, corroborating previous researches (Wang et al. 2003; Aravena-Román et al. 2012). For *P. shigelloides*, no specific primers have been developed for the PCR-based detection of virulence genes and only phenotypical analyses have been performed (Fariñas et al. 2005). Thus, the same primers used for the detection of virulence genes in *Aeromonas* spp. were tested with *P. shigelloides*, because of the similarities between these bacterial genera, but none of the PCR products were compatible with the size expected for these genes, confirming the identification performed by the Vitek 2 system and the specificity of these primers to the *Aeromonas* genus.

Finally, scarlet ibises and their environment harbour waterborne and foodborne bacterial pathogens for humans and other animals. Hence, these birds and their environments should be continuously monitored for the presence of these microorganisms and measures for the prevention of infections should be implemented in order to minimize the zoonotic risks

for humans who handle these animals and visitors of zoological facilities.

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Compliance with ethical standards See Methods.

Conflicts of interest None.

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