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1 **Short contribution**

2

3 **Detection of *Chlamydiaceae* in ocular swabs from Australian pre-export feedlot sheep**

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15

16 **Abstract**

17 Infectious Ovine Keratoconjunctivitis (IOK) is a contagious ocular disease of sheep. A range of  
18 organisms have been observed as the aetiological agents of IOK. In this study, the presence  
19 of chlamydial pathogens (*C. pecorum*, *C. abortus*, *C. psittaci*) in conjunctival swabs was tested  
20 for. The swabs were collected from sheep with varying grades of IOK in an Australian pre-  
21 export feedlot. The sheep had been rejected from a shipment because of the eye disease. The  
22 relative contribution of chlamydial pathogens to IOK and the rejection of animals was  
23 evaluated.

24

25 In total, 149 conjunctival swabs were taken from rejected sheep (IOK Grades 1 to 6; n=126)  
26 as well as those with healthy eyes (Grade 0; n = 23). Screening for chlamydial pathogens was  
27 done using species-specific qPCR assays. Chlamydial DNA was detected in 35.6% (53/149) of  
28 conjunctival samples. *C. pecorum* was the most predominant species with an overall  
29 prevalence of 28.9% (43/149). *C. psittaci* prevalence was 6.7% (10/149). Both organisms were  
30 detected in healthy as well as IOK-affected eyes. All swabs tested negative for *C. abortus*.

31

32 The results from this study demonstrate that *Chlamydia* spp. can be readily detected in sheep  
33 presenting with IOK. The zoonotic *C. abortus* was not detected in any of the samples in this  
34 study, providing further evidence to the suggestion that this pathogen remains absent from  
35 Australia. Although the exact contribution of *Chlamydia* spp. in the IOK pathogenesis is  
36 unclear, such studies are anticipated to be of benefit to Australian domestic and live export  
37 production systems.

38

39 **Keywords:** Sheep; Infectious keratoconjunctivitis; *Chlamydia*; species-specific qPCR;  
40 *Chlamydia pecorum*; *Chlamydia psittaci*

41

42 **Abbreviations:** IOK, Infectious Ovine Keratoconjunctivitis; qPCR, quantitative PCR; HRM, high  
43 resolution melt; CI, confidence interval.

44 **Introduction**

45 Infectious Ovine Keratoconjunctivitis (IOK), also known as *pinkeye*, is a highly transmissible  
46 ocular disease of sheep that has a worldwide distribution. IOK is characterised by an acute  
47 conjunctivitis, blepharospasm, lacrimation, and hyperaemia, followed by varying degrees of  
48 corneal opacity and ulceration<sup>1</sup>. A range of organisms, including *Mycoplasma* spp. (notably *M*  
49 *conjunctivae*), *Moraxella ovis*, and *Chlamydia* spp. have been observed as the aetiological  
50 agents of IOK<sup>1-4</sup>.

51

52 In Australia, besides the concerns for animal welfare, diseases such as IOK can also cause  
53 significant economic loss to the ovine feedlot and export industry associated with animal  
54 rejections from shipments<sup>2</sup>. A study characterising the ocular flora associated with IOK as well  
55 as the flora in healthy eyes in pre-export feedlot sheep in Western Australia, identified a range  
56 of microorganisms. *Mycoplasma* spp. and *Moraxella ovis* were the pathogens most closely  
57 associated with IOK. However, it was also observed that healthy animals shed these same  
58 pathogens, representing a possible a risk to other sheep <sup>2</sup>. At the time of the study, no testing  
59 was performed for *Chlamydia* spp.

60

61 Over the past decade, several studies have demonstrated that the obligate intracellular  
62 bacterial pathogen, *Chlamydia pecorum*, can cause economically significant diseases such as  
63 conjunctivitis and arthritis in Australian sheep, as recently reviewed <sup>4</sup>. *Chlamydia abortus*, the  
64 important cause of ovine enzootic abortion in small ruminants elsewhere in the world is  
65 seemingly absent in Australia<sup>5</sup>. The avian pathogen, *Chlamydia psittaci* infects birds and has  
66 occasionally been documented in mammals, including humans<sup>6,7</sup>. While laboratory detection  
67 of chlamydial infections in domestic sheep production is primarily limited to *C. pecorum*, some  
68 countries importing Australian livestock for breeding purposes require *C. abortus* testing,  
69 despite no confirmatory evidence of *C. abortus* infection in Australian animals. This latter  
70 testing relies primarily on commercially available assays with low specificity and hence high  
71 cross-reactivity to other *Chlamydiae*<sup>8</sup> as well as other bacteria <sup>5</sup>. The result is that the specific  
72 prevalence and impact of these chlamydial infections on the health of live export animals is  
73 unknown.

74

75 In this study, retrospective screening for several chlamydial pathogens (*C. pecorum*, *C.*  
76 *abortus*, *C. psittaci*) in conjunctival swabs collected from pre-export rejected sheep with  
77 varying grades of IOK from an Australian pre-export feedlot was performed. It was  
78 hypothesised that chlamydial pathogens are commonly detected in IOK and such infections  
79 may contribute to the IOK disease process and are therefore associated with rejection of  
80 animals before export.

81

## 82 **Materials and methods**

83 One hundred and twenty seven conjunctival swabs were collected from sheep with naturally  
84 occurring IOK that arrived at a pre-export feedlot in Western Australia. The sheep were  
85 selected from a group of sheep that had been identified as unsuitable for shipment on arrival  
86 at the feedlot within the previous three days. This group was made up of animals with a  
87 variety of ailments, not just ocular lesions. Those with ocular lesions were identified initially  
88 and then a further selection was taken from this group to be included in the study. The  
89 conjunctival swabs were taken from sheep with varying degrees of IOK ranging from epiphora  
90 (weeping eye) (Grade 1; n = 32), conjunctivitis and scleral injection (Grade 2; n = 49), corneal  
91 oedema (Grade 3; n = 28), corneal ulceration (Grade 4; n = 9), corneal neovascularisation  
92 (Grade 5; n = 7) and to chronic eye damage (Grade 6; n = 1). This swab collection was followed  
93 by heterotrophic bacterial isolation and identification. Cotton tipped swabs were placed  
94 between the globe and the conjunctiva and gently rotated for 15 seconds. The study was  
95 carried out with approval from Murdoch University's Animal Ethics Committee (AEC  
96 R2613/13).

97 Twenty-three sheep with healthy "normal" eyes, showing no clinical signs of IOK, were  
98 selected and sampled from Murdoch University's teaching flock. These animals were selected  
99 from the merino adult breeding ewe group. The animals selected had no history of ocular  
100 disease in the past twelve months and there had been no history of recent treatment with  
101 antibiotics. These conjunctival swabs taken from sheep with healthy, clinically unaffected  
102 eyes were used as a control in our study (Grade 0; n = 23).

103

104 The swabs (n=149) were processed by vortexing and heating to 95°C , followed by DNA  
105 extraction using a QIAmp DNA kit (Qiagen, Doncaster, Australia)<sup>9</sup>. The use and testing of these

106 swabs was considered and approved for exemption by the University of The Sunshine Coast  
107 (USC) Animal Ethics Committee (AN/E/14/01).

108

109 All 149 samples were screened for chlamydia using species-specific qPCR assays. *C. pecorum*-  
110 specific qPCR targeted a 209 bp region of the *C. pecorum* CpecG\_0573 gene characterised  
111 with a high-resolution melt (HRM) of  $77.5 \pm 0.5$  °C, while *C. psittaci*-specific qPCR assay  
112 targeted a 263 bp fragment of the Cps\_ORF607 gene characterised with a melt of  $79 \pm 0.5$  °C  
113 <sup>10</sup>. The *C. abortus*-specific qPCR assay targeted a 190 bp of the Cab\_ORF581 gene<sup>11</sup>,  
114 characterised with a melt of  $77 \pm 0.5$  °C. Each assay was calibrated using known standards of  
115 *C. pecorum*, *C. psittaci* and *C. abortus* target amplicons serially diluted from  $10^6$  to  $10^0$   
116 copies/ $\mu$ l. The amplicons were generated by conventional PCR from purified genomic DNA of  
117 the *C. pecorum* E58 bovine isolate, *C. psittaci* CR009 parrot isolate and *C. abortus* B577 ovine  
118 isolate, respectively. Each sample was tested in duplicate, with negative (dH<sub>2</sub>O) and positive  
119 controls included in each amplification assay. Samples with <10 copies/ $\mu$ l (and/or >Ct 33) and  
120 below the signature melt threshold were considered negative.

121

122 To evaluate the relationship between each IOK grade and chlamydial positivity, statistical  
123 analyses was performed using the Chi-squared test for r x c contingency table with 95%  
124 confidence. To compare the species prevalence proportions, we used the two tailed 2-sample  
125 z-test with 95% confidence, as implemented in the online epidemiological calculator software  
126 EpiTools (<http://epitools.ausvet.com.au/content.php?page=StatisticsHome>) <sup>12</sup>.

127

## 128 **Results**

129 Chlamydial DNA was detected in 35.6% (53/149) of conjunctival samples. *C. pecorum* was the  
130 most predominant species with *C. pecorum*-specific PCR screening revealing an overall  
131 prevalence of 28.9% (43/149). *C. pecorum* DNA was detected in swabs from animals with  
132 “healthy” unaffected eyes as well as all grades of ocular pathology except for Grade 5 (Table  
133 1). Excluding the Normal grade samples, *C. pecorum* prevalence in IOK samples was 28.5%  
134 (36/126). There was no statistically significant association between *C. pecorum* positivity and  
135 IOK severity.

136

137 *C. psittaci* was detected in a total of 6.7% (10/149) samples from both normal as well as IOK  
138 affected eyes. The relationship between *C. psittaci* positivity and different IOK grades was also  
139 not statistically significant (Table 1). Detection of *C. psittaci* in animals with IOK was only 5.6%  
140 (10/126), significantly lower than the overall prevalence of 28.5% for *C. pecorum* in IOK (P  
141 <0.0001).

142

143 All swabs tested negative for *C. abortus*.

144 **Conclusion**

145 The results from this study demonstrate that *Chlamydia* spp. and, in particular, *C. pecorum*  
146 can be readily detected in sheep presenting with IOK, but also in animals with no evidence of  
147 ocular disease. Consistent with recent pilot molecular studies<sup>8</sup> of Australian sheep, no  
148 evidence for *C. abortus* infection could be found.

149

150 The high number of *C. pecorum* positives is consistent with the reported endemicity of *C.*  
151 *pecorum* infections in Australian sheep<sup>4, 8, 13</sup>. The majority of these infections are typically  
152 reported to be asymptomatic in adult animals, while serious disease (in a form of  
153 keratoconjunctivitis, polyarthritis and/or encephalomyelitis) appears to be mainly limited to  
154 lambs and calves<sup>8, 13</sup>. Whilst the results of this study show that chlamydial organisms can be  
155 shed from both healthy as well as clinically affected eyes of sheep, there was no obvious  
156 statistical relationship between their presence in healthy sheep as well as the different stages  
157 of IOK severity (Table 1). The exact contribution of *C. pecorum* to pathogenesis of these cases  
158 of IOK is unclear and disease in animals is likely to be multi-factorial, including co-infections  
159 with other microorganisms, the prevailing environmental conditions and host-related factors.

160

161 The detection of *C. psittaci* was surprising as revealed a new livestock host for this pathogen  
162 in Australia, but not completely unexpected. Infections in Australian horses have been  
163 recently documented for the first time and have been linked to reproductive loss in mares<sup>7</sup>.  
164 Molecular typing of these cases suggested that infection spill-over from psittacine birds may  
165 be the source<sup>7</sup>. Globally, *C. psittaci* infections have been documented in asymptomatic sheep  
166 as well as sheep with ocular disease before<sup>3, 14</sup>. At present, the genetic identity of these ovine  
167 *C. psittaci* strains is mainly unknown, however limited molecular typing revealed that ovine  
168 *C. psittaci* strains are also closely related to the psittacine and other birds<sup>7</sup>. On the basis of  
169 PCR testing alone, this result should be treated with caution with further work required to  
170 establish whether these results are evidence of *C. psittaci* conjunctival infection or simply  
171 exposure to *C. psittaci* DNA present in the environment potentially contaminated with bird  
172 feces.

173

174 In a pilot study, it was shown that commercially available *C. abortus*-specific assays  
175 demonstrate low specificity, with high rates of seropositivity in animals with concurrent and



176 highly prevalent *C. pecorum* infection in the absence of any detectable *C. abortus* infection<sup>8</sup>.  
177 As such, the use of these assays may be contributing to unnecessary rejection of animals  
178 where *C. abortus* testing is required. The failure to detect any *C. abortus* positive samples  
179 from the current study is consistent with this recent pilot work . An obvious weakness of this  
180 approach is that conjunctival sampling is not optimal for the detection of *C. abortus*, although  
181 *C. abortus* conjunctival infections have been documented<sup>3, 15</sup>. Further and expanded studies  
182 of *C. abortus* prevalence in Australian livestock are required to resolve any uncertainty over  
183 *C. abortus* infection, particularly those including paired sampling of sera and mucosal sites  
184 (conjunctiva, reproductive organs, rectum) for chlamydial detection. Such studies are  
185 anticipated to be of benefit to Australian domestic and live export production systems.

186

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194

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198

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242

Table 1. *C. pecorum*, *C. psittaci* and *C.abortus* detection in the eye swabs taken from sheep with varying IOK grades.

IOK affected eye grade	No. of samples	<i>C. pecorum</i> (%)	Chi square* (P value, 95% CI)	<i>C. psittaci</i> (%)	Chi square* (P value, 95% CI)	<i>C. abortus</i> (%)
0 - Normal	23	7/23 (30.4)	-	3/23 (13.0)	-	0/23 (0.0)
1 - Epiphora	32	8/32 (25.0)	0.33	0/32 (0.0)	N/A	0/32 (0.0)
2 - Conjunctivitis	49	16/49 (32.6)	0.18	2/49 (4.1)	0.19	0/49 (0.0)
3 - Oedema	28	9/28 (32.7)	0.21	4/28 (14.3)	0.06	0/28 (0.0)
4 - Ulceration	9	2/9 (22.2)	0.57	1/9 (11.1)	0.5	0/9 (0.0)
5 - Neovascularisation	7	0/7 (0.0)	N/A^	0/7 (0.0)	N/A	0/7 (0.0)
6 - Damage	1	1/1 (100.0)	0.23	0/1 (0.0)	N/A	0/1 (0.0)
<b>Total</b>	<b>149</b>	<b>43 (28.9)</b>		<b>10 (6.7)</b>		<b>0 (0.0)</b>

\*: IOK grade compared to normal; ^N/A: not applicable.