Occurrence, Diversity, and Host Association of Intestinal 
Campylobacter, Arcobacter, and Helicobacter in Reptiles

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Abstract

Campylobacter, Arcobacter, and Helicobacter species have been isolated from many vertebrate hosts, including birds, mammals, and reptiles. Multiple studies have focused on the prevalence of these Epsilonproteobacteria genera in avian and mammalian species. However, little focus has been given to the presence within reptiles, and their potential zoonotic and pathogenic roles. In this study, occurrence, diversity, and host association of intestinal Epsilonproteobacteria were determined for a large variety of reptiles. From 2011 to 2013, 444 cloacal swabs and fecal samples originating from 417 predominantly captive-held reptiles were screened for Epsilonproteobacteria. Campylobacter, Arcobacter, and Helicobacter genus specific PCRs were performed directly on all samples. All samples were also cultured on selective media and screened for the presence of Epsilonproteobacteria. Using a tiered approach of AFLP, atpA, and 16S rRNA sequencing, 432 Epsilonproteobacteria isolates were characterized at the species level. Based on PCR, Campylobacter, Arcobacter, and Helicobacter were detected in 69.3% of the reptiles; 82.5% of the chelonians, 63.8% of the lizards, and 58.0% of the snakes were positive for one or more of these genera. Epsilonproteobacteria were isolated from 22.1% of the reptiles and were isolated most frequently from chelonians (37.0%), followed by lizards (19.6%) and snakes (3.0%). The most commonly isolated taxa were Arcobacter butzleri, Arcobacter skirrowii, reptile-associated Campylobacter fetus subsp. testudinum, and a putative novel Campylobacter taxon. Furthermore, a clade of seven related putative novel Helicobacter taxa was isolated from lizards and chelonians. This study shows that reptiles carry various intestinal Epsilonproteobacteria taxa, including several putative novel taxa.

Citation: Gilbert MJ, Kik M, Timmerman AJ, Severs TT, Kusters JG, et al. (2014) Occurrence, Diversity, and Host Association of Intestinal Campylobacter, Arcobacter, and Helicobacter in Reptiles. PLoS ONE 9(7): e101599. doi:10.1371/journal.pone.0101599

Editor: Jan S. Suchodolski, GI Lab, United States of America

Received November 4, 2013; Accepted June 9, 2014; Published July 2, 2014

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Funding: The authors have no support or funding to report.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Campylobacter, Arcobacter, and Helicobacter species occupy a broad vertebrate host range and have been isolated from birds, mammals, and reptiles. These Epsilonproteobacteria genera are often found as commensals in the digestive tract, but some species are associated with disease in specific hosts [1–3]. The diverse genus Arcobacter also contains species free-living in the environment and species associated with non-vertebrate hosts [4]. Campylobacter, Helicobacter, and some Arcobacter species are mainly associated with endotherm mammalian vertebrates, which is reflected in an optimal growth temperature of 30–42°C for most of the bacterial species [5]. However, Campylobacter, Arcobacter, and Helicobacter species have also been isolated from ectothermic reptiles [4,6–10], which show considerable fluctuations in their body temperatures, often resulting in a lower average body temperature compared to most endotherm vertebrates [11]. A genetically distinct variant of Campylobacter fetus has been found in reptiles and humans that had direct or indirect contact with reptiles [6–8,12,13]. This reptile-associated C. fetus has been shown to cause infection in humans with underlying disease [12,13]. Few bacterial species have been recognized as primary pathogens in reptiles [14,15], and currently no cases have been reported of Campylobacter or Arcobacter species causing disease in reptiles, although a fatal septicemia in a pancake tortoise (Malacochersus toruensis) associated with an unidentified Helicobacter species has been described [9].

The role of reptiles in the epidemiology of Epsilonproteobacteria is currently unknown. Therefore, the aim of this study was to determine occurrence, diversity, and host association of intestinal Epsilonproteobacteria in reptiles.

Materials and Methods

Ethics statement

The procedures conducted on the reptiles in the study were merely combined with veterinary diagnostic and therapeutic acts and were therefore not considered to cause any additional discomfort. Consequently, the study was not considered an animal experiment under the Dutch Experiments on Animals Act (1977), making an assessment by an animal ethics committee unnecessary. The sampling was performed in strict accordance with the Dutch
Law for Practicing Veterinary Medicine (1990). Fecal samples and cloacal swabs were collected as part of routine post mortem examination and diagnostic fecal examination for Campylobacter and other infectious agents. Sampling of live animals was performed by a veterinarian specialized in reptiles and amphibians (MK, Diplomate of the European College of Zoological Medicine, Herpetology). All owners provided consent for use of the samples collected from the reptiles.

Sample collection and preparation
From 2011 to 2013, a total of 444 cloacal swabs (n = 363) and fecal samples (n = 81) were collected from 417 healthy, diseased, or dead reptiles (Table S1). Free-living (n = 2), recently wild-caught (n = 56), and captive-held animals (n = 359) were included. Sampled animals belonged to 97 unique species of 27 families (Table S1). Diversity was high amongst sampled animals and the reptilian orders Squamata (comprising suborders Lacertilia (lizards) and Serpentes (snakes)) and Testudines (chelonians) were covered. Samples were obtained from three zoos (n = 170), a veterinary pathology division (n = 77), a veterinary clinic specialized in reptiles and amphibians (n = 102), a veterinary microbiological diagnostic laboratory (n = 5), a pet shop specialized in reptiles and amphibians (n = 56), and private owners (n = 34); all located in the Netherlands. For 27 animals two samples per animal were included.

Cloacal swabs (Amies charcoal 114C and 116C; Copan, Brescia, Italy) were extracted in 500 μl phosphate buffered saline (PBS); fecal samples were diluted in PBS until a fluid suspension was obtained. Of the each suspension, 240 μl was used directly for culturing and 100 μl was stored at −80°C with 10 mM EDTA for DNA extraction and direct genus specific PCR analysis.

Direct genus specific PCR analysis of samples
On all samples Campylobacter, Arcobacter, and Helicobacter genus specific PCRs were performed. For DNA isolation, 50 μl of lysis buffer (Cobas PCR Female Swab Sample Kit, Roche Molecular Systems Inc., Branchburg, USA) was added to 50 μl of each sample, followed by freezing at −80°C for 24 h and immediate heating at 95°C for 15 min in order to release the DNA and to inactivate DNAses. DNA was then extracted from this 100 μl sample on MagNA Pure96 (Roche), using the Roche MagNA Pure96 and Viral NA Small Volume Kit with the Viral NA Universal SV extraction protocol according to the manufacturer’s instructions. Final elution was in 100 μl, and 2 μl of these eluates was used as input for all PCRs. The Arcobacter, Campylobacter, and Helicobacter genus specific PCRs were performed as described previously [16–18], with slight adaptations. The Arcobacter genus specific PCR was performed using Go Taq Hot Start Green Master Mix (Promega, Madison, USA); total reaction volume was 25 μl and the PCR was run for 40 cycles on a 2720 Thermal Cycler (Applied Biosystems). Of each reaction, 10 μl was analyzed on a 1% agarose gel for the 1223 bp amplicon. Both Campylobacter and Helicobacter genus specific PCRs were performed using a LightCycler 480 II (Roche) and were run 45 cycles. LightCycler 480 Probes Master (Roche) was used for the Campylobacter genus specific PCR in a total reaction volume of 25 μl; LightCycler 480 SYBR Green I Master (Roche) was used for the Helicobacter genus specific PCR in a total reaction volume of 15 μl. Using the LightCycler 480 software (Roche), the Campylobacter and Helicobacter genus specific PCRs were analyzed based on absolute quantification and melting curves (range 85.5–87°C), respectively. Arcobacter butzleri strain SX–460, Campylobacter fetus strain NCTG 10842, and Helicobacter pylori strain J99 were used as reference strains. A subset of the amplicons was sequenced to validate the PCR results.

Culturing
From each of the sample suspensions, 10 μl was plated on each of four different media: blood agar (Colombia agar with 5% sheep blood), CCD agar, Preston agar and Skirrow agar (Oxoid, Lunsmeer, The Netherlands). Additionally, a membrane filtration method was used; 200 μl suspension was applied on a cellulose filter membrane (ME26; 50 mm; pore size 0.6 μm; Whatman, Dassel, Germany) placed on a blood agar plate, incubated at atmospheric conditions at 37°C for one hour, after which the filter membrane was discarded. All agar plates were incubated in a microaerobic atmosphere containing hydrogen (83.3% N₂, 7.1% CO₂, 3.5% H₂, and 6% O₂) at 37°C and screened for colonies showing Epsilonproteobacteria-like morphology after 24 h, 48 h, and after a week of incubation. Selected colonies were colorless, whitish, greyish, or brownish, often small, slow growing, and flat, with or without the tendency to spread and showing no or little hemolysis on blood containing media. The number and diversity of colonies showing Epsilonproteobacteria-like morphology varied considerably for each sample and determined the number of selected colonies per sample. On average 9.0 (±2.6) colonies per sample were subcultured onto a fresh blood agar plate, incubated at 37°C under microaerobic conditions until growth was visible, and Gram-stained. Gram-negative bacteria showing helical or curved rod-shaped microscopic morphology typical for most Epsilonproteobacteria were selected and stored at −80°C.

For molecular analysis of the isolates, bacterial genomic DNA of all isolates was extracted following the Gram-negative bacteria protocol of either the High pure PCR template preparation kit (Roche Diagnostics, Almere, The Netherlands) or Gentra Puregene yeast/bacteria kit (Qiagen, Venlo, The Netherlands).

AFLP
Amplified fragment length polymorphism (AFLP) was used to analyze diversity amongst the isolates and for preliminary species identification by inclusion of well-characterized Campylobacter, Arcobacter, and Helicobacter strains. AFLP was performed on all isolates as described by Dunn et al. [19], with 20 ng genomic DNA for each 14 μl restriction/ligation reaction mixture. Fingerprint data were created with a 3730 DNA analyzer (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands) and clustered (pairwise similarities; curve-based Pearson correlation; UPGA clustering) using BioNumerics v.6.6 (Applied Maths, Sint-Martens-Latem, Belgium). For each sample one representative isolate per distinct fingerprint cluster (≥75% similarity) was selected for subsequent atpA and 16S rRNA sequencing.

atpA and 16S rRNA sequencing for species identification of isolates
For further characterization and species identification of the isolates atpA sequencing was performed. The atpA (or uncA) locus, encoding the ATPase synthase α subunit, is part of the MLST-scheme for Campylobacter [20]. In Epsilonproteobacteria, atpA is conserved and has been used to improve Campylobacteraceae and Helicobacteraceae species identification by 16S rRNA sequencing [21], thereby making it a good candidate for (sub)species identification of the isolates. Two degenerate primer sets targeting atpA from a broad range of Campylobacter, Arcobacter, and Helicobacter species were: atpAF/R [20] and HFatpF/R [22]. For isolates for which atpA could not be sequenced, 16S rRNA encoding DNA sequencing was used using the 16S rRNA targeting primers 27F and 1492R [23]. Each PCR amplification mixture contained 2.5 μM each primer, 1x Go Taq Hot Start Green Master Mix
methods (22.1%) than based on genus specific PCRs (69.3%). Campylobacter positive by genus specific PCR (all Epsilonproteobacteria) Testudinidae, these animals made up 80.4% (45/56) of the 18.2% (76/417) of the sampled reptiles belonged to the Epsilonproteobacteria. Amongst Testudines, terrestrial chelonians of the Epsilonproteobacteria Testudines 37.0% (57/154), followed by Lacertilia 19.6% (32/163). Occurrence of Campylobacter taxon 1 was isolated from Testudines 15.6% (24/154) and Laceritia 6.1% (10/163). Occurrence of C. fetus taxon 1 was highest in Testudines 7.1% (11/154), followed by Laceritia 5.3% (8/154) and Serpentes 3.0% (3/100). Arcobacter species were isolated from 7.9% (33/417) of the reptiles, predominantly from Testudines (Table 1). Species isolated were Arcobacter butzleri, Arcobacter cryaerophilus, and Arcobacter skirrowii. A. butzleri was isolated from Laceritia, Serpentes, and Testudines while A. skirrowii was exclusively isolated from Testudines.

Compared to Arcobacter and Campylobacter, Helicobacter occurrence was low, 4.8% (20/417), but diversity was high. All isolates belonged to seven unknown taxa and were obtained from Laceritia and Testudines (Table 1). A 16S rRNA based neighbor-joining dendrogram shows that these seven taxa cluster together, but apart from other Helicobacter species isolated from mammals and birds (Figure 1). Within this cluster, three distinct clusters are separated by deep branching. Interestingly, these clusters suggest Helicobacter host association: five out of six taxa obtained from Laceritia clustered together, whereas Helicobacter taxon 2, obtained from Testudines, clustered with a previously described Helicobacter species isolated from a pancake tortoise (Malacochersus tornieri) [9]. In addition to Campylobacter, Arcobacter, and Helicobacter taxa, isolates of an unknown Sulfurospirillum taxon were obtained from a Malaysian giant turtle (Oritia bosmensis) (Table 1).

For each putative novel taxon, 16S rRNA sequences have been deposited in GenBank and accession numbers have been provided (Table S2). The detection rates for each culturing method based on sampled animals were: filter method 85.9% (79/92), CCD Preston agar. Although some Campylobacter and Arcobacter taxa (21 positive animal for each taxon) could be isolated using the filter method, CCD, Preston, and Skirrow agar. Although some Helicobacter isolates were obtained from Preston and Skirrow agar, the majority (53/59) was isolated from blood agar or by the filter method. The Sulfurospirillum isolates were obtained by the filter method and from Preston agar.

### Discussion

Epsilonproteobacteria were present in all reptilian orders examined. Chelonians showed highest Epsilonproteobacteria occurrence and diversity. Based on direct genus specific PCR analysis, Campylobacter occurrence was highest in all reptilian (sub)orders, followed by Helicobacter and Arcobacter. The majority of isolated Epsilonproteobacteria belonged to the genera Campylobacter and Arcobacter. Helicobacter was isolated less frequently. One undescribed Sulfurospirillum taxon was isolated. All currently described Sulfurospirillum species were isolated from environmental sources, potentially making this the first Sulfurospirillum taxon isolated from an animal host. Most commonly isolated taxa were Campylobacter taxon 1 and Campylobacter fetus taxon 1. Campylobacter taxon 1 has been proposed as a novel species, Campylobacter ‘iguaniorni’ (Cig) (unpublished data). The 16S rRNA sequences of Cig were homologous to a recently described Campylobacter strain isolated from a leopard.
Table 1. Number of animals positive for each *Epsilonproteobacteria* taxon per reptilian (sub)order.

|                          | Lacertilia (*n* = 163) Animals (%) | Serpentes (*n* = 100) Animals (%) | Testudines (*n* = 154) Animals (%) | Total (*n* = 417) Animals (%) |
|--------------------------|-------------------------------------|-----------------------------------|-------------------------------------|-----------------------------|
|                          | PCR (%)                             | Culturing (%)                     | PCR (%)                             | Culturing (%)               |
| **Epsilonproteobacteria**|                                     |                                   |                                     |                             |
| Arcobacter genus         | 23 (14.1)                           | 6 (3.7)                           | 13 (13.0)                           | 2 (2.0)                     | 47 (30.5)                   | 25 (16.2)                   | 83 (19.9)                   | 33 (7.9)                    |
| Arcobacter butzleri      | 6 (3.7)                             |                                    | 2 (2.0)                             |                             | 11 (7.1)                    |                             | 19 (4.6)                    |                             |
| Arcobacter cryaerophilus | 1 (0.6)                             | 0 (0.0)                           | 9 (5.8)                             |                             |                             | 9 (5.8)                     |                             |                             |
| Arcobacter skirrowii     | 0 (0.0)                             | 0 (0.0)                           |                                    |                             | 14 (9.1)                    |                             | 14 (3.4)                    |                             |
| Campylobacter genus      |                                     |                                   |                                     |                             |                             |                             |                             |                             |
| Campylobacter fetus taxon 1* | 9 (5.5)                         | 3 (3.0)                           | 1 (0.6)                             |                             | 1 (0.6)                     |                             | 23 (5.5)                    |                             |
| Campylobacter fetus taxon 2* | 0 (0.0)                           | 0 (0.0)                           | 1 (0.6)                             |                             | 1 (0.6)                     |                             | 1 (0.2)                     |                             |
| Campylobacter hyointestinalis | 0 (0.0)                         | 0 (0.0)                           | 9 (5.8)                             |                             |                             | 9 (5.8)                     |                             |                             |
| Campylobacter taxon 1*   | 10 (6.1)                            | 0 (0.0)                           | 24 (15.6)                           |                             |                             | 34 (8.2)                    |                             |                             |
| Campylobacter taxon 2*   | 0 (0.0)                             | 0 (0.0)                           | 1 (0.6)                             |                             |                             | 1 (0.6)                     |                             |                             |
| Helicobacter genus       |                                     |                                   |                                     |                             |                             |                             |                             |                             |
| Helicobacter taxon 1*    | 1 (0.6)                             | 0 (0.0)                           | 8 (5.2)                             |                             | 8 (5.2)                     |                             | 163 (39.1)                  | 20 (4.8)                    |
| Helicobacter taxon 2*    | 0 (0.0)                             | 0 (0.0)                           | 8 (5.2)                             |                             | 8 (5.2)                     |                             |                             |                             |
| Helicobacter taxon 3*    | 4 (2.5)                             | 0 (0.0)                           | 0 (0.0)                             |                             | 0 (0.0)                     |                             | 4 (1.0)                     |                             |
| Helicobacter taxon 4*    | 1 (0.6)                             | 0 (0.0)                           | 0 (0.0)                             |                             | 0 (0.0)                     |                             | 1 (0.2)                     |                             |
| Helicobacter taxon 5*    | 1 (0.6)                             | 0 (0.0)                           | 0 (0.0)                             |                             | 0 (0.0)                     |                             | 1 (0.2)                     |                             |
| Helicobacter taxon 6*    | 1 (0.6)                             | 0 (0.0)                           | 0 (0.0)                             |                             | 0 (0.0)                     |                             | 1 (0.2)                     |                             |
| Helicobacter taxon 7*    | 4 (2.5)                             | 0 (0.0)                           | 0 (0.0)                             |                             | 0 (0.0)                     |                             | 4 (1.0)                     |                             |
| Sulfurspirillum taxon*    | 0 (0.0)                             | 0 (0.0)                           | 1 (0.6)                             |                             | 1 (0.6)                     |                             | 1 (0.2)                     |                             |
| **Total****              | 104 (63.8)                          | 32 (19.6)                         | 58 (58.0)                           | 3 (3.0)                     | 127 (82.5)                  | 57 (37.0)                   | 289 (69.3)                  | 92 (22.1)                   |

*n*, total of sampled animals;
*1*, putative novel species or subspecies based on 16S rRNA sequence and AFLP fingerprint;
**1**, not sum of *Epsilonproteobacteria* positive animals in case of multiple *Epsilonproteobacteria* taxa per animal.

doi:10.1371/journal.pone.0101599.t001
tortoise (*Stigmochelys pardalis*) [10]. *Campylobacter fetus* taxon 1 has recently been described as a novel subspecies, *Campylobacter fetus* subsp. *testudinum* (Cft) [24]. Cft is homologous to the previously described reptile-associated *C. fetus* [6-8,12,13] and similar...
with disease, but due to the low number of animals this should be interpreted with care. Little is known about pathogenicity in reptiles of other Epsilonproteobacteria taxa, although a fatal septicaemia in a pancake tortoise (Malacochersus tornieri), associated with a Helicobacter species related to Helicobacter taxon 2, isolated from spur-thighed tortoises (Testudo graeca) and Russian tortoises (Agrionemys horsfieldii), has been described [9]. The majority of Epsilonproteobacteria species isolated from reptiles during this study (C. fetus, C. hyointestinalis, A. butzleri, A. cryaerophilus, and A. skirrowii) have been shown to be potentially pathogenic in humans [1, 25, 26]. Although for reptilian C. fetus there appears to be an association between contact with reptiles and infection in humans [6, 12, 13], to this date, no such association has been described for the other Epsilonproteobacteria species. The growing number of reptiles kept as pets in some European countries [27] and increased farming of reptiles, predominantly freshwater turtles for human consumption, in Asian countries [28], might increase the risk of reptilian derived Epsilonproteobacteria infections in humans.

It has to be noted that most samples were obtained from captive-held reptiles, which may lead to other transmission dynamics and an intestinal microbiota composition distinct from free-living reptiles, as has been shown for other vertebrates [29]. Indeed, AFLP indicated that unrelated reptile species having direct or indirect contact (same housing, same zoo, or same shipment) can harbor the same specific Epsilonproteobacteria variants, suggesting horizontal transmission (data not shown). Fecal-oral transmission is likely, especially in captive animals and species displaying coprophagy, although a common feed related origin cannot be excluded. Particularly chelonians of the Testudinidae family commonly display coprophagy, presumably to supplement their intestinal microbiota and optimize digestion of their herbivorous diet. This behavior could partly explain the frequent detection rates in reptiles as found in this study have been reported [8]. A demarcated Campylobacter species distribution was observed in this study; species commonly found in mammals and birds, such as C. jejuni, C. coli, and C. lari, were not found in reptiles. The reverse was observed for Cig and Clt, which were isolated from reptiles, but have not (Cig) or rarely (Clt) been isolated from endothermic vertebrates. This differential distribution of Campylobacter species likely reflects the body temperature of the host, which is fluctuating and on average lower in ectothermic reptiles (with a mean of 22°C), which might be an adaptation to their reptilian hosts. Many Epsilonproteobacteria cluster with isolates originating from lizards and a cluster with isolates based on 16S rRNA phylogeny the taxa were separated in a reverse was observed for Cig and Cft, which were isolated from reptiles, but have not (Cig) or rarely (Cft) been isolated from free-living reptiles, as has been shown for other vertebrates [29]. Indeed, AFLP indicated that unrelated reptile species having direct or indirect contact (same housing, same zoo, or same shipment) can harbor the same specific Epsilonproteobacteria variants, suggesting horizontal transmission (data not shown). Fecal-oral transmission is likely, especially in captive animals and species displaying coprophagy, although a common feed related origin cannot be excluded. Particularly chelonians of the Testudinidae family commonly display coprophagy, presumably to supplement their intestinal microbiota and optimize digestion of their herbivorous diet. This behavior could partly explain the frequent

| Taxon | Blood agar | Filter | CCD | Preston | Skirrow | Total |
|-------|------------|--------|-----|---------|---------|-------|
|       | Animals (%) | Animals (%) | Animals (%) | Animals (%) | Animals (%) | Animals (%) |
| Arcobacter butzleri | 0 (0.0) | 12 (63.2) | 7 (36.8) | 4 (21.1) | 9 (47.4) | 19 (20.2) |
| Arcobacter cryaerophilus | 0 (0.0) | 9 (90.0) | 4 (40.0) | 5 (50.0) | 4 (40.0) | 10 (10.9) |
| Arcobacter skirrowii | 1 (7.1) | 13 (92.9) | 7 (50.0) | 3 (21.4) | 3 (21.4) | 14 (15.2) |
| Campylobacter fetus taxon 1* | 0 (0.0) | 14 (60.9) | 16 (69.6) | 8 (34.8) | 10 (43.5) | 23 (25.0) |
| Campylobacter fetus taxon 2* | 0 (0.0) | 0 (0.0) | 1 (100.0) | 1 (100.0) | 0 (0.0) | 1 (1.1) |
| Campylobacter hyointestinalis | 2 (22.2) | 3 (33.3) | 6 (66.7) | 6 (66.7) | 2 (22.2) | 9 (9.8) |
| Campylobacter taxon 1* | 3 (8.8) | 18 (52.9) | 9 (26.5) | 18 (52.9) | 8 (23.5) | 34 (37.0) |
| Campylobacter taxon 2* | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1 (100.0) | 1 (1.1) |
| Helicobacter taxon 1* | 0 (0.0) | 1 (100.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1 (1.1) |
| Helicobacter taxon 2* | 1 (12.5) | 8 (100.0) | 0 (0.0) | 0 (0.0) | 1 (12.5) | 8 (8.7) |
| Helicobacter taxon 3* | 2 (50.0) | 4 (100.0) | 0 (0.0) | 1 (25.0) | 1 (25.0) | 4 (4.3) |
| Helicobacter taxon 4* | 0 (0.0) | 1 (100.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1 (1.1) |
| Helicobacter taxon 5* | 0 (0.0) | 1 (100.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1 (1.1) |
| Helicobacter taxon 6* | 0 (0.0) | 1 (100.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1 (1.1) |
| Helicobacter taxon 7* | 3 (75.0) | 4 (100.0) | 0 (0.0) | 0 (0.0) | 2 (50.0) | 4 (4.3) |
| Sulphaspirillum taxon* | 0 (0.0) | 1 (100.0) | 0 (0.0) | 1 (100.0) | 0 (0.0) | 1 (1.1) |
| Total** | 12 (13.0) | 79 (85.9) | 45 (48.9) | 43 (46.7) | 37 (40.2) | 92 (100.0) |

*, putative novel species or subspecies based on 16S rRNA sequence and AFLP fingerprint; **, not sum of Epsilonproteobacteria positive animals in case of multiple Epsilonproteobacteria taxa per animal.

doi:10.1371/journal.pone.0101599.t002
occurrence and high diversity of intestinal Epsilonproteobacteria in these chelonians. Nevertheless, the majority of Epsilonproteobacteria species were also isolated from recently caught free-living reptiles. Studies comparing Salmonella carriage in captive-held and free-living reptiles showed that occurrence was lower in the latter [30,31], but additional sampling of free-living reptiles would be necessary to elucidate the native Epsilonproteobacteria carrier status. In addition to this, analysis of additional samples obtained from species of the excluded orders Rhynchocephalia and Crocodilia is required to obtain a more precise estimation of the intestinal Epsilonproteobacteria occurrence in reptiles.

The methods used in this study might have influenced the observed intestinal Epsilonproteobacteria occurrence, as described below. A total of 69.1% (PCR) and 22.3% (culturing) of the cloacal swabs, and 55.6% (PCR) and 16.0% (culturing) of the fecal samples was positive for Epsilonproteobacteria. Although there was no statistical difference between the detection rates for cloacal swabs and fecal samples (two tailed Fisher exact test; P = 0.03 (PCR) and P = 0.23 (culturing)), it is uncertain whether these detection rate differences are inherent to the sample type or merely a result of stochastic processes. Of 27 animals two samples were included, which potentially increased the detection rate: 12 (PCR) and 22 (culturing) cases contained two negative samples; eight (PCR) and three (culturing) cases contained one positive sample; seven (PCR) and two (culturing) cases contained two positive samples. The culturing temperature was 37°C, which may have been too high for culturing potential Campylobacter, Arcobacter, and Helicobacter from reptiles with low preferred body temperatures. A subset of samples was cultured at both 30°C and 37°C to determine the effect of temperature on growth rate and species composition. Although a lower growth rate was observed at 30°C, colony composition appeared similar at both temperatures (data not shown). The culture media were selected to promote Epsilonproteobacteria growth and inhibit growth of non-Epsilonproteobacteria. Although most of the antimicrobial containing media used are optimized for C. jejuni and C. coli, the results of the filter method showed high agreement with media containing antimicrobials for all isolated Campylobacter and Arcobacter taxa (positive animals >1). Most Helicobacter taxa were isolated using media without antimicrobials. The discrepancy in detection rates between culturing and direct genus specific PCR was most prominent in the Helicobacter genus, suggesting that the culturing methods were suboptimal for Helicobacter. Also, non-motile Epsilonproteobacteria species, such as C. gracilis, will likely not pass through the filter. Microaerobic conditions are preferred by most culturable Epsilonproteobacteria species [5], although some species, such as C. gracilis and C. rectus, prefer anaerobic conditions. The presence of hydrogen, formate, or succinate as an electron source is essential for growth of some Campylobacter species. Furthermore, only 10 µl (≈2%) of the fecal suspension was plated on selective media, and a selection of colonies displaying Epsilonproteobacteria-like morphology was isolated. Since not all Epsilonproteobacteria display the typical helical or curved rod shape, screening microscopically for this feature might lead to an underestimation of the true occurrence.

As expected, direct genus specific PCRs resulted in higher detection rates compared to culturing. Nearly all samples positive for Epsilonproteobacteria by culturing were positive by PCR as well. Members of the Campylobacter genus were most frequently detected by both PCR and culturing. Remarkably, members of the Arcobacter genus were detected least frequently by PCR, whereas members of the Helicobacter genus were detected least frequently by culturing. This discrepancy indicates that the Arcobacter and Helicobacter PCRs likely have different sensitivities and that both genera show differential growth on the media used. Indeed, many members of the Helicobacter genus are considered fastidious organisms [2]. Since Epsilonproteobacteria were detected on genus level by PCR and higher detection rates were observed by PCR than by culturing, fastidious or non-culturable species might not have been identified, which could have led to an underestimation of the true Epsilonproteobacteria diversity. Ideally, one PCR detecting all Epsilonproteobacteria directly in crude samples would overcome the potential differences in sensitivities between PCRs. Although a PCR-RFLP method targeting 16S rRNA of most Campylobacter, Arcobacter, and Helicobacter species has been described [32], this assay is designed for species identification of isolates, and not for direct assessment of samples. While molecular methods are useful, isolates obtained by culturing are preferred for further analysis, such as examination of epidemiology, diversity, and phenotypic characters, making both methods complementary.

The polyphasic approach based on direct genus specific PCR and culturing was useful to determine intestinal Epsilonproteobacteria occurrence and diversity from genus to subspecies level. Our results show that reptiles comprise a significant reservoir for Epsilonproteobacteria. A clad of related putative novel Helicobacter taxa appears to be specifically associated with reptiles. Arcobacter butzleri, Arcobacter skrjavici, Campylobacter fetus subsp. testudinum, and Campylobacter “iguianum” represent a major portion of the Epsilonproteobacteria isolated from reptiles, chelonians in particular. The Campylobacter taxa are thus far predominantly found in reptiles, which may constitute their primary reservoir.

Supporting Information

Table S1 Number of samples, animals, and detected Epsilonproteobacteria for all reptilian species included in this study. Epsilon, Epsilonproteobacteria; A, Arcobacter genus; Ab, Arcobacter butzleri; Ac, Arcobacter cyanophophilus; As, Arcobacter skrjavici; C, Campylobacter genus; C1, Campylobacter fetus taxon 1; C2, Campylobacter fetus taxon 2; Ch, Campylobacter hyointestinalis; C1, Campylobacter taxon 1; C2, Campylobacter taxon 2; H, Helicobacter genus; H1, Helicobacter taxon 1; H2, Helicobacter taxon 2; H3, Helicobacter taxon 3; H4, Helicobacter taxon 4; H5, Helicobacter taxon 5; H6, Helicobacter taxon 6; H7, Helicobacter taxon 7; S, Sulfurospirillum taxon. (XLSX)

Table S2 GenBank accession numbers of the 16S rRNA sequences for all putative novel Epsilonproteobacteria taxa isolated during this study. *, 16S rRNA sequence deposited at GenBank prior to this study. (XLSX)

Acknowledgments

We kindly thank Mark de Boer (Rotterdam Zoo), Dennis de Haan, Warren Spencer (Artis Royal Zoo), Arendu Fljune (Herpetological Center Holland), Job Stumpel, and Carlos Voogdt for their valuable contribution to sample collection, Abigail Kouden and Linda van der Graaf-van Bloois (Utrecht University) for technical support, and Rebekah Keesler (Utrecht University) for proof reading.

Author Contributions

Conceived and designed the experiments: MJG, JGK BD JAW. Performed the experiments: MJG MK AJT TTS JAW. Analyzed the data: MJG TTS JG BD JAW. Wrote the paper: MJG BD JAW.
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