Legione Alistair (Orcid ID: 0000-0003-2407-4496)

Title: *Chlamydia psittaci*: a suspected cause of reproductive loss in three Victorian horses

Running title: *C. psittaci* and equine abortion in Victoria

Rumana Akter[a,b], Andrew W. Stent[c], Fiona M. Sansom[a], James R. Gilkerson[a], Chelsie Burden[d], Joanne M. Devlin[a*^], Alistair R. Legione[a^] and Charles M. El-Hage[a^]

[a] Asia Pacific Centre for Animal Health, The Melbourne Veterinary School, The University of Melbourne, Parkville, Victoria, 3010 Australia

[b] Department of Medicine (RMH), The University of Melbourne, Parkville, Victoria, 3010 Australia

[c] The Melbourne Veterinary School, The University of Melbourne, Werribee, Victoria, 3030 Australia

[d] Goulburn Valley Equine Hospital, 905 Goulburn Valley Hwy, Congupna, Victoria, 3633 Australia

^These authors contributed equally

*Corresponding author: devlinj@unimelb.edu.au

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Abstract

Chlamydia psittaci was detected by PCR in the lung and equine foetal membranes of two aborted equine foetuses and one weak foal from two different studs in Victoria, Australia. The abortions occurred in September 2019 in two mares sharing a paddock north east of Melbourne. The weak foal was born in October 2019 in a similar geographical region and died soon after birth despite receiving veterinary care. The detection of *C. psittaci* DNA in the lung and equine foetal membranes of the aborted or weak foals, and the absence of any other factors that are commonly associated with abortion or neonatal death, suggest that this pathogen may be the cause of the reproductive loss. The detection of *C. psittaci* in these cases is consistent with the recent detection of *C. psittaci* in association with equine abortion in New South Wales. These cases in Victoria show that *C. psittaci*, and the zoonotic risk it poses, should be considered in association with equine reproductive loss in other areas of Australia.

Keywords: *Chlamydia psittaci,* equine, abortion, Victoria

Case report introduction

Chlamydia psittaci causes a zoonotic disease known as psittacosis [1]. The disease in humans is characterized by fever, malaise, myalgia and atypical pneumonia [2]. Birds are commonly infected and a major feature of *C. psittaci* infection in birds is its ready transmission to mammalian species, including humans [3, 4]. Human psittacosis is frequently reported worldwide and mainly occurs as a result of direct or indirect interactions with infected birds or bird excreta [2, 5, 6].

In addition to humans, *C. psittaci* has been detected in other mammals including ruminants and horses [7-10]. In animals, respiratory, intestinal, arthritic and reproductive diseases can occur due to *C. psittaci* infection [11]. Horses are considered to be occasional hosts of *C. psittaci* and infection can result in pneumonia, conjunctivitis, polyarthritis or abortion [9, 11]. Until recently, *C. psittaci* infection has often been overlooked amongst the differential diagnoses of equine reproductive loss, with only limited epidemiological studies conducted to examine the associations between *C. psittaci* and equine abortion. Within this last decade however, several reports of *C. psittaci* as the aetiological agent of equine abortion have been published [11-15]. These reports have included the description of *C psittaci*

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infection in cases of equine abortions and/or neonatal illness, with associated cases of human disease, in New South Wales, Australia.

In this case series, we report three cases of equine abortion or neonatal death associated with detection of *C. psittaci* in foetal and/or equine foetal membranes. To our knowledge these are the first reports of Australian equine abortions/reproductive loss in association with *C. psittaci* outside of New South Wales and highlights the importance of considering *C. psittaci,* and the zoonotic risk that it presents, in association with equine reproduction loss in other areas of Australia.

Clinical features

History and clinical findings

A 7-year-old Friesian mare aborted a 9.5 month old foetus (Foetus ID 3341) in September 2019. Two weeks later another mare (also Friesian) residing in the same paddock also aborted (Foetus ID 3345). The gestational age of mare and the age of foetus in the second abortion event were not recorded. The vaccination status of the mares was unknown. No predisposing factors for abortion were identified.

In October 2019, a thoroughbred mare delivered a weak foal (Foal ID 3350) which died 24 hours later despite resuscitative treatment for suspected neonatal isoerythrolysis. The one-day-old thoroughbred foal displayed clinical signs of disease consistent with neonatal isoerythrolysis, including weakness, bradycardia, bradypnoea, low temperature (35˚C), congested mucous membranes and cold extremities. The two farms were located North East of Melbourne, 75 km apart from each other.

Diagnostic sample collection and DNA extraction

Foetal tissues (lung, liver, thymus, spleen and equine foetal membranes) were initially submitted to the AsiaPacific Centre for Animal Health (APCAH) in Melbourne, Australia for equine herpesvirus-1 (EHV-1) and 4 (EHV-4) testing using PCR. Following negative EHV-1 and EHV-4 results the samples were tested for the presence of *C. psittaci* DNA. For this, DNA from individual tissues was extracted by a Kingfisher robot with a MagMAX™ Core Nucleic Acid Purification Kit (Thermo Fisher Scientific)

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according to the manufacturer instructions. Extracted DNA was then stored at -80°C before being used as template in a PCR to detect *C. psittaci.*

C. psittaci screening and genotyping

Individual foetal tissues were tested by targeting a 460 bp region of the 16S rRNA gene of *Chlamydiaceae* using quantitative PCR as described previously [16]. The PCR was performed in a total volume of 25 µL containing 1× Green GoTaq Flexi Buffer (Promega), 2 mM $MgCl_2$ (Promega), 0.2 mM of each deoxynucleotide triphosphate (Bioline), 2 µM of each primer (16SG-F TGATGAGGCATGCAAGTC, 16SG-R TTACCTGGTACGCTCAAAT), 1.5 U of GoTaq DNA polymerase (Promega), 10 µM of SYTO 9 fluorescent nucleic acid stain (Invitrogen) and 5 µL DNA template, with Milli-Q filtered water to reach final volume. Positive control reactions used DNA extracted from the target sequences of *C. psittaci* and *C. pecorum* cloned into pGEM-T (Promega) . Milli-Q filtered water was used as template in negative control reactions. The limit of the assay was determined by testing ten-fold serial dilutions from 10^8-10^1 copies of the targeted plasmid in triplicate. Thermal cycling conditions were the same as described previously [16].The curves produced from control samples of *Chlamydia* DNA were used to compare the profile of the melting curve for each clinical sample. Samples were considered to be positive if the Ct value was < 35 and the profile of the melt curve was consistent with those generated from positive control samples. The genome copies of *Chlamydia* in the reactions were quantitated from the qPCR results.

Multi-locus sequence typing (MLST) was performed on *C. psittaci* positive samples according to the previously developed scheme by Pannekoek et al [17]. Fragments of seven housekeeping genes (*gatA, oppA, hflX, gidA, enoA, hemN* and *fumC*) of *Chlamydiales* were amplified and sequenced using the oligonucleotide primers previously published on the *Chlamydiales* MLST website (https://pubmlst.org/chlamydiales/info/MLST_primers_2012.pdf) [18]. The PCR of each targeted gene was performed as described previously [19]. All PCRs were performed in a T100 Bio-Rad thermal cycler. The amplified PCR products (381 – 635 bp in length) were visualised by UV transillumination after agarose gel electrophoresis.

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PCR products were purified from the PCR reaction mixtures using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instruction and eluted in 30 µL Tris-EDTA buffer. The quantity of purified DNA was estimated using a spectrophotometer (NanoDrop Technologies) and sequenced using Big Dye Terminator (BDT) v3.1 (Life Technologies) according to manufacturer's instructions. The purified products were sent to Australian Genome Research Facility (AGRF) for capillary separation. Geneious bioinformatics software version 11.1.4 (Biomatters) was used to trim, manually curate and align all obtained sequences [20]. Nucleotide sequences were compared with publicly available sequences in the Genbank database [21] using the NCBI Nucleotide Basic Local Alignment Search Tool (BLASTN) online algorithm [22]. The online *Chlamydiales* MLST database (https://pubmlst.org/chlamydiales) was used to determine the allelic designation for each locus of the *C. psittaci* sequences , and the sequence types (STs) were identified using the subsequent allelic profile. The alignment of concatenated MLST fragments was used to generate a phylogenetic tree. A phylogenetic tree with 500 bootstraps and GTR+I model was generated with PhyML [23] using multiple sequence MAFFT [24] alignment of positive strains, as implemented in Geneious.

Results and Discussion

C. psittaci DNA was detected by qPCR in lung and equine foetal membrane samples from foetus ID 3341, in the equine foetal membranes from foetus ID 3345 and in the lung from foal ID 3350. Non-infectious causes of abortion such as twin pregnancy, foetal malformations, umbilical cord strangulations and uterine torsion were not observed. The presence of *C. psittaci*, the exclusion of other pathogens (EHV-1 and EHV-4 were not detected by PCR) and the absence of any apparent non-infectious causes of abortion in these three cases suggests that *C. psittaci* was the cause of the abortion or foal death and indicates that this pathogen is a threat to equine industries beyond NSW in Australia where it has been detected previously. The load of *C. psittaci* in positive samples (mean = 1.27×10^7 genome copies/µL of extract) was comparable to the highest levels detected in foetal tissue in an epizootic of *C. psittaci* equine reproductive loss in NSW [14] further suggesting that *C. psittaci* was the causative agent, rather than an incidental detection, in these Victorian cases.

Previous studies have used histopathological examination to confirm *C. psittaci* infection in association with cases of equine abortion or reproductive loss [14]. Histopathological analysis was attempted in

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these current cases, however the tissues had been stored at -70°C without any fixative and the cellular detail was insufficiently preserved for detection of lesions. Investigation of future cases of equine abortion events would be aided by histopathological examination of appropriately stored tissues, but it is important to note that in previous reports of *Chlamydia*-associated abortion in mares, 31% of positive cases did not display any histological lesions, while a further 20% only displayed mild lymphohistiocytic placentitis that could easily be masked by autolysis [11]. These findings indicate that routine histological assessment is not a sensitive method for detection of *C. psittaci* infection, and other ancillary testing should be used to support the diagnosis.

When MLST analysis was applied to the *C. psittaci* positive samples they were identified as belonging to ST24. This same ST has previously been detected in humans, Australian parrots, including wild parrots in Victoria, and other *C. psittaci* equine abortion cases in Australia. [25-28] (Figure 1). The findings from the molecular analyses conducted in this case report are consistent with Australian native parrots being the reservoir of equine *C. psittaci* infection in Australia [14, 25, 26]. Information relating to direct or in-direct contact between Australia native parrots and the horses in this case report is not available. A greater number of Australian white ibis (*Threskiornis Molucca*) in the area had been noted during the spring of 2019 by one of the authors (CB). *C. psittaci* have been detected in species of ibis in Europe, [29] but to date the ST24/6BC genotype has not been specifically detected.

The highly virulent 6BC clade of *C. psittaci* detected in equine abortion samples in Australia is a concern for public health. Recently, human *C. psittaci* infections have been reported after contact with equine foetal membranes within an Australian veterinary school and hospital in NSW. The cases identified in this report were at least 200 km south of the southernmost NSW property, and the first reported in Victoria [14, 30]. Measures such as the use of appropriate personal protective equipment and biosecurity procedures are recommended to decrease the risk of human infection. These actions may also help in preventing spread of infection among horse populations.

Conclusion

This report adds to the previous detections this decade of equine psittacosis in New South Wales and highlights the importance of including *C. psittaci* in routine diagnostic testing in cases of equine

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abortion or reproductive loss across different regions of Australia. As *C. psittaci* is zoonotic this report also underscores the importance taking appropriate protective measures when dealing with cases of equine abortion or reproductive loss in order to avoid human infection and disease.

Conflict of interest

The authors declare that they have no competing interests.

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Figure Legends

Figure 1: PhyML phylogenetic tree generated with 500 bootstraps using the alignment of the concatenated MLST fragment sequences (3098 bp) of Australian and international strains of *C. psittaci*. *C. psittaci* sequences from this study are shown in bold. The host species is denoted by different colours. Bootstrap values greater than 60 are shown on the tree nodes. Previously published sequences were retrieved from the MLST database [18].

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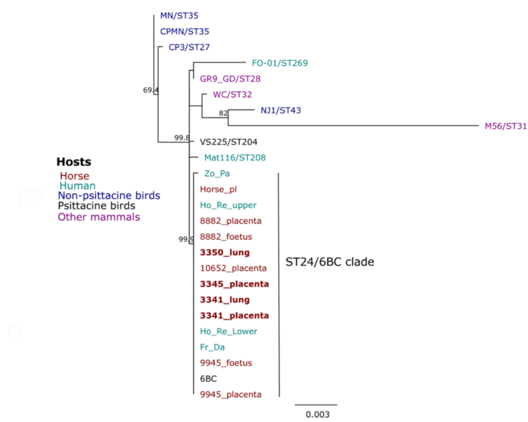

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