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Review

Recent progress in research on animal chlamydioses – A summary of COST Action 855 (2003-2008)

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Introduction

COST (European Co-Operation in the field of Scientific and Technical research) Action 855 “Animal Chlamydioses and the Zoonotic Implications” was set up in order to promote and enable cooperation between scientists in veterinary research to address issues relating to chlamydial infections in animals and also their zoonotic potential. To achieve this goal, working groups were tasked to focus on five specific objectives: (1) the development and validation of new and existing diagnostic tests; (2) the field evaluation of diagnostic tests; (3) the assessment of the zoonotic risks associated with animal chlamydioses; (4) to conduct basic research to improve our understanding of chlamydial pathogenesis, virulence factors and associated immune responses; and (5) to develop new vaccines for controlling chlamydial infection. Through the research activities of the member groups of this European network substantial advances have been made in these areas and presented at the annual conferences in Dublin (2003), Budapest (2004), Siena (2005), Edinburgh (2006), Pulawy (2007) and now culminates with this final meeting in Aarhus. Here we review some of the scientific highlights that have been achieved over the course of the 5 years of the Action.

New diagnostic methods

Recent advances in laboratory diagnosis of chlamydial infections have been reviewed by members of COST 855 (Sachse et al., 2008). Real-time PCR has been recognised as a powerful technique for rapid, specific and sensitive laboratory diagnosis of chlamydial infections. New real-time PCR assays have been developed and evaluated for *Chlamydomphila (C.) psittaci*, the causative agent of psittacosis (Geens et al., 2005; Heddema et al., 2006a;

Pantchev et al., 2008), as well as *C. pecorum* and *C. pneumoniae* (Markey et al., 2005; Markey et al. 2007), *C. abortus* (Livingstone et al., 2008; Pantchev et al., 2008) and the family *Chlamydiaceae* (Ehricht et al., 2006; Godin et al., 2006). DNA microarray technology has been shown to possess a great potential for species identification of chlamydiae (Sachse et al., 2005). In a collaborative study to validate this technology, the microarray findings were compared with real-time PCR, conventional PCR, immunohistochemistry and other tests. The DNA microarray assay was shown to be highly accurate with good performance parameters, demonstrating its suitability for routine diagnosis (Borel et al., 2008). To explore the genotype repertoire and epidemiology of *C. psittaci* strains in the Netherlands, an *ompA* gene sequence-based approach was suggested (Heddema et al., 2006b). Geens et al. (2005) developed a new *C. psittaci* genotype-specific real-time PCR and demonstrated the occurrence of an additional avian genotype, designated E/B. A new DNA microarray assay has been developed for direct genotyping of *C. psittaci* strains from clinical samples, which was shown to detect all currently accepted genotypes, as well as subgroups and so far untyped strains (Sachse et al., 2007, 2008). With the availability of the genome sequence of *C. psittaci* 6BC, the typing method termed Multiple Loci Variable Number of Tandem Repeats (VNTR) Analysis (MLVA), which is based on the detection of tandem repeat polymorphisms, has been used successfully to characterise the molecular diversity among *C. psittaci* isolates and assess its usefulness for future epidemiological investigations (Laroucau et al., 2008a). Similar studies are ongoing for the species of *C. pecorum* and *C. abortus* (Laroucau et al., 2008d). Recently developed real-time PCR methods specific for *Parachlamydia* and *Waddlia*, respectively, were applied to investigate the presence of these organisms in ruminant abortion (Casson et al., 2008; Ruhl et al., 2008). Species-specific immunohistochemistry protocols using antibodies against *Parachlamydia* and *Waddlia* have been established, demonstrating the agent within the placental lesions (Borel et al., 2007). The protocols used are suitable for routine diagnosis and should complete the current examination procedure on bovine abortion cases.

The tissue microarray technology was validated in combination with immunohistochemistry by testing antibodies to differentially expressed proteins in an IFN- γ -induced model of chlamydial persistence (Borel et al., 2006). A newly developed nested PCR-enzyme immunoassay with an internal inhibition control has proved to be highly sensitive and specific for demonstrating chlamydial DNA in birds (Van Loock et al., 2005b). A recently developed serological test for ovine chlamydioses has been shown to detect infection much earlier in pregnancy than existing tests, which should allow appropriate control measures to be applied earlier, thus limiting the environmental spread of infection to other animals (Livingstone et al., 2005). To improve serological monitoring of flocks free of *C. abortus*, a new test for simultaneous detection of *C. pecorum* based on recombinant antigens is being developed (Rodolakis et al., 2006). Several new commercial serological tests have been evaluated using sera from experimentally infected sheep and field sera (Vretou et al., 2007; Rekiki et al., 2006b; Wilson et al., 2006). A recent field study, the first of its kind, compared antibody titres in latently infected, diseased and vaccinated animals over a two-year period (Gerber et al., 2007). In another study, a comparison of 3 commercial serological assays with CFT and 4 'in-house' ELISAs has been conducted to evaluate their specificity and sensitivity in detecting *C. abortus* and *C. pecorum* infected sheep (Wilson et al., 2008). Additionally, a recombinant antibody ELISA suitable for birds, pigs and other animals has been developed and compared to established tests (Vanrompay et al., 2004; Verminnen et al., 2006). Finally, Ortega et al. (2007b) compared several protocols of DNA purification for the diagnosis of OEA from paraffin-embedded samples by PCR. The results showed that PCR from paraffin-embedded sections can be a useful tool for analysis of field samples.

Research on pathogenesis, immune response and virulence factors

A number of peculiarities associated with chlamydial infections make research in pathogenesis particularly difficult, e.g. the absence of classical virulence factors, the wide variety of clinical manifestations (from subclinical or mild to acute), the tendency towards chronicity, as well as poorly understood host immune response.

Research of COST 855 member groups has focused on the identification and characterisation of inclusion membrane proteins in *C. abortus*, which are known as virulence factors involved in the pathogenesis of the disease. Vretou et al. (2006) identified and partially characterised the first inclusion membrane protein of *C. abortus*, which was designated Inc766 and represents a potential virulence factor. Inc766 has been shown to form dimers and high order oligomers (Vretou et al., 2008a) and to decorate cytoplasmic extra-inclusion vesicles (Vretou et al., 2008b). One of the projects has focused on the chlamydial type III secretion system and its contribution to chlamydial virulence (Geens et al., 2004; Beeckman et al., 2008). Further studies are being conducted to characterise effector proteins and to elucidate their possible role in chlamydial pathogenesis. Examination of the transcriptional response associated with in vitro models of chronic infections has also improved our understanding of the regulation of the chlamydial intra-cellular developmental cycle and has identified new molecular features of the persistent state of *C. pneumoniae* and *C. psittaci* (Polkinghorne et al., 2006; Goellner et al., 2006a). Recent work on host cell processes triggered by chlamydial infection revealed that *C. psittaci* was capable of modulating host cell apoptosis. In particular, inhibition of host cell apoptosis may facilitate permanent colonisation of host tissue by the pathogen and ensure the long-term survival of persistent *C. psittaci* within the host cell (Goellner et al., 2006b).

Studies investigating the persistence and shedding of *C. abortus* in post abortion sheep at oestrus and subsequent lambing by real-time PCR have revealed low numbers of organisms suggesting that they do not impact significantly on disease epidemiology either through mechanical or venereal transmission by the ram or through the products of lambing (Livingstone et al., 2008). A mathematical/computational epidemiological model for *C. abortus* infection in sheep has been constructed to simulate disease progression and in order to understand the underlying processes that drive transmission (Milne et al., 2008).

A series of experimental infection studies clearly demonstrated the pathogenic interaction between *C. psittaci* and other respiratory pathogens, such as the avian metapneumovirus (Van Look et al., 2006a), *Escherichia coli* (Van Look et al., 2006b), and *Ornithobacterium rhinotracheale* (Van Look et al., 2005a) in the turkey respiratory disease complex.

Following the completion of the *C. abortus* genome (Thomson et al., 2005), an 11k *C. abortus* microarray for pathogenesis studies has been designed and developed in conjunction with Agilent Technologies, and used to investigate strain variation in the field. Through comparative genomics the genome has revealed significant variation in two families of proteins, the polymorphic membrane proteins or pmps (autotransporter proteins of the type V secretion system) and the TransMembrane Head or TMH proteins (inclusion membrane proteins) and it has been suggested that it is variation in these proteins that are responsible for the niche specificity of this pathogen (Thomson et al., 2005). A *C. abortus* genome web resource has been developed (Longbottom et al., 2005, 2006) and is now available on the Moredun Research Institute website (<http://bioweb-2.mri.sari.ac.uk/cab/>). The site can be used to both search and browse the genome using graphical views. Another project is addressing virulence genes of *C. abortus* identified from the total genome sequence of strain AB7 to compare them with those of vaccine strain 1B and mutant strains (Rodolakis et al., 2006). Vretou et al. (2006) identified and partially characterised the first inclusion membrane protein of *C. abortus*, which was designated Inc766 and represents a potential virulence factor.

Using a murine model, several aspects of the immune response against *C. abortus* infection were investigated focusing on the role of NK cells (Buendía et al., 2004), or CD4⁺ and CD8⁺ T cells (Martínez et al., 2006), respectively. It was concluded that CD8⁺ T cells may play a

role in the regulatory control of CD4⁺ T cell response and may have a direct cytotoxic or IFN- γ -mediated effect on infected cells. Also, Buendia et al. (2007) have developed a new model of intranasal infection of *C. abortus*, which is useful for testing the protection offered by different vaccines against *C. abortus*. Furthermore, knowledge of the pathogenesis of *C. abortus* experimental infection in the natural host has been considerably extended (Navarro et al., 2004). The development of lesions in placental tissues following experimental infection of sheep with *C. abortus* have been described early in infection (Maley et al., 2008). Comparison of the foetal and maternal inflammatory responses in the ovine placenta showed that the foetal response is innate in character, while the maternal response represented an acquired, chlamydia-specific response (Sammin et al., 2006) and it is this acquired response of the ewe that prevents the organism reaching the placenta in subsequent pregnancies (Sammin et al., 2005). Innate immune sensory mechanisms (e.g. Toll-like receptors) and defence mechanisms (anti-proteases, defensins) are likely to have an important role in limiting chlamydial infection in the early stages. The expression of ovine Elafin and Secretory Leukocyte Protease (SLPI) Inhibitor, both of which possess anti-microbial and anti-inflammatory properties, has been demonstrated in the female reproductive tract. SLPI has been shown to be expressed in the uterus late in pregnancy and so may form part of the innate defence against *C. abortus* (Wheelhouse et al., 2006). The pattern of the cytokine interferon-gamma (IFN- γ) mRNA expression and its production in foetal and maternal components of the placenta during the advanced stages of chlamydial placentitis were systematically examined to elucidate the host response to enzootic abortion of ewes (Worrall et al., 2008). The epidemiological importance of chlamydia-associated abortion in small ruminants was analysed in Italy, Hungary, Ireland and other countries (Masala et al., 2005; Cafiero et al., 2006; Szeredi et al., 2006; Markey, 2006). A serological study was undertaken to estimate the prevalence of *C. abortus* in samples of sheep and goats originating from Greek organic farms which had outbreaks of abortion.

The effect of natural and experimental chlamydial infection on lung function parameters in pigs has been examined (Sachse et al., 2004; Reinhold et al., 2005). A group of subclinically infected calves, i.e. natural carriers of *Chlamydophila* spp., was shown to display significantly deteriorated health parameters, such as lower body weight, elevated body temperature, altered blood parameters and other signs associated with chronic effects on animal health at a subclinical level, when compared to chlamydia-free calves (Reinhold et al., 2008). Even in the absence of overt of clinical symptoms, respiratory chlamydial infection appeared to be associated with chronic inflammation of lungs and airways, and pulmonary dysfunctions persisted in calves until the age of 7 months (Jaeger et al., 2007). A retrospective survey on the importance of chlamydial infections in cases of bovine abortion was conducted in Switzerland (Borel et al., 2006). Investigations are underway to determine the prevalence of chlamydial infections in cattle and pigs in Great Britain, Sweden, Germany and other European countries and to assess the impact of these infections on infertility and abortion, as well as animal health and performance.

The suitability of a horse model for the study of human COPD was addressed by Theegarten et al. (2008). *C. psittaci* and *C. abortus* were present in the lung of both clinically healthy horses and those with RAO. Immunohistochemistry revealed acute chlamydial infections with inflammation in RAO horses, whereas mostly persistent chlamydial infection and no inflammatory reactions were seen in clinically healthy animals.

In vitro infections of amoebae with *C. abortus* have been undertaken to study their interaction and to elicit the potential role of amoebae in the epidemiology of chlamydioses (Wirz et al., 2005).

Development of new vaccines for improved control of chlamydial infections

At present, few commercial vaccines for animal chlamydioses are commercially available,

and some of those in use are known to have considerable limitations (Longbottom and Livingstone, 2006). Members of COST 855 have conducted vaccination studies based on DNA vaccines, recombinant MOMP and subunit membrane fractions. *Cp. psittaci* DNA vaccination significantly protected turkeys against severe clinical disease and significantly reduced chlamydial excretion, as well as the lesions caused by this pathogen, whereas recombinant MOMP vaccination induced lower protective immune responses (Van Loock et al. 2004; Verminnen et al., 2005). The use of adjuvants like vitamin D₃ and CpG motifs was evaluated, but they did not significantly enhance protective immune responses following DNA vaccination (Verminnen et al., 2005; Loots et al., 2006). A DNA vaccination protein boost protection study in sheep against *C. abortus* infection has been completed and shown to generate both cellular and humoral immune responses (Frew et al., 2005), although these responses did not appear to be protective. The choice of antigen, adjuvant and delivery method requires further investigation to improve protective efficacy (Longbottom & Livingstone, 2005). The protective efficacies of a purified native soluble MOMP preparation and a purified recombinant MOMP preparation were assessed in a *C. abortus* challenge mouse model and compared to vaccination with live EBs (Livingstone and Longbottom, 2006b). The protective immunity of a vaccine based on the *C. abortus* groEL gene was tested and evaluated in a murine model (Hechard et al., 2004). Members of COST 855 are currently conducting a study on serological evaluation of titres against *C. abortus* after vaccination in OEA-affected and OEA-negative sheep flocks to collect and evaluate data for future schemes to control outbreaks of this disease. Using novel adjuvants, new inactivated vaccines against *C. abortus* have been tested. The data indicate a good performance in sheep (García de la Fuente et al., 2004). Caro et al. (2005) presented a new co-infection model using *Nippostrongylus brasiliensis* and *C. abortus* to analyse the protection induced by experimentally inactivated vaccines and new adjuvants, as well as the influence of an established Th2 immune response on the outcome of vaccination. The role of PMNs and NK cells in protection against *C. abortus* infection conferred by different vaccines was studied by Ortega et al. (2006). The results open up new perspectives to study the innate immune mechanisms underlying effective protection after vaccination.

A new model of animal infection has been developed in the hamster: this model has been used to evaluate the immune protection induced by recombinant antigens against a challenge infection by *C. pneumoniae* (Sambri et al., 2004; Finco et al., 2005).

Assessment of zoonotic risks associated with animal chlamydioses

The Technical Annex of COST 855 stated that specific data needed to be collected and published to assess the current epidemiological situation concerning zoonotic transmission of chlamydiae in Europe. Laboratories of the participating countries have conducted diagnostic surveys in flocks of poultry (Verminnen et al., 2008) and sheep, herds of cattle, pet animals (Harkinezhad et al., 2007; Vanrompay et al., 2007) and others to evaluate the prevalence of chlamydial agents. Cases of chlamydioses in persons connected with the respective farm, production unit or household are being included in several ongoing studies.

A severe outbreak of psittacosis in a poultry flock in Germany led to infection of 24 individuals with one death (Gaede et al., 2008). Molecular diagnostic examinations revealed involvement of two different genotypes of *Chlamydophila psittaci*, i.e. A and E/B. The high zoonotic potential of genotype A requires particular attention.

Although poultry breeding is economically important in France, few data are available concerning the prevalence of *C. psittaci* and cases of zoonotic transmission. In 2006 and 2007, several outbreaks of human psittacosis linked to ducks or psittacines were investigated by the French National Public Health Surveillance Centre (InVS) and the National Reference Centre (human aspects), and by Veterinary Services and AFSSA (animal aspects). Medical and veterinary epidemiological surveys were carried out, which included diagnostic

confirmation by serological and/or PCR testing (Laroucau et al., 2007). Whenever possible, samples were typed by PCR-RFLP and by MLVA, and animal and human samples were compared (Laroucau et al., 2008b; Laroucau et al., 2008c). Considering the potential severity of the human disease and the recurrence of epidemic episodes in various professional contexts, a two-year prospective descriptive study of human psittacosis, which was coordinated by the InVS, was started in January 2008. The aim of this study is to determine the incidence of hospitalised human cases and the frequency of grouped cases, as well as to define the risk of exposures for the patients. Additionally, the analysis of the strains isolated from humans and animals, as well as the description of breeding characteristics and working conditions will improve our knowledge of risk factors for animal-to-human transmission. Ultimately, this study will allow the introduction of improved prevention and control measures (Laroucau et al., 2008c).

The potential health hazards caused by urban pigeons as carriers of *C. psittaci* infection have been addressed by COST 855 (Magnino et al., 2006; Magnino et al., 2008). There is an urgent need for more epidemiological studies, which include cases of zoonotic transmission to contact persons, as well as regular exchange of data and experience among experts and health authorities of European countries. In this context, studies on urban pigeons, wild birds and poultry have been conducted in Croatia, Macedonia, Bulgaria and other countries to assess the zoonotic risk (Prukner-Radovic et al. 2004, 2005; Martinov, 2006). The availability of swabs collected for the French bird flu surveillance offered the opportunity to study the prevalence of avian chlamydiosis among a heterogeneous bird population. In this study based on the molecular detection of *Chlamydiae* by real-time PCR from cloacal and/or tracheal swabs, 7% of the samples were diagnosed positive, which corresponded to 11% of the birds studied. While most of the restriction patterns were genotype B or E-like, in some samples atypical patterns were observed (Laroucau et al., 2007).

Furthermore, *C. psittaci* was demonstrated to be present in aborted fetal membranes from mares (Szeredi et al., 2005). This new finding indicates another possible source of human infection. The significance of chlamydiae in male genital tracts and ejaculates of ruminants and pigs has been studied to assess the potential role of venereal transmission (Kauffold et al., 2006a, 2006b; Teankum et al. 2006, 2007).

A recent study has focused on *Chlamydiales* in guinea pigs and their zoonotic potential (Lutz et al., 2006; Wohlgroth-Lutz et al., 2006). A flock of diseased guinea pigs was found to be infected with *C. caviae*. The owner of the guinea pig flock was found to be infected (*Parachlamydia acanthamoebae* was also detected), as were in-contact animals (cat and rabbit). This suggests that zoonotic infection with *C. caviae* should be considered and appropriate advice given to owners of guinea pigs. The role of chlamydiae as a possible cause of mass deaths of free-ranging amphibians and frogs in Switzerland has been studied (Blumer et al., 2005).

Cattle abortion of unknown infectious etiology still remains a major economic problem. Thus, late-term bovine abortion cases were investigated for possible new abortigenic agents, such as *Waddlia* and *Parachlamydia*, resulting in the first description of *Parachlamydia* in the setting of bovine abortion in Switzerland (Borel et al., 2007). As *Parachlamydia* may be involved in lower respiratory tract infection in humans, caution should be taken when handling bovine abortion material because of the potential zoonotic risk.

The association between infection with *C. psittaci* and ocular adnexal lymphomas of the MALT (mucosa-associated lymphoid tissue)-type (OAML) in humans has been studied in a patient, owner of a *C. psittaci*-infected canary, who developed two metachronous *C. psittaci*-associated lymphomas (Ferreri et al., 2007b). The response to *C. psittaci*-eradicating antibiotic therapy with doxycycline has been assessed in patients with advanced-stage ocular adnexal MALT lymphoma. After therapy, *C. psittaci* DNA has no longer been detectable by PCR in all six treated patients, and durable complete or partial regression of lesions were

achieved in three patients (Ferreri et al., 2007a). The detection of viable and infectious *C. psittaci* in conjunctival swabs and/or peripheral blood mononuclear cells (PBMC) in a proportion of OAML patients has been also reported (Ferreri et al., 2008).

Final Remarks

In its 4 years of existence, the Action has attracted the participation of more than 100 chlamydia researchers from 18 COST member states (Belgium, Bulgaria, Croatia, Germany, Former Yugoslav Republic of Macedonia, France, Great Britain, Greece, Hungary, Ireland, Israel, Italy, the Netherlands, Poland, Slovenia, Sweden, Switzerland, Spain) and 6 other countries (Australia, Argentina, Bosnia, China, Japan USA). Continuous exchange and interaction between research groups have led to a measurable increase in quantity and quality of chlamydia research in Europe. The publications of COST 855 scientists have contributed to raising the awareness of chlamydial infections, as well as their economic and zoonotic dimensions in many European countries. The major scientific achievements of this network will be published in a special issue of the journal *Veterinary Microbiology* later this year.

Taking advantage of the financial support of the COST organisation, numerous activities of the participating researchers have created an active and efficient network, which will continue to function way beyond its official conclusion.

References

The list of references is available at

<http://www.vetpathology.uzh.ch/forschung/CostAction855/publications/publicationsCOST855.pdf>