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Characterisation of *Chlamydia pneumoniae* and other novel chlamydial infections in captive snakes

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ABSTRACT

Chlamydiosis has been described in both free-ranging and captive reptiles. The infection usually manifests as granulomatous inflammation in inner organs such as spleen, heart, lung and liver but might also occur in asymptomatic reptiles. The aim of this study was to investigate and characterise *Chlamydia pneumoniae* and potential other novel chlamydial infections in the choana and cloaca samples of 137 clinically healthy captive snakes from six private collections. Forty eight samples from 29 animals were found to be positive by a *Chlamydiaceae* family-specific qPCR. By *Chlamydia* species-specific ArrayTube Microarray, 43 samples were positive, with 36 of these being identified as *C. pneumoniae*. The prevalence of *Chlamydia* ranged from 5 to 33%. PCR and sequencing of the *Chlamydiales* 16S rRNA signature sequence of 21 *Chlamydia* positive samples revealed the presence of seven novel 16S rRNA genotypes. BLAST-n and phylogenetic analysis of the near-full length 16S rRNA gene sequence of each of these novel 16S rRNA sequences revealed that five genotypes share closest sequence identity to 16S rRNA sequences from *C. pneumoniae* (98.6–99.2%), suggesting that these sequences are novel *C. pneumoniae* strains. One genotype is 96.9% similar to *C. pneumoniae* strains suggesting it may originate from a yet undescribed chlamydial species within the genus *Chlamydia*. This study further highlights the broad host range for *C. pneumoniae* and suggests that reptiles may still contain a significant and largely uncharacterised level of chlamydial genetic diversity that requires further investigation.

1. Introduction

The *Chlamydiae* are a unique phylum of intracellular bacteria that are ubiquitous in the environment and are able to infect and cause disease in a wide range of hosts. In recent years there has been an expansion of this phylum and, as such, it now consists of nine families including a range of novel "*Chlamydia*-like" organisms that share obligate intracellular lifestyles and a similar developmental cycle but otherwise a diverse range of morphologies, host specificities and disease outcomes (Taylor-Brown et al., 2014).

Chlamydiosis has been described in both free-ranging and captive reptilian hosts including puff adders, boas, chameleons, crocodiles, frogs and tortoises (Homer et al., 1994; Bodetti et al., 2002; Jacobson et al., 2002; Soldati et al., 2004; Hotzel et al., 2005; Huchzermeyer et al., 2008). The infection typically manifests as inflammatory lesions in affected organs (granulomatous inflammation) (Howerth, 1984; Homer et al., 1994). Organs commonly affected are the spleen, heart, lung and liver, and diagnosis is usually on the basis of visualisation of the inclusions by light or electron microscopy. In many cases, proliferative pneumonia was observed prior to euthanasia or natural death of reptiles (Bodetti et al., 2002), and limited cases have reported inflammation or lesions in the gastrointestinal tract, wasting disease, necrotising mycocarditis, necrotising enteritis and splenitis (Bodetti et al., 2002; Jacobson et al., 2002; Jacobson et al., 2004; Cope et al., 2014).

Although some studies identified *Chlamydia psittaci* (Huchzermeyer et al., 1994; Robertson et al., 2010) or novel *Chlamydia*-like organisms (Soldati et al., 2004) as the aetiological agent in reptilian

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chlamydiosis, *Chlamydia pneumoniae* appears to the most widespread (Bodetti et al., 2002; Vlahovic et al., 2006). However, while human and marsupial *C. pneumoniae* strains are well characterised (Roulis et al., 2013), reptilian strains are not.

Detection of asymptomatic carriers in association with a lethal case of granulomatous inflammation of the heart, liver and splenopancreas of a horned viper (*Vipera ammodytes ammodytes*) caused by *C. pneumoniae* prompted the investigation and characterisation of the diversity of chlamydiae strains circulating in clinically inconspicuous captive snakes in Switzerland.

2. Methods

2.1. Snake collections

The six private collections (arbitrarily named collections 1–6), included in this study, contributed samples from 51, 11, 17, 17, 17 and 24 exotic snakes, respectively. Collections 1 and 4 provided samples from snakes belonging to the families *Viperidae, Colubridae, Pythonidae* and *Elapidae*. Collections 5 and 6 included snakes belonging to *Boidae* and *Pythonidae* families, while collection 6 also included Vipers. Collections 2 and 3 provided samples from Vipers only. Owners 1–3 trade snakes while the other owners and their collections have no contact. One snake, in collection 1, was sampled at two time points (Table 2).

2.2. Sample collection and DNA extraction

An arbitrary selection of 137 snakes from six collections (272 samples in total) was sampled by swabbing the cloaca and choana and extracting DNA according to Borel et al. (2008).

2.3. Chlamydial screening

A *Chlamydiaceae* family-specific qPCR targeting the 23S rRNA gene was carried out in 96-well microtitre plates on all samples ($n = 272$), and positive samples were then subjected to an ArrayTube Microarray targeting 23S rRNA (described in Ehricht et al., 2006). The AT Microarray chip carried 36 *Chlamydiales* probes, five positive controls and one internal staining control. Sample DNA was amplified and biotin labelled prior to hybridisation according to Borel et al. (2008). Hybridisation patterns were assigned on the basis of the most intense signal, with the provision that all other probes of the same species were among the 10 most intense signals.

A PCR targeting the 298 bp *Chlamydiales* signature sequence of the 16S rRNA gene was conducted on 23 samples using 16SIGF and 16SIGR primers (Everett et al., 1999), followed by amplicon sequencing. One sample corresponding to each genotype was chosen for near full length (approximately 1400 bp) 16S rRNA sequencing using the 16SIGF primer paired with the 16SB1 primer (Hosokawa et al., 2006), followed by amplicon sequencing.

2.4. Phylogenetic analysis

Resulting sequences were first compared to chlamydial sequences previously deposited in GenBank using BLAST (Altschul et al., 1990), then further analysis was conducted in Geneious v.7.1 (Kearse et al., 2012). Sequences were aligned using the Geneious algorithm, and phylogenetic trees were constructed using MrBayes, with a HKY85 substitution model.

3. Results

A total of 137 snakes from six collections were tested at both the cloaca and choana to ascertain the presence and diversity of chlamydial infections in captive snakes in six collections in Switzerland.

3.1. Detection of chlamydiae in captive snakes

The presence of chlamydiae in this population of snakes ranged from 5.9% to 33.3% in the six collections (Table 1a). Initially, a *Chlamydiaceae* specific qPCR and *Chlamydia* species-specific ArrayTube microarray were conducted to screen for the presence of chlamydiae. By qPCR, 48 samples from 29 animals were positive for *Chlamydiaceae*, and of these, the chlamydial species could be identified in 43 samples by ArrayTube microarray (Table 1b). By *Chlamydiaceae* qPCR, four snakes were positive at the choana only, six snakes were positive at the cloaca only and 19 snakes were positive at both sites. By ArrayTube, six animals were positive at the choana only, seven were positive at the cloaca only and 15 were positive at both sites (Table 1b). By *Chlamydia* ArrayTube microarray, the hybridisation pattern indicated clear species identity of *C. pneumoniae* at the cloaca of six animals, the choana of four animals and at both sites in 13 animals, *Chlamydia muridarum* at both sites from one snake and the genus *Chlamydia* (chlamydial species could not be determined) in three samples from two animals. An additional four samples from four animals (two choana and two cloaca samples) had inconclusive or uncharacteristic AT hybridisation patterns (Table 1b). *C. pneumoniae* was detected in all six collections, and it was the sole *Chlamydia* detected in collections 3, 4 and 6. No chlamydia was detected in snakes belonging to the *Elapidae*.

3.2. Chlamydial identity by PCR and sequencing

Further molecular characterisation of chlamydial strains circulating in these six collections was performed. A 279–286 bp sequence could be fully resolved from 21 of the 23 samples following direct sequencing of the PCR product in both directions. BLAST-n analysis revealed that for 20 of these sequences, closest sequence similarity (95.0–99.0% nucleotide identity) could be found to one of the two 'Uncultured *Chlamydia*' sequences (accession numbers GQ507439.1 and GQ507442.1), *C. pneumoniae* strains or a chlamydial sequence isolated from a tortoise (accession number AY845424.1; Table 2). The last sequence shared 99.0% nucleotide identity with *C. muridarum* (Table 2). No sequences shared 100% nucleotide similarity with any sequences in the database.

Multiple sequence alignment of these short novel sequences against other representative 16S rRNA sequences from species in each chlamydial family lead to the designation of seven unique genotypes from the 21 PCR amplified sequences (genotypes 1–7).

Two unique sequences (genotypes 1 and 2) were found in seven samples each, differing from each other by one base. These sequences were both most similar to 'Uncultured *Chlamydia*' isolates (97.5–98.5%) and *C. pneumoniae* (97.9–98.5%). A third

Table 1a
The presence of chlamydial infection in captive snakes from six collections in Switzerland.

Collection	Number of snakes sampled	Number of samples	Number of samples positive by qPCR	Number of samples positive by AT
1	51	98	18	17
2	11	22	5	3
3	17	34	9	9
4	17	34	1	1
5	17	36	11	11
6	24	48	4	4
Total	137	272	48	45