**Identification of *Parachlamydiaceae* DNA in nasal and rectal passages of healthy dairy cattle**

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Running title: Parachlamydial carriage in healthy cattle

**Abstract**

Aims: The order Chlamydiales comprises a broad range of bacterial pathogens and endosymbionts, which infect a wide variety of host species. Within this order, members of the family *Parachlamydiaceae*, which includes *Parachlamydia* and *Neochlamydia* species, have been particularly associated with infections in both humans and cattle, including having a potential pathogenic role in cases of bovine abortion. While the route of transmission has yet to be defined, it has been hypothesised that asymptomatic carriage and contamination of the immediate environment may be a route of inter-animal transmission. We investigated the asymptomatic carriage of *Chlamydia*-related organisms in healthy cattle.

Methods & Results: DNA was isolated from nasal and rectal swabs obtained from 38 healthy dairy heifers.  A Chlamydiales sp. 16S rRNA qPCR was performed on each sample. A total of 18/38 nasal samples and all 38/38 rectal samples were identified as positive for Chlamydiales sp. Each positive sample was sequenced confirming the presence of DNA bel/span>onging to the *Parachlamydiaceae*.

Conclusions: The presence of *Parachlamydiaceae* DNA in nasal and rectal swab samples of healthy cattle provides evidence for the asymptomatic carriage of parachlamydial organisms within cattle.

Significance & Impact of the Study: The study provides evidence of potential routes of environmental contamination that could provide a route for inter-animal and animal transmission of *Parachlamydiaceae*.

Keywords

Veterinary; PCR; Molecular detection; infection; shedding cattle;  Chlamydiales; *Parachlamydiaceae*.

**Introduction**

Members of the order Chlamydiales are Gram-negative obligate intracellular bacteria, which share similar biphasic developmental cycles and infect a wide range of animal species. In mammals, the most recognised clinical infections are associated with members of the *Chlamydiaceae*, including *Chlamydia trachomatis* (*C. trachomatis*) in humans, *C. psittaci* in avian species and *C. abortus* and *C. pecorum* in ruminant livestock (Wheelhouse and Longbottom 2012). In cattle, chlamydial infections are associated with a wide range of disease aetiologies, including abortion, endometritis, polyarthritis, encephalomyelitis, pneumonia and mastitis. In recent years, however, several novel species of *Chlamydia*-related organisms have emerged as potential disease causing organisms in cattle. These species include *Waddlia chondrophila (W. chondrophila)*, an organism isolated originally from an aborted bovine fetus in the USA (Dilbeck et al. 1990), and members of the *Parachlamydiaceae* (including *Parachlamydia* and *Neochlamydia* species) and *Rhabdochlamydiaceae* (*Rhabdochlamydia* species), whose presence has been reported in aborted bovine placentas in Switzerland and the UK using both molecular and immunohistochemical techniques (Borel et al. 2007; Ruhl et al. 2009; Wheelhouse et al. 2010; Wheelhouse et al. 2012).

In addition to overt pathogenesis, apparently healthy cattle may harbour sub-clinical chlamydial infections that can be transmitted between animals and which have been hypothesised to have a negative impact on performance (Jee et al. 2004; Poudel et al. 2012). Fecal shedding has been identified as an important mode of transmission of *Chlamydia* between cattle, however, it has also been suggested that the organisms may also be shed in other secretions, such as ocular and nasal discharges (Longbottom and Coulter 2003; Reinhold et al. 2011). To date, studies have been restricted to investigations into the transmission and effects of *Chlamydiaceae* species, particularly *C.abortus*, *C. psittaci* and *C. pecorum* (Reinhold et al. 2011). In a follow-on study on a farm in which a series of *Parachlamydia*-positive abortions had been diagnosed (Deuchande et al. 2010), parachlamydial DNA was identified in the cattle drinking water, including sequences identical to those found within the cattle tissues (Wheelhouse et al. 2011). This raised the possibility of transmission of these organisms between animals through nasal and rectal shedding and consequential contamination of their immediate environment, including drinking water supplies. Given this potential for intra-animal transmission of *Chlamydia*-related organisms, the current study was carried out to investigate the potential shedding of chlamydial organisms from the nasal and rectal passages of a cohort of apparently healthy dairy cattle.

**Animals, Materials & Methods**

**Animal sampling and welfare**

Nasal and rectal swab samples were taken from 38 non-pregnant 14-15 month old Friesian-Holstein heifers, using blue polystyrene breakpoint shaft, viscose tip swab, in tube (Technical Service Consultants Ltd., Heywood, Lancashire, UK), which were stored at -20ºC until analysis. All animals were purchased from the same initial source in Spring 2013. Each animal was Bovine Herpes Virus-1 antibody negative and Bovine Viral Diahorea Virus antigen negative and had been assessed by an experienced veterinary surgeon to be clear of any clinical signs of illness prior to sampling. The animals were housed communally within a cattle shed in two groups with equal numbers of animals and had shared access to the same mains drinking water plus hay/concentrate diet for a period of 70 days of acclimation prior to sampling. Sampling was performed in strict accordance with the requirements of' the Animals (Scientific Procedures) Act 1986. The experimental protocol was approved by the Moredun Animal Welfare Ethical Review Body (Permit number: E03/13; approved on 15 Jan 2013).

**DNA extraction & PCR**

Swabs were vigorously vortexed in 1 ml PBS and all liquid removed following centrifugation at 18,000 x g for 5 min. DNA was extracted from the pellet using a DNeasy® Blood and Tissue Kit (Qiagen Ltd., Crawley, UK).

To determine the presence of chlamydial DNA a pan-Chlamydiales qPCR targeting the 16S rRNA gene was performed. The qPCR assays were performed  using the forward primer panCh16F2 (5′-CCGCCAACACTGGGACT-3′), the reverse primer panCh16R2 (5′-GGAGTTAGCCGGTGCTTCTTTAC-3′) and the probe panCh16S (5′-FAM-CTACGGGAGGCTGCAGTCGAGAATC-BHQ1-3′) targeting a fragment of approximately 207 to 215 bp in the 16S rRNA gene (Lienard et al. 2011). Assays were performed in a total volume of 20 µl, using the Quanta Toughmix Low ROX (Quanta BioSciences, Inc.,Gaithersburg, USA), 0.1 µM primer (Exiqon, Vedbaek, Denmark), 0.1 µM probe (Integrated DNA Technologies, Iowa, USA), molecular-biology-grade water (Promega, Southampton, UK), and 1 µl DNA. The cycling conditions were 3 min at 95°C, followed by 50 cycles of 15 s at 95°C, 15 s at 67°C and 15 s at 72°C. The PCR products were detected with an Applied Biosystems7500 real time PCR system (ThermoFisher Scientific, Loughborough, UK).

Quantiﬁcation was achieved using a standard curve derived using a recombinant plasmid control containing the cloned 16S rRNA sequence from *Parachlamydia acanthamoebae* strain Bn9 (ATCC) in pGEM-T (Promega, Southampton, UK) as described (Wheelhouse et al. 2014). Ten-fold dilutions (107 copies to 1 copy/µl) were used to establish a standard curve for quantification. Negative controls containing molecular grade water, the standard curve, and samples were all analyzed in triplicate.

**Sequence analysis**

PCR products were purified using ISOLATE PCR and Gel Kit (Bioline, UK). Sequencing was performed using the primers panFseq (5′-CCAACACTGGGACTGAGA-3′) and panRseq (5′-GCCGGTGCTTCTTTAC-3′) by dideoxy chain termination / cycle sequencing on an ABI 3730XL DNA sequencer (Eurofins Genomics, Ebersberg, Germany).

Mutational distances between sequences and a haplotype network were estimated in the ‘pegas’ package version 1.0 in R 4.1.1 (Paradis 2010) from 33 rectal and five nasal collected chlamydial DNA sequences aligned with MUSCLE version 3.8.31 (Edgar 2004) (R script, Supplementary File1). The haplotype network was created using a kimura 80 model (the default in pegas), whereas percentage identity between haplotypes was calculated from raw number of sites differing between clusters. Clustering of samples into potential molecular operational taxonomic units was performed with VSEARCH version 2.13.6 (Rognes et al. 2016) at 98% identity with parameters “--cluster\_fast --id 0.98 --centroids --uc --clusterout\_id --clusters --consout --otutabout --profile -–sizeout.” Finally, the vsearch-defined centroid nucleotide sequence for each cluster was searched for using NCBI nucleotide BLAST against the ‘nt’ database ([https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE TYPE=BlastSearch](https://blast.ncbi.nlm.nih.gov/Blast.cgi?PARAMS=xik_8pm9ZzwpUbLNczRu3JtwwPRcqGtHtJspRDP3kM5zZTjydJtYcHs9QfqctJu9KVhQBN)) with default parameters. Sequences were also classified to genus-level using the RDP Classifier ([https://rdp.cme.msu.edu/classifier/classifier.jsp](https://mc.manuscriptcentral.com/jamicro?DOWNLOAD=TRUE&PARAMS=xik_25BbBYWWo5qnBd9s4ZS7ZqxNQGRFj38Cyy3BunaiG6MMCUjJrgV81yksrpy2fkEUiv55EyChoWTiUxQuBfwvoUXXDcGMhDKoDgD4swhnazxgPBDv8hfS6J1t4CD69GNZXnso2Lh7TEvxPnCpjPdMvSjPc7TEeK2LHzq4WY5BxJJk9BSRdr13haNxRoMbn5uN3jhoyPVgcnkPCGvMTKfQsodv6w5e9Qtsxg3xnGw2huFpnJCeRtvjC6ecjiJCzHiQC8wY8wN)) (Wang et al. 2007) and IDTAXA ([http://www2.decipher.codes/Classification.html](https://mc.manuscriptcentral.com/jamicro?DOWNLOAD=TRUE&PARAMS=xik_KqX7XiQvopYcHuVmC4Z34qFpo4siAhMwFieJwttkxRDXtmM4i7TMZJiDDUYmU9YcRDr7uCV2siyiKdCK7jGvGV4bjacXcsQWj9b2TUBxtDVYsM7UN5PLz3uqk3YaoAwAL8E5tW2qMzzjY9Ve8yVhSSWcPMRtnEPtx4iA6vaooWp5tH9DBpsGEjUoJTwaRRwoKSRP6UdVnALDUAQMFCgNBfj5Jw5JwwtYag1gRWdMKPeenjjsCSGDN4yNZsAyCmUYpvSDrvsBFeDenftqyshiVuqgRzMU)) (Murali et al. 2018). Sequences were classified using the RDP database version 16 with a confidence threshold of 50%, recommended for sequences below 250 bp (Wang et al. 2007) for both tools.

**Statistics**

Genome copy numbers determined from the nasal and rectal swabs by qPCR against a standard curve were compared using a two-tailed Mann-Whitney U (Wilcoxon rank-sum) test. All statistical analyses were carried out using the SPSS v23 statistical package (IBM).

**Results**

**Real-time qPCR**

PCR analysis revealed the presence of chlamydial DNA in 18/38 (47%) nasal and 38/38 (100%) of rectal swab samples. Quantification of the genome copy numbers detected from each of the swabs demonstrated a 75-fold greater number of 16S genomic copies extracted from rectal swabs than nasal swabs (median 855 (min 0; max 1.33 x 104) vs 1.08 x 105 (min 4.93 x 104; max 2.28 x 105); P<0.001, [Figure 1](https://mc.manuscriptcentral.com/jamicro?DOWNLOAD=TRUE&PARAMS=xik_2vwB7wTu47qn4dXuZf3ZPSo7vi9eVAygQQmPW2yTy2xcMpC65zpRntcqrGNPYJixLWeHMfdhWCtrz6LwxB3NAjbwZnpcD91rfTjLDXP4H2peC7r52VGFYEdzzN9rTUP37an24RUgAUPrJuhYPeK3tqGL2tg5H6BCQ4fZNVYdHQvRfVaP9T1yRdk3bbaBZWNkpYMjwWgxWaJkGV7N3S4XyZiZLDh" \t "_imageProof)).

**Sequence analysis**

Of the 56 PCR positive samples, sequence information was obtained for 45 samples from both forward and reverse sequencing primers, ranging in size from 123-212 bp in length though only 38 (33, nasal; 5, rectal) high quality sequences of greater than 150bp were included in subsequent analyses. As per repository guidelines only the four sequences greater than 200 bp were submitted to NCBI (accession numbers KF651170-KF651173). For consistency and due to variability in sequence lengths 37 sequences were aligned and trimmed (~163-165bp analogous to nucleotides 314-477 of the 16S gene (AF366365.1) of the *P. acanthamoebae* str. Hall’s coccus prior to downstream analysis. Sequence 15R which had a length of 156bp across the same region was also also included.

Haplotype clustering resulted in 18 clusters of sequences, with none of the five nasal sequences clustering with another sequence. Sequence similarity was high and the largest cluster was of nine rectal sequences which was separated from the next largest clusters of six sequences by only one mutation. Two of the nasal sequences were most distant from the next closest sequence cluster with 8 and 5 mutations separating them respectively ( [Figure 2](https://mc.manuscriptcentral.com/jamicro?DOWNLOAD=TRUE&PARAMS=xik_mM1QbBeN2rQXaRtbVLpiWFCG6ZuvoRpAGU7YnRqKFRoSCN4gc12dFZHy2QeyZBCr4U7RMzdwDSFS5EWAMZgFzBjSGrVvknnciRkQZfLvaJqJvFWRPNhow9JEweuMXZZKmhwy4NtML1fLLzga7vLK1qzT2uWkLxAy8CK4XMi6bNopz4iohDn51AnxaBXDf7ZaxqsgHavJgEszbNToQeSesmJd8N" \t "_imageProof) & Supplementary [Figure 1](https://mc.manuscriptcentral.com/jamicro?DOWNLOAD=TRUE&PARAMS=xik_2vwB7wTu47qn4dXuZf3ZPSo7vi9eVAygQQmPW2yTy2xcMpC65zpRntcqrGNPYJixLWeHMfdhWCtrz6LwxB3NAjbwZnpcD91rfTjLDXP4H2peC7r52VGFYEdzzN9rTUP37an24RUgAUPrJuhYPeK3tqGL2tg5H6BCQ4fZNVYdHQvRfVaP9T1yRdk3bbaBZWNkpYMjwWgxWaJkGV7N3S4XyZiZLDh" \t "_imageProof), haplotype network of nasal and rectal sequences). Percentage identity was accordingly high between haplotypes, with a lowest of 92.7% between groups I and IV (Supplementary Table S1, pairwise mutational distances and percentage similarity between haplotypes). Results between the haplotype network ([Figure 2](https://mc.manuscriptcentral.com/jamicro?DOWNLOAD=TRUE&PARAMS=xik_mM1QbBeN2rQXaRtbVLpiWFCG6ZuvoRpAGU7YnRqKFRoSCN4gc12dFZHy2QeyZBCr4U7RMzdwDSFS5EWAMZgFzBjSGrVvknnciRkQZfLvaJqJvFWRPNhow9JEweuMXZZKmhwy4NtML1fLLzga7vLK1qzT2uWkLxAy8CK4XMi6bNopz4iohDn51AnxaBXDf7ZaxqsgHavJgEszbNToQeSesmJd8N" \t "_imageProof)) and the sequence distance differ slightly due to the evolutionary model used to calculate distance in the network versus raw mutational differences for sequence similarity.

Vsearch clustered our 38 sequences into five groups (Supplementary Table S2), the largest of which contained 33 sequences. This cluster contained 32 sequences of rectal origin and one nasally sampled sequence. Of the remaining four clusters, one cluster contained two nasal sequences and the remaining three clusters were composed of one sequence each (i.e singletons). Blast results for a representative sequence (or centroid) for each vsearch cluster are given in (Supplementary Table S3), and results highlighted in bold are mentioned in the discussion.  RDP Classifier places all sequences to the *Parachlamydiaceae* family, however, not all sequences were placed into the *Parachlamydiaceae* family by IDTAXA, as 8 were not assigned to a family (Table 1). Full output for each program can be found in Supplementary Tables S4-S5.

**Discussion**

The potential of *Chlamydia*-related organisms to cause disease in cattle was initially suggested by the isolation of *W. chondrophila* in 1989 (Dilbeck et al, 1990). However, while the sporadic nature of *W. chondrophila* identification has suggested a limited impact on cattle health, parachlamydial species have been implicated in a significant number of clinical cases of bovine abortion and respiratory disease (Taylor-Brown et al. 2015). Although this has suggested a hitherto unrecognised cause of livestock disease the absence of marked pathophysiology after experimental respiratory infection in cattle with *Parachlamydia* implies that the organisms exhibit lower levels of pathogenicity than *Chlamydia sp*. such as *C. psittaci* (Lohr et al. 2015; Lohr et al. 2016) . However, while the prevalence of these organisms seems to suggest endemicity or in the absence of clinical disease potentially a commensal role, the source of infection has largely remained conjecture. The results of the current study are the first to date to demonstrate the presence of parachlamydial DNA in nasal and rectal swabs in apparently healthy cattle suggesting asymptomatic carriage and potential shedding of parachlamydial organisms into the farm environment. Furthermore, the significantly higher levels of parachlamydial DNA in rectal swabs is also consistent with fecal shedding as a primary route of transmission consistent with other chlamydial species in cattle (Ostermann et al. 2013).

The suggestion that cattle could be potential sources of parachlamydial transmission came from a previous study which demonstrated the presence of parachlamydial DNA in cattle drinking water (Wheelhouse et al. 2011). The presence of parachlamydial DNA in water was not unusual as many of the known *Parachlamydiaceae* were initially isolated from water sources as endosymbionts of amoebal species (Greub and Raoult 2002). However, the absence of parachlamydial DNA in the bore-hole water source indicated the potential for contamination from within the cattle sheds, including the cattle themselves. The current study while not definitively demonstrating the presence of live organisms in mucosal sites of the cattle does support the hypothesis that cattle themselves may be the source of parachlamydial infection.

Although both classifier programs compared amplicons against the same database, IDTAXA was less specific in its classification of our sequences than RDP Classifier. This is likely due a more conservative underlying approach to sequence classification of our short sequences. Blast results of our 5 vsearch clusters revealed all of them best-match *Parachlamydia* species as for IDTAXA and RDP Classifier analyses. Four of the vsearch clusters had the same best-scoring match (accessions beginning KX451 in Supplementary Table S3), including the group containing the majority of sequences (32/38). This was to *Parachlamydiaceae* detected in *Ixodes ricinus* ticks (Hokynar et al. 2016), concordant with other studies implicating ticks in the transmission of *Parachlamydia* and other *Chlamydia*-related bacteria in multiple mammalian species (Taylor-Brown and Polkinghorne 2017). The only cluster that did not best match *Ixodes-*derived *Parachlamydiaceae* contained a single nasal cluster and was most similar to *Parachlamydia* sequences detected in domestic water supply in Switzerland (Lienard et al. 2017) (Accessions beginning JX083), with a secondary hit to a *Protochlamydia* (*Parachlamydiaceae*) species identified from a Japanese hot spring (Sampo et al. 2014). While the short sequence lengths analysed in this study limit the degree of certainty with which we can make identifications beyond the family or genus level, these results tentatively suggest that water could be the source of at least some of the parachlamydial DNA identified in the nasal samples. However, while we previously demonstrated the presence of parachlamydial DNA in cattle drinking water related to an abortion storm (Wheelhouse et al. 2011) many of the nasal samples in this current study could not be sequenced and the animals in this study had only recently been brought onto the farm this hypothesis remains conjecture and worthy of further study.

In summary, this report demonstrates the presence of *Parachlamydiaceae* DNA in nasal and rectal swab samples of healthy cattle providing evidence for the asymptomatic carriage of parachlamydial organisms within cattle and potential routes of environmental contamination that could provide a route for inter-animal transmission. Given the almost ubiquitous identification of parachlamydial DNA in these animals and the previously identified numbers of parachlamydial positive bovine abortions, these data suggests that there is a need for further systematic investigations to determine the true causative role of *Parachlamydiaceae* sp. in bovine abortion.

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**Conflict of interest**

No conflict of interest declared

**Authors contribution statement**

NW, DL, MD conceived the original idea and designed the study. NW, ML and AF were involved in sampling and carried out the molecular analyses. JH undertook the DNA sequence analysis. NW and JH wrote the manuscript with support from ML, AF, DL and MD.

**Data availability statement**

The data that support the findings of this study are available from the corresponding author upon reasonable request. Sequences KF651170-KF651173 are freely available from NCBI Genbank (https://www.ncbi.nlm.nih.gov/genbank/).

References

Borel, N., Ruhl, S., Casson, N., Kaiser, C., Pospischil, A. and Greub, G. (2007) *Parachlamydia spp*. and related *Chlamydia*-like organisms and bovine abortion. *Emerg Infect Dis* **13**, 1904-1907.

Deuchande, R., Gidlow, J., Caldow, G., Baily, J., Longbottom, D., Wheelhouse, N., Borel, N. and Greub, G. (2010) *Parachlamydia* involvement in bovine abortions in a beef herd in Scotland. *Vet Rec* **166**, 598-599.

Dilbeck, P.M., Evermann, J.F., Crawford, T.B., Ward, A.C., Leathers, C.W., Holland, C.J., Mebus, C.A., Logan, L.L., Rurangirwa, F.R. and McGuire, T.C. (1990) Isolation of a previously undescribed *Rickettsia* from an aborted bovine fetus. *J Clin Microbiol* **28**, 814-816.

Edgar, R.C. (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* **32**, 1792-1797.

Greub, G. and Raoult, D. (2002) *Parachlamydiaceae*: potential emerging pathogens. *Emerg Infect Dis* **8**, 625-630.

Hokynar, K., Sormunen, J.J., Vesterinen, E.J., Partio, E.K., Lilley, T., Timonen, V., Panelius, J., Ranki, A. and Puolakkainen, M. (2016) *Chlamydia*-Like Organisms (CLOs) in Finnish Ixodes ricinus Ticks and Human Skin. *Microorganisms* **4**.

Jee, J., Degraves, F.J., Kim, T. and Kaltenboeck, B. (2004) High prevalence of natural *Chlamydophila* species infection in calves. *J Clin Microbiol* **42**, 5664-5672.

Lienard, J., Croxatto, A., Aeby, S., Jaton, K., Posfay-Barbe, K., Gervaix, A. and Greub, G. (2011) Development of a new *Chlamydiales*-specific real-time PCR and its application to respiratory clinical samples. *J Clin Microbiol* **49**, 2637-2642.

Lienard, J., Croxatto, A., Gervaix, A., Levi, Y., Loret, J.F., Posfay-Barbe, K.M. and Greub, G. (2017) Prevalence and diversity of *Chlamydiales* and other amoeba-resisting bacteria in domestic drinking water systems. *New Microbes New Infect* **15**, 107-116.

Longbottom, D. and Coulter, L.J. (2003) Animal chlamydioses and zoonotic implications. *J Comp Pathol* **128**, 217-244.

Lohr, M., Prohl, A., Ostermann, C., Liebler-Tenorio, E., Schroedl, W., Aeby, S., Greub, G. and Reinhold, P (2015). A bovine model of a respiratory *Parachlamydia acanthamoebae* infection. Pathog Dis. **73**, 1-14

Lohr, M., Prohl, A., Ostermann, C., Diller, R., Greub, G. and Reinhold, P. (2016). Effect of *Parachlamydia acanthamoebae* on pulmonary function parameters in a bovine respiratory model. Vet J. **213**, 9-15

Murali, A., Bhargava, A. and Wright, E.S. (2018) IDTAXA: a novel approach for accurate taxonomic classification of microbiome sequences. *Microbiome* **6**, 140.

Ostermann, C., Rüttger, A., Schubert, E., Schrödl, W., Sachse, K. and Reinhold, P. (2013).

Infection, disease, and transmission dynamics in calves after experimental and natural challenge with a Bovine *Chlamydia psittaci* isolate. PLoS One. 2013 May 14;8(5):e64066

Paradis, E. (2010) pegas: an R package for population genetics with an integrated-modular approach. *Bioinformatics* **26**, 419-420.

Poudel, A., Elsasser, T.H., Rahman Kh, S., Chowdhury, E.U. and Kaltenboeck, B. (2012) Asymptomatic endemic *Chlamydia pecorum* infections reduce growth rates in calves by up to 48 percent. *PLoS One* **7**, e44961.

Reinhold, P., Sachse, K. and Kaltenboeck, B. (2011) *Chlamydiaceae* in cattle: commensals, trigger organisms, or pathogens? *Vet J* **189**, 257-267.

Rognes, T., Flouri, T., Nichols, B., Quince, C. and Mahe, F. (2016) VSEARCH: a versatile open source tool for metagenomics. *PeerJ* **4**, e2584.

Ruhl, S., Casson, N., Kaiser, C., Thoma, R., Pospischil, A., Greub, G. and Borel, N. (2009) Evidence for *Parachlamydia* in bovine abortion. *Vet Microbiol* **135**, 169-174.

Sampo, A., Matsuo, J., Yamane, C., Yagita, K., Nakamura, S., Shouji, N., Hayashi, Y., Yamazaki, T., Yoshida, M., Kobayashi, M., Ishida, K. and Yamaguchi, H. (2014) High-temperature adapted primitive *Protochlamydia* found in *Acanthamoeba* isolated from a hot spring can grow in immortalized human epithelial HEp-2 cells. *Environ Microbiol* **16**, 486-497.

Taylor-Brown, A. and Polkinghorne, A. (2017) New and emerging chlamydial infections of creatures great and small. *New Microbes New Infect* **18**, 28-33.

Taylor-Brown, A., Vaughan, L., Greub, G., Timms, P. and Polkinghorne, A. (2015) Twenty years of research into *Chlamydia*-like organisms: a revolution in our understanding of the biology and pathogenicity of members of the phylum Chlamydiae. *Pathog Dis* **73**, 1-15.

Wang, Q., Garrity, G.M., Tiedje, J.M. and Cole, J.R. (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol* **73**, 5261-5267.

Wheelhouse, N., Coyle, C., Barlow, P.G., Mitchell, S., Greub, G., Baszler, T., Rae, M.T. and Longbottom, D. (2014) *Waddlia chondrophila* infects and multiplies in ovine trophoblast cells stimulating an inflammatory immune response. *PLoS One* **9**, e102386.

Wheelhouse, N., Howie, F., Gidlow, J., Greub, G., Dagleish, M. and Longbottom, D. (2012) Involvement of *Parachlamydia* in bovine abortions in Scotland. *Vet J* **193**, 586-588.

Wheelhouse, N., Katzer, F., Wright, F. and Longbottom, D. (2010) Novel chlamydia-like organisms as cause of bovine abortions, UK. *Emerg Infect Dis* **16**, 1323-1324.

Wheelhouse, N. and Longbottom, D. (2012) Endemic and emerging chlamydial infections of animals and their zoonotic implications. *Transbound Emerg Dis* **59**, 283-291.

Wheelhouse, N., Sait, M., Gidlow, J., Deuchande, R., Borel, N., Baily, J., Caldow, G. and Longbottom, D. (2011) Molecular detection of *Chlamydia*-like organisms in cattle drinking water. *Vet Microbiol* **152**, 196-199.

Table 1. RDP Classifier and IDTAXA classification of nasal and rectal swab sequences. Both tools were used the RDP 16S rRNA training set 16 as a reference.

**Figure legends**

[Figure 1](https://mc.manuscriptcentral.com/jamicro?DOWNLOAD=TRUE&PARAMS=xik_2vwB7wTu47qn4dXuZf3ZPSo7vi9eVAygQQmPW2yTy2xcMpC65zpRntcqrGNPYJixLWeHMfdhWCtrz6LwxB3NAjbwZnpcD91rfTjLDXP4H2peC7r52VGFYEdzzN9rTUP37an24RUgAUPrJuhYPeK3tqGL2tg5H6BCQ4fZNVYdHQvRfVaP9T1yRdk3bbaBZWNkpYMjwWgxWaJkGV7N3S4XyZiZLDh" \t "_imageProof). Box and whisker plot of total Chlamydiales genomic copy number in nasal and rectal samples analysed by 16S qPCR (see Materials and Methods). \*\*\* P<0.001 significantly higher rectal vs nasal genomic copy number in healthy cattle (two-tailed Mann-Whitney U (Wilcoxon rank-sum) test).

[Figure 2](https://mc.manuscriptcentral.com/jamicro?DOWNLOAD=TRUE&PARAMS=xik_mM1QbBeN2rQXaRtbVLpiWFCG6ZuvoRpAGU7YnRqKFRoSCN4gc12dFZHy2QeyZBCr4U7RMzdwDSFS5EWAMZgFzBjSGrVvknnciRkQZfLvaJqJvFWRPNhow9JEweuMXZZKmhwy4NtML1fLLzga7vLK1qzT2uWkLxAy8CK4XMi6bNopz4iohDn51AnxaBXDf7ZaxqsgHavJgEszbNToQeSesmJd8N" \t "_imageProof) . Haplotype network of nasal and rectal sequences. Haplotype size is indicated by circle diameter and the label, rectal and nasal haplotypes were coloured blue and red respectively. Mutational distance between haplotypes is indicated by small black circles along nodes.

**Supplementary Figures**

Supplementary Figure S1. [Figure 1](https://mc.manuscriptcentral.com/jamicro?DOWNLOAD=TRUE&PARAMS=xik_2vwB7wTu47qn4dXuZf3ZPSo7vi9eVAygQQmPW2yTy2xcMpC65zpRntcqrGNPYJixLWeHMfdhWCtrz6LwxB3NAjbwZnpcD91rfTjLDXP4H2peC7r52VGFYEdzzN9rTUP37an24RUgAUPrJuhYPeK3tqGL2tg5H6BCQ4fZNVYdHQvRfVaP9T1yRdk3bbaBZWNkpYMjwWgxWaJkGV7N3S4XyZiZLDh" \t "_imageProof) with labels given as Roman numerals corresponding to haplotype groups allowing the figure to be matched to distances given in Supplementary Table S1.

**Supplementary Files**

Supplementary File S1. R-code used to generate haplotype network ([Figure 2](https://mc.manuscriptcentral.com/jamicro?DOWNLOAD=TRUE&PARAMS=xik_mM1QbBeN2rQXaRtbVLpiWFCG6ZuvoRpAGU7YnRqKFRoSCN4gc12dFZHy2QeyZBCr4U7RMzdwDSFS5EWAMZgFzBjSGrVvknnciRkQZfLvaJqJvFWRPNhow9JEweuMXZZKmhwy4NtML1fLLzga7vLK1qzT2uWkLxAy8CK4XMi6bNopz4iohDn51AnxaBXDf7ZaxqsgHavJgEszbNToQeSesmJd8N" \t "_imageProof)).

**Supplementary Tables**

Supplementary Table S1. Distance matrix between the 18 haplotypes estimated in pegas. The top half gives percentage similarity and the bottom half mutational distance. The haplotypes can be matched back to [Figure 2](https://mc.manuscriptcentral.com/jamicro?DOWNLOAD=TRUE&PARAMS=xik_mM1QbBeN2rQXaRtbVLpiWFCG6ZuvoRpAGU7YnRqKFRoSCN4gc12dFZHy2QeyZBCr4U7RMzdwDSFS5EWAMZgFzBjSGrVvknnciRkQZfLvaJqJvFWRPNhow9JEweuMXZZKmhwy4NtML1fLLzga7vLK1qzT2uWkLxAy8CK4XMi6bNopz4iohDn51AnxaBXDf7ZaxqsgHavJgEszbNToQeSesmJd8N" \t "_imageProof) through Supplementary [Figure 1](https://mc.manuscriptcentral.com/jamicro?DOWNLOAD=TRUE&PARAMS=xik_2vwB7wTu47qn4dXuZf3ZPSo7vi9eVAygQQmPW2yTy2xcMpC65zpRntcqrGNPYJixLWeHMfdhWCtrz6LwxB3NAjbwZnpcD91rfTjLDXP4H2peC7r52VGFYEdzzN9rTUP37an24RUgAUPrJuhYPeK3tqGL2tg5H6BCQ4fZNVYdHQvRfVaP9T1yRdk3bbaBZWNkpYMjwWgxWaJkGV7N3S4XyZiZLDh" \t "_imageProof) which labels haplotypes by Roman numerals.

Supplementary Table S2. Vsearch clusters of nasal and rectal sequences.

Supplementary Table S3. Blast results of Vsearch centroid sequences against the nucleotide database (nt). Sequences discussed in the text are bolded. Top sequences originating from our own NCBI submission for this work were not considered.

Supplementary Table S4. RDP Naive Bayesian rRNA Classifier tool output for nasal and rectal sequences against RDP 16S rRNA training set 16.

Supplementary Table S5. IDTAXA tool output for nasal and rectal sequences against RDP 16S rRNA training set