**The novel ‘*Candidatus* Amphibiichlamydia ranarum’ is highly prevalent in invasive exotic bullfrogs (*Lithobates catesbeianus*)**

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## Summary

**Knowledge concerning microbial infectious diseases in the current amphibian crisis is rudimentary and largely limited to ranavirosis and chytridiomycosis. The family *Chlamydiaceae* is gaining attention as a common cause of disease in amphibians and may harbour new and emerging amphibian pathogens. We identified a novel species of Chlamydiales (Candida- tus *Amphibiichlamydia ranarum*) with a prevalence of 71% in exotic invasive bullfrog tadpoles (*Lithobates catesbeianus*) from an introduced population in the Netherlands. The sequence of a 1474 bp 16S rRNA gene fragment showed that the novel taxon forms a well-defined clade with ‘*Candidatus* Amphibiichlamy- dia salamandrae’ within the *Chlamydiaceae* family. Although none of the tadpoles examined showed signs of clinical disease, urgent evaluation of its pathogenic potential for native amphibian species is required.**

## Introduction

Exotic invasive bullfrogs (*Lithobates catesbeianus*) are considered to exert a considerable negative impact on native amphibian communities (Lever, 2003; Ficetola *et al*., 2007). Bullfrogs are a notorious source of the infec- tious diseases chytridiomycosis and ranavirosis, affecting amphibian populations globally (Garner *et al*., 2006; Sharifian-Fard *et al*., 2011). However, little is known regarding the presence of other microbial agents that might be transferred to native amphibians. The family *Chlamydiaceae* is gaining attention as a common cause of disease in amphibians and is associated with high mortality rates in a variety of amphibian species. Of the order Chlamydiales, the presence of the species *Chlamydia psittaci, C. pneumoniae, C. abortus, C. suis* and the novel genus ‘*Candidatus* Amphibiichlamydia salamandrae’ has been demonstrated in amphibians (Newcorner *et al*., 1982; Wright, 1996; Mutschmann, 1998; Berger *et al*., 1999; Reed *et al*., 2000; Hotzel *et al*., 2001; Blumer *et al*., 2007; Martel *et al*., 2012). This new genus may represent a clade within the chlamydiae adapted to amphibians, pos- sibly containing new and emerging amphibian pathogens. In this study, we examined whether and which species of Chlamydiales occur in invasive and reproducing exotic bullfrog populations in Belgium (Hoogstraten, Antwerp)

and the Netherlands (Baarloo, Noord Limburg).

## Results and discussion

Using PCR for the detection of the 16S rRNA gene of Chlamydiales (Everett *et al*., 1999), we demonstrated the presence of Chlamydiales DNA in 71% of the 200 livers from Dutch tadpoles examined but in none of those from the 200 Belgian tadpoles. A 1473 bp 16S rRNA gene fragment was sequenced from 10 of these tadpoles using the primers 16SF and 16SR, described by Everett (2000). This sequence was identical for all tadpoles and shared more than 90% nucleotide identity with known members of the order Chlamydiales and 91% identity with the ref- erence 16S rRNA gene sequence of *C. abortus* B577 (Accession No. D85709).

Immunofluorescently stained (IMAGEN Chlamydia kit, Oxoid, Basingstoke, UK) impression smears from tadpole

**Fig. 1.** Immunocytochemically stained (IMAGEN Chlamydia kit, Oxoid, Basingstoke, UK) impression smears from tadpole livers.

livers revealed the sporadic presence of strongly fluores- cent aggregates associated with anuran cells in the liver in 7/20 (35%) of the livers examined, resembling Chlamy- diales in host cells (Fig. 1). PCR confirmed the presence of Chlamydiales DNA in these livers that showed positive fluorescent staining. An additional 10/20 livers were also PCR positive but did not yield a positive result on immu- nofluorescent (IF) staining. The three remaining IF nega- tive samples were also PCR negative. Excision of 100 fluorescent particles using laser capture microdissection (Vandewoestyne *et al*., 2012) with subsequent PCR and sequencing confirmed colocalization of the novel Chlamy- diales DNA sequence with fluorescent particles. Despite intensive searching, transmission electron microscopic (TEM) examination of PCR and IF positive samples did not result in the detection of Chlamydiales like organisms. In amphibians infected with the closely related Candida- tus *Amphibiichlamydia salamandrae*, the presence of Chlamydiales like organisms in the host cells could be clearly demonstrated using TEM and IF revealed the pres- ence of numerous positively stained inclusions in the host cells. These animals exhibited marked clinical signs (Martel *et al*., 2012). The absence of clinical signs, low numbers of IF positive cells and absence of relevant TEM findings suggest low-level infections in the tadpoles or perhaps even symbiosis in the present study (Horn, 2008). However, the consistent PCR and sequencing find- ings combined with IF demonstrate the presence of a novel Chlamydiales taxon, despite the lack of relevant morphological data. Based on the sequences obtained, the novel taxon can be identified as a member of the *Chlamydiaceae* (Everett *et al*., 1999), with highest 16S rRNA similarity to ‘*Candidatus* Amphibiichlamydia sala- mandrae’ (95%). Neighbour-joining analysis (Kodon; Applied Maths, Sint-Martens-Latem, Belgium) showed that, together with ‘*Candidatus* Amphibiichlamydia sala- mandrae’, the novel taxon forms a distinct branch in the well-supported monophyletic clade with the genera *Chlamydia* and *Candidatus* Clavochlamydia salmonicola (family *Chlamydiaceae*) (Fig. 2). The discovery of this

second species suggests the genus *Amphibiichlamydia* to contain species adapted to amphibians. Further studies should focus on the ability of this genus to cross verte- brate taxon barriers as recently shown for *C. pneumoniae* (Mitchell *et al*., 2010).

The presence of the novel taxon in the bullfrogs tad- poles was not associated with obvious clinical disease, resembling results obtained by Blumer and colleagues (2007), who found a prevalence of 2.5% of Chlamydiales in 126 *Rana temporaria* without a clear link with disease. However, its closest relative ‘*Candidatus* Amphibiichlamy- dia salamandrae’ has been associated with severe disease and high mortality (Martel *et al*., 2012). Infection trials using an isolate of the novel taxon should clarify its pathogenic potential to bullfrogs and native amphibian species. However, attempts to isolate this novel species on a variety of cell lines have failed so far. Until the pathogenic potential of these novel Chlamydiales is evaluated, we recommend to include diagnostics for Chlamydiales in any outbreak of amphibian disease, both in captive and in wild populations. Indeed, this study dem- onstrates our current lack of knowledge of amphibian pathogens, other than the few well-studied ones such as chytridiomycosis or ranavirosis (Duffus, 2009).

**Description of ‘*Candidatus* Amphibiichlamydia ranarum’**

‘*Candidatus* Amphibiichlamydia ranarum’ [Am.phi. bi.i.chla.my’dia. N.L. n. Amphibia name of host class; L. fem. n. *Chlamydia* name of bacterial taxon; N. L. fem. n. *Amphibiichlamydia Chlamydia* from an amphibian; ra.na’rum L. gen. n. from frogs].

The provisional taxon ‘*Candidatus* Amphibiichlamydia ranarum’ contains cell-associated bacteria that infect frogs of the genus *Lithobates* in freshwater environments. The 16S rRNA gene of ‘*Candidatus* Amphibiichlamydia ranarum’ has been deposited in the GenBank with Acces- sion number JN402380 and shows phylogenetic affinity towards the family *Chlamydiaceae*.

26 24 22 20 18 16 14 12 10 8 6

4 2 0

# Candidatus Fritschea eriococci AY 140911 Candidatus Fritschea bemisae AY 140910 Simkania negevensis U68460

100.00

100.00

99.6

100.00

100.00

100.00

87.9 100.00

41.5

100.00

87.9

90.9

96.7

100.00

55.0

57.5

100.00

100.00

63.7

100.00

44.7

93.6

97.2

100.00

Candidatus Rhabdochlamydia porcellionis AY 223862 Candidatus Rhabdochlamydia crassificans AY 928092 Candidatus Clavochlamydia salmonicola EF577392 Candidatus Amphibiochlamydia ranarum JN402380 Candidatus Amphibiichlamydia salamandrae JN392919 Candidatus Amphibiichlamydia salamandrae JN392920 Chlamydia muridarum D85718

Chlamydia suis U73110 Chlamydia trachomatis D89067 Chlamydia psittaci AB001778 Chlamydia abortus AB001783 Chlamydia caviae D85708

Chlamydia felis D85701

JN402380

# Chlamydia pneumoniae L06108

Chlamydia pecorum D88317 Estrella lausannensis EU074225

Criblamydia sequanensis DQ124300 Waddlia malaysiensis AY 184804 Waddlia chondrophila AF042496 Parachlamydia acanthamoebae Y 07556 Protochlamydia naegleriophila DQ632609 Protochlamydia amoebophila AF083615 Neochlamydia hartmannellae AF177275

Candidatus Piscichlamydia salmonis AY 462244 Verrucomicrobium spinosum X90515

**Fig. 2.** Topology of the novel species ‘C*andidatus* Amphibiichlamydia salamandrae’ within the phylogenetic tree obtained by neighbour joining and based on 16S rRNA gene data from representative species. Maximum parsimony and UPGMA analyses yielded cladograms with the same topology (results not shown). Numbers show the percentage of times each branch was found in 1000 bootstrap replicates. The tree has been rooted with *Verrucomicrobium spinosum* as outgroup.

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