Quantitative Detection of *Chlamydia psittaci* and *C. pecorum* by High-Sensitivity Real-Time PCR Reveals High Prevalence of Vaginal Infection in Cattle

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Bovine vaginal cytobrush specimens were analyzed for the presence of *Chlamydia* spp. by a high-sensitivity, high-specificity quantitative PCR. The 53% prevalence of low-level *Chlamydia psittaci* and *C. pecorum* genital infection detected in virgin heifers suggests predominantly extragenital transmission of *Chlamydia* in cattle and conforms to the high seroprevalence of anti-*Chlamydia* antibodies.

Over the last 40 years, evidence has accumulated to suggest the ubiquitous presence of infections with intracellular bacteria of the genus *Chlamydia* in cattle and other livestock species. Despite some improvement in diagnostic techniques, our understanding about the prevalence and pathogenetic significance of these infections, succinctly reviewed by Shewen (11) in 1980, has not substantially changed since that time. In cattle, enzyme-linked immunosorbent assay examinations of sera for the antibody against *Chlamydia psittaci* suggest a high level of exposure to *C. psittaci* (6, 8, 10). The application of nested PCR to bovine clinical specimens substantiated such widespread, but mostly clinically inapparent, presumably low-level infections (3, 7), similar to the findings for human *C. pneumoniae* infections (1). However, due to high technical demands, these PCR methods were rarely transferred from research settings to systematic epidemiological investigations and diagnostic use. A simple, highly specific, fluorescent-probe-based single-tube LightCycler quantitative PCR (qPCR) platform was recently developed for the detection of *Chlamydia* DNA. This platform optimizes sample nucleic acid preservation, extraction, and recovery, as well as qPCR methodology, for maximum sensitivity in the detection of *Chlamydia* (2). This has opened the possibility for the sensitive routine diagnosis of chlamydial infection as well as for systematic epidemiological studies. Such investigations would benefit from both the high sensitivity and the ability to ascertain quantitative differences in chlamydial burdens between animals, which may be required to understand disease mechanisms. In this initial epidemiological application of the *Chlamydia* qPCR platform, we addressed the question of chlamydial infection of the bovine genital tract in animals that had not previously been exposed to the possibility of sexual transmission of chlamydiae. We report here a high prevalence of genital tract infection with *C. psittaci* and *C. pecorum* in clinically normal virgin cattle.

A herd of 51 virgin Holstein heifers, 14 to 16 months old, was sampled four times at weekly intervals. These animals were clinically normal, but low-grade vaginitis was common. Vaginal cytobrush specimens (Histobrush; Fisher Scientific, Suwanee, Ga.) were obtained by 10-s rotation in the vaginal vestibulum, brushes were immediately transferred to 400 μl of RNA-DNA stabilization reagent (Roche Applied Science, Indianapolis, Ind.) in a 1.5-ml screw-cap microcentrifuge tube, samples were stirred, and the brushes were cut off. Brushes were removed after a 5-min centrifugation at 3,000 × g at room temperature; nucleic acids were extracted by glass fiber binding (High Pure PCR template kit; Roche Molecular Biochemicals, Indianapolis, Ind.) and eluted two times at 72°C in 20 μl of 0.1 mM EDTA–10 mM Tris-HCl, pH 8.4. Extractions with the High Pure kit were performed after the direct addition of 10% proteinase K solution to stabilized specimens. Fluorescence resonance energy transfer (FRET) qPCRs for the detection and typing of *Chlamydia* 23S rRNA and of the *C. psittaci* B577 omp1 and *C. pecorum* omp1 genes were performed as described previously (2, 5) with the following modifications: the *Chlamydia* 23S rRNA qPCR used the genus-specific primers CHL23SUP (5′-GGGTTGTAGGGTYGAGRAIA WRRGATC-3′) and CHL23SDN (5′-GAGAGTGCTTCC CCAGATTARACTA-3′), the Lightcycler Red 640 probe CHL23LCR (5′-LCRed640-CCTGAGTAGRRCTAGACCHL23SDN-3′), and the *C. psittaci*-specific carboxyfluorescein probe CP23FLU (5′-ACGAARAAACCGTAKGTGACCTAWTCGAT-FAM-3′), the *C. psittaci*- and *C. pecorum*-specific carboxyfluorescein probe CP23FLU (5′-ACGAARAAACCGTAKGTGACCTAWTCGAT-FAM-3′). All Lightcycler Red 640 probes were used at a concentration of 0.2 μM, the carboxyfluorescein probes were used at 0.1 μM, and Platinum Taq polymerase (Invitrogen, Carlsbad, Calif.) was used at 1.5 U per 20-μl qPCR (2). Thermal cycling for the 23S rRNA or omp1 qPCRs, respectively, was performed in a step-down protocol of 6 cycles for 12 s at 64 or 60°C, for 11 s at 72°C, and for 0 s at 95°C; 9 cycles for 12 s at 62 or 58°C, for 11 s at 72°C, and for 0 s at 95°C; 3 cycles for 12 s at 60 or 56°C, for 11 s at 72°C, and for 0 s at 95°C; and 40 cycles for 8 s at 54 or 50°C. Fluorescence acquisition was performed for 11 s at 72°C and for 0 s at 95°C (2).

In an enzyme-linked immunosorbent assay for a peptide of the *C. psittaci* major outer membrane protein (6), the sera of
The 39 specimens were found to be Chlamydia but not in both the 23S rRNA and omp1 multiple times by the 23S rRNA or presence of data on the true prevalence of chlamydiae in cattle, for the detection of chlamydiae and, consequently, in the ab-
ture of qPCR-positive versus -negative results, (ii) evaluation of the concordance of species identification between the pos-
tive 23S rRNA and omp1 qPCRs, and (iii) considering the results of the parallel analysis of the control specimens in-
cluded in each batch of cytobrush specimens. First, an import-
ant strength of the qPCR method is that detection specificity is confirmed not only by the appearance of a fluorescent signal during amplification but also by melting point analysis of the saturation amplification products (Fig. 1). These data indicated exquisite, virtually 100% specificity without unspecific background. For instance, both omp1 qPCRs used the same Chlamydia genus-specific primers and Lightcyler Red 640 probe but the exchange of the single C. psittaci B577 probe for the C. pecorum probe and vice versa completely abolished the fluorescent signal for the respective noncognate species (Fig. 1). Similarly, three polymorphic nucleotides in the 54-bp probe binding region allowed for species differentiation in the Chla-
mydia 23S rRNA qPCR (Fig. 1). This fluorescent signal was much more specific and easier to differentiate from background binding than the fluorescent antibody binding used for the detection of low-level Chlamydia-infected cell cultures. Second, chlamydial species identification in the 23S rRNA and omp1 qPCR assays was 89% concordant. This suggests virtually 100% concordant species identification, considering the random detection of targets in 5-µl-sample DNA aliquots given the Poisson distribution of single target genes at the observed low target concentrations (12) and given the high probability of the presence of both chlamydial species in cyto-
brush specimens from heifers with mixed infection. Third, throughout the course of this study, we did not observe positive amplification in any negative control specimen, presumably because the closed, single-tube format eliminated product carryover. Collectively, these data suggest 100% specificity of the Chlamydia qPCRs.

The question of the relative sensitivity of the qPCRs has been addressed earlier (5). Compared to the optimized 23S rRNA qPCR method, the standard diagnosis of chlamydial infection by cell culture isolation is approximately 300 times less sensitive (5). At the low concentration of chlamydiae found in the vaginal cytobrush specimens, cell culture methods would have been uniformly negative. Nested PCR methods are also capable of detecting single copies of target genes (1, 7, 12). However, the overall sensitivity of investigations by nested PCR methods might have been lower due to the suboptimal

table 2. Reexamination of 39 discordant specimens with multiple qPCRs

<table>
<thead>
<tr>
<th>qPCR result</th>
<th>omp1 qPCR positive</th>
<th>omp1 qPCR negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>23S rRNA qPCR positive</td>
<td>18</td>
<td>20</td>
<td>38</td>
</tr>
<tr>
<td>23S rRNA qPCR negative</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>19</td>
<td>20</td>
<td>39</td>
</tr>
</tbody>
</table>

a DNA from vaginal cytobrush specimens had been initially examined by a single Chlamydia 23S rRNA, C. psittaci B577 omp1, and C. pecorum omp1 qPCRs. Thirty-nine discordant specimens identified as Chlamydia positive by one qPCR but as negative by the others or positive in both 23S rRNA and omp1 qPCRs but discordant in Chlamydia species identification were reexamined by reamplification by the respective negative qPCR four or five times. Specimens were assayed multiple times to compensate for the random distribution of single target genes in 5-µl-sample DNA aliquots at the observed low target concentra-
tions (12). Specimens were scored as positive if any single of the multiple qPCRs was positive.
preservation and concentration of DNA. The approximately twofold-increased detection frequency of chlamydiae in this study by the 23S rRNA qPCR, compared to that by the omp1 qPCR, is consistent with the shorter target sequence of the 23S rRNA qPCR, which increases amplification efficiency (7). Earlier Chlamydia omp1 qPCR methods that were not optimized for low target concentrations are approximately 20-fold less sensitive than the 23S rRNA qPCR and probably would not have detected Chlamydia in these heifers either (5).

Given our estimate of 100% specificity of the qPCR assays, we do not assume false-positive results in our study and calculated therefore a 100% predictive value for positive results (4). The absolute sensitivity and predictive value of negative results require data on the true prevalence of chlamydial shedding in cattle. We observed an apparent 4-week period prevalence of 53% and thus assumed a minimum 53% true 4-week period prevalence. Serological data in this and earlier studies indicate that all animals have been infected with Chlamydia (6), suggesting that a maximum 100% true prevalence of chlamydial shedding can be approached, given a high sampling density over an extended time period. Our unpublished preliminary data in cattle indicate similar chlamydial detection rates for other samples, such as milk or nasal and conjunctival cytobrush specimens. This suggests that sampling frequency and duration, more than the anatomic sampling site, are critical in establishing the true prevalence of chlamydial infection. Assuming 53, 75, and 98% true 4-week period prevalence of chlamydial shedding in the heifers in this study, the absolute sensitivity values were 96.4, 76, and 67.6%, respectively, for the 23S rRNA qPCRs and 62.8, 58.5, and 56.2% for the omp1 qPCRs. The predictive values of negative results under these assumptions were 96, 52, and 4%, respectively, for the 23S rRNA qPCRs and 60, 32.5, and 2.5% for the omp1 qPCRs.

Our application of the Chlamydia 23S rRNA and omp1 qPCR detection platforms yielded valuable information on the epidemiology of bovine chlamydial infection. Both the high sensitivity and quantitative aspects of the method proved valuable. The high sensitivity of the qPCR enabled us to detect low levels of chlamydiae in cattle with subclinical or low-grade vaginitis. These data conform to the high seroprevalence of chlamydial infection in cattle. The high frequency of chlamydial genital infection in virgin heifers suggests that extragenital transmission, most likely by social interaction such as mutual licking and possibly by inhalation, is the predominant mode of spread of chlamydial infection in cattle. These results have a direct impact on our understanding of the epidemiology of Chlamydia in cattle and confirm the ubiquitous nature of bovine chlamydial infection.

The ubiquitous presence of two chlamydial species in cattle begs a number of questions. (i) Are individual animals long-term persistently infected, or do we observe mainly short-lived reinfections? (ii) What is the impact on herd health and production? (iii) What is the significance for public health? It is clear that we need to understand chlamydial infection as a continuum, ranging from severe overt disease to low-grade inflammatory change, with little, if any, difference between clinical signs of low-level infections with different chlamydial species. The question of persistence versus reinfection is difficult to answer in the current epidemiological situation. Most likely, some animals will harbor and shed the organisms for an extended period of time and continuously reinfect their herd mates while the majority of the herd will eliminate chlamydiae more rapidly. Understanding the balance between these response patterns and the impact of low-level infection on herd health and production will require investigations into the interaction of chlamydial infection with herd nutrition, population density, and host genetics. The significance of high-dose infection with animal chlamydiae has long been recognized for human ornithosis and for human abortion caused by infection with C. psittaci contracted from aborting ewes (9, 11). We do not know the public health significance of human exposure to the pervasive low-level infections of cattle. A preponderance of evidence suggests that specific chlamydial strains are associated with the long-term infection of specific mammalian hosts,
such as *C. pneumoniae* with humans. Given the close association of humans with low-level *C. psittaci* and *C. pecorum*-infected livestock, and the frequent identification of human infection with *C. pneumoniae* but not with *C. psittaci* or *C. pecorum*, it appears likely that low-level chlamydial infection of cattle only transiently crosses over into human hosts, if at all, and does not cause overt disease.

REFERENCES