

## Choanal and cloacal aerobic bacterial flora in captive green iguanas: a comparative analysis

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Received May 15, 2014

Accepted October 22, 2014

### Abstract

The aims of this study were to characterize the choanal and cloacal aerobic bacterial flora in healthy captive green iguanas and to compare it with the bacterial flora of the biofilm present in the water container of each terrarium. Samples were collected from the choana and the cloaca of 20 healthy captive adult green iguanas and from the biofilm of 15 water containers. The final identification of aerobic bacteria was performed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *Salmonella* positive samples were serotyped. The most common strains observed at each test location were from 1) choanae: *Staphylococcus* spp., *Enterobacter cloacae* and *Comamonas testosteroni*; 2) cloacae: *Citrobacter* spp., *Salmonella* spp. and *Corynebacterium* spp.; and 3) biofilms: *Pseudomonas* spp., *Salmonella* spp. and *Acidovorax* spp. We showed that apart from *Salmonella* spp., the choanal and cloacal bacterial flora differed from the microorganisms present in the biofilm of the animal's water container. These data revealed that healthy captive adult green iguanas harbored several aerobic bacterial strains that in immunosuppressed reptiles may act as opportunistic pathogens. Also, several of the aerobic bacteria identified in samples are potential zoonotic agents. Characterization of the normal background flora in captive reptiles and their environment can contribute to an understanding of the spread of bacterial contamination and the risk of potential zoonotic diseases for people in contact with these animals.

*Reptiles, biofilm, matrix-assisted laser desorption ionization time-of-flight mass spectrometry, serotype*

Previous studies in clinically healthy captive lizards showed a predominance of *Pseudomonas* spp., *Aeromonas* spp., *Serratia* spp. and *Enterobacter* spp. in the oral cavity of green iguanas (Barten 2002) and *Citrobacter* spp., *Enterobacter* spp., *Escherichia coli*, *Klebsiella oxytoca*, *Salmonella* spp., *Pseudomonas aeruginosa*, *Corynebacterium* spp., *Staphylococcus* spp., and *Streptococcus* spp. in the cloaca of giant lizards (Martínez Silvestre et al. 2003). These studies have helped us to understand the normal bacterial flora in captive lizards and to interpret the significance of bacterial strains in ill individuals.

Pet green iguanas are kept for many years in indoor terrariums. This situation leads us to consider the influence of the bacterial population in the biofilm that forms on the water container that is used by the lizard to drink from and bathe in, on the bacterial community of the lizard.

The aims of this study were to characterize and compare the aerobic bacterial flora between the choanal and cloacal sites in healthy captive adult green iguanas, and with the bacteria of the water container in their terrariums. This information will explore the hypothesis that the biofilm in water containers may perpetuate and spread bacterial contamination between animals and between terrariums. Furthermore, these data will contribute to knowledge of the normal background flora in pet iguanas and their environment, and for understanding the risk of potential zoonotic diseases for people in contact with these animals.

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Table 1. Bacterial strains isolated from healthy green iguanas and water containers.

Green iguana	Sex	Choana	Cloaca	Water container	Biofilm
No. 1	Male	<i>Staphylococcus capitis</i> , <i>Comamonas testosteroni</i>	<i>*Corynebacterium</i> spp.	A	<i>Escherichia coli</i> , <b><i>Salmonella</i> II 13,22:z29:1,5</b>
No. 2	Female	<i>Staphylococcus capitis</i> , <i>Enterobacter cloacae</i> , <i>Comamonas testosteroni</i>	<i>Staphylococcus capitis</i> , <i>Morganella morganii</i> , <i>Comamonas testosteroni</i>		
No. 3	Male	negative finding	<i>*Corynebacterium</i> spp., <i>Enterobacter aerogenes</i> , <i>Ochrobactrum gallinifaecis</i> , <i>Citrobacter freundii</i> , <b><i>Salmonella</i> IIIb 6,14:z10:z</b>	B	<i>*Chryseobacterium</i> spp., <i>Pseudomonas alcaligenes</i>
No. 4	Female	negative finding	<i>Proteus vulgaris</i> , <i>Citrobacter freundii</i> , <b><i>Salmonella</i> II 13,22:z29:1,5</b>		
No. 5	Male	<i>Staphylococcus capitis</i> , <i>Enterobacter cloacae</i>	<i>*Corynebacterium</i> spp., <i>Citrobacter freundii</i> , <b><i>Salmonella</i> IV 45:g:z51:-</b>	C	<i>Pseudomonas putida</i> , <i>Acidovorax delafieldii</i> , <b><i>Salmonella</i> IV 45:g:z51:-</b>
No. 6	Male	<i>Staphylococcus capitis</i> , <i>Staphylococcus sciuri</i>	<i>*Corynebacterium</i> spp., <i>Citrobacter freundii</i>	D	<i>Pseudomonas alcaligenes</i>
No. 7	Male	<i>Ewingella americana</i> , <i>Serratia liquefaciens</i> , <i>Citrobacter freundii</i>	<i>Citrobacter freundii</i> , <i>Escherichia coli</i>	E	<i>*Bacillus</i> spp., <i>Kocuria carniphila</i>
No. 8	Female	**Gram-negative bacilli	<i>*Staphylococcus</i> spp., <i>Alcaligenes faecalis</i> , <i>*Acinetobacter</i> spp., <i>Escherichia coli</i> , <b><i>Salmonella</i> II 13,22:z29:1,5</b>		
No. 9	Male	<i>Staphylococcus capitis</i>	<i>Delftia acidovorans</i> , <i>Citrobacter freundii</i> , <b><i>Salmonella</i> IIIb 6,14:z10:z</b>	F	<i>*Acidovorax</i> spp., <i>Pseudomonas alcaligenes</i>
No. 10	Male	**Gram-positive cocci	<i>Citrobacter freundii</i>	G	<i>*Acidovorax</i> spp., <i>Pseudomonas alcaligenes</i> , <i>Escherichia coli</i> , <b><i>Salmonella</i> II 13,22:z29:1,5</b>
No. 11	Female	<i>Staphylococcus capitis</i>	<i>*Corynebacterium</i> spp., <i>Alcaligenes faecalis</i> ssp. <i>faecalis</i> , <i>Enterobacter aerogenes</i> , <i>Citrobacter freundii</i> , <i>Ewingella americana</i> , <b><i>Salmonella</i> II 13,22:z29:1,5</b>		
No. 12	Female	negative finding	**G-positive bacilli, <i>Brevundimonas diminuta</i> , <i>Citrobacter freundii</i>	H	<i>Pseudomonas aeruginosa</i> , <i>Pseudomonas alcaligenes</i>

Table 1. Bacterial strains isolated from healthy green iguanas and water containers.

Green iguana	Sex	Choana	Cloaca	Water container	Biofilm
No. 13	Male	<i>Staphylococcus warneri</i> , negative finding	<i>Pseudomonas aeruginosa</i> , <b>Salmonella IIIb 6,14:z10:z</b> <i>Pseudomonas chlororaphis</i>	I	<i>Pseudomonas putida</i>
No. 14	Male	negative finding	<i>Staphylococcus warneri</i> , <i>Escherichia coli</i>	J	<i>Comamonas testosteroni</i> , <i>Stenotrophomonas maltophilia</i> , <i>Citrobacter freundii</i> , <b>Salmonella IV 45:g,z51:-</b>
No. 15	Female	negative finding	<i>Citrobacter koseri</i> , <i>Citrobacter freundii</i>	K	<i>*Acidovorax</i> spp., <i>Pseudomonas putida</i>
No. 16	Female	<i>Staphylococcus capitis</i> , <i>Staphylococcus xylosum</i> negative finding	<i>Bacillus firmus</i> , <i>*Achromobacter</i> spp., **Gram-negative bacilli	L	<i>Comamonas testosteroni</i> , <i>Escherichia coli</i>
No. 17	Female	negative finding	<i>Alcaligenes faecalis</i> , <i>Pseudomonas aeruginosa</i> , <i>Citrobacter freundii</i>	M	<i>Pseudomonas montelii</i>
No. 18	Male	<i>Staphylococcus capitis</i>	<i>Citrobacter freundii</i> , <b>Salmonella IIIb 6,14:z10:z</b>	N	<i>Comamonas testosteroni</i> , <i>Pseudomonas alcaligenes</i> , <b>Salmonella II 13,22:z29:1,5</b>
No. 19	Female	negative finding	<i>*Corynebacterium</i> spp., <i>Morganella morganii</i> , <i>Providencia rettgeri</i> , <i>Citrobacter freundii</i> , <b>Salmonella II 13,22:z29:1,5</b>	O	<i>*Kocuria</i> spp., <i>Pseudomonas</i> sp.
No. 20	Male	negative finding	<i>Citrobacter freundii</i>		

\* Not identified at species level by MALDI TOF MS \*\* Not identified by MALDI TOF MS

## Materials and Methods

Animals and water containers

Twenty green iguanas (*Iguana iguana*), 11 males and 9 females, aged 10 to 14 years were included in this study. The green iguanas were acquired when young (6 months old) from the breeding program of the local zoo. They were kept in indoor terrariums for over 9 years on 12 h light regime, including UVB light source, at a temperature between 24 and 35 °C. The main food was fresh dandelion leaves, cabbage, carrots, cucumbers and apples. Newspaper was used as substrate which was exchanged at least × 3 a week. Ten individuals (5 males and 5 females) were kept in pairs, and the other 10 were kept individually. Before sample collection, iguanas were clinically examined including haematology, plasma chemistry analyses, and parasitological examination of faecal samples. Within the period of the study, iguanas were healthy and sexually inactive. The animals were housed and handled in agreement with the Branch Commission for Animal Welfare of the Ministry of Education, Youth and Sports of the Czech Republic (accreditation No. 25-2013, MSMT-19018/2013-1, MSMT3XBZJE).

Each terrarium had a water container for drinking and bathing. The containers were cleaned every second day by scrubbing with a brush and rinsing with water. A thick and sticky layer known as biofilm was noted on the walls of the containers. A biofilm is a microbial community that is able to adhere to surfaces, it can resist removal by standard husbandry washing methods and it can survive for long periods in the environment.

### Sample collection

Iguanas were manually restrained and sterile cotton swabs (Copan Italia S.p.A, Italy) were: a) gently rubbed along the choana, b) gently inserted and twirled into the cloaca. Water container samples were collected by wiping the biofilm using a

sterile cotton swab. All samples were transported to bacteriological laboratory in Amies transport medium (Copan Italia S.p.A, Italy).

#### Bacterial isolation and identification

The samples were cultured on blood agar supplemented with 5% sheep blood (Columbia Agar Base, Oxoid, Ltd., England) and MacConkey agar (Oxoid, Ltd., England). All samples were aerobically incubated at 25 °C for 24–72 h. Cultures with mixed populations were subcultured on the same media. For *Salmonella* spp. detection, cloacal and biofilm samples were additionally incubated in buffered peptone water (Oxoid, Ltd., England) at 37 °C overnight, then selectively enriched in modified semisolid Rappaport Vassiliadis medium (Oxoid, Ltd., England) at 41.5 °C for 24 (48) h. Selected colonies were subcultured on xylose-lysine-deoxycholate agar (Oxoid, Ltd., England) and brilliant green agar (Oxoid, Ltd., England) at 37 °C for 24 h. The final identification of all isolated bacteria was performed by MALDI-TOF MS (MALDI Biotyper, Bruker Daltonics, Germany). Concordance studies show 99.1% and 99.4% agreement between MALDI TOF MS and conventional gold standard 16S rRNA gene sequencing (Cherkaoui et al. 2010).

*Salmonella* strains were serotyped by slide agglutination testing using two commercial O- and H- antisera (BioRad, France; Denka Seiken, Japan). Serotypes were designated according to the principles of the Kauffmann-White-Le Minor scheme (Grimont and Weill 2007).

## Results

### Choana and cloaca

Twenty one strains of aerobic bacteria were isolated from choanae (Table 1). The common strains were *Staphylococcus* spp. (*S. capitis*, *S. xylosus*, *S. warneri*, *S. sciuri*) (52.4%), *Comamonas testosteroni* (9.5%) and *Enterobacter cloacae* (9.5%). Fifty seven strains of aerobic bacteria were obtained from cloacae. The common strains were *Citrobacter* spp. (*C. freundii*, *C. koseri*) (26.3%), *Salmonella* spp. (15.8%) and *Corynebacterium* spp. (10.5%). *Salmonella enterica* was detected in 9 of 20 individuals and strains belonged to subspecies *salamae* (II), *diarizonae* (IIIb) and *houtenae* (IV). Each serotype was found in multiple individuals (Table 1). All 5 pairs of green iguanas had at least 1 aerobic bacterial species in common. However, no pairs shared the same *Salmonella* serotype.

### Biofilm in water containers

Thirty three strains of aerobic bacteria were obtained from biofilms (Table 1). The common strains were *Pseudomonas* spp. (*P. alcaligenes*, *P. putida*, *P. aeruginosa*, *P. monteilii*) (36.4%), *Salmonella* spp. (15.2%) and *Acidovorax* spp. (*A. delafieldii*) (12.1%). *Salmonella enterica* was detected in 5 of 15 water containers and the strains belonged to subspecies *salamae* (II) and *houtenae* (IV) (Table 1). Unexpectedly, the bacteria in the biofilms were not found in any of the choanae of the iguanas in the same terrarium. Apart from *Salmonella* spp., biofilms and cloacae also did not share the same bacteria, whereas 3 of the 20 iguanas had in their cloacae the same serotypes of *Salmonella* as the biofilm.

## Discussion

The results of this investigation demonstrate that the aerobic bacterial flora in the choanae and cloacae of healthy captive green iguanas was composed of diverse microbial spectra. Those known to be capable of causing opportunistic infection in malnourished reptiles or kept at suboptimal environmental temperatures are: *Acinetobacter* spp., *Stenotrophomonas maltophilia*, *Pseudomonas* spp., *Citrobacter* spp., *Proteus vulgaris*, *Enterobacter* spp., *Serratia* spp., and *Salmonella* spp. (Mader 1998; Harris and Rogers 2001; Mehler and Bennett 2003). Thus, animals could be at risk if they develop concurrent illness or if husbandry standards deteriorate.

Of the bacteria considered to pose a potential health hazard to immunosuppressed and/or immunocompromised humans (Johnson-Delaney 2006), the following were identified in our cohort of healthy captive green iguanas: *Stenotrophomonas maltophilia*, *Pseudomonas*

spp., *Citrobacter* spp., *Enterobacter* spp., *Escherichia coli*, *Proteus* spp., *Serratia* spp., and *Salmonella* spp. These species were present in our study with prevalence rates over 53.2% and are thus a potential zoonotic risk.

*Salmonella* species are known to be associated with ectotherms and also occur in their captive environment. It has been well documented that reptiles may spread *Salmonella* intermittently for a long time without showing any clinical signs of disease (Burnham et al. 1998; Mader 1998; Mitchell and Shane 2000). The narrow spectrum of *Salmonella* serotypes detected in the current study could be a consequence of the terrariums being isolated in the same room for many years. This could limit serotype exposure and allow cross contamination between individuals through husbandry. The *Salmonella* serotype II 13,22:z<sub>29</sub>:1,5 was found in 4 green iguanas in the current study, has previously been described in a pet turtle in Japan and free living tortoises in Morocco (Hidalgo-Vila et al. 2008; Lee et al. 2009). Serotype IIIb 6,14:z10:z was found in 4 green iguanas and has been reported in captive snakes in the United States (Goupil et al. 2012). The serotype IV 45:g,z<sub>51</sub>:- was found in 1 green iguana, has been reported to be responsible for salmonellosis in humans after contact with a green iguana, bearded dragon and gecko (Woodward et al. 1997; Bertrand et al. 2008).

In this study, apart from the more widespread presence of *Salmonella* species, the choanal and cloacal aerobic bacterial flora differed from those present in biofilm of water containers. This may reflect the true distribution or be an artifact arising from the technique of biofilm sampling. Abrasive sponge swabbing of the highly adherent biofilm may release and so aid detection of more bacteria. Alternatively, choanal, cloacal, and biofilm environments likely provide different conditions that are optimal for different bacteria. The choanal and cloacal bacterial species showed some overlap with previous studies in other reptiles (Barten 2002; Martínez Silvestre et al. 2003); this may be due to species variation, exposure or housing and husbandry differences.

We have shown that the high throughput MALDI TOF MS technology can benefit studies of microorganisms in reptiles and the captive environment, allowing accurate identification at the species level of many Gram-positive and Gram-negative bacterial strains. Nevertheless, a user of this technology should be aware that it is limited to the detection of strains represented in the MALDI-TOF MS database at any one time and to those that can be cultured.

Animals in the current study were housed in the same room and cared for by the same staff. Investigation of the choana, cloaca, and biofilm in reptiles kept by the public in different households, and as a consequence under varied conditions, would better represent the zoonotic potential posed by reptile to owners in the community, and make an interesting comparison with the current isolated collection. Future studies will also be extended to identify anaerobic bacteria and fungi in captive green iguanas.

#### Acknowledgements

We thank Dr. Corinne Lendon BVSc, BSc, PGCert L&T, PhD for her valuable suggestions, critical comments and correction of English, Doc. MVDr. Renata Karpíšková, PhD and Mgr. Petra Myšková at the Veterinary Research Institute, Brno, Czech Republic for determination of *Salmonella* serotypes and technicians at the Avian and Exotic Animal Clinic for the professional care of green iguanas. The project received partial funding from the Faculty of Veterinary Medicine (Institucionalni vyzkum FVL-2013).

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