CHLAMYDIAL INFECTIONS

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ENDEMIC *CHLAMYDIA PECORUM* INFECTIONS REDUCE MILK PRODUCTION IN DAIRY COWS VIA INFLAMMATORY LIVER INJURY

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Introduction

Infection with *Chlamydia* (*C.*) *abortus* and *C. pecorum* is ubiquitous in cattle worldwide, with high seroprevalence rates approaching 100% in some investigations (1). While acute chlamydial infections have been associated with distinct clinical diseases, the vast majority of *Chlamydia* spp. infections in cattle, particularly of low-level infections frequently detected after introduction of sensitive PCR methods, are not associated with obvious clinical disease (2, 3), and the health impact of these ubiquitous subclinical infections remains unknown.

Mastitis, the inflammation of the mammary gland, is the most prevalent production disease in dairy cows and causes the greatest economic losses in animal agriculture (4). We have shown earlier that vaccination against *C. abortus* and *C. pecorum* temporarily eliminates clinically inapparent bovine mastitis associated with chlamydial infection in cattle (5). Nevertheless, questions have remained about the mechanisms by which these *Chlamydia* spp. infections cause mastitis and milk production losses. In particular, is the localized effect of chlamydial infection of the mammary gland the dominant factor, or is it the systemic effect of circulating inflammatory mediators released from different sites of infection such as the respiratory or intestinal tract (6)? The present study investigated in detail the magnitude, type, kinetics, and cyclical characteristics of the health effects of low-level chlamydial infections on bovine milk and serum parameters. Results show that systemic effects on the liver dominate the mechanisms by which chlamydial infections reduce milk production in high-performance dairy cows.

Methods

The study was performed in a 140-head dairy herd of 75% Holstein and 25% Jersey cows at the EV Smith Dairy Unit of the Alabama Agricultural Experiment Station in Shorter, AL, USA. Beginning 2 weeks post-calving until week 20 of lactation, individual animals were sampled in a 2-week interval. In total, 17 Holstein cows were sampled over the study duration of 40 weeks. At each sampling time point, milk yield from each mammary gland quarter was determined, and milk samples, vaginal cytobrush swabs, and serum were collected. From selected cows, mammary gland biopsies were obtained by use of Tru-Cut® biopsy needles (Becton Dickinson and Company, Franklin Lakes, NJ, USA). All animal procedures were approved by the Auburn University IACUC.

Each quarter milk sample was examined by bacteriological culture for standard mastitis pathogens. Somatic cell counts (SCC) were determined by laser-based flow cytometry of ethidium bromide-stained DNA of nuclei of somatic cells. Milk protein and fat were determined by mid infrared (2-15 µm wavelength) absorption spectroscopy.

DNA from all milk and vaginal cytobrush swabs was extracted by glass fiber absorptoon and elution, and examined by real-time *Chlamydia* 23S rRNA fluorescence resonance energy transfer real-time PCR on the LightCycler® platform (Roche Applied Science, Indianapolis, IN, USA) for the presence of *Chlamydia* spp. Melting curve analysis was used to differentiate *Chlamydia* species as described before (7).

Serum samples were analyzed for IgM antibodies against *C. pecorum* by chemiluminescent ELISA using a lysate of *C. pecorum* EBs as antigen (2). Bovine serum amyloid A (SAA), an acute phase protein, was determined by solid phase sandwich ELISA (Tri-Delta Diagnostics

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Inc., Boonton Township, NJ, USA). Albumin, total protein, and cholesterol were determined by colorimetric and enzymatic colorimetric assays by a Cobas c 311 analyzer (Roche, Indianapolis, IN, USA). Serum globulin was calculated as total protein minus albumin.

Statistical analyses were perfomed with the SAS/STAT 9.1® (SAS Institute Inc., Cary , NC) and Statistica 7.0 (StatSoft, Inc., Tulsa, OK) software packages. Probability of binary outcome of total chlamydial detection and milk chlamydial detection (negative/positive) was modeled as a function of milk production and composition, immune parameters, and principal components using multiple logistic regression (8). Principal component analysis (PCA) based on the parameter correlation matrix was used to reduce the dimensionality of the data set and to identify new meaningful underlying variables. The principal components were then used to perform a disjoint cluster analysis to delineate natural clusters present in the cases (9). Significant differences in production parameters and immune parameters between clusters were determined using Tukey's unequal HSD test.

Results

Prevalence of chlamydial infection. All cows had high concentrations of IgM antibodies against *C. pecorum* in all serum samples tested. Of the total of 17 cows, 12 (70%) were positive in any one of the specimens for *Chlamydia* spp by 23S rRNA gene FRET real-time PCR. All positive specimens showed very low numers of 1-2, and occasionally up to 40 chlamydiae per PCR. Melting curve analysis unambiguously identified *C. pecorum* in all positive specimens. In 11/12 (92%) of the positive cows, *C. pecorum*. was detected in the vaginal mucous membrane, and 2 of these 11 cows (18%) were also positive in milk; however only one out of the 12 *Chlamydia*-positive cows (7%) was positive only in a milk sample from one mammary gland quarter. Overall, *C. pecorum* was detected in 12% of the total vaginal mucous membrane swabs, and only in 0.7% of all quarter milk samples. Biopsies were obtained from all four udder quarters of seven cows from the study herd that had been designated for culling. None of the 28 mammary tissue samples showed evidence of mammary gland colonization by *Chlamydia* spp. Therefore, relatively infrequent and seemingly random detection of very low numbers of *C. pecorum* genomes, primarily at vaginal epithelium, was combined with persistently high and fluctuating anti-*C. pecorum* IgM levels. This suggests frequent episodes of re-infection by *C. pecorum* and indicates an asymptomatic endemic infection of the study herd with *C. pecorum*.

The working hypothesis of this investigation was that the main effect of chlamydial infection on milk would be due to interstitial colonization of the mammary gland. However, the rare detection of chlamydiae in milk samples strongly indicates that chlamydial colonization of the bovine mammary gland under conditions of an endemic *C. pecorum* infection of a herd is rare (<1% of udder quarter samples). The corollary of this finding is that the influence of chlamydial infection on the mammary gland would primarily result from systemic effects of mucosal infections rather than from local effects of direct udder colonization by *Chlamydia* spp. To elucidate the effects of systemic chlamydial infections, milk yield and milk components across the mammary gland quarters were combined in a weighted approach to represent the total milk production of the cow in subsequent analyses.

Association of *C. pecorum* infection with milk production. *Chlamydia* detection (negative/positive) in any specimen was regressed in a multiple logistic regression model against milk yield, milk SCC, milk fat, milk protein, serum anti-*Chlamydia* IgM, and SAA. Of all parameters in this model, positive chlamydial PCR detection significantly associated with higher daily milk yield (36.24 vs 29.41 lb, P=0.004), higher milk fat (2.81 vs 2.66%, P=0.034), and lower milk protein (2.90% vs. 3.20%, P<0.001). Chlamydial detection in milk associated with higher milk SCC (5.31 vs. 5.09 $\log_{10}$ SCC, P<0.0001) and lower milk protein (2.73 vs. 3.05%, P<0.0001). Thus, detectable chlamydial infection was more frequently

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observed in higher producing cows, and detection of *C. pecorum* in this endemic herd infection suggested lower resistance rather than lower production of these cows. However, chlamydial infection of the mammary gland as detected in milk had a significant added effect on the inflammatory status of the mammary gland as compared to the systemic effect of chlamydial infection that did not increase, on its own, milk SCC.

Hypothesis-free data exploration by principal component and cluster analyses. Principal component analysis was used to help in understanding the cumulative effect of infection and the response to it (detection of chlamydiae and other bacterial mastitis pathogens, anti-chlamydial IgM, SAA) on the milk production outcomes (yield, SCC, fat, protein) in this study. Three principal components (PC) explain 70% of the total variance: 1) Production loss index explains 32% of variability in the dataset and the linear combination of the original variables is largely a contrast of milk yield with SCC and protein content. 2) Anti-*Chlamydia* immune index explains 21% of the variance and is primarily weighted on the anti-chlamydial IgM. 3) Inflammation index explains 17% of the variance and is positively weighted on SAA and negatively weighted on milk fat. Cluster analysis separated all 170 cases (17 cows × 10 samplings) into 4 discrete clusters. Clusters 1 and 3 are dominant and relevant with 68 and 87 observations that are delineated by high and low anti-*Chlamydia* immune index, respectively. Clusters 2 and 4 represent a total of 15 observations from cows with bacterial mastitis. To extract the influence of chlamydial infection, or the response to it, *C. pecorum*-positive cases in clusters 1 and 3 without bacterial mastitis were studied.

Table 1. Production and immune parameters of *C. pecorum*-positive cluster 1 and 3 cases without mastitis caused by other bacterial mastitis pathogens.

	Cluster 1		Cluster 3	
	Mean	95% CI	Mean	95% CI
Milk lb*	42.21	39.88 - 44.54	36.35	34.35 - 38.34
Log SCC/ml*	4.71	4.52 - 4.90	5.13	4.95 - 5.31
Fat %*	3.05	2.85 - 3.26	2.40	2.18 - 2.61
Protein %	2.77	2.71 - 2.83	2.88	2.81 - 2.95
SAA, µg/ml	51.70	25.95 - 77.45	55.25	33.80 - 76.71
C. pec. IgM, rlu*	2453	2082 - 2824	973	753 - 1193

* Differences between corresponding variables in clusters 1 and 3 are significant ($P<0.01$)

Table 1 indicates that cows in cluster 1 have significantly higher anti-*C. pecorum* serum IgM than those in cluster 3. Milk production in cluster 1 is characterized by significantly higher yield and fat and lower SCC than in cluster 3. Thus, a low immune response to chlamydial infection, as indicated by serum anti-*C. pecorum* IgM, but not the infection itself, associates with highly significant milk production losses. Since these losses are associated with mucosal, but largely not mammary gland chlamydial infections, they are likely precipitated by circulating inflammatory mediators and their influence on the bovine liver, the central metabolic organ in high-performance dairy cows (10, 11).

To test this hypothesis, additional serum parameters were analyzed. Serum cholesterol in dairy cows is an indicator of liver production of cholesterol transport proteins and is elevated in healthy and high milk yield- and fat-producing dairy cows as compared to low-producing cows under inflammatory stress, and is considered a "negative acute phase protein" (10, 11). Serum globulin is reduced in high-producing cows and is considered a surrogate of acute phase proteins with extended serum half-life (10). **Table 2** indicates that in fact high producing cows in cluster 1 have highly significantly elevated serum cholesterol and highly significantly reduced serum globulin levels as compared to low-producing cluster 3 cows. These results strongly suggest that clinically asymptomatic chlamydial infection in cows with

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low anti-chlamydial immunity results in significant inflammatory stress on the liver and highly significantly reduces milk quality and production efficiency by up to 20%.

Table 2. Surrogate parameters for liver function ("negative acute phase protein", cholesterol) and acute phase proteins (globulin) of *C. pecorum*-positive cluster 1 and 3 cases without mastitis caused by other bacterial mastitis pathogens.

	Cluster 1		Cluster 3	
	Mean	95% CI	Mean	95% CI
Cholesterol, mg/dl*	279.42	251.58 – 307.26	208.67	176.34 – 240.99
Globulin, mg/ml*	3.62	3.50 - 3.74	4.37	3.98 – 4.76

* Differences between corresponding variables in clusters 1 and 3 are significant ($P<0.01$)

Discussion

The results of this study shift the paradigm for the mechanisms of asymptomatic chlamydial dairy herd disease from the local effect of an isolated mammary gland infection to a systemic effect mediated largely by metabolic stress on the liver by circulating inflammatory mediators released from mucosal infection sites. The fact that it is the immune response to the chlamydial infection that determines the outcome, rather than the omnipresent infection itself, suggests a promising potential for immunoprophylactic and immunotherapeutic intervention to ameliorate the profound production losses incurred by endemic chlamydial infection of dairy cows.

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