Arch Microbiol (2016) 198:315–324
DOI 10.1007/s00203-016-1192-x

ORIGINAL PAPER

The emergence of epitheliocystis in the upper Rhone region: evidence for *Chlamydiae* in wild and farmed salmonid populations

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Received: 3 November 2015 / Revised: 17 December 2015 / Accepted: 12 January 2016 / Published online: 22 January 2016

Abstract We present the first study comparing epitheliocystis in a wild and farmed salmonid in Europe. Sampling three tributaries to the Lake Geneva, including one from headwaters to river mouth, revealed an unequal distribution of epitheliocystis in brown trout (*Salmo trutta*). When evaluated histologically and comparing sites grouped as wild versus farm, the probability of finding infected trout is higher on farms. In contrast, the infection intensities, as estimated by the number of cysts per gill arch, were higher on average and showed maximum values in the wild trout. Sequence analysis showed the most common epitheliocystis agents were *Candidatus* Piscichlamydia salmonis, all clustering into a single clade, whereas *Candidatus* Clavichlamydia salmonicola sequences cluster in two closely related subspecies, of which one was mostly found in farmed fish and the other exclusively in wild brown trout, indicating that farms are unlikely to be the source of infections in wild trout. A detailed morphological analysis of cysts using transmission electron microscopy revealed unique features illustrating the wide divergence existing between *Ca.* P. salmonis and *Ca.* C. salmonicola within the phylum *Chlamydiae*.

Keywords Brown trout · *Candidatus* Piscichlamydia salmonis · *Candidatus* Clavichlamydia salmonicola · River · Farm

Communicated by Erko Stackebrandt.

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Introduction

Epitheliocystis is a bacterial disease affecting more than 90 wild and farmed fish species worldwide, both in marine and freshwater (Paperna and Sabnai 1980; Lewis et al. 1992; Nowak and LaPatra 2006; Abowei and Briyani 2011; Stride et al. 2013a, b, 2014). Lesions occur on skin and gills in larval stages, but are largely restricted to gills in adult fish. Macroscopically, they present as white nodules which vary widely in size, from as large as 400 µm (Fehr et al. 2013) and readily visible to the naked eye, down to 10 µm (Schmidt-Posthaus et al. 2012) and only detectable histologically. Histologically, the infection was first described as intracellular cysts causing cell hypertrophy in epithelial cells of blue gills (*Lepomis macrochirus*) by bacteria assigned to the *Chlamydozoaceae* on the basis of morphology in electron micrographs (Hoffman et al. 1969). The gill pathologies as well as morphologies in electron micrographs observed in diverse fish hosts since this publication are manifold, often being interpreted in terms of chlamydial developmental cycles (Paperna 1977; Crespo et al. 1999, 2001; Nylund et al. 1998). Only with the advent of molecular data has it become clear that a diverse spectrum of intracellular bacteria, and not only *Chlamydia*, is associated with this disease (e.g., Draghi et al. 2004, 2007; Mitchell et al. 2013; Toenshoff et al. 2012).

The first molecular evidence for members of the phylum *Chlamydiae* causing epitheliocystis came from infections of salmonids of the genus *Salmo* that were named accordingly *Candidatus* Piscichlamydia salmonis (Draghi et al. 2004) and *Candidatus* Clavichlamydia salmonicola (Karlsen et al. 2008). In addition to farmed marine salmon (*Salmo salar*; Draghi et al. 2004), *Ca.* P. salmonis has also been found in farmed freshwater Arctic char (*Salvelinus umbla*; Draghi et al. 2007). By contrast, *Ca.* C. salmonicola appears to be freshwater specific, found in farmed salmon and in wild brown trout (*Salmo trutta*; Karlsen et al. 2008; Mitchell et al. 2010), and has been observed to be lost upon transfer of infected farmed salmon to marine cages (Mitchell et al. 2010).

In Switzerland, epitheliocystis has been found sporadically in wild brown trout (Schmidt-Posthaus et al. 2001, 2012), caused by both *Ca.* P. salmonis and *Ca.* C. salmonicola (Schmidt-Posthaus et al. 2012). It is still unknown whether epitheliocystis also occurs in farmed brown trout, whether a horizontal infection takes place between farmed and wild fish and whether one or the other population serves as a primary source of infection. Therefore, the aims of this study were to investigate whether epitheliocystis is present in brown trout reared in farms receiving water from a river harboring epitheliocystis positive trout and whether there are indications for a horizontal transmission of epitheliocystis agents between wild and farmed fish, namely whether the same bacteria species are found in farmed and wild animals. Histopathology and polymerase chain reaction (PCR) were performed to investigate the presence and prevalence of epitheliocystis, histopathology was used to judge infection intensity and gill pathology, and sequencing was applied to evaluate and compare the genotypes of bacteria in wild and farmed fish. Finally, transmission electron microscopy was used to identify epitheliocystis agents based on their characteristic morphologies and independently of the molecular data.

Materials and methods

Sample collection

The neighboring Rhone tributaries the Venoge (length 44 km long), Aubonne (12 km) and Boiron (15 km) enter Lake Geneva, the largest lake in Switzerland, along its northern shores. These medium-sized rivers are regularly controlled by fisheries authorities, and the Venoge also has farms rearing brown trout for stocking purposes along the upper and middle reaches.

In 2014, electro-fishing was performed to collect brown trout from two sites in the Aubonne, three in the Boiron and five sites in the Venoge (Fig. 1). Venoge sites were one headwater location (river site Cuarnens), one midriver location (river site Eclépens) and three downriver locations (river sites Cossonay, Les Isles and Embouchure), with the latter close to the river mouth. Three fish farms which receive water from the Venoge were included in the study:

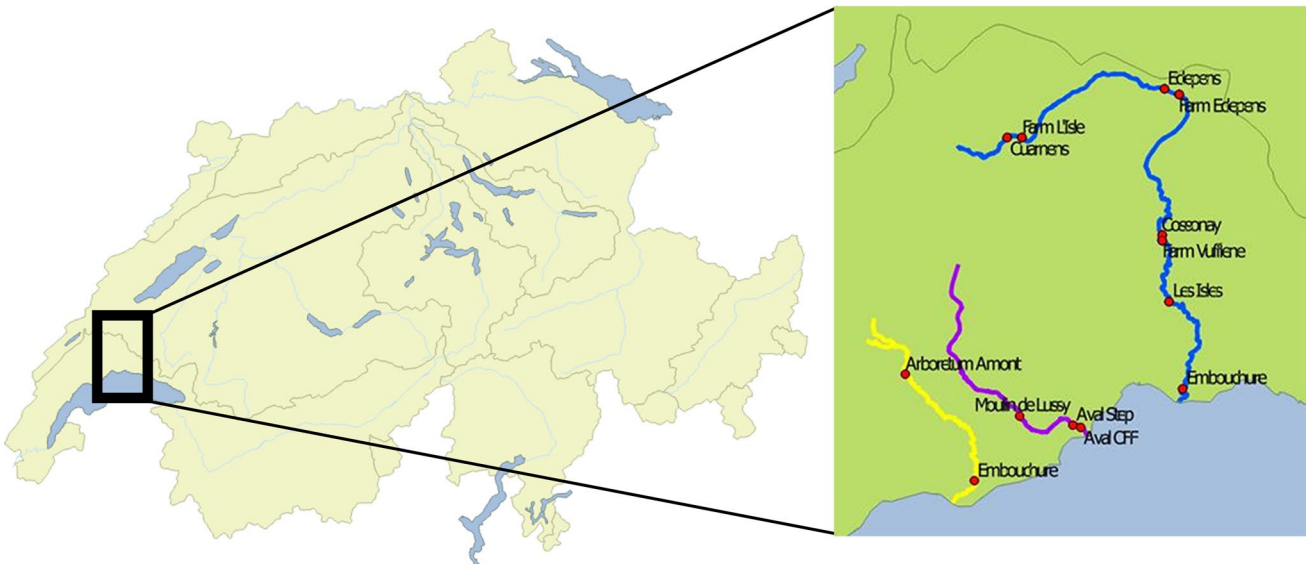

Fig. 1 Map of Switzerland. Three tributaries to the Lake Geneva, Venoge (Cuarnens: headwater site, farm L'Isle: headwater farm, Eclépens: midriver site, farm Eclépens: midriver farm, Cossonay: downriver site, farm Vufflene: downriver farm, Les Isles: downriver and Embouchure: river mouth site), Aubonne (Arboretum Amont: headwater site and Embouchure: downriver site) and Boiron (Moulin de Lussy: midriver site, Aval Step and Aval CFF: two downriver sites)

one headwater (fish farm L'Isle), one midriver (fish farm Eclépens) and one downriver (fish farm Vufflene) (Fig. 1). The three farms are located within 3 km downriver of the corresponding Venoge sampling sites. The Venoge is the sole water source for two farms mid- and downstream, with the headwater farm supplemented by spring water; however, this water drains into the Venoge without any treatment, as shown in Fig. 1.

All river sites were sampled once between September and October, and the fish farms were sampled twice, firstly in July and secondly between September and October. Twenty-five trout per river site and fish farm, respectively, were collected and euthanized by an overdose of tricaine methanesulfonate (MS-222®, Argent Chemical Laboratories, Redmond, USA). Lengths of the brown trout were measured. A standard necropsy was performed, and macroscopical changes were recorded. On the left body side, three gill branches were removed, the first one was fixed in 10 % buffered formalin for histopathology, the second branch was preserved in RNAlater (Sigma-Aldrich, Missouri, USA), and the third branch was fixed in 2 % glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4 for electron microscopy (EM).

Histopathology

Gill branches were routinely processed for paraffin embedding, sectioned at 4 µm and stained with hematoxylin and eosin (H&E). Gill pathology was investigated, and the following parameters were encountered: edema, mainly pronounced as epithelial lifting on the lamellae, infiltration with macrophages and lymphocytes in the area of epitheliocystis cysts and lamellar fusion due to epithelial proliferation, mainly in the basal part of the lamellae. Severity of each parameter was classified as 0 (no lesion), 1 (mild lesions), 2 (moderate lesions) or 3 (severe lesions).

The samples were examined for the presence of epitheliocystis, and if present, the number of cysts per gill branch was counted.

Cysts were assigned as *Ca. P. samonis* or *Ca.* C. salmonicola following the descriptions in Schmidt-Posthaus et al. 2012.

16SrRNA gene PCR and sequencing

DNA from gills from RNAlater was extracted using the QIAGEN DNAeasy Blood and Tissue kit (Qiagen, Hilden, Germany) and stored at −20 °C until use.

PCR was performed initially to generate a 300 bp chlamydial signature sequence using the following primers 16SigF: 5′-CGG CGT GGA TGA GGC AT-3′ and 16Sig R: 5′-TCA GTC CCA GTG TTG GC-3′ (Everett et al. 1999). Positive gill samples from the first screening were further investigated by 16S rRNA gene PCR to generate a long fragment (1520 bp) using primers 16SigF 5′-TCA GTC CCA GTG TTG GC-3′ and 16SB1 rev 5′-TAC GGY TAC CTT GTT ACG ACT T-3′.

All PCR reactions were performed in individual 50 µl reaction mixtures containing 24.2 µl water, 5 µl PCR Buffer Roche® 10× ($MgCl_2$ 20 mM), 5 µl $MgCl_2$ (25 mM), 5 µl dNTP (10 mM), 2.5 µl each primer (10 µM), 5 µl DNA and 0.8 µl Fast Star Taq (Roche, Basel, Switzerland).

The signature amplification used 45 cycles with annealing at 54 °C and extension at 72 °C for 90 s. The 1520 bp amplification was the same but with an annealing temperature of 52°. PCR products were visualized after agarose electrophoresis by a BioDoc-IT Imaging System UVP.

Samples that showed positive results for the 1520 bp product were purified using the MinElute PCR Purification Kit (Qiagen, Hilden, Germany) and immediately cloned using the TOPO TA Cloning® Kit (pCR®2.1-TOPO® vector) (Invitrogen, California. USA) and One Shot TOP10 chemically competent *E. coli* (Invitrogen, California, USA).

Plasmids from individual clones were purified using the QIAPrep Spin Miniprep Kit (Qiagen, Hilden, Germany), and those with the amplicon inserts were identified using Eco RI (Biolabs, Massachusetts, USA) according to manufacturer's instructions. Positive clones were capillary-sequenced by Microsynth (Balgach, Switzerland). The resulting reads were assembled [CLC Main Workbench 7.0.2 (CLC bio, Qiagen)], compared using BLASTN against the Genbank database and used to create alignments with reference sequences [using Muscle and PhyML v3 within Seaview v4 (Gouy et al. 2010)]. Representative 16S rRNA gene sequences have been deposited in EMBL Nucleotide Sequence Database under accession numbers LN995843 to LN995859.

Electron microscopy

Gill branches fixed with 2.5 % glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.5 at 4 °C and originating from fish positive in histology for epitheliocystis and in PCR for *Chlamydiales*, were prepared for embedding into epoxy resin and for TEM according to standard procedures. Gill sections containing epitheliocystis lesions were selected from epoxy resin blocks using semithin sections (1 µm) stained with toluidine blue (Sigma-Aldrich, Missouri, USA). Ultrathin sections (80 nm) were mounted on copper grids (Merck Eurolab AG, Dietlikon, Switzerland), contrasted with uranyl acetate dihydrate (Sigma-Aldrich, USA) and lead citrate (Merck Eurolab AG, Dietikon, Zurich, Switzerland) and investigated using a Philips CM10 transmission electron microscope. Images were processed with Imaris 7.7.1 (Bitplane, Zurich, Switzerland) and assembled for publication using Adobe Photoshop.

Statistics

The prevalence of epitheliocystis infections was evaluated using data obtained by histopathology and PCR. Histopathology data were further used to analyze associations between epitheliocystis morphologies observed and pathological changes using Chi2 tests. As the infection intensity was not normally distributed, a Kruskal–Wallis ANOVA was used to test differences between wild fish and farmed fish populations, different pathological changes and different morphologies. All analyses were carried out using NCSS Statistical Software (Kaysville, Utah, USA).

Additionally, the prevalence of infection in farm versus wild fish was evaluated using Logistic Regression for (1) all farms and wild fish in two groups and (2) the three fish farms compared to their corresponding three river sites separately. In all tests, P values <0.05 were considered significant.

Results

Histopathology of epitheliocystis

Two different morphologies were readily distinguished in the epitheliocystis lesions from brown trout in this study based on histology (Fig. 2a, b) and on electron microscopy (Fig. 3a–h). Figure 2a shows *Ca.* P. salmonis-like cysts with a dark basophilic amorphous center and clear halo. Infection was associated with a mild to moderate hyperplasia of epithelial cells, subepithelial edema and mild to moderate infiltration with mainly lymphocytes. Figure 2b shows lightly basophilic granulated cysts, presumably representing *Ca.* C. salmonicola. Each cyst was classified according to the above-mentioned criteria in accordance with Schmidt-Posthaus et al. 2012, and the morphologies of cysts per gill branch were classified as type 1 (indicative for *Ca.* P. salmonis), type 2 (indicative for *Ca.* C. salmonicola) and mixed (both types 1 and 2) morphology.

The differences in morphology between the cyst types evident in light microscopy are even more striking when viewed in the transmission electron microscope (TEM). Representative panels of *Ca.* P. salmonis (Fig. 3a, c, e, g) and *Ca.* C. salmonicola (Fig. 3b, d, f, h) cysts are presented at increasing magnification in parallel to illustrate this point. Whereas the *Ca.* P. salmonis cysts are filled with bacteria all at a uniformly similar stage of development from either replicating forms (Fig. 3a) to what we presume are the infectious particles or elementary bodies (EBs) (Fig. 3g), the *Ca.* C. salmonicola cysts show a developmental cycle more closely resembling that of the family *Chlamydiaceae*, with replicating forms aligned along the inclusion membrane (Fig. 3b, d), adjacent intermediate bodies (Fig. 3f), and then the characteristic "head-and-tail" inferred infectious forms (Karlsen et al. 2008; Schmidt-Posthaus et al. 2012) (Fig. 3h) furthest from the inclusion membrane. We can observe the host cell to be still relatively well intact and identifiable as a mitochondria-rich or chloride cell. The host cell for *Ca.* P. salmonis is also likely to be a mitochondria-rich cell, evident in very early stage inclusions (not shown), but undergoes much greater changes in morphology during infection with many long interdigitating processes connecting with surrounding cells and an electron-dense

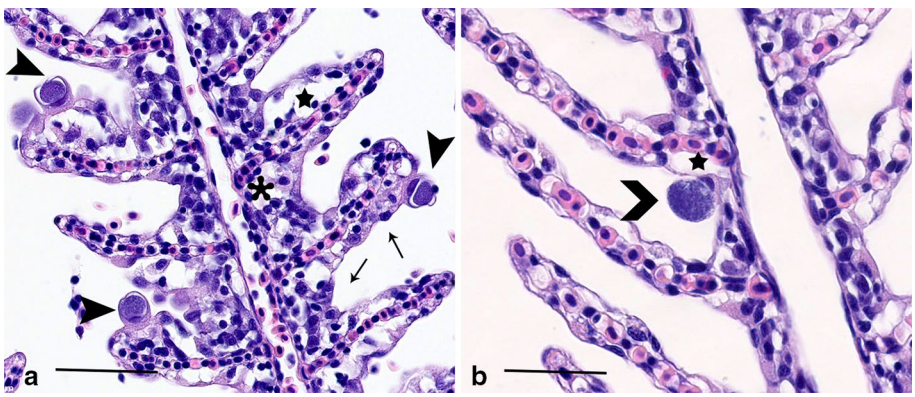

Fig. 2 Histologic appearance of *Ca.* P. salmonis (**a**) and *Ca.* C. salmonicola (**b**) in gills of brown trout (*Salmo trutta*). **a** *Ca.* P. salmonis cysts (*closed arrowheads*) affecting epithelial cells on the gill lamellae, leading to a mild to moderate hypertrophy of affected cell with bacteria forming a basophilic homogenous center surrounded by a clear halo and translocation of the host nucleus to the margin of the cell. Infection is associated with mild to moderate hyperplasia of epithelial cells (*arrows*), subepithelial edema (*star*) and moderate subepithelial infiltration with lymphocytes and fewer macrophages (*asterisk*). *Bar* 50 μm. **b** *C. salmonicola* cyst (*open arrowhead*) at the base of the lamella, showing a more granular basophilic appearance and no halo; host cell nucleus is translocated to the margin of the cell. Infection leads to moderate hypertrophy of affected epithelial cell; infection is associated with mild subepithelial edema (*star*) and no other pathology. *Bar* 50 μm

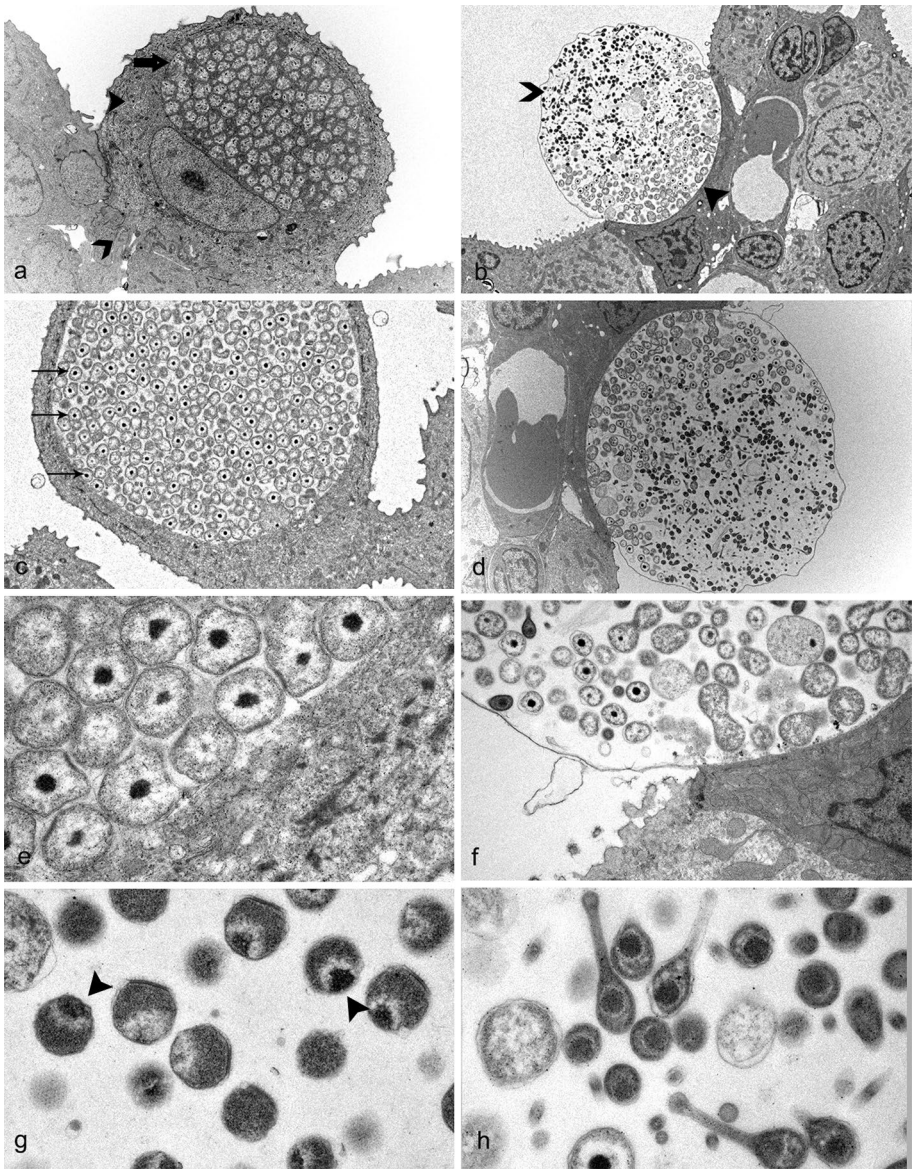

Fig. 3 Transmission electron microscopy of *Ca.* P. salmonis (**a**, **c**, **e**, **g**) and *Ca.* C. salmonicola (**b**, **d**, **f**, **h**). Early stage *Ca.* P. salmonis perinuclear cyst (**a**) filled with amorphous replicating bacteria (*arrow*) embedded in a thickened disorganized host cell cytoplasm (*closed arrowhead*) with the cell anchored to adjacent cells through tightly interdigitating processes (*open arrowhead*). A later stage *Ca.* P. salmonis cyst (**c**), now containing bacteria with condensed nucleoids (e.g., *arrows*), shown at higher magnification in (**e**). Infectious particles of *Ca.* P. salmonis from an end-stage inclusion are shown in (**g**) with an electron-dense nucleoid (*arrowhead*) opposite a flattened cap with associated projections, arranged in an orthogonal array. A *Ca.* C. salmonicola cyst with thin surrounding cell membrane in mitochondria-rich cell with irregularly shaped nucleus at the base of a secondary lamella (**b**). Replicating *Ca.* C. salmonicola are closest (**d**) or aligned along the host cell side of the inclusion membrane (*closed arrowhead*) (**f**), with non-dividing intermediary forms exhibiting electron-dense nucleoids (*open arrowhead*) and a well-organized host cell cytoplasm densely packed with mitochondria. The characteristic head-and-tail infectious particles (**h**) are furthest from the host cell nucleus and have a rounded condensed nucleoid in the head with filamentous material extending along the inside of the tail

cytoplasm with darkly staining features, resembling the tubular structures of the closely related *Ca*. Actinochlamydia clariae (Steigen et al. 2013).The *Ca.* P. salmonis EBs also show similarities with those of *Ca*. A. clariae, with an electron-dense nucleoid opposite a flattened cap with associated projections, arranged in an orthogonal array (Fig. 3g), which are also evident on earlier developmental forms (Fig. 3e).

These different morphologies permit a ready distinction between *Ca.* P. salmonis and *Ca.* C. salmonicola and form the basis for studies of prevalence in farmed and wild brown trout.

Table 1 Prevalence and intensity of epitheliocystis in brown trout originating from river and farm sites

River	River site	Farm site	Location	Date of sampling	n	Prevalence (%)		Intensity (n cysts) median ± 95 % CI
						Histology (n)	PCR	
Venoge	Cuarnens		Headwater river site	September 2014	25	8 (2)	0	37.5 ± 38
		L'Isle	Headwater farm site	July 2014	25	0 (0)	44	0
		L'Isle	Headwater farm site	September 2014	25	0 (0)	4	0
	Eclépens		Midriver site	September 2014	25	20 (5)	100	6 ± 12
		Eclépens	Midriver farm site	July 2014	25	56 (14)	100	7 ± 38
		Eclépens	Midriver farm site	October 2014	25	60 (15)	100	1 ± 17
	Cossonay		Downriver site	September 2014	25	20 (5)	100	24 ± 116
		Vufflene	Downriver farm site	September 2014	25	36 (9)	88	1 ± 3
		Vufflene	Downriver farm site	October 2014	25	4 (1)	100	1 ± 1
	Les Isles		Downriver site	September 2014	25	32 (8)	83	15.5 ± 53
	Embouchure		Rivermouth site	September 2014	25	8 (2)	100	12.5 ± 15
Aubonne	Arboretum Amont	–	Headwater site	September 2014	25	4 (1)	40	nd
	Embouchure	–	Downriver site	September 2014	25	12 (3)	nd	nd
Bioron	Moulin de Lussy	–	Midriver site	July and September 2014	50	10 (5)	32	nd
	Aval Step	–	Downriver site	July and September 2014	50	4 (2)	44	nd
	Aval CFF	–	Downriver site	July and September 2014	50	18 (9)	64	nd

Levels of epitheliocystis infection of brown trout in both river and farm sites of the Venoge, Aubonne and Boiron during summer and early autumn of 2014. An average number of cysts present per gill branch show that wild fish have more cysts than those from the fish farms except for the river site Eclépens and farm Eclépens, where the values are similar

Prevalence and intensity of epitheliocystis

To determine the degree to which epitheliocystis is present in wild salmonid populations and how this relates to disease in farmed fish, we examined whether epitheliocystis is present in farmed and wild brown trout in a small, well-defined river system, the Venoge, which is a 44-km tributary of the Rhone flowing into Lake Geneva, together with five additional sites for sampling wild fish on the neighboring rivers, the Aubonne and the Boiron. Three fish farms on the Venoge, all receiving untreated water directly from the river, were paired with closely situated upstream river sites (Fig. 1). Samples (n = 25) were collected from each site as described in methods, and first histological examination of the gills was used to estimate epitheliocystis prevalence.

When evaluated histologically, the prevalence of epitheliocystis in farmed and wild brown trout varied from 0 % (headwater farm L'Isle) to 60 % (midriver farm Eclépens) (Table 1). Comparing sites grouped as river versus farms, the probability of finding infected fish is higher on farms (Logistic Regression, OR 1.25, $P = 0.054$). However, a pairwise comparison of river versus farm at the same location revealed that the number of infected fish was only higher in midriver farm Eclépens (Logistic Regression, OR 5.1, $P = 0.0113$ and OR 6.00, $P = 0.0055$ for every sampling date, respectively), with no statistically significant differences for the remainder of the locations. Indeed, the probability of finding infected fish in this farm is much higher than at any other site (OR 4.7 Logistic Regression, $P = 0.0000$).

In the tributaries not containing any fish farms (Aubonne and Boiron), prevalence measured by histology varied between 4 % (headwater site Arboretum Amont) and 12 % (downstream site Embouchure) in the Aubonne and between 4 % (downstream site Aval Step) and 18 % (downstream site Aval CFF) in the Boiron (Table 1).

We also attempted to evaluate prevalence using chlamydial signature PCR (Table 1). These data gave the prevalence of epitheliocystis from 0 (headwater river site Cuarnens) to 83–100 % (remainder of farm and river sites), with chlamydial-free sites not correlating with those diagnosed by histology. As this PCR selects for all bacteria within the phylum Chlamydiae, including those not in recognizable cysts, it is considerably more sensitive than histological examination. Each technique was performed on different, yet adjacent, gill arches, further explaining possible non-correlating results. For these reasons, and for comparison to previous studies, we used our histological results as the standard for the prevalence analysis.

We measured the intensity of infection by counting the number of cysts per gill arch (Table 1). When comparing sites grouped as river versus farm (Fig. 4), the number of cysts was found to be higher in fish from river sites (Kruskal–Wallis ANOVA, $P = 0.0009$) with the highest values (up to

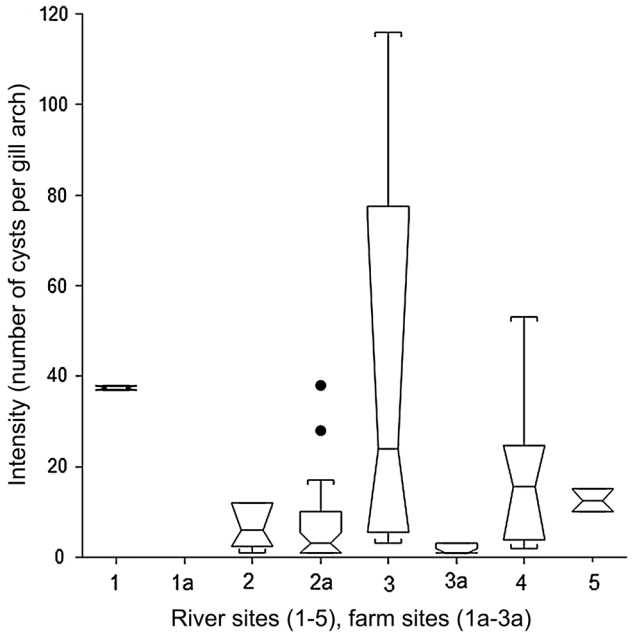

Fig. 4 *Box plot* showing the infection intensities (number of cysts per gill arch) in brown trout from the river sites and farms from the Venoge. Number 1 is the headwater site Cuarnens, and 1a is the corresponding farm site L'Isle, which in the *box plot* shows no data because it was negative. Number 2 is the midriver site Eclépens and 2a the corresponding farm Eclépens and similarly for 3 (Cossonay) and 3a (fish farm Vufflene). River site 4 (Les Isles) is downriver and river site 5 (Embouchure) is at the river mouth

116 cysts) in midstream river site 3 and the highest median value in the river headwater site. In contrast, the lowest values were in the midriver farm site Vufflene.

We then combined these data with the two different cyst morphologies observed, corresponding to *Ca.* P. salmonis and *Ca.* C. salmonicola. The number of cysts per gill arch was highest in mixed infections, followed by those for *Ca.* P. salmonis and lowest for *Ca.* C. salmonicola morphology (Kruskal–Wallis ANOVA, $P = 0.0003$) (Fig. 5a). Taken together, the highest infection intensities were mixed infections at river sites (Fig. 5b). There is no association between the intensity of infection as measured by number of cysts and pathology.

Candidatus Clavichlamydia salmonicola was found to occur more often on farm sites (Logistic Regression, OR 1.68, $P = 0.0058$) with the commonest occurrence being in midriver farm Eclépens. Mixed infections were more often found on river sites (weaker association).

Sequencing data

Nearly full-length 16S rRNA gene sequences were produced from the gill samples. The sequencing data revealed *Ca.* P. salmonis (46 sequences) and *Ca.* C. salmonicola (28 sequences) to be the dominant chlamydial species detected in both wild and farmed brown trout in the Rhone tributaries. This is depicted as an overview in Fig. 6 relative to other published chlamydial epitheliocystis agents. The 46 *Ca.* P. salmonis 16S rRNA gene sequences obtained all fall within a single clade around the reference sequence from epitheliocystis-affected salmon from Norway (Fig. 6b) separated by only 1-5 SNPs from the reference (AY462244; Draghi et al., 2004) over 1400 bp. The *Ca.* C. salmonicola sequences

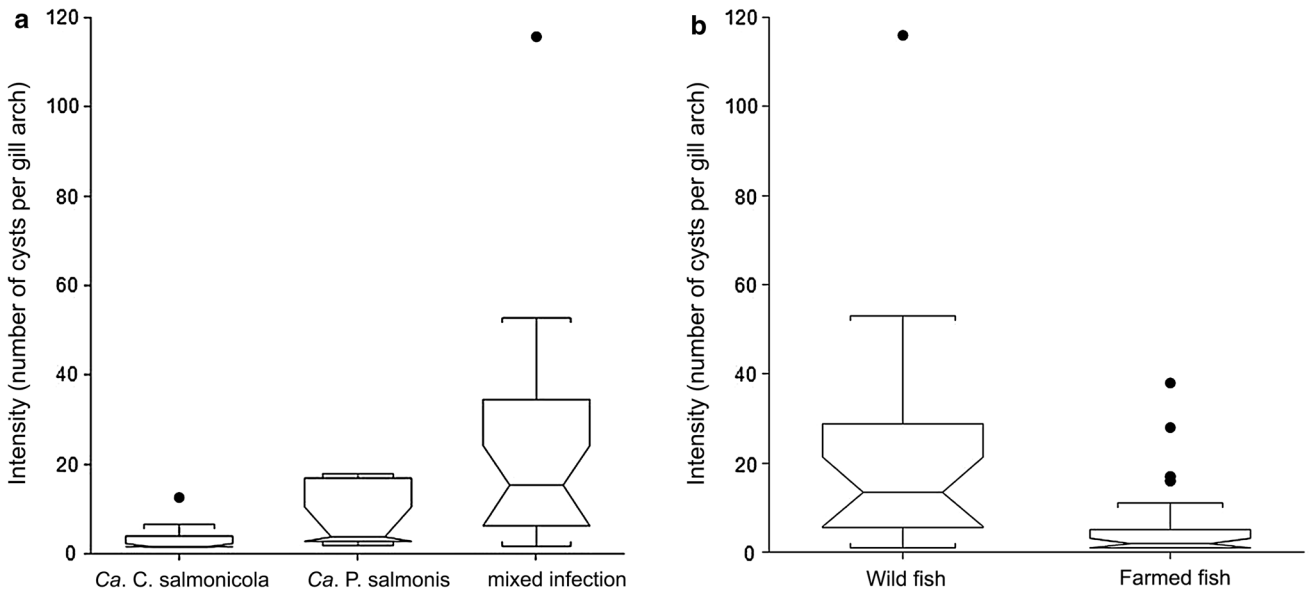

Fig. 5 *Box plot* showing mixed infections (**a**) have the highest number of cysts, with single infections with either *Ca.* P. salmonis or *Ca.* C. salmonicola having lower infection intensities. The *box plot* in **b** shows a higher number of cysts present in wild fish than in farmed fish

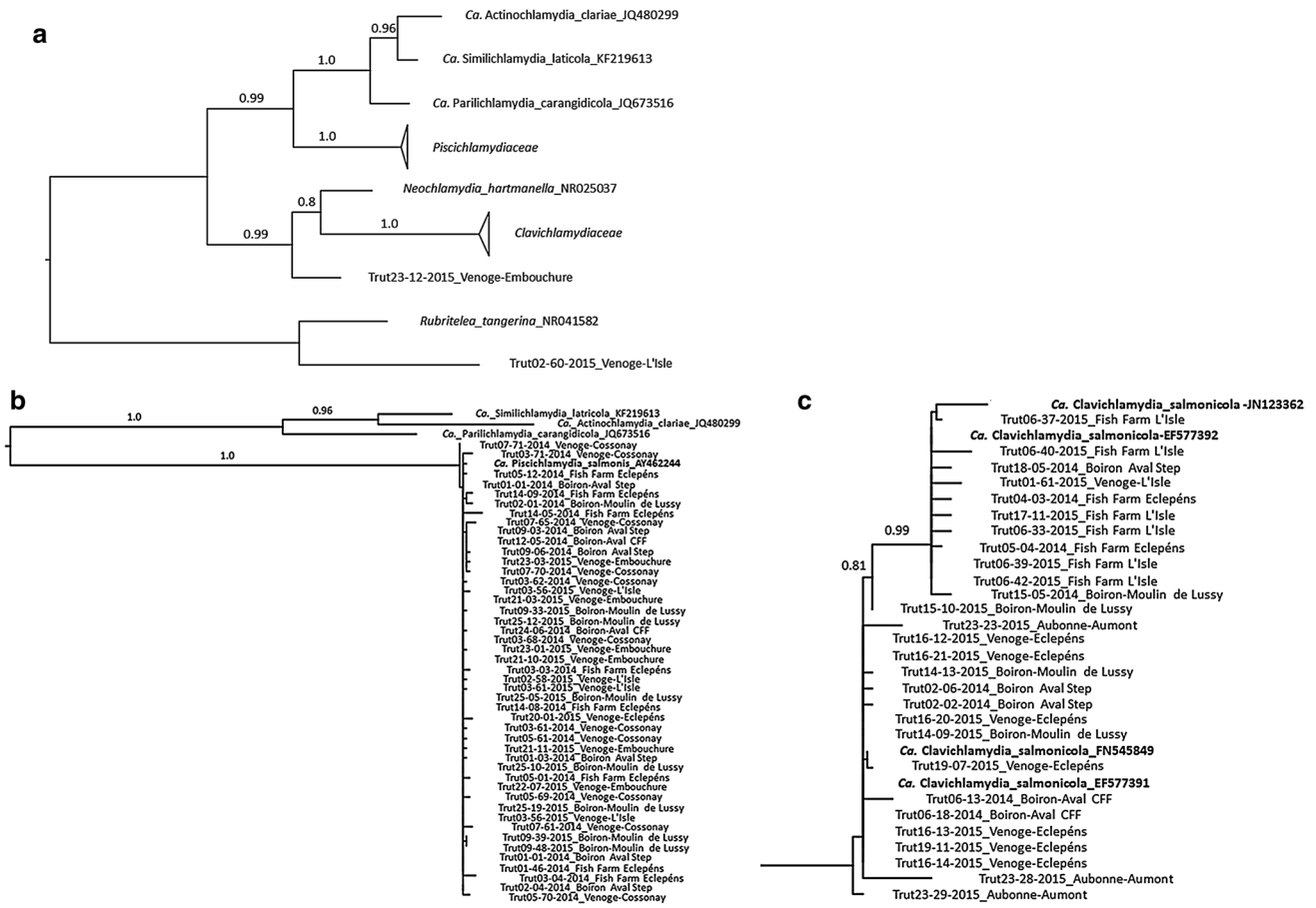

Fig. 6 **a** Phylogenetic tree rooted on *Rubritalea* showing an overview of the sequences partitioning into the *Piscichlamydiaceae* clade (46 of 76) or *Clavochlamydiaceae* (28 of 76, two closely related clades), plus two additional sequences (2 from 76) from the Venoge. The nomenclature describes the fish number-clone-year_site. Also included for comparison are published sequences for epitheliocystis agents within the *Neochlamydiaceae*, *Similichlamydiaceae*, *Parilichlamydiaceae* and *Actinochlamydiaceae* families. **b** Expanded clade of phylogenetic tree from 6a which shows the *Ca.* P. salmonis sequences partitioning into a single clade around the reference sequence from salmon, Norway. **c** Expanded clade of phylogenetic tree from 6a, expanded to show the separation of *Ca.* C. salmonicola into two clades, separated by at least 6 SNPs. The upper clade is dominated by *Ca.* C. salmonicola sequences from two fish farms of the Venoge, but also includes three sequences from wild brown trout of the Boiron and the Venoge, along with reference sequences from farmed Norwegian salmon (JN123362) and wild Norwegian brown trout (EF577392). In the lower clade, Irish *Ca.* C. salmonicola reference sequence from freshwater farmed salmon (FN545849) along with a Norwegian reference sequence from salmon (EF577391) group together with sequences from *Ca.* C. salmonicola of wild brown trout of the Venoge, Aubonne and Boiron. Bootstrap values are only shown if >50 %

are more diverse and partition into two clades (Fig. 6c). One of these clades contains all the sequences obtained from farmed brown trout, along with three isolates from wild brown trout which share over 99 % nucleotide identity (2 SNPs over 1294 bp) to a wild brown trout isolate from Norway (EF577392) and 9 SNPs over 1301 bp compared to a Norwegian isolate from farmed Atlantic salmon (*Salmo salar*; JN123362). The other clade contains further 17 *Ca.* C. salmonicola sequences, which share over 99 % identity to the reference sequence EF577391 from farmed Atlantic salmon from Norway (4 SNPs over 1244 bp; Karlsen et al., 2008) or FN545849 from Ireland (4 SNPs over 1249 bp; Mitchell et al., 2010).

Two additional chlamydial sequences were also detected and are shown in the phylogeny. Of these, one (Trut23-12-2015_Venoge-Embouchure) shows 93 % nucleotide identity to *Neochlamydia hartmannellae* and the second (Trut02-60-2015_Venoge-L'Isle) 91 % identity to members of the marine bacteria *Rubritaleaceae* which, as with the *Chlamydiae*, are members of the *Verrucomicrobia* super phylum.

Figure 6b and c shows that single fish can be infected by different clones and also mixed infections with members of *Ca.* P. salmonis and *Ca.* C. salmonicola were present, supporting our histological findings showing mixed infections on one gill branch in single brown trout.

Discussion

Epitheliocystis in northern hemisphere salmonids of the genera *Salmo* and *Salvelinus* appears to be mainly caused by three bacterial species: *Ca.* P. salmonis, *Ca.* C. salmonicola and *Ca*. Brachiomonas cysticola (Draghi et al. 2004, 2010; Mitchell et al. 2010; Schmidt-Posthaus et al. 2012; Toenshoff et al. 2012; Mitchell et al. 2013; Contador et al. 2015). Of these, only *Ca.* C. salmonicola appears to be exclusively freshwater in origin, whereas *Ca*. B. cysticola and *Ca.* P. salmonis are found in both freshwater and seawater hosts: Atlantic salmon (*Salmo salar*), brown trout (*Salmo trutta*), Arctic char (*Salvelinus umbla*) and Lake trout (*Salvelinus namaycush*). These bacterial species occur in a circumpolar distribution in Europe as well as in North America, but with no reports for the southern hemisphere or in salmonids of the genus *Oncorhynchus* until now.

Most of the data on epitheliocystis in salmonids are derived from studies in farmed fish, with the exceptions being wild brown trout, shown to be infected with *Ca C. salmonicola* (Karlsen et al. 2008; Schmidt-Posthaus et al. 2012) or *Ca.* P. salmonis (Schmidt-Posthaus et al. 2012). Previous to this study, no data have been collected for wild and farmed salmonids within the same stretch of river.

With this current study, we substantiate our previous data (Schmidt-Posthaus et al. 2012) showing that brown trout are a host for *Ca.* P. salmonis. *Ca.* P. salmonis is ubiquitously distributed in Atlantic salmonid hosts in marine and freshwater of the northern hemisphere. Here we could show that all sequences to date are closely related and form a single clade, including our data shown in this study. *Ca.* P. salmonis was found to be the major epitheliocystis agent, as judged by histology and sequencing and, when present in mixed infections with *Ca.* C. salmonicola, was also responsible for the heaviest epitheliocystis burdens, both in farmed and wild fish. Against our initial expectations, heaviest intensity of infection was observed in wild brown trout rather than farmed fish, despite the fish in the farms being cultivated in water taken directly from the river system (Venoge). The farms investigated were producing brown trout for restocking of the wild population and as such were running lower stocking densities than those commonly used in grow out facilities for food production. The constant water levels in the holding ponds along with the regular feeding regimes may well result in improved welfare and better health (Segner et al. 2011) than the wild population, especially during the summer months, when fluctuating river levels and food availability may pose greater stress on the young fish. This is particularly striking when the data from wild trout collected from site 3 are compared with those from the adjacent farm, site 3a where infection intensity was higher in wild trout compared to farmed fish. As we previously observed (Schmidt-Posthaus et al. 2012), epitheliocystis has local concentrations and species patterns. The highest prevalences were observed in the middle reaches of the Venoge, with the headwaters and the site closest to the entry into Lake Geneva having the lowest levels. This might be due to the influence of the above-located fish farms, but further studies are needed to confirm this hypothesis.

Until now, *Ca.* P. salmonis, *Ca.* C. salmonicola and *Ca*. Brachiomonas cysticola have only been found in salmonids of the genera *Salmo* and *Salvelinus*, but not in *Oncorhynchus, i.e.*, Pacific salmonids. Rainbow trout (*Oncorhynchus mykiss*) are commonly farmed, so it is indeed surprising that epitheliocystis has not yet to be reported in this host. Should *Oncorhynchus* sp. not be a susceptible host for the epitheliocystis agents we described, this would be an important point to add to discussions of the load placed on the environment and the local *Salmo* populations by farms culturing these species. What is urgently needed are more wide ranging studies of other potential vertebrate and invertebrate hosts for *Ca.* P. salmonis and *Ca.* C. salmonicola. This would aid our understanding of how local concentrations of these agents develop and may also aid in efforts to isolate these agents which unfortunately still remain uncultivated.

We have extended the previous single description of a *Ca.* C. salmonicola species infecting brown trout, to include new strains represented by nearly full-length 16S rRNA gene sequences, closely related to both the *Ca.* C. salmonicola strains previously described. Whether these new strains have different infectious behavior must await their isolation and characterization; however, they have provided a useful molecular tool to distinguish wild and farm populations in the Venoge, implying that the sources of infection are local and distinct, and that if transmission is trout to trout, then this is likely to be through contact rather than indirectly via the water column. This is especially interesting as fish in the farms are siblings from mother animals caught in the same river and transported to the farms. Therefore, also vertical transmission is unlikely. There is an urgent need to study the wider environment, including other fish species and also possible invertebrate or protist hosts. An excellent study into epitheliocystis in cleaner wrasse associated with Atlantic salmon farms in Norway has already taken up this challenge (Steigen et al. 2015), showing that this is possible.

Acknowledgments We thank the fish farmers at the Venoge who provided the brown trout for examination. Further we thank Aurelie Rubin and the team of the Centre for Fish and Wildlife Health, Bern for help during the sampling. This study was partly financed by the ESKAS-Bundesstipendien für ausländische Studierende, Kunstschaffende und Forschende, Bern, Switzerland.

Compliance with ethical standards

Conflict of interest The authors declare they have no competing interests and they were notified about the content of the manuscript.

Animal rights statement All animals were treated according to the Swiss welfare regulations (permission number: 2871).

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