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Minireview

MINIREVIEW

Twenty years of research into *Chlamydia*-like organisms: a revolution in our understanding of the biology and pathogenicity of members of the phylum *Chlamydiae*

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One sentence Summary: This manuscript reflects on our progress in understanding the biology, evolution, adaptation and pathogenesis of Chlamydia-like organisms, 20 years following their initial identification and description.
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ABSTRACT

Chlamydiae are obligate intracellular bacteria that share a unique but remarkably conserved biphasic developmental cycle that relies on a eukaryotic host cell for survival. Although the phylum was originally thought to only contain one family, the *Chlamydiaceae*, a total of nine families are now recognized. These so-called *Chlamydia*-like organisms (CLOs) are also referred to as 'environmental chlamydiae', as many were initially isolated from environmental sources. However, these organisms are also emerging pathogens, as many, such as *Parachlamydia* sp., *Simkania* sp. and *Waddlia* sp., have been associated with human disease, and others, such as *Piscichlamydia* sp. and *Parilichlamydia* sp., have been documented in association with diseases in animals. Their strict intracellular nature and the requirement for cell culture have been a confounding factor in characterizing the biology and pathogenicity of CLOs. Nevertheless, the genomes of seven CLO species have now been sequenced, providing new information on their potential ability to adapt to a wide range of hosts. As new isolation and diagnostic methods advance, we are able to further explore the richness of this phylum with further research likely to help define the true pathogenic potential of the CLOs while also providing insight into the origins of the 'traditional' chlamydiae.

Key words: chlamydiae; bacterial pathogenesis; amoeba; genomics; phylogeny

INTRODUCTION

The *Chlamydiae* are an assemblage of bacteria that are united by their unique developmental cycle and obligate intracellular lifestyle. *Chlamydiae* depend on a eukaryotic host cell for their replication, which takes place in an inclusion inside the host cell, and for their dispersal, occurring following cell lysis. The genus *Chlamydia* remains the most widely studied of the *Chlamydiae*, as until the 1990s it was thought to be the only

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family of the order *Chlamydiales* (Kahane *et al.*, 1993; Everett, Bush and Andersen 1999), and is comprised of 11 species that are well-recognized pathogens of humans and animals. *Chlamydia trachomatis* is the leading cause of trachoma, which can lead to blindness if left untreated (Taylor *et al.*, 2014). The same organism is also the most prevalent cause of sexually transmitted diseases worldwide (Bebear and de Barbeyrac 2009). Another human pathogen, *C. pneumoniae*, causes respiratory infections in humans, but can also cause disease in a range of animals including horse and frogs (Roulis, Polkinghorne and Timms 2013). Blindness and infertility caused by *C. pecorum* have contributed to the decline in koala populations (Polkinghorne, Hanger and Timms 2013) and this same pathogen can cause arthritis in cows and sheep (Fukushi and Hirai 1992). While some chlamydial species are specific to their hosts, others, such as *C. abortus* and *C. psittaci*, pose a zoonotic threat (Beeckman and Vanrompay 2009).

While our knowledge of the diversity and significance of members of the family *Chlamydiaceae* has been well established thanks to more than 50 years of intensive biological and medical research, studies over the last 20 years have also revealed that this family only represents the 'tip of the iceberg' in terms of diversity within the phylum *Chlamydiae*. In this, we are referring to the recent explosion of the description of eight additional families of genetically related obligate intracellular bacteria including (i) the most well documented, the *Parachlamydiaceae*, consisting of five genera [*Parachlamydia* (Amann *et al.*, 1997; Everett *et al.*, 1999), *Neochlamydia* (Horn *et al.*, 2000), *Protochlamydia* (Collingro *et al.*, 2005b), *Mesochlamydia* (Corsaro *et al.*, 2013) and *Metachlamydia* (Corsaro *et al.*, 2010)] that have been detected in a wide range of hosts; (ii) *Waddliaceae*, a monophyletic family, containing two species, *Waddlia chondrophila* (Rurangirwa *et al.*, 1999) and *W. malaysiensis* (Chua *et al.*, 2005); (iii) the *Simkaniaceae*, containing four reported species (*Simkania negevensis* (Kahane *et al.*, 1993), *Fritschea bemisiae*, *F. eriococci* (Thao *et al.*, 2003; Everett *et al.*, 2005) and the recently proposed *Syngnamydia venezia* (Fehr *et al.*, 2013); (iv) the *Rhabdochlamydiaceae*, containing two species in the genus *Rhabdochlamydia* (Kostanjsek *et al.*, 2004; Corsaro *et al.*, 2007), as well as an additional species *Renichlamydia lutjani* (Corsaro and Work 2012) among a number of uncultured isolates; (v) the *Criblamydiaceae* contains two genera, *Estrella* and *Criblamydia*, both recovered from river water (Thomas, Casson and Greub 2006; Lienard *et al.*, 2011b); and (vi) the *Piscichlamydiaceae* (Draghi *et al.*, 2004), *Clavichlamydiaceae* (Karlsen *et al.*, 2008) (originally denoted as *Clavochlamydiaceae*) and *Parilichlamydiaceae* (Stride *et al.*, 2013b), three families whose members have been isolated solely from fish.

Formally and informally, these new families are often collectively referred to as '*Chlamydia*-like organisms' (CLOs), '*Chlamydia*-related bacteria' or 'environmental chlamydiae'. These names have historical precedence but also significant shortcomings. For example, the use of the term '*Chlamydia*-like' is due to the fact that the chlamydial developmental cycle is remarkably conserved across the phylum *Chlamydiae* and indeed similar to the *Chlamydia* genus. However, there are some significant biological differences between the different members of the phylum. Equally, the term 'environmental chlamydiae' does not do them justice, as although most of the founding isolates from each new CLO species have come from environmental samples (Kahane *et al.*, 1993; Amann *et al.*, 1997; Collingro *et al.*, 2005b; Thomas *et al.*, 2006; Lienard *et al.*, 2011b), as will be discussed in this review, several species clearly do cause disease in both humans and animals. For the purpose of this review, we will nevertheless collectively refer to these organisms as CLOs.

Research efforts by an initially small but growing number of research groups around the world have revealed a previously unprecedented range of terrestrial and aquatic hosts that can be infected by CLOs, ranging from humans and warm-blooded terrestrial vertebrates to fish, reptiles, amphibians and down to eukaryotic microorganisms such as amoeba. The diverse host range of CLOs has had an unfortunate consequence for their study, as their apparently strict intracellular nature has prevented culturing for most of the species that infect 'non-model' organisms. As a result, many taxa still retain their candidatus status.

It has been estimated that the members of the genus *Chlamydia* (*Chlamydiaceae* family) and the CLOs (*Parachlamydiaceae* family) diverged more than 700 million years ago, from a last common ancestor that also resided within a host cell (Greub and Raoult 2003). This realization has meant that molecular and cell biology studies of CLOs should be viewed as an opportunity to 'look into the window of the past' for members of the *Chlamydiaceae*. Indeed, cell biology studies and a growing number of genome sequence efforts have revealed amazing new insights into the adaptations that chlamydiae have evolved to survive and subvert their diverse host cell environments.

Given the significant advances in our understanding of the diversity of CLOs since their first description nearly 20 years ago, this review will provide an opportunity to reflect on the discovery of each new CLO and examine current knowledge on their biology, phylogeny and genetics. This review will also discuss the potential role of CLOs in human and animal disease and highlight potential avenues for further exploration of these unique, ubiquitous and enigmatic organisms.

HISTORICAL OVERVIEW OF CLO RESEARCH

Given the relatively recent (20 years) description of CLOs, we have elected to describe the most significant events in the description and classification of the CLOs chronologically (Fig. 1).

Initial isolation and identification of CLOs

The first CLO to appear in the literature was a previously undescribed obligate intracellular bacterium (WSU-86-1044^T) isolated from an aborted bovine foetus (Dilbeck *et al.*, 1990). The organism exhibited a development cycle and two distinct morphological forms that resembled that of *Chlamydiae* (Kocan *et al.*, 1990), but did not share antigenic determinants with *Chlamydiae*. As such, it was suggested to belong to the *Rickettsiae*, until further taxonomic assignment could be given.

Three years later, a cell-culture contaminant able to grow in a range of cultured cells and possessing a 5–7 day developmental cycle with morphological characteristics similar to that of *Chlamydia* was described as microorganism 'Z' (Kahane *et al.*, 1993). In 1995, the 16S rRNA encoding gene of 'Z' was shown to be 83% identical to members of the *Chlamydiaceae*, and thus 'Z' was postulated to belong to a novel genus, *Simkania* (Kahane, Metzer and Friedman 1995). Human exposure to this organism was subsequently shown to be widespread (Kahane *et al.*, 1998; Friedman *et al.*, 1999).

Shortly following the description of 'Z', clinical and environmental *Acanthamoeba* specimens were found to be infected with organisms physiologically resembling *Chlamydiae* (Fritsche *et al.*, 1993; Amann *et al.*, 1997). Using the approach taken by Kahane *et al.* for 'Z', the authors demonstrated 86–87% 16S rDNA nucleotide similarities with other chlamydiae, and this organism was proposed as 'Candidatus Parachlamydia

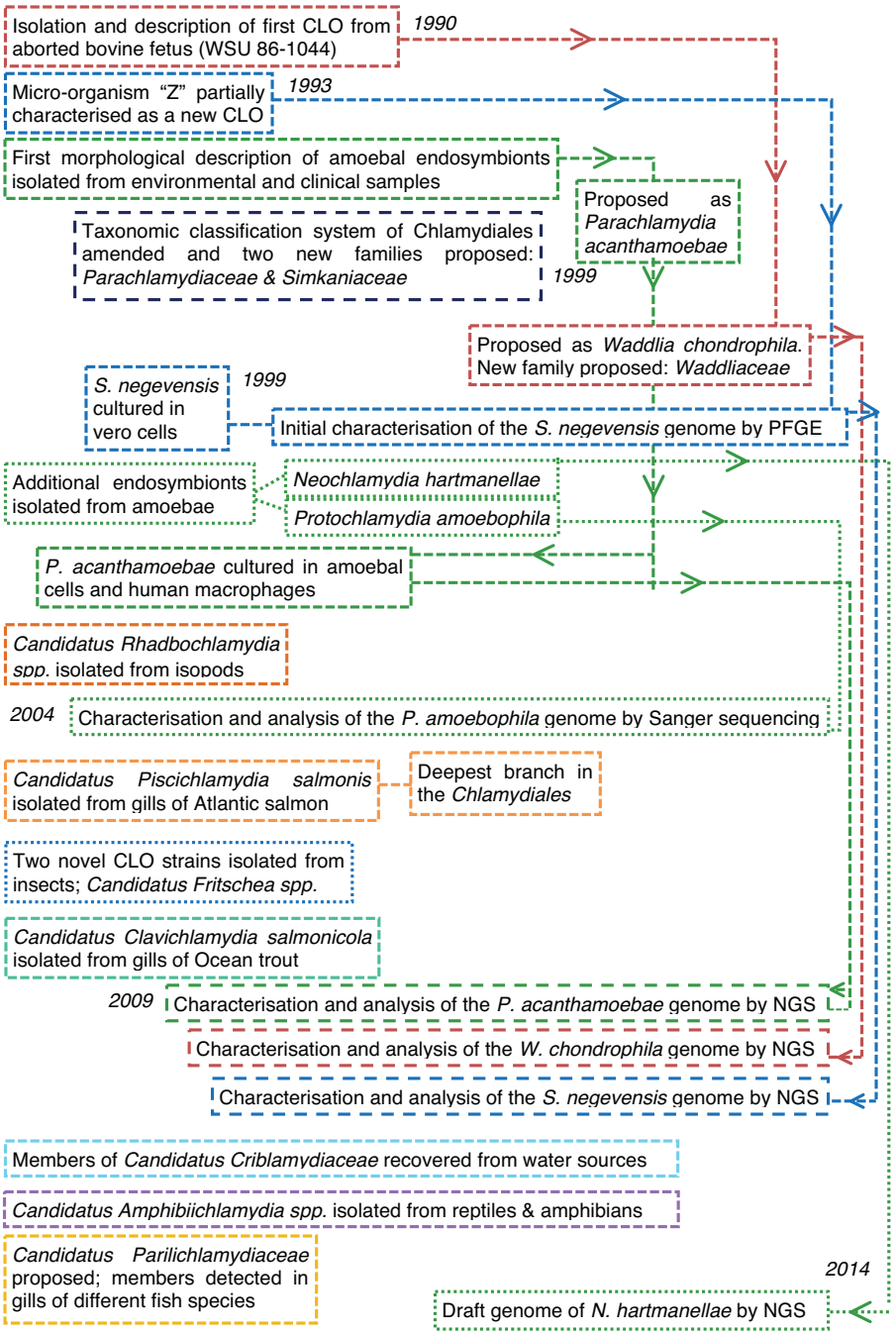

Figure 1. Significant events in the detection, isolation and identification of CLOs over the last 20 years. Genome sequencing events are also included. Boxes and lines are coloured based on families: red, *Waddliaceae*; blue, *Simkaniaceae*; green, *Parachlamydiaceae*; dark orange, *Rhabdochlamydiaceae*; pale orange, *Piscichlamydiaceae*; yellow, *Parilichlamydiaceae*; pale green, *Clavichlamydiaceae*; pale blue, *Criblamydiaceae*. Dashed lines represent events regarding the type strains of these families, while dotted lines represent events regarding other species in these families. Arrowed lines join events corresponding to the same family. Some years are included for context. PFGE, Pulsed-field gel electrophoresis; NGS, Next-generation sequencing.

acanthamoebae' (Everett *et al.*, 1999). The discovery of this bacterium suggested for the first time that amoebae could potentially act as environmental reservoirs for pathogens belonging to the *Chlamydiae* phylum.

Amended taxonomic classification of *Chlamydiae*

The identification and description of multiple strains related to but distinct from members of the *Chlamydiaceae* prompted an amendment to the taxonomic classification system for the *Chlamydiae*, and this system separated 'Z', WSU-86 and *P. acanthamoebae* from the *Chlamydiaceae* at the family level. As such, these strains became the type species for three new families: *S. negevensis* as the type species for the genus *Simkania* and family *Simkaniaceae* (Everett *et al.*, 1999; Kahane *et al.*, 1999), *W. chondrophila* as the type species for the family *Waddliaceae* (Rurangirwa *et al.*, 1999) and *P. acanthamoebae* as the type species for *Parachlamydiaceae* (Everett *et al.*, 1999) (Figs 1 and 2).

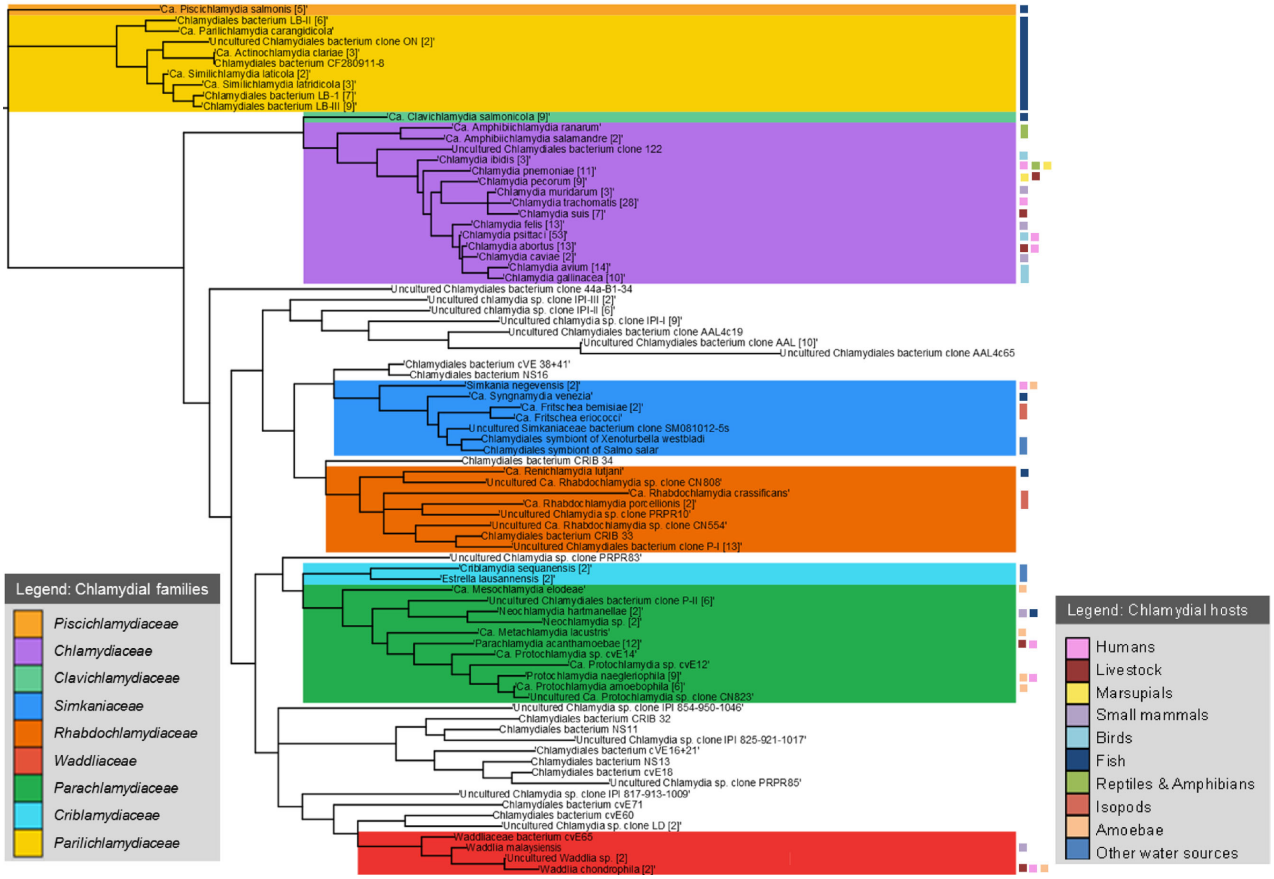

Figure 2. Order *Chlamydiales* phylogenetic tree based on near-full-length 16S rRNA gene sequences. Sequences for the 16S rRNA gene were obtained from Genbank, NCBI. Sequences longer than 1100 bp were included for analysis. Unclassified isolates with sequences >1100 bp were also included to capture the entire chlamydial diversity. Consensus sequences of related isolates were generated prior to alignment in Geneious, and the number of sequences used for each consensus sequence is represented in square brackets. Phylogenetic tree was generated in Geneious; MrBayes tree using a HKY85 substitution model. Chlamydial families were coloured using iTOL (see the legend). Coloured squares or rectangles represent chlamydial hosts or sources which these organisms have been isolated from (see the legend). Uncoloured clades represent the diversity of the as yet uncharacterized sequences, which are mostly comprised of environmental isolates.

The year 1999 marked a watershed, with an explosion of publications describing CLOs in more depth than seen previously. The initial genetic characterization of the first CLO, *S. negevenis* strain ZT (Kahane *et al.*, 1999) followed culture in Vero cells, which coincided with the culturing of *W. chondrophila* (WSU-86) and the description of its 16S rRNA gene sequence (Rurangirwa *et al.*, 1999). Given the diversity of hosts and ecological niches CLOs had been isolated from, researchers were prompted to further investigate the presence of these bacteria in other amoebae and subsequently, other potential reservoirs. Additional amoebal endosymbionts were thus described and classified into the *Parachlamydiaceae* (*Neochlamydia* sp., *Protochlamydia* sp.), and, soon after their initial description, they were shown to be able to survive and replicate in both amoebal cells and human macrophages (Greub, Mege and Raoult 2003). Concomitantly, new species were identified from an arthropod: *Ca.* Rhabdochlamydia porcellionis (Kostanjsek *et al.*, 2004), originally thought to be a *Rickettsia* (Radek 2000) and a fish, *Ca.* Piscichlamydia salmonicola (Draghi *et al.*, 2004) and these formed new, distinct lineages in the *Chlamydiales*, with *P. salmonis* representing the deepest branch (Fig. 2). In 2005, two novel strains that were identified in cattle-associated insects were described as a new genus (*Ca.* Fritschea) in the *Simkaniaceae* (Everett *et al.*, 2005).

Water sources have proven to be a rich source of CLO diversity, with an additional two species described in 2006, *Criblamydia sequanensis* (Thomas *et al.*, 2006), and 2011, *Estrella lausannensis* (Lienard *et al.*, 2011b), both part of a novel family, the *Criblamydiaceae*. Moreover, two new members of the *Parachlamydiaceae* family were also retrieved from water samples (Corsaro *et al.*, 2010, 2013). Fish have also been found to play host to a number of CLOs, and these have been characterized over the last 10 years (genera *Piscichlamydia, Clavichlamydia, Renichlamydia, Parilichlamydia, Similichlamdyia and Syngnamydia)* (Draghi *et al.*, 2004; Karlsen *et al.*, 2008; Corsaro and Work 2012; Fehr *et al.*, 2013; Stride *et al.*, 2013a,b,c).

CLOs in the genomics era

In the absence of genome sequencing data, a direct result of the non-cultivable status of most of these CLOs, 16S rRNA gene sequencing was the most widely used tool for phylogenetic analyses (Fig. 2), with nearly all genetic information on CLOs limited to sequencing of near full-length fragments of this ribosomal RNA gene. This changed in 2004 with the sequencing of the genome of *P. amoebophila* (Horn *et al.*, 2004) which, as will be discussed later, presented the first opportunity for researchers to pry open the genetic secrets of CLOs. The genomes of the

type species of *Parachlamydiaceae*, *Waddliaceae* and *Simkaniaceae* were subsequently characterized and analysed in 2009, 2010 and 2011 (Fig. 1), respectively (Greub *et al.*, 2009; Bertelli *et al.*, 2010; Collingro *et al.*, 2011), and bioinformatics methods have allowed the 'Pan-genome' of the *Chlamydiae* to be investigated (Collingro *et al.*, 2011). Mostly recently, the draft genome of *Neochlamydia hartmanellae* was made available (Ishida *et al.*, 2014), making the *Parachlamydiaceae* the best described CLO family to date.

BIOLOGICAL DIVERSITY OF CLOs

Growth, morphology and developmental cycle

The existence of a unique biphasic developmental cycle is common among all chlamydiae. The simplified developmental cycle begins with endocytosis of the infectious elementary bodies (EBs) into the host cell. Here, they reside within a cytoplasmic inclusion, which facilitates condensation of the DNA and conversion from the EB to the reticulate body (RB). The RBs replicate and de-differentiate back to EBs, lysing the inclusion and host cell, perpetuating the infectious cycle.

Members of the *Parachlamydiaceae* have a particular preference for intracellular growth in free-living amoebal hosts from environmental origins. The developmental cycle and growth of several species in the family *Parachlamydiaceae* have been well studied revealing that (i) developmental stages of *P. acanthamoebae* appear to be similar to that of other chlamydiae, (Greub and Raoult 2002), (ii) they can be endosymbiotic or lytic to free-living amoeba, depending on temperature (Greub, La Scola and Raoult 2003) and (iii) they are capable of growing in a range of cells from amoebae to human macrophages and pneumocytes (Greub *et al.*, 2003; Casson *et al.*, 2006) (Fig. 3). Additionally, a third developmental body has been described for these CLOs; the crescent bodies are reported to be an infectious stage akin to EBs and are associated with prolonged incubation time (Greub and Raoult 2002). The different morphology is thought to reflect a different composition of the parachlamydial cell wall compared to that of the *Chlamydiaceae* (Greub and Raoult 2002). Unique to this family, *P. acanthamoebae, P. amoebophila* and *N. hartmanellae* have also been observed outside the inclusion, residing in the cytoplasm of their amoebal hosts (Greub and Raoult 2002).

Cell biology studies have revealed that (i) *W. chondrophila* can proliferate within human macrophages and induce cell lysis (Goy and Greub 2009) (Fig. 3), rapidly evading host cell endocytic pathways (Croxatto and Greub 2010), and (ii) host cell mitochondria are recruited to the *Waddlia*-containing vacuoles along with the endoplasmic reticulum (ER), suggestive of a requirement of these organelles in *Waddlia* intracellular replication (Croxatto and Greub 2010). Interestingly, no cytopathic effect was observed in endometrial cells, but this appears to be replaced by the development of aberrant bodies (Kebbi-Beghdadi, Cisse and Greub 2011), a biological phenomenon that was also observed when *Waddlia* bacteria were treated with penicillin derivatives, suggesting that chlamydial peptidoglycan is important for bacterial division (Jacquier *et al.*, 2014). Besides amoebal cells, *W. chondrophila* replicates well in fish epithelial and gonad cell lines exhibiting a 2–6 day lifecycle (Kebbi-Beghdadi, Batista and Greub 2011), and is also capable of replicating in ovine trophoblast cells, where it elicits an inflammatory immune response (Wheelhouse *et al.*, 2014), as well as Vero cells and pneumocytes (Kebbi-Beghdadi *et al.*, 2011). Studies such as these provide strong evidence for a pathogenic role of these bacteria in animals and humans.

Similar to *W. chondrophila*, *S. negevensis* interacts with the infected host cell ER forming a single vacuolar system between the bacteria-containing vacuole and this organelle (Mehlitz *et al.*, 2014). This process appears to be directly regulated by the bacteria's interference of stress signalling pathways of the ER. *Simkania negevensis* has been successfully cultured in a range of epithelial and endothelial cell lines (Kahane *et al.*, 1999, 2007), and demonstrates a significantly longer developmental cycle time than the *Chlamydiaceae* and other CLOs, reaching a growth plateau at 2–3 days, while the cytopathic effect lasts for 12 or more days (Kahane *et al.*, 1999). Kahane, Dvoskin and Friedman (2008) have also shown transmission from *Simkania*-infected *Acanthamoeba* to a macrophage cell line, which resulted in death of the amoebae, highlighting a potential route of transmission for these CLOs. In contrast to *P. amoebophila*, *S. negevensis*

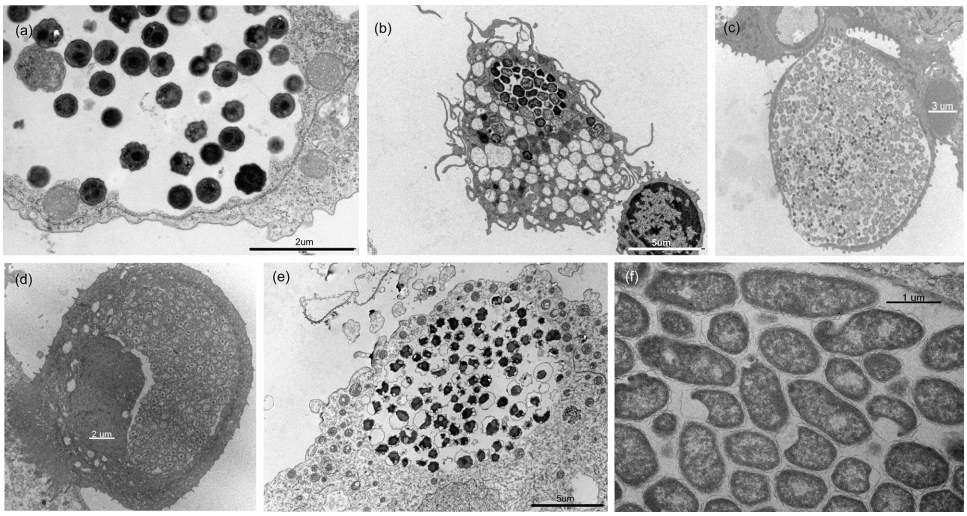

Figure 3. Electron micrographs of selected CLOs, demonstrating differences in cell morphology of EBs and RBs. *Parachlamydia acanthamoeba* (a) in *A. polyphaga* (15 000 × magnification), *W. chondrophila* (b) within a macrophage at 16 h post-infection (4500 × magnification), *Ca.* Clavichlamydia salmonicola (c) and *Ca.* Piscichlamydia salmonicola (d) in gill epithelial cells of Brown trout, *E. lausannensis* (e) within *Acanthamoeba commandonii* at 48 h post-infection (4500 × magnification) and *Ca.* Syngnamydia venezia (f) in gill epithelial cells of broad-nosed pipefish. Scale bars are shown in individual images. Note the diversity in EB and RB morphologies among different CLOs and the number of chlamydial cells in each inclusion.

survives encystment (Kahane *et al.*, 2001), a mechanism likely to assist survival and later dissemination. Another member of the *Simkaniaceae*, *S. venezia* has an ovular EB shape and shares a rippled outer membrane with other *Simkaniaceae* species (Fehr *et al.*, 2013) (Fig. 3).

Estrella lausannensis and *C. sequanensis* have been studied using amoebal co-culture (Thomas *et al.*, 2006; Lienard *et al.*, 2011b). *Estrella lausannensis*, the first member of the *Chlamydiales* to be cultured in *Dictyostelium discoideum*, a genetically tractable soil amoebae (Lienard *et al.*, 2011b), potentially provides a novel model system for studying CLO biology, given the huge numbers of mutants available. Both species exhibit star-like EBs (Fig. 3), but this has been since shown to be an artefact of fixative methods used (Rusconi *et al.*, 2013). Neither species has been successfully cultured in a mammalian cell line, but *E. lausennensis* is capable of replicating in fish cell lines, albeit poorly (Kebbi-Beghdadi *et al.*, 2011).

While most CLOs exhibit reasonably spherical EBs, members of the *Rhabdochlamydiaceae* possess distinct rod-shaped EBs that are contained by a five-layer cell wall and include translucent oblong structures in their cytoplasm (Kostanjsek *et al.*, 2004; Corsaro *et al.*, 2007). Similar to other CLOs, these species exhibit an additional intermediate body.

Host range and phylogeny

Following the initial isolation of *Simkania* sp. from cell culture contaminants, this species has been isolated from hosts as diverse as humans (Kahane *et al.*, 1998; Friedman *et al.*, 1999; Jelocnik *et al.*, 2013), amoebae (Kahane *et al.*, 2001) and reptiles (Soldati *et al.*, 2004). Two strains of an additional candidatus genera, *Ca.* Fritschea spp. have been isolated from insects (Thao *et al.*, 2003; Everett *et al.*, 2005), while water sources, including drinking water, have also been described as reservoirs for *Simkania* spp. (Kahane *et al.*, 2004, 2007). Expanding the host range of this family even further, an endosymbiont of a deuterosome has been described (Israelsson 2007), as has a novel agent of epitheliocystis in a broad-nosed pipefish (Fehr *et al.*, 2013) (Fig. 2). The varied host range of this family offers a myriad of avenues for further exploration of this family as pathogens of a range of hosts.

Members of the *Parachlamydiaceae* appear to be ubiquitous among amoebae, with amoebal hosts including *Acanthamoeba castellanii* and *A. polyphaga* (Greub and Raoult 2002). In the environment, *Parachlamydiaceae*-containing amoebae have been retrieved from activated sludge (Collingro *et al.*, 2005a), hot springs (Sampo *et al.*, 2014) and soil (Fritsche *et al.*, 1993), while clinical specimens such as corneal and nasal samples have been found to harbour *Parachlamydia* spp. (Fritsche *et al.*, 1993; Corsaro, Valassina and Venditti 2003). Two members of the *Parachlamydiaceae*, *Ca.* Metachlamydia lacustris (Corsaro *et al.*, 2010) and *Ca.* Mesochlamydia elodeae (Corsaro *et al.*, 2013) were initially isolated from aquatic amoebae and demonstrate high host specificity in contrast to other *Parachlamydiaceae*, which can grow in *Acanthamoeba* species. Interestingly, these species co-inhabit the amoebae with members of either alpha-proteobacteria or beta-proteobacteria (Corsaro *et al.*, 2010), and such close association could offer opportunities for genetic exchange.

Conversely, the taxonomic diversity of *Chlamydiae* that infect fish is a reflection of the taxonomic diversity in the hosts. Two candidatus families, *Piscichlamydiaceae* and *Parilichlamydiaceae*, represent the most distantly related members of the *Chlamydiae* phylum (Fig. 2). Interestingly, *Ca.* Clavichlamydia sp., another fish pathogen, is more closely related to the *Chlamydiaceae* than the above-mentioned families. The diversity of chlamydiae isolated from fish has also extended to the *Simkaniaceae* and *Rhabdochlamydiaceae* (Corsaro and Work 2012; Fehr *et al.*, 2013). The initial proposal that each fish species may harbour a unique lineage is likely to reflect undersampling, as *Piscichlamydia* and *Clavichlamydia* were found together in the same brown trout (Schmidt-Posthaus *et al.*, 2012). The ongoing identification of novel CLOs in fish suggests that we are only just beginning to appreciate the full host range of aquatic CLOs (Stride, Polkinghome and Nowak 2014), and, with an estimated 26 000 fish species worldwide, the potential for novel CLOs is indeed vast.

Finally, a large biodiversity of CLOs has been detected in ticks from Switzerland and Algeria (Croxatto *et al.*, 2014). A possible transmission of these bacteria by ticks however remains to be confirmed.

DNA sequencing and bioinformatics to illustrate the diversity of CLOs

Beyond the use of 16S rRNA sequencing as the first tool for the characterization, classification and phylogenetic analysis of the recently described CLOs, researchers are beginning to make use of the ever-expanding metagenomic and amplicon datasets that are available in diverse databases to discover new CLOs. Recently, Lagkouvardos *et al.* (2014) used advanced, high stringency bioinformatic analyses to interrogate over 22 000 high quality, non-redundant chlamydial 16S rRNA gene sequences found in diverse databases. Using near-full-length sequences and a conservative clustering approach, 17 family-level lineages supported by two or more isolates were identified, and this number increased to 28 when considering families represented by only one isolate. Similar analysis of the V4–V6 region of the 16S rRNA gene in over 12 000 amplicons resulted in 181 putative families each supported by at least two isolates. At the species level, a potential 1161–2276 OTUs were represented, depending on the bioinformatic method used (Lagkouvardos *et al.*, 2014).

Ecological analyses revealed the majority of OTUs belonging to marine and freshwater environments and only a small proportion arising from terrestrial environments (2%) (Lagkouvardos *et al.*, 2014). As exciting as the prospect of potentially 200 or so chlamydial families is, real progress will only ensue when we utilize this information to answer biological, genetic, ecological and evolutionary questions wherever possible. Considerations to be made when novel sequences are isolated include (i) what are the health implications for humans or animals?, (ii) is there a zoonotic risk associated with this isolation? and (iii) could this isolation represent a potential reservoir or vector? Given the isolation of a vast number of CLOs from environmental sources (water, soil) and traditional vectors (protozoa, insects, bats), there is no doubt that these sources have the capacity to act as reservoirs and/or vectors of potentially 'environmental-pathogenic' chlamydiae.

Genomics

While researchers are largely restricted by the uncultivable status of most CLOs, in its absence, the advent of next-generation sequencing (NGS) technologies has not only revolutionized our understanding of the biology of both member species in the *Chlamydiaceae* but also CLOs (Bachmann *et al.*, 2014). Members of the *Chlamydiaceae* are notable for their high degree of genomic synteny (Stephens *et al.*, 1998; Read *et al.*, 2000). On the contrary, little to no synteny is observed within the *Parachlamydiaceae*, for which the genomes of four species are available, and between the *Simkaniaceae*, *Parachlamydiaceae* and *Waddliaceae* when

Table 1. Features of sequenced genomes of CLOs compared to representatives of the *Chlamydiaceae*. Note the significant difference in genome size of the CLOs, coupled with the increased number of unique genes.

Family, strain	Genome characteristics				Reference(s)
	Size (Mbp)	Plasmid size (Kbp)	Predicted CDSs	Unique predicted CDSs	
Chlamydiaceae					
C. trachomatis D/UW-3/CX	1.0	7.5	894	255	(Stephens *et al.*, 1998)
C. pneumoniae AR39	1.2	Np^	1052	186	(Kalman *et al.*, 1999; Read *et al.*, 2000)
Simkaniaceae					
S. negevensis 'Z'	2.5	132	2519	1340	(Collingro *et al.*, 2011)
Waddliaceae					
W. chondrophila WSU 86–1044	2.1	15.5	1934	438	(Bertelli *et al.*, 2010)
W. chondrophila 2032/99	2.1	Np	2028	595	(Collingro *et al.*, 2011)
Parachlamydiaceae					
P. amoebophila UWE25	2.4	Np	2031	1093	(Horn *et al.*, 2004)
P. acanthamoebae Hall's coccus	2.9	Np	2809	Nr	(Greub *et al.*, 2009)
P. acanthamoebae UV-7	3.1	Np	2788	1277	(Collingro *et al.*, 2011)
Neochlamydia S13	3.2	Np	2832	1030	(Ishida *et al.*, 2014)

^Present in some other strains of *C. pneumoniae*
Np, Not present; Nr, Not reported

compared with the *Chlamydiaceae* (Collingro *et al.*, 2011). This lack of synteny probably reflects the evolutionary distance between these families and their different evolutionary trajectories as symbionts of protozoa and pathogens of animals (Horn *et al.*, 2004; Nunes and Gomes 2014).

CLOs possess uncharacteristically large genomes for intracellular bacteria (Table 1). Yet, despite the increase in genome size, the lengths of the coding regions and proportion of coding regions are rather conserved, with the notable exception of *P. amoebophila* which encodes more than 50 large leucine-rich proteins, which evolved by serial duplication and include some proteins exhibiting significant similarity with mammalian Nod (nucleotide-binding oligomerization domain) proteins (Eugster, Roten and Greub 2007). These large genomes likely reflect the different ecology of CLOs, which may use amoebae as a melting pot for genetic transfer (Greub 2009). Like their *Chlamydiaceae* brethren, over half of the CDSs in the CLO genomes have an as yet unknown function. Collingro *et al.* (2011) identified 560 'core genes' common across the *Chlamydiaceae* and the CLOs with genomes available. More recently, similar analysis identified members of 304 protein families to belong to the 'core' gene set of the *Chlamydiae* (Psomopoulos *et al.*, 2012), while larger genomes correlated with a higher number of unique genes (Table 1). Among the conserved genes, many are housekeeping genes that are presumably involved in the highly conserved intracellular lifestyle and unique developmental cycle (Collingro *et al.*, 2011), as well as basic genetic processing roles such as transcription and translation (Psomopoulos *et al.*, 2012). Importantly, the core gene set also contains members of all 100 clusters of orthologues, which are conserved among all intracellular bacteria. Additional species-specific proteins are predicted to be involved in transport and metabolism, and this likely correlates to a less strict or more recent obligate association with the host, and/or a vital requirement to adapt to changing conditions (Nunes and Gomes 2014).

The type three secretion system (T3SS) is a gene cassette common to several Gram-negative bacteria that confers the ability to sense eukaryotic cells and secretes effector proteins in order to fuse with the host cell membrane, and thus infect the cell via a needle-like injection mechanism. A high number of structural and chaperone components of the T3SS are conserved between the CLOs and the *Chlamydiaceae*, including inner membrane proteins and needle formation proteins (Bertelli *et al.*, 2010; Collingro *et al.*, 2011). However, the *Chlamydiaceae* genomes also encode some flagellar proteins that are missing from the CLO genomes which could have implications for intracellular survival (Collingro *et al.*, 2011). Despite many structural components of the T3SS being encoded by the CLOs, many effectors recognized in the *Chlamydiaceae* are not. Of particular interest are the Inc proteins (inclusion membrane proteins), which have virtually no homologues in other microorganisms. Just three Inc proteins are conserved throughout the *Chlamydiae* (Collingro *et al.*, 2011) and the differences in sequence homology could indicate differing degrees of virulence and/or success at infecting a range of cell types. Additionally, TARP, a translocated actin-recruiting protein, which is translocated by the T3SS and thus implicated in chlamydial invasion in the *Chlamydiaceae* (Lane *et al.*, 2008), is absent from the CLO genomes, suggesting a host cell entry mechanism distinct from that of the *Chlamydiaceae*. Taken together, the

conservation of T3SS structural proteins coupled with the lack of homologous effector proteins indicates that all chlamydiae share some mechanisms for host cell entry but differ greatly in their host survival mechanisms, perhaps reflecting the varying pressures placed on these organisms by their diverse range of host cells.

The chlamydial outer membrane is crucial for host cell adhesion and invasion. The *Chlamydiaceae* possess a unique outer membrane complex, the main component of which is the major outer membrane protein (MOMP). *Simkania* encodes 35 MOMP-like proteins, while *Waddlia* encodes 11 (Bertelli *et al.*, 2010; Collingro *et al.*, 2011). *Parachlamydia* and *Neochlamydia* are almost devoid of MOMP-like proteins, while *Protochlamydia* appears to have replaced them with structurally similar porin proteins (Heinz *et al.*, 2009, 2010). Similarly, a lower number of Pmps are encoded in all CLO genomes (Bertelli *et al.*, 2010; Collingro *et al.*, 2011; Ishida *et al.*, 2014). Despite low-sequence homologies, structural similarities show that although the outer membrane proteins are a highly diverse group of proteins, they are nonetheless conserved throughout the *Chlamydiae*. This again reflects the previously acknowledged versatility of the environmental chlamydiae and their hosts compared to the *Chlamydiaceae*, and can probably account for some of the differences seen in the EB and RB morphology.

Homologous of a number of *Chlamydiaceae* virulence factors are encoded by *Simkaniaceae*, *Parachlamydiaceae* and *Waddliaceae* (Collingro *et al.*, 2011). The *Chlamydia* protease-like activity factor, CPAF, previously thought to be common to all *Chlamydiae*, is missing from only the *Simkania* genome. As it is thought to interfere with major histocompatibility complex expression and has roles in intracellular vacuole formation (Bednar *et al.*, 2011), its absence could signify a reduced pathogenicity of *Simkania*. However, two proteases homologous to proteins present in CPAF are encoded by *Simkania*; whether these proteins could compensate for CPAF remains unknown but this evidence would support *Simkania* as a pathogen.

A sizeable number of proteins containing eukaryotic domains are seen in the *Simkaniaceae*, *Parachlamydiaceae* and *Waddliaceae* genomes. In other intracellular bacteria, the presence of eukaryotic domains has been associated with intracellular proliferation in protozoan and mammalian hosts (Habyarimana *et al.*, 2008), offering more clues to pathogenesis mechanisms of these organisms.

The expanding number of available chlamydial sequences has allowed researchers to explore the evolutionary history of the *Chlamydiae* by tracing the acquisition of particular genes. Some authors suggest that the altered gene order seen in CLOs reflects a gene reorganization following divergence of environmental and pathogenic chlamydiae. Distribution of the CDSs in the *P. amoebophila* UWE25 genome suggests gene acquisition over a number of lateral gene transfer events, most of which occurred prior to the divergence from pathogenic chlamydiae (Horn *et al.*, 2004). Gene exchanges may have been facilitated by the presence of a DNA conjugative system encoded in a genomic island (Pam100G) on the chromosome of *P. amoebophila* (Greub *et al.*, 2004). Similar analysis also suggests that the last common ancestor was intracellular but less dependent on host metabolism (Horn *et al.*, 2004). Indeed, some CLOs such as *W. chondrophila* encode as many as 12 different amino acids (Bertelli *et al.*, 2010).

CLOs AS EMERGING PATHOGENS

A major driver of research interest in CLOs has been the repeated association between these bacteria and a range of different diseases in humans and animals, highlighting their potential as emerging bacterial pathogens but also illustrating that direct evidence for the majority of CLOs as pathogens is lacking (Table 2).

S. negevensis: an agent of respiratory illness

Following the initial isolation of *Simkania* 'Z' from cell culture contaminants, this CLO has been associated with respiratory disease in humans by both serological and molecular methods. Detection of IgG antibodies to *S. negevensis* indicated a previous infection in 37–62% of pneumonia patients in two separate cohorts, with a small proportion of those indicating a current acute infection (Lieberman *et al.*, 1997; Friedman *et al.*, 2006). Kahane *et al.* (1998) observed a 25% prevalence rate in bronchiolitis patients by culture and/or PCR. Further, the drinking water was postulated to be the source of infection in a cohort of children with pneumonia, based on immunoassay and culture, as well as *S. negevensis* 16S rRNA sequence amplification from 76% of nasopharyngeal swabs and corresponding drinking water (Kahane *et al.*, 2007). Drinking water supplies and wastewater in Israel also tested positive for *S. negevensis* antigens, often in association with amoebic antigens (Kahane *et al.*, 2004). Other studies have found a range of prevalence rates in healthy populations (Friedman *et al.*, 1999; Husain *et al.*, 2007), suggesting the potential opportunistic nature of this organism. *In vitro* studies have also demonstrated successful *Simkania* growth in several epithelial cell types in which an inflammatory response was also observed (Kahane *et al.*, 2007). More recently, *S. negevensis* antibodies were also detected in association with gastrointestinal symptoms (Donati *et al.*, 2013).

Parachlamydia spp: mucosal pathogens in humans and animals

The pathogenicity of *P. acanthamoebae* was first suspected when the bacteria was isolated from an amoeba recovered from the water of a humidifier implied in an outbreak of fever (Birtles *et al.*, 1997). Since then, a growing body of evidence has suggested that these CLOs may be pathogenic to humans and a variety of animal hosts. Like *S. negevensis*, in humans, molecular and serological studies have linked infections of *Parachlamydia* spp. to respiratory disease (Greub 2009). Additional reports of this bacterium in respiratory samples have backed up speculation of this bacterium as a respiratory pathogen (Greub *et al.*, 2003; Lamoth *et al.*, 2011) (Table 2).

The permissivity of pneumocytes, lung fibroblasts and macrophages to *P. acanthamoebae* (Greub *et al.*, 2003; Casson *et al.*, 2006) has also been demonstrated, adding support for the pathogenic potential of these bacteria and also indicating a potential route of dissemination through the body. Further strengthening the argument for *Parachlamydia* as a respiratory pathogen, both a murine and bovine model of parachlamydial respiratory disease (Casson *et al.*, 2008; Lohr *et al.*, 2014) have been established, fulfilling the third and fourth of Koch's postulates.

There is a growing body of evidence to suggest that *Parachlamydia* spp. may also be linked to adverse pregnancy outcomes in ruminants (Borel *et al.*, 2007; Barkallah *et al.*, 2014) (Table 2) and, moreover, in humans (Baud *et al.*, 2007). The prevalence of *Parachlamydia* spp. associated with abortion in cattle has been studied extensively in Europe, with initial Swiss studies showing prevalence of over 60% in placental lesions by immunohistochemistry (Borel *et al.*, 2007), while further studies have demonstrated slightly lower prevalence rates (Ruhl *et al.*, 2008,

Table 2. CLOs are emerging pathogens of a range of terrestrial and aquatic hosts. CLO diseases most commonly involve epithelial cells. Different diagnostic methods are available for different CLOs. Diagnostic methods: PCR, Polymerase chain reaction (followed by amplicon sequencing), ELISA, Enzyme linked immunosorbent assay; TEM, Transmission electron microscopy, ISH, In situ hybridization; IHC, Immunohistochemistry; MIF, Microimmunoflourescence; HE, Histopathological examination; WB, Western blot; IEM, Immunoelectron microscopy.

Family, species	Host	Disease	Detection method(s)	Reference(s)
Simkaniaceae				
S. negevensis	Humans	Respiratory disease	Culture, PCR, ELISA	(Kahane *et al.*, 1998; Friedman *et al.*, 1999, 2006)
S. venezia	Fish	Epitheliocystis	TEM, ISH, PCR	(Fehr *et al.*, 2013)
Uncultured *Simkaniaceae*	Reptiles	Granulomatous inflammation	IHC, PCR	(Soldati *et al.*, 2004)
Parachlamydiaceae				
P. acanthamoebae	Humans	Respiratory disease	PCR, IHC, MIF	(Greub *et al.*, 2003; Lamoth *et al.*, 2011)
		Adverse pregnancy outcomes	PCR	(Baud *et al.*, 2009)
	Cattle	Respiratory disease	PCR	(Wheelhouse, Longbottom and Willoughby 2013)
		Adverse pregnancy outcomes	PCR, IHC, HE	(Borel *et al.*, 2007; Blumer *et al.*, 2011; Wheelhouse *et al.*, 2012; Barkallah *et al.*, 2014)
Uncultured *Parachlamydiaceae*	Humans	Respiratory disease	PCR	(Corsaro *et al.*, 2002)
P. naegleriophila	Humans	Respiratory disease	PCR	(Haider *et al.*, 2008)
Neochlamydia sp.	Cats	Ocular disease	PCR, IHC	(von Bomhard *et al.*, 2003)
	Arctic charr	Epitheliocystis	HE, ISH, TEM, PCR	(Draghi *et al.*, 2007)
Waddliaceae				
W. chondrophila	Humans	Adverse pregnancy outcomes	WB, PCR, IHC	(Baud *et al.*, 2007, 2011, 2014)
	Cattle	Adverse pregnancy outcomes	PCR, IHC,	(Blumer *et al.*, 2011; Barkallah *et al.*, 2014)
Rhabdochlamydiaceae				
R. crassificans	Cockroach	Body swelling	PCR	(Corsaro *et al.*, 2007)
Rhabdochlamydia sp.	Humans	Respiratory disease	PCR	(Lamoth *et al.*, 2009; Niemi, Greub and Puolakkainen 2011)
R. lutjani	Blue-striped snapper	Epitheliocystis	HE, PCR	(Corsaro and Work 2012)
Clavichlamydiaceae				
C. salmonicola	Atlantic salmon	Epitheliocystis	HE, ISH, TEM, PCR	(Karlsen *et al.*, 2008; Mitchell *et al.*, 2010; Schmidt-Posthaus *et al.*, 2012)
	Brown trout	Epitheliocystis	HE, TEM, PCR	(Schmidt-Posthaus *et al.*, 2012)
Piscichlamydiaceae				
P. salmonis	Atlantic salmon	Epitheliocystis	HE, IEM, TEM, ISH, PCR	(Draghi *et al.*, 2004; Schmidt-Posthaus *et al.*, 2012)
	Arctic charr	Epitheliocystis	HE, TEM, IEM, ISH, PCR	(Draghi *et al.*, 2010; Schmidt-Posthaus *et al.*, 2012)
Parilichlamydiaceae				
P. carangidicola	Yellowtail kingfish	Epitheliocystis	HE, TEM, PCR	(Stride *et al.*, 2013b)
S. laticola	Barramundi	Epitheliocystis	HE, ISH, PCR	(Stride *et al.*, 2013b)
S. latridicola	Striped trumpeter	Epitheliocystis	HE, ISH, PCR	(Stride *et al.*, 2013a)
A. clariae	African catfish	Epitheliocystis	HE, ISH, TEM, PCR	(Steigen *et al.*, 2013)

2009). In Scotland, prevalence has been reported at around 20%, with a higher prevalence by PCR detection (Wheelhouse *et al.*, 2012). Blumer *et al.* (2011) also found the presence of CLOs as mixed infections with *Chlamydiaceae* or other CLOs in ruminants.

Considering *Parachlamydia* has been isolated from both ruminant foetal tissue and human respiratory samples, this bacterium potentially poses a zoonotic threat, particularly in individuals who have contact with livestock. Interestingly, in a study of healthy individuals, detection of *Parachlamydia* sp. was associated with interaction with farm animals (Baud *et al.*, 2009), supporting a potential role of this bacteria as a zoonotic agent. Further, *Parachlamydia* and other CLOs have been isolated from cattle drinking water (Wheelhouse *et al.*, 2011), suggesting a possible source of infection and mode of transmission for this pathogen to cattle and potentially humans. Maternal-foetal

transmission of *Parachlamydia* has also been demonstrated in a case study and was postulated to be a result of zoonotic transmission (Baud *et al.*, 2009).

W. chondrophila: an abortifacient

As previously mentioned, *W. chondrophila* was originally isolated from an aborted bovine foetus (Dilbeck *et al.*, 1990) and has since been described as an abortigenic agent in a number of studies on adverse pregnancy outcomes in cattle throughout Europe, using both molecular and serological methods (Dilbeck-Robertson *et al.*, 2003; Borel *et al.*, 2007) (Table 2). The disparity between reports from different authors highlights the prospect of an unknown determinant of susceptibility to infection and/or progression to disease.

In humans, *W. chondrophila* has been reported in association with miscarriage and other adverse pregnancy outcomes in up to 30% of cohorts studied (Baud *et al.*, 2007, 2014). *Waddlia chondrophilawas* also recently shown to multiply inside endometrial cells (Kebbi-Beghdadi *et al.*, 2011). At 96 h post-infection, the bacteria transform into persistent enlarged aberrant bodies that could be linked to recurrent episodes of miscarriage. Studies that show some evidence of acute or previous *Waddlia* infection in women who have miscarried suggests a possible reactivation of a latent asymptomatic infection, further strengthening this argument (Baud *et al.*, 2014). While initial studies focused on cervicovaginal swabs, a recent study detected *W. chondrophila* in a placenta from miscarriage by both PCR and IHC (Baud *et al.*, 2011), providing convincing evidence of a pathogenic role of *Waddlia* in abortion. What does remain to be established is the route of entry and transmission of this pathogen, as well as the underlying mechanism of pathogenesis. Studies of well water suggest that this could be one potential reservoir (Codony *et al.*, 2012), while other authors hypothesize that routes of entry could be sexual transmission or via the bloodstream following a respiratory infection (Baud *et al.*, 2014), or acquired following contact with animals (Baud *et al.*, 2007).

Piscichlamydia, Parilichlamydia, Similichlamydia, Clavichlamydia, Actinochlamydia: epitheliocystis agents in wild and cultured fish

The term epitheliocystis was coined after cyst-like inclusions were observed in gill epithelial cells of the Bluegill (Hoffman *et al.*, 1969), though this phenomenon was originally reported as 'Mucophilosis' (Plehn 1920). To date, epitheliocystis has been reported from over 90 species of fish globally (Stride *et al.*, 2014) (Table 2), including both wild and cultured fish from marine and freshwater environments. Initially, it was believed that the same aetiological agent caused epitheliocystis in all fish species, but as early as 1977 it was recognized that these agents demonstrated a high degree of host specificity (Zachary and Paperna 1977). Fredericks and Relman's postulates (Bebear and de Barbeyrac 2009) have been fulfilled (as opposed to Koch's, as no *in vitro* culture system is available for these organisms) for a number of CLOs. *Candidatus* Piscichlamydia salmonis was the first to be comprehensively described as the epitheliocystis agent in farmed Atlantic salmon (Draghi *et al.*, 2004). *Candidatus* Clavichlamydia salmonicola has been detected in Atlantic salmon and Brown trout species, both species belonging to the same genus *Salmo* (Karlsen *et al.*, 2008; Mitchell *et al.*, 2010; Schmidt-Posthaus *et al.*, 2012), while *Ca.* Parilichlamydia carangidicola and *Ca.* Actinochlamydia clariae were independently proposed as founding species of a novel family causing disease in Yellowtail kingfish and African catfish, respectively (Steigen *et al.*, 2013; Stride *et al.*, 2013b). An additional CLO, *Renichlamydia lutjani*, was identified in a blue-striped snapper from Hawaii (Corsaro and Work 2012) and its role as a purely epitheliocystis agent is questionable due to its detection in inner organs as opposed to the gills or skin. On the other hand, few epitheliocystis studies have included internal organs in the analysis, an aspect needing closer attention in future as this would have implications for disease progression and dissemination. In addition to these characterized organisms, short *Chlamydiales* 16S rRNA sequences have also been detected in association with cases of epitheliocystis in a diverse range of fish species, including Leopard shark (Polkinghorne *et al.*, 2010), Eagle ray (Camus *et al.*, 2013), Leafy sea dragon and Silver perch (Meijer *et al.*, 2006). For all of these agents but also for the species proposed in these novel CLO families, virtually nothing is known about their transmission and acquisition, and whether the utilization of a reservoir such as amoebae could explain the diverse, widespread nature of these agents and disease.

CLO DIAGNOSTICS

There have been two major challenges in demonstrating that members of the CLO group can cause disease in humans and animals. Firstly, they are very difficult to grow *in vitro* and secondly, species-specific reagents are generally lacking for most CLOs, raising questions over the interpretation of diagnostic results in screening surveys. Practically, cell culture is not routinely available in diagnostic laboratories. It may, however, prove useful to isolate new species, particularly if they involve the use of a variety of cell lines including different types of free-living amoeba. Indeed, free-living amoebae represent an ideal tool for culturing strict intracellular microbes when applicable (Kebbi-Beghdadi and Greub 2014), and this method has been used successfully to not only isolate a number of these organisms but also to study their biology and interactions with their hosts (Horn *et al.*, 2000; Thomas *et al.*, 2006; Lienard *et al.*, 2011b).

PCR is now the main approach used in most clinical diagnostic laboratories for the diagnosis of infections due to viruses and/or strict intracellular bacteria. 16S rRNA PCR assays were widely used during the first years of CLO research (Kahane *et al.*, 1993; Corsaro, Venditti and Valassina 2002). Pan-*Chlamydiales* primer sets based on the 16S rRNA gene have proven successful for detecting chlamydial DNA in both clinical and environmental samples (Everett, Hornung and Andersen 1999; Ossewaarde and Meijer 1999). These PCRs were often not very specific or sensitive, the latter due to a requirement to amplify > 500 bp gene fragments and the need to assess amplification by agarose gel electrophoresis (Corsaro and Greub 2006). Nested PCR strategies have been utilized to increase the specificity of these PCRs. Recently, these first generation PCRs have been replaced by several real-time PCRs targeting 16S or 23S rRNA, which have proven to be more specific and more sensitive, detecting fewer than five genome copies (Corsaro and Greub 2006). To increase species specificity, additional targets have also been used including the gene encoding the ATP-ADP translocase, which is present only in *Rickettsiales* and *Chlamydiales* (Greub and Raoult 2003) and the *secY* gene (Lienard *et al.*, 2011b). Other authors have opted for PCR assays targeting characteristic indel signatures in essential genes (Griffiths, Petrich and Gupta 2005), which could have diagnostic applications. Nevertheless, since the diversity of *Chlamydiales* is still largely unknown, the development of a pan-*Chlamydiales*

quantitative PCR (Lienard *et al.*, 2011a) represents an important tool for screening clinical and environmental samples for CLOs.

Apart from molecular diagnosis, serology still remains useful not only for epidemiological purposes but also to assess the pathogenic potential of these novel CLOs. Immunofluorescence is generally considered as the gold standard for the *Chlamydiaceae* (Dowell *et al.*, 2001) with cutoff for positivity of 1/64 for IgG and 1/32 for IgM (Corsaro and Greub 2006). Western-blotting, also not amenable for large-scale testing, has been used to confirm positive immunofluorescence (Greub *et al.*, 2003; Baud *et al.*, 2007), and this should ideally be performed to confirm specificity. However, there is a clear need for species-specific ELISA tests for CLOs. Early studies on *Simkania* prevalence applied an ELISA to a cohort of pneumonia patients and successfully showed seropositivity (Friedman *et al.*, 1999). Such a test is not yet available for all the CLOs, although for both *Parachlamydia* and *Waddlia*, immunogenic proteins have been identified through genome sequencing that might be used to develop a specific and sensitive ELISA (Greub *et al.*, 2009; Kebbi-Beghdadi *et al.*, 2012). Perhaps not surprisingly, there is remarkably little cross-reactivity between members of different family level lineages when immunofluorescence is performed (Casson, Entenza and Greub 2007). The same is true for immunohistochemistry, with antibodies raised against CLOs appearing to be highly specific (Borel *et al.*, 2007; Casson *et al.*, 2007). Immunofluorescence alongside *in situ* hybridization of short CLO-specific sequences is of crucial importance for demonstrating the presence of suspected CLO pathogens in lesions and thus confirming their suspected pathogenic role.

CLO RESEARCH—WHERE TO FROM HERE?

As we rapidly approach the 20th anniversary of the identification of the first CLO, *Simkania*, it is staggering to think how research into CLOs has changed our understanding of the genetic diversity, host range and biology of this unique phylum of obligate intracellular parasites.

In terms of where CLO research should be headed, a review of the CLO literature quickly reveals that there is a sampling bias toward environmental sources. Primary targets for expanded screening for CLOs should potentially focus on the estimated 26 000 species of marine and fresh water vertebrates, the immediate precursors to land animals. This area is clearly underrepresented, and could be key in understanding the evolutionary history of these enigmatic organisms. This largest group of vertebrate hosts has well developed innate and humoral immune systems and, as such, likely provided the immediate training ground for the evolution of many *Chlamydiaceae* and their specialization for air-breathing land animals.

The sequencing of the full genomes of several members of the current families has added significant credibility to this group of organisms. A next step would be to characterize a selection of CLOs at the strain level, as has been done for numerous species in the *Chlamydiaceae* (Bachmann *et al.*, 2014). Applying a comparative genomics approach to CLO strains isolated from diverse environments, hosts and reservoirs would provide further clues to the acquisition and loss of genes in response to adaptation to a diverse range of environments, with the common thread being the restrictive intracellular environment. While our ability to sequence the genomes of non-cultivable CLOs makes this prospect challenging, recent breakthroughs in culture-independent genome analysis of human *C. trachomatis* strains may hold promise to overcome these limitations (Seth-Smith *et al.*, 2013a,b). In doing so, genome analysis could also inform novel approaches to cultivate these bacteria with their enhanced metabolic capabilities potentially giving researchers opportunities to establish the first host-free culture methods for chlamydiae. In addition, some CLOs may prove to be interesting model organisms for studying chlamydial biology. In this regard, *Waddlia* are particularly promising since they replicate faster and exhibit slightly larger bacterial cells than *Chlamydiaceae* and grow in amoebae such as *D. discoideum*, a genetically tractable eukaryote (Tosetti, Croxatto and Greub 2014).

As already mentioned, there is a clear need to develop better species-specific detection and diagnostic methods, especially for serology. One significant step forward would be the development of novel PCR targets for each group. There is a challenge here though, as first it will be necessary to uncover their true biodiversity (and hence sequence diversity), before it will be possible to develop accurate diagnostic tools that may be used in large-scale epidemiological studies. However, the recent availability of a pan-*Chlamydiales* broad-range PCR targeting the 16 rRNA encoding gene (Lienard *et al.*, 2011a) has at least the advantage to more easily tackle the whole diversity present in a given sample, even if at a low copy number.

While research efforts begin to better characterize the farthest reaches of this phylum, it is inevitable that much of the field's attention will remain on the CLOs' namesakes, the members of the *Chlamydiaceae*. With the advent of genome sequencing and genetic manipulation technologies (Wang *et al.*, 2011; Humphrys *et al.*, 2013), researchers are beginning to unravel how members of the *Chlamydiaceae* interact with and manipulate their intracellular niche. Nevertheless, major questions still remain over what are the exact determinants of tissue tropism or host specificity for many of these chlamydial species. While some question marks remain over the pathogenic potential of CLOs, the fact that they are the closest relatives of the 'traditional' chlamydiae and that the host range of these organisms is significantly expanded makes them a tantalizing target for such studies. In doing so, research into CLOs will undoubtedly continue to revolutionize our understanding of all members of this biologically fascinating bacterial phylum.

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