CHLAMYDIAL INFECTIONS

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RECENT ADVANCES IN THE KNOWLEDGE OF ANIMAL CHLAMYDIAL INFECTIONS

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Summary

Application of improved PCR and serological detection methods has confirmed and further expanded the notion that chlamydial infections in domestic and feral animals are highly prevalent and caused by diverse chlamydial species. In particular, new findings indicate that *C. pneumoniae* has an extremely wide host spectrum, ranging from humans to cold-blooded vertebrates, and that *Chlamydia*-like organisms, phylogenetically positioned outside the cluster of the nine classical pathogenic *Chlamydiaceae* species, can be found in numerous host species. The first genome sequences of animal chlamydial species suggest that *tox*, a chlamydial toxin, and tryptophan synthesis and nucleotide salvaging genes are associated with pathogenic mechanisms and tissue tropisms of animal chlamydiae. Epidemiological and experimental inoculation studies indicate that the majority of animal chlamydial infections are endemic and clinically inapparent, but enhance respiratory lesion severity during superinfection with other infectious agents, and negatively affect in subtle ways resistance to other pathogens, lung function, fertility, or milk production. Studies in mouse models and original hosts suggest the possibility of controlling animal chlamydial infections with vaccines. A future challenge for veterinary chlamydiology will be the unraveling of the functional consequences of these endemic chlamydial infections, and the use of vaccines to investigate the disease mechanisms as well as control chlamydial infections by use of vaccines instead of antibiotics.

Genome sequences of animal chlamydial species

The *C. muridarum* genome was published in 2000 as the first genome of an animal chlamydial species that is of great comparative interest to human *C. trachomatis* (28). The *C. muridarum* genome is highly similar to the *C. trachomatis* D genome, but has also significant differences that potentially explain the different host and tissue tropism. The main differences of *C. muridarum* are the loss of tryptophan synthesis genes, different nucleotide salvaging pathway genes, and in particular three copies of a toxin gene *tox* that is very similar to the cytotoxic enterobacterial *Efa1* gene of enterohemorrhagic *E. coli* or clostridial large cytotxins (LCT). The cytotoxicity of these genes is related to their interaction with actin that causes the disassembly of the actin cytoskeleton, and analysis of the cytotoxicity of *C. muridarum* and *C. trachomatis* has demonstrated cytopathic effects in epithelial cells that are indistinguishable from that of LCTs and thus potentially attributable to the *tox* genes (1).

The next animal chlamydial genome determined was that of *C. caviae* in 2003 (29), followed by *C. abortus* in 2005 (38). Interestingly, the genomes of these species, while again highly syntenic, differ critically with respect to the above putative host, tissue and/or disease determinants ("niche-specific" genes): *C. caviae* encodes a tox ortholog, and tryptophan synthesis and nucleotide salvaging genes, while *C. abortus* encodes none of these. This might explain why *C. abortus* can i, be propagated easily in many cultured cells and to higher yields than other chlamydiae; ii, be found in high numbers in infected host tissue; iii, colonizes macrophages effectively; and iv, rapidly spreads systemically after mucosal inoculation (6, 19, 23). Genome sequencing of the remaining 4 animal chlamydial species - *C. psittaci*, *C. felis*, *C. pecorum*, and *C. suis* - is currently underway, and the distribution of these and other

potential "niche-specific" genes will be of great interest for attempts to explain host, tissue, and disease tropisms of these animal chlamydial species. Initial data indicate that *C. pecorum* carries multiple copies of the *tox* gene, consistent with the high cytotoxicity of *C. pecorum* (27).

Improved detection methodology

Nucleic acid amplification - A major reason for the better understanding and increased detection of chlamydial infections in animals has been the introduction of nucleic acid detection methodology, particularly by PCR, since the early days after introduction of this technique. These methods continue to be improved in several aspects. Sampling into guanidinium-based buffers for maximum preservation of nucleic acids and optimum nucleic acid extraction have vastly improved detection of low numbers of chlamydial genomes in specimens (8, 9). Real-time PCR techniques have not only the benefit of determination of copy number of chlamydial targets, but also allow routine high-throughput PCR assays, reduce the risk of false positives through product carry-over contamination by virtue of the single tube method, and typically also increase detection sensitivity (9, 12, 13). The combination of PCR amplification and low-density single-tube microarrays has resulted in a rapid, accurate, and flexible typing format for use in routine detection and multiplex typing of chlamydial amplicons (33).

Antibody detection - Reliable ELISA methods continue to improve previously unreliable methods, particularly complement fixation test, for determination of antibodies against animal chlamydial species. In particular, recombinant fragments of the *C. abortus* POMP90 protein as antigens in ELISAs successfully differentiate *C. abortus* and *C. pecorum* infection in small ruminants (24), and peptide epitopes of this protein hold the promiuse of even more specific assays (45). Another effective approach is the use of a monoclonal antibody against variable domain 1 of *C. abortus* in a competitive ELISA format for detection of *C. abortus* antibodies in sheep for population monitoring in pooled serum samples (5).

Epidemiology

Collectively, these improvements in detection have expanded epidemiological data, and confirmed high incidences of chlamydial infections with up to 70% or more PCR-positive carriers in the populations examined, particularly in cattle and swine (8, 21, 30), but also in feral pigs (16) and marsupials (25). Szeredi *et al*. (37) examined 77 cases of equine abortion from 49 farms in Hungary, and detected *C. psittaci* in 83% of the cases. Further microbial and pathological investigations determined that the chlamydial infection was the most likely cause of 11 abortions (14% of the cases), but was not etiologically involved in the remaining abortions. Gaede *et al*. (12) determined that real-time PCR was the detection gold standard when compared to chlamydial isolation by culture or antigen detection by ELISA. They found typically low threshold cycles (high numbers of chlamydial genomes) in specimens from poultry or sheep and goats and high threshold cycles (low genome copy number) in cattle and swine specimens. From these data they concluded that chlamydial infections show epidemic behavior in poultry and small ruminants, but are endemic in cattle and swine. Using a real-time PCR approach that allowed simultaneous quantification and differentiation of chlamydial 23S rRNA sequences, Jee *at al.* (21) studied acquisition and prevalence of chlamydial infection in calves for a 12-week time period post-partum. This study showed that calves were born free of chlamydiae, but despite strict separation through single housing started to acquire both *C. abortus* and *C. pecorum* infections within 2 weeks post partum. The cohort size of calves at any given time point in the study correlated in quadratic regression with chlamydial detection such that doubling of cohort size was associated with a four-fold increase in infection frequency and intensity. This investigation demonstrated the profound

influence of population density (crowding) on prevalence and intensity of animal chlamydial infections.

Ever increasing host range of highly prevalent and diverse animal chlamydiae

PCR-based epidemiological investigations in a wide variety of domestic and feral animal populations not only continue to find frequent, but also surprisingly diverse chlamydial infections. In a feral pig population in Germany, nested PCR detected 57% *Chlamydia*-positive animals, mainly infected with *C. psittaci*, but also with *C. abortus* and *C. suis*. In a recent survey of cats with ocular inflammation von Bomhard *et al*. (44) found in 12% of the cats *C. felis*, but surprisingly in additional 39% non-*C. felis* chlamydial DNA. DNA sequencing of the amplification products revealed 16S rRNA sequences that were 99% identical to *Neochlamydia hartmannellae*, and amebic endosymbiont. Investigations of exotic, cold-blooded animals also reveal diverse *Chlamydia*-like DNA sequences such as those found in the nasal discharge of 10% of examined tortoises (17). These atypical *Chlamydia*-like sequences clustered outside the present family *Chlamydiaceae* with the closest relative being *C. pecorum*. A chlamydial isolate from an African frog was identified as a distinct genotype of *C. pneumoniae* (18), thus clearly expanding the host range of *C. pneumoniae* to cold-blooded animals (3). All species of marsupials, the native Australian mammals, continue to be a wellspring of new chlamydiae. Devereaux *et al*. (11) found in koalas in addition to the known respiratory pathogenic *C. pneumoniae* and mainly urogenital pathogenic *C. pecorum* nine novel *Chlamydiales* genotypes that are clustered together with other *Chlamydia*-like bacteria within a second lineage separate from the known *Chlamydiaceae* species. Bodetti *et al*. (4) examined five species of wild Australian mammals and found in addition to known *C. pecorum* and *Parachlamydia* sequences 10 new *Chlamydiales* genotypes. Overall, the ability to find, with competent techniques, frequently detected novel chlamydial DNA sequences as well as the corresponding organisms in virtually any host species strongly suggests that no living single- or multicellular eukaryotic organism is free of these profoundly diverse and well-adapted obligate intracellular parasites.

Vaccines against animal chlamydioses

Generally accepted chlamydial diseases that call for prophylaxis by vaccination are presently abortion in small ruminants, the respiratory disease complex of turkeys in which *C. psittaci* infection is an important component, and conjunctivitis and respiratory infection in cats. The potential value of vaccination for prevention of chlamydiosis in pet birds (psittacosis, ornithosis) remains under discussion. While both attenuated live and inactivated vaccines against *C. felis* are in use for pet cats, little has been reported about their efficacy. Live and inactivated *C. abortus* vaccines for prevention of sheep/goat abortion are available, but there is nevertheless considerable interest in development of improved vaccines of higher efficacy against *C. abortus* or *C. psittaci* that also allow discrimination between vaccinated and naturally infected animals. To this end, several groups have used models of mouse infection with *C. abortus* or of the original host infection with *C. psittaci* to identify vaccine candidate antigens or to test delivery modalities of vaccines.

Vaccine-mediated protection from *Chlamydia*-induced disease - Vanrompay *et al.* (42, 43) used genetic immunization to vaccinate turkeys, the original host, with *C. psittaci ompA*, and achieved significant reduction in chlamydial shedding, and virtually complete protection from disease after respiratory challenge with *C. psittaci*. While maternal antibodies against the natural *C. psittaci* infection reduced vaccine-mediated antibody production in this trial, cell-mediated immunity was unaltered and protection was achieved (42). Berri *et al*. (2) successfully tested a live vaccine of an attenuated, temperature-sensitive strain of *C. abortus* for mouse protection against abortion caused by intraperitoneal inoculation of a Springbok

abortion isolate of *C. abortus*. This live vaccine also showed evidence for heterologous protection by significantly reducing *C. pecorum* colonization of placentas after challenge inoculation of mice with *C. pecorum* (31).

Candidate antigens for subunit vaccines - Héchard *et al*. (14, 15) tested the protective efficacy of the *C. abortus ompA* and *groEL* genes by genetic immunization, but did not obtain significant reduction in chlamydial loads or protection of fetuses after intraperitoneal challenge inoculation of pregnant vaccinated mice with *C. abortus*. To identify vaccine candidates in a screen of the whole *C. abortus* genome, Stemke-Hale *et al*. (36) used expression library immunization of mice with pools of plasmids, starting with 80,000 random inserts of the *C. abortus* genome, followed by respiratory challenge with *C. abortus*. The primary selection criterion for protective plasmid pools was prevention of lung disease. Protective plasmid pools were divided, and re-screened until individual candidates were identified. Nine gene fragments were isolated that conferred protection. Four of the five fragments that mediated better protection than the live control vaccine encode portions of cytosolic *C. abortus* proteins, not membrane proteins, which have been generally thought to be the best candidates for chlamydial subunit vaccines. These genes and their corresponding proteins were used in a cattle vaccine trial for protection from *C. abortus*-induced infertility and evidence for effective protection was obtained, although significance was not reached due to small group size ($p = 0.12$).

Vaccine inactivation and adjuvantation - In a systematic approach, Caro *et al*. (7) examined the influence of the mode inactivation of *C. abortus* elementary bodies and of the type of adjuvant on the protective efficacy of a killed vaccine against intraperitoneal challenge with *C. abortus*. They found that best reduction in chlamydial organism load on day 4 after inoculation was mediated by a killed vaccine prepared by inactivation of chlamydial organisms by binary ethylenimide and adjuvanted with QS-21, a purified *Quillaja saponaria* saponin, or with Montanide 773. These data may also be helpful in formulating future subunit vaccines.

Chlamydial infections and economically important livestock production diseases

Historically, the existence of latent chlamydial infections in livestock had been well recognized, but their impact was not understood because of the difficulty detecting these infections and the resulting uncertainty about the overall prevalence of chlamydial infections in animals (34). Improved diagnostics, both by serology and PCR-detection of chlamydial DNA, have changed the historical perception that detectable chlamydial infection in animals is typically associated with clinically apparent, frequently severe disease, such as enteritis and pneumonia in birds with ornithosis, or abortion, conjunctivitis, polyarthritis, or encephalomyelitis in ruminants. Veterinary chlamydiologists are now faced with the conundrum of a high frequency of detectable chlamydial infections, but a dearth of significantly associated clinical disease manifestations (4, 5, 8, 21).

Demonstration of health effects of these widespread, low-level endemic chlamydial infections represents a challenge, but also a great opportunity for a contribution to an improved understanding of such infections that similarly affect humans, such as in the association of *C. pneumoniae* with atherosclerotic lesions. Health effects of subclincial infections may have a devastating impact on livestock productivity and farm income, if these infections affect the respiratory and intestinal tract of young animals in which they may reduce growth rates, or the reproductive organs of adult animals in which they may affect fertility or milk production. Some of the most interesting advances in veterinary chlamydiology come from “production medicine” studies that address the subtle health effects of these chlamydial infections.

Respiratory *C. psittaci* infection in turkeys - Vanrompay *et al*. (40, 41) examined the interaction of *C. psittaci* with avian pneumovirus (AVP) and *Ornithobacterium rhinotracheale* (ORT), all critical pathogens observed in the respiratory disease complex of turkeys. The pattern of seroreactivities indicates that herds of growing turkeys experience 2 waves of *C. psittaci* infection, clinically observed at 3-6 and at 8-12 weeks of age, caused by diverse *C. psittaci* serovars (40). Each first infection wave was accompanied by an AVP infection, and closely followed by an ORT infection starting at the age of 6-8 weeks. When *C. psittaci*-infected turkeys were experimentally exposed to APV, superinfection exacerbated respiratory disease during the acute phase of *C. psittaci* infection, but not the minor respiratory irritation of post-acute *C. psittaci* infection. Single acute *C. psittaci* or APV infections caused less clinical disease than dual acute infections (41). Superinfection of acute and post-acute *C. psittaci*-predisposed turkeys with a respiratory-pathogenic *E. coli* isolate exacerbated acute disease and increased *C. psittaci* excretion in both acute and post-acute infections (D. Vanrompay, personal communication). These studies demonstrated the pre-disposing role of chlamydiae for other bacterial and viral infections, and the disease exacerbation caused by combined acute chlamydial and other bacterial or viral infections.

Respiratory chlamydial infections in pigs and calves - Reinhold et al. (20, 30, 32) studied experimental respiratory infection of pigs with *C. suis*, and the consequences of naturally acquired lung infections in pigs infected with *C. suis* or *C. abortus*, and in calves infected *C. abortus* and/or *C. pecorum*. Experimental exposure to aerosolized *C. suis* caused acute bronchiolitis and interstitial pneumonia that significantly affected lung function (30, 32). Respiratory disease symptoms such as severe dispnoea, wheezing, and shortness of breath were comparable to acute viral infection or human asthma exacerbation. Lung function in both pigs and calves was evaluated non-invasively by impulse oscillometry, in which external sound pressure signals are superimposed onto the airflow of spontaneously breathing subjects (35). The sound frequency allows testing of respiratory mechanics for different regions of the respiratory tract, with low frequencies (3 Hz) penetrating deeper than higher frequencies (15 Hz). Functionally, *C. suis* infection resulted in significant distal airway obstruction between days 3-5 after inoculation, and proximal airway obstruction one week after challenge (30). Natural occurence of *Chlamydiaceae* in the respiratory tract of pigs that were *Chlamydia*-seronegative was not associated with functional changes in the growing lungs between 5-27 weeks of age. In contrast, natural *C. abortus* and/or *C. pecorum* lung infection of calves without clinical disease symptoms associated with significantly increased airway resistance as compared to calves without PCR-detectable chlamydial infection (20). The fact that chlamydial infection in calves, but not in pigs, associated with airway obstruction is likely related to the difficulty in performing impulse oscillometry in pigs, which tend to hyperventilate and require sedation, while calves are naturally relaxed during lung function testing. These sensitive lung function tests have quantitatively demonstrated the effects of subclincial as well as clinically manifest chlamydial infections on lung function, and have shown the potential for modeling *C. pneumoniae*-associated human asthma exacerbation (26) in a natural host of the chlamydial agent rather than in a mouse model that is poorly suited for functional testing.

Bovine fertility - DeGraves *et al*. (10) investigated the effects of controlled re-infection on the fertility of cattle naturally pre-exposed to *C. abortus*. All animals had high pre-challenge levels of IgM, IgG, IgG1, and IgG2 serum antibodies against ruminant *C. abortus* in a chemiluminescent ELISA. Twenty virgin heifers were estrus synchronized with prostaglandin F2, artificially inseminated 2-3 days later, and challenged immediately by intra-uterine administration of 0, 10^4, 10^5, 10^6, or 10^8 inclusion forming units (IFU) of *C. abortus*. Ten heifers were estrus-synchronized, inseminated, and uterine-challenged 2 weeks later. These

animals were also indirectly exposed to *C. abortus* infection (cohort challenged) by contact with their previously challenged cohorts. Pregnancy was determined by rectal palpation 42 days after insemination. No animal showed signs of clinical disease. One hundred percent, 83%, 50%, 66%, and 0% of heifers were pregnant after uterine challenge with 0, 10^4, 10^5, 10^6, or 10^8 IFU of *C. abortus*, respectively. Fifty percent and 65% of heifers were pregnant with or without cohort challenge, respectively. Uterine inoculum dose and cohort challenge, or alternatively a negative pregnancy outcome (infertility), correlated highly significantly with a rise in post-challenge over pre-challenge anti-*C. abortus* IgM. Logistic regression significantly modeled that the uterine *C. abortus* inoculum causing infertility is 8.5-fold higher for heifers without cohort exposure and 17-fold higher for heifers with high IgM than for heifers with cohort exposure or with low IgM. This investigation demonstrated that an asymptomatic, circulating, non-sexually transmitted herd infection by *C. abortus* has a profound negative influence on the fertility of cattle bred at this time (22).

Chlamydial infection of the bovine mammary gland - Another approach at analyzing the effect of clinically inapparent chlamydial infections in cattle was taken by Uhe *et al.* (39). Mastitis is the economically most important disease in animal agriculture, affecting both milk quantity and quality. Most cases of mastitis in dairy cattle are clinically inapparent, and typical mastitis pathogens such as *Streptococcus agalactiae* are detected only in a fraction of the cases. Subclinical mastitis is nevertheless of major interest to "production medicine" because of the large impact on profit margins of dairy farms. Infections with *C. abortus* and *C. pecorum* are ubiquitous in cattle, and have been experimentally and clinically associated with bovine mastitis. In a prospective cohort study in a herd of 140 Holstein dairy cows they examined the influence of chlamydial infection detected by PCR on subclinical inflammation of the bovine mammary gland as characterized by elevated somatic cell counts (SCC) in milk. SCCs are a sensitive quantitative indicator of inflammation, and 10^5 somatic cells per ml milk are considered the upper limit for a healthy bovine mammary gland. All cows had serum antibodies against *Chlamydia*, and 49% of the cows were positive for *C. abortus* on day 0 on at least one PCR of a conjunctival or vaginal swab from day 0 of the experiment. *Chlamydia* infection and below-median anti-chlamydial serum antibody levels significantly associated with bovine subclincal mastitis in this investigation (39). An intervention approach by perturbation of the immune response to *C. abortus/C.pecorum* was used to further examine induction, and immune-mediated reduction, of mastitis caused by chlamydial infection. All dairy cows had established immunity to chlamydiae, and serologically- and/or PCR-demonstrated chlamydial infection. They received two doses of an inactivated Alum-Quil-A-based vaccine of *C. abortus/C. pecorum* elementary bodies (therapeutic vaccination) or a mock vaccine on days 0 and 35 of the investigation. This vaccination highly significantly reduced milk SCC (***Figure 1***), thus reduced bovine mastitis, and increased anti-chlamydial antibody levels, but did not reduce shedding of *Chlamydia* bacteria. *Chlamydia* vaccination also resulted in improved relative body condition of dairy cows after 10 weeks. The disease-protective effect was maximal 10 weeks after vaccination, and lasted for additional 4 weeks. Vaccination with the *C. abortus/C. pecorum* vaccine, the mock-

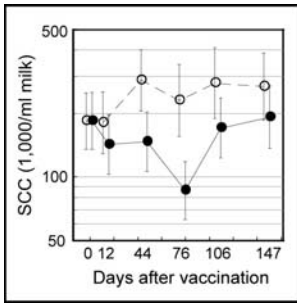

Figure 1. **Effect of *Chlamydia* vaccination on milk somatic cell counts (SCC).** ● *Chlamydia* vaccine, ○ mock vaccine, data were normalized for identical day-0 means of *Chlamydia*- and mock-vaccinated animals; means ± 95% confidence interval. *Chlamydia*-vaccinated cows (vaccine on days 0 and 35) have significantly lower milk SCC than mock-vaccinated cows ($p = 0.007$ for all combined time points after day 0, repeated measures ANOVA).

vaccine, or an unrelated vaccine against Bovine Viral Diarrhea virus resulted in highly significant transient increase in chlamydial shedding in milk, presumably mediated by the vaccine adjuvant. This investigation demonstrated an etiological involvement of the ubiquitous chlamydial infections in bovine mastitis, a herd disease of critical importance for the dairy industry. Furthermore, it shows the potential for transient improvement of chlamydial disease by therapeutic vaccination. The economically desirable, therapeutic *Chlamydia* vaccination of cattle for disease mitigation may well also serve as testing ground for use of chlamydial vaccines in humans.

Conclusions

The ever increasing data from epidemiological surveys, both of domestic and of feral animals, indicate that chlamydial infection of animals, by classical *Chlamydaceae* species as well as *Chlamydia*-like organisms, is the rule rather than the exception. PCR detection typically indicates low numbers of the organisms, and the vast majority of these infections are without obvious clinical symptoms, suggesting overall mostly endemic infections. The dominant maintenance mechanism of chlamydial infections in the host populations appears to be frequent and clinically inapparent re- or superinfection coupled with the slow elimination of these agents by host immunity. Only if several epidemiological risk factors coincide, such as stress imposed on a susceptible, high-density host population, do these infections build up to become clinically manifest. Emerging data, though, indicate that the inapparent infections are not innocuous, but do cause minor inflammatory reactions and increase susceptibility to viral and bacterial superinfection. While clinically manifest chlamydial diseases are rare and affect only a small fraction of, or infrequently the whole, host population, inapparent infections affect in subtle ways every member of the population. For that reason, subclinical chlamydial infections are probably economically more important in livestock than classical chlamydial diseases. The future challenge for veterinary chlamydiology will be to dissect the impact of the endemic animal chlamydial infections, and devise strategies to ameliorate their negative effects. Vaccines against animal chlamydiae have the potential to be used as perturbation tools in such studies, as well as instruments for control of animal chlamydial infections, potentially substituting for presently widely used antibiotics.

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