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Recent developments in the laboratory diagnosis of chlamydial infections

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Abstract

There are two main approaches to diagnosing infections by *Chlamydia* and *Chlamydophila* spp. in mammals and birds. The first involves the direct detection of the agent in tissue or swab samples, while the second involves the serological screening of blood samples for the presence of anti-chlamydial antibodies. Ultimately, the test that is used is dependent on the types of samples that are submitted to the diagnostic laboratory for analysis.

The present paper gives an overview on methodologies and technologies used currently in diagnosis of chlamydial infections with emphasis on recently developed tests. The performance characteristics of individual methods, such as the detection of antigen in smears and in pathological samples, the isolation of the pathogen, various antibody detection tests and DNA-based methods utilising conventional and real-time PCR, as well as DNA microarray technology are assessed, and specific advantages and drawbacks are discussed. Further, a combination of a specific real-time PCR assay and a microarray test for chlamydiae is proposed as an alternative reference standard to isolation by cell culture.

Keywords: *Chlamydia*; *Chlamydophila*; antigen detection; antibody detection; DNA amplification tests; specificity; sensitivity

Abbreviations:

C., *Chlamydophila*; CFT, complement fixation test; DFA, direct fluorescent antibody test; EB, elementary body; ELISA, enzyme-linked immunosorbent assay; cELISA, competitive ELISA; rELISA, recombinant ELISA; LPS, lipopolysaccharide; mAb, monoclonal antibody; MIF, microimmunofluorescence test; MOMP, major outer membrane protein; MZN, modified Ziehl-Neelsen stain; OEA, ovine enzootic abortion; PCR, polymerase chain reaction; rRNA, ribosomal RNA; POMP, polymorphic outer membrane protein; VD, variable domain

1. Introduction

1.1 Historic overview and taxonomic classification

Although the first scientific report on avian chlamydiosis dates back to the 19th century (Ritter, 1879) and chlamydiae were first described as the causative agents of trachoma one hundred years ago (Halberstaedter and von Prowazek, 1907), it took several decades until a generally accepted nomenclature evolved.

In the first half of the 20th century, no less than seven attempts, including three repetitive ones, were made to classify, define and designate the members of the psittacosis-lymphogranuloma-venereum-trachoma (PLT) group. Other names for these bacteria included Bedsonia, Miyagawanella, Halprowia, ornithosis-, trachoma-inclusion-conjunctivitis- (TRIC), psittacosis-lymphogranuloma venereum- (PLV), and psittacosis - ornithosis - mammalian - pneumonitis- agents. The term '*Chlamydia*' (Greek χλαμύς = cloak) appeared in the literature in 1945.

With the advent of electron microscopy and tissue culture techniques in the 1960s, it became evident that chlamydiae were not viruses. In a major taxonomic reclassification, Page introduced the genus *Chlamydia* within the family *Chlamydiaceae* and the order *Chlamydiales* (Page, 1966). Only two species were known until the 1980s, i.e. *Chlamydia trachomatis* and *Chlamydia psittaci*. These bacteria were distinguished by chlamydia-like biochemical characteristics, morphology and developmental replication (Page, 1968). Strains of *Chlamydia trachomatis* were identified by their accumulation of glycogen in inclusions and their sensitivity to sulfadiazine. In contrast, *Chlamydia psittaci* strains did not accumulate glycogen and were usually resistant to sulfadiazine. The introduction of this classification was a milestone in chlamydial taxonomy, as it abandoned the concept of reliance on presumed host, tissue preference and serology in grouping these organisms.

Up until 1999, this group of obligate intracellular bacteria comprised four species, i.e. *Chlamydia trachomatis*, *Chlamydia psittaci*, *Chlamydia pneumoniae*, and *Chlamydia pecorum* (Herring, 1993). With the advancement of nucleic acid-based characterisation methods in the 1990s, it became evident that particularly *Chlamydia trachomatis* and *Chlamydia psittaci* represented rather heterogeneous species. This had found its expression in the introduction of three biovars and 12 serovars for *Chlamydia trachomatis* and 12 serovars for *Chlamydia psittaci*. Furthermore, DNA-DNA hybridisation experiments revealed remarkably low sequence homology (30 %) between the genomes of the mouse and trachoma biovars of *Chlamydia trachomatis*, while the relatedness among *Chlamydia psittaci* isolates from different host animals ranged from 93 to 30 % (Storz and Kaltenboeck, 1993a).

Extensive DNA sequence analysis led (Everett et al., 1999a) to reassess genetic relatedness in the order *Chlamydiales* and propose taxonomic reclassification. According to this proposal, the family *Chlamydiaceae* comprises two genera, *Chlamydia* and *Chlamydophila* (C.), with a total of nine presumably host-associated species, i.e. *Chlamydia trachomatis*, *Chlamydia suis*, *Chlamydia muridarum*, *C. psittaci*, *C. abortus*, *C. felis*, *C. caviae*, *C. pecorum*, and *C. pneumoniae*. This classification had initially been based on sequence data of 16S and 23S rRNA genes, as well as the *ompA* gene, which encodes the major outer membrane protein (MOMP). Later, additional sequence data, based on the genes of GroEL chaperonin, KDO-transferase, small cysteine-rich lipoprotein and 60 kDa cysteine-rich protein (*ompB*), confirmed the new taxonomy (Bush and Everett, 2001).

Since its publication, the revised taxonomy has been adopted by many chlamydiologists, particularly those working in veterinary medicine, but it is still facing opposition from some of the chlamydia research community. From the authors' point of view, the subdivision of the very heterogeneous former species *Chlamydia psittaci* into four new species represents a major practical advantage of the new classification. Notably, the causative agents of the two

most important animal chlamydioses with zoonotic potential are now considered to be separate species, i.e. *C. psittaci* and *C. abortus*. However, as more strains are being examined using ever more sophisticated tests, the host range of individual chlamydial species may well turn out to be broader than anticipated.

1.2 Importance of individual chlamydial pathogens

Most members of the family *Chlamydiaceae* represent agents of important animal and/or human diseases (Storz and Kaltenboeck, 1993b). Avian strains of *C. psittaci* cause psittacosis/ornithosis, a systemic disease of acute, protracted, chronic or subclinical manifestation occurring in psittacine birds, domestic poultry and wild fowl (Vanrompay et al., 1995). The infection is transmissible to humans, the symptoms being largely non-specific and influenza-like, but severe pneumonia, endocarditis and encephalitis are also known (Crosse, 1990).

Enzootic abortion in sheep and goats, also called Ovine Enzootic Abortion (OEA) or Enzootic Abortion of Ewes (EAE), is caused by *C. abortus*. The disease has a major economic impact as it represents the most important cause of lamb loss in sheep in parts of Europe, North America and Africa (Longbottom and Coulter, 2003). This serious and potentially life-threatening zoonosis can also affect pregnant women after contact with lambing ewes, leading to severe febrile illness in pregnancy and loss of the foetus (Buxton, 1986; Kampinga et al., 2000; Pospischil et al., 2002a).

In cattle *C. pecorum*, *C. abortus* and *C. psittaci* are found in connection with infections of the respiratory and genital tracts, and can cause enteritis, arthritis, encephalomyelitis (Storz and Kaltenboeck, 1993b), as well as endometritis and hypofertility (Wittenbrink et al., 1993).

Chlamydioses in pigs are associated with four different species: *Chlamydia suis*, *C. pecorum*, *C. abortus* and *C. psittaci*. A widely held view is that chlamydiae may act in concert

with other agents in multifactorial infectious diseases, such as abortions in sows (Thoma et al., 1997), polyarthritis in piglets, diarrhoea in pigs (Pospischil and Wood, 1987) and genital disorders in boars (Szeredi et al., 1996). Other relevant animal diseases include conjunctivitis in cats caused by *C. felis* (von Bomhard et al., 2002, 2003), respiratory disorders and abortion in horses caused by *C. pneumoniae* and *C. psittaci*, respectively. *C. pneumoniae* has also been isolated from diseased koalas and frogs.

Apart from the diseases in the animals mentioned above, chlamydiae are responsible for a number of diseases in humans, e.g. trachoma, sexually transmitted infection of reproductive organs (*Chlamydia trachomatis*), as well as respiratory infection in adults and cardiovascular disease (*C. pneumoniae*).

1.3 Detection of chlamydiae

It should be emphasised that a presumptive diagnosis of chlamydial infection, particularly in farm animals and birds, can often be made on the basis of history, clinical symptoms and presenting pathology. Thus, these as well as other potential factors, such as the possibility of other infectious agents or non-infectious causes being involved, need to be considered before the samples are passed on for testing. Ultimately, however, accurate diagnosis requires confirmation through laboratory investigations.

Being obligate intracellular bacteria, chlamydiae require tissue culture techniques to be isolated and propagated. As culture in permanent cell lines or embryonated hens' eggs is necessary to demonstrate the viability of a field strain and also facilitates detailed characterisation by molecular and biochemical methods, it is still widely regarded as the gold standard in chlamydial diagnosis (Thejls et al., 1994). However, there are difficulties associated with this technique that have stimulated the development of a large variety of

direct detection and identification assays targeting LPS or protein antigen, antibodies or DNA. These issues will be discussed in the following sections.

There are essentially two main approaches to diagnosing chlamydial infections in mammals and birds. The first involves the direct detection of the agent in tissue or swab samples, which is described in **Sections 2, 4 and 5**, while the second involves the serological screening of blood samples for the presence of anti-chlamydial antibodies, and which is covered in detail in **Section 3**. Ultimately the test that is used is dependent on the types of samples that are submitted to the diagnostic laboratory for analysis.

By way of example, the authors suggest that the following steps are considered when diagnosing chlamydiae or other infectious agents from submitted ruminant placental specimens and/or aborted foetuses, although the order of the steps will be subject to variation in different laboratories depending on the presenting situation:

- i) Characterisation of the type of abortion (e.g. early abortion, late-term abortion, mummified foetus).
- ii) Consideration of the disease history of the herd/flock (first or repeated case of abortion, number of animals affected).
- iii) Taking of blood samples from the affected animals for serological screening (for the exclusion of other abortigenic agents such as *Brucella abortus*, Bovine Herpesvirus infection).
- iv) Inspection of the placenta for gross pathology (if available).
- v) Sampling of placental specimens and foetal organs for further investigation (microbiology, histopathology, molecular biology).
- vi) Conducting routine bacteriological investigations (e.g. smear preparation, culture).
- vii) Histopathological investigation of the placenta and/or foetal organs.
- viii) Antigen detection within the lesions (immunohistochemistry).

ix) Antigen detection/confirmation/differentiation by immunoassay and/or molecular techniques.

x) In accordance with the specific requirements of individual countries, report notifiable disease.

In step vii, it is important to ensure that the diagnosed infectious agent is related to the lesion present and is not present as a contaminant, since placental samples are taken from a contaminated environment. In many cases it will not be possible, or indeed be necessary, to adhere to all of these steps, for example, if only swab samples taken from the vagina or from the coats of aborted foetuses are submitted, steps iii-v and vii-viii will be omitted.

In the present paper, recently published diagnostic methods and tests are reviewed, their performance characteristics are assessed, and the specific advantages and drawbacks are discussed.

2. Antigen detection

2.1. Introductory remarks

The presence of antigen in tissue and swab samples can be demonstrated through the preparation and cytochemical staining of smears (**Section 2.2**). In veterinary pathology laboratories, antigen detection in clinical tissue samples (e.g. placentae from cases of abortion) is generally accomplished on submitted formalin-fixed or formalin-fixed and paraffin-embedded tissue specimens using various histochemical and immunohistochemical staining techniques (**Section 2.3**). Other approaches include the use of various immunoassays that have been developed, including enzyme-linked immunosorbent assays (ELISAs) and fluorescent antibody tests (FATs) (**Section 2.4**). However, although these tests successfully demonstrate the presence of chlamydial organisms, they generally do not allow the

identification of the respective species, serotype or subtype involved. This is because many of the tests involve the use of non-specific cytochemical stains or of mAbs based on *Chlamydiaceae*-specific antigens, such as chlamydial LPS.

Historically, isolation of the pathogen is considered the ‘gold standard’ and most sensitive method of detection for diagnosing chlamydial infection. Isolation involves the cultivation of organisms from clinical samples in either embryonated hen’s eggs, cell culture or, to a lesser degree, laboratory animals (**Section 2.5**). A confirmatory diagnosis can then be made either by the cytochemical staining of smears prepared from the infected egg yolk sac material or the immunohistochemical staining of infected cells. The major disadvantage of this approach is that it is wholly dependent on the adequate storage and transportation of the biological samples to ensure the viability of the organisms. Where samples are submitted for isolation and may be subject to delay it is recommended that they are maintained at 4°C in a suitable transport medium, such as sucrose/phosphate/glutamate or SPG medium supplemented with foetal bovine serum, antibiotics, and a fungal inhibitor (Spencer and Johnson, 1983). Depending on the type of sample submitted for analysis and the detection method employed, there may also be contamination issues caused by other Gram-negative bacterial species that can result in false-positive reactions and thus inaccurate diagnosis. In addition, the isolation approach is also time consuming and requires both specialist expertise and culture facilities. There are also safety issues that need to be taken into consideration when handling these pathogens as at least two of the veterinary pathogens, *C. psittaci* and *C. abortus*, are zoonotic and can cause severe infection in humans, which in the case of *C. abortus* is also lethal for the unborn foetus (Longbottom and Coulter, 2003). However, despite precautions and limitations, it is clear that isolation is still of extreme importance in the characterisation of individual strains from an epidemiological viewpoint, and allows strains to be properly typed to unambiguously identify the species or serotype/subtype

involved in the infection. Indeed, in early studies, the inclusion morphologies and growth characteristics of *Chlamydia psittaci* (old nomenclature, now *C. psittaci*) isolates derived from several animal species following their cultivation in cell culture was the basis of their division into eight biotypes (Spears and Storz, 1979).

Most recently, molecular based methods, utilising PCR and DNA microarray technology, have been developed that can detect chlamydial nucleic acid prepared from tissue and swab specimens. These techniques, which have revolutionised the typing and identification of specific species and strains will be covered in detail in **Sections 4** and **5**, respectively.

2.2. Smears

Where chlamydial infection is suspected in mammals or birds, appropriate clinical samples or specimens can be taken for the preparation of smears for rapid diagnosis of infection. In the case of *C. abortus* infection in sheep, smears can be prepared from placental membranes and cotyledons showing typical OEA-associated lesions, or from swabs taken from the vagina at the time of abortion or from the moist coats of aborted foetuses and lambs (Longbottom and Coulter, 2003). For *C. psittaci* infection in birds, smears can be prepared from faeces, cloacal swabs, conjunctival scrapings, although pharyngeal or nasal swabs are preferred (Andersen, 2004). Impression smears can also be prepared from tissue samples obtained from liver, spleen, kidney, lung and pericardium. In cats and koalas, organisms may be detected from conjunctival, urogenital or genital swabs (Canfield et al., 1991; Volopich et al., 2005).

Prepared smears can be stained for detection of chlamydiae using one of several staining procedures, for example, modified Machiavello, modified Gimenez, Giemsa, or modified Ziehl-Neelsen (MZN) (Stamp et al., 1950; Aitken and Longbottom, 2004; Andersen, 2004). MZN is considered the most satisfactory method and stained smears from positive samples,

examined by high-power microscopy, demonstrate small coccoid elementary bodies stained red/pink against a counter-stained blue or green cellular background (**Figure 1A**). Under dark-ground illumination, the elementary bodies appear pale green. Although this method works well in cases of heavily infected tissues, samples with low levels of infection where EBs only appear singly can be easily overlooked. Furthermore, in the case of suspected *C. abortus* infection, when a history of infection or placental pathology is lacking, care must be taken to avoid confusion with the rod-shaped rickettsia *Coxiella burnetii*, which can also cause abortion and has similar staining characteristics.

Fluorescent antibody tests using *Chlamydiaceae*-specific anti-LPS antibodies or species-specific mAbs to MOMP, which are either directly conjugated with fluorescein or combined with a fluorescein-conjugated anti-mouse antiserum, improve the sensitivity of detection of chlamydial EBs in smears. More recently, a streptavidin-biotin method of staining *C. abortus* smears has also been reported as being more sensitive and specific than the MZN technique (Szeredi and Bacsadi, 2002). However, where numbers are too low for easy detection, greater success is likely to be achieved using immunoassays (**Section 2.4**) or DNA-based detection methods such as conventional or real-time PCR (**Section 4**).

2.3. Pathological specimens

Following the submission of tissue samples to the diagnostic laboratory for analysis, chlamydiae can be demonstrated in histological preparations using a variety of staining procedures. A simple method involves the histochemical staining of thin tissue sections ($\leq 4 \mu\text{m}$) with Giemsa after fixation in fluids such as Bouin and Carnoy (Stamp et al., 1950). Dark-ground methylene blue staining, which has been shown to be a more reliable method for detecting *C. abortus* in foetal membranes than Giemsa, can also be used (Dagnall and

Wilsmore, 1990). However, both these techniques are non-specific and can cross-react with other bacterial species, therefore, care must be taken with interpretation of results.

Immunohistochemical staining procedures that utilise mAbs directed against chlamydial surface antigens, such as LPS or MOMP, are more sensitive and produce more striking results in comparison to histochemical staining. A direct immunoperoxidase method that has been developed for detecting *C. abortus* in formalin-fixed tissues has been shown to be a very rapid and sensitive test (Finlayson et al., 1985; Kunz et al., 1991; Zahn et al., 1995), however this initially requires the direct conjugation of the antibody with the enzyme horse radish peroxidase. An alternative approach, as described previously, is to use an indirect method that involves the use of a fluorescein-conjugated anti-mouse antiserum in combination with the mAb. Enhanced labelling can be achieved using the more complex streptavidin-biotin method using formalin-fixed, paraffin-embedded tissues (**Figure 1B**) (Szeredi et al., 1996; Buxton et al., 2002). This approach of immunoperoxidase staining of formalin-fixed, paraffin-embedded or cryostat sections of various tissues is commonly used for detecting chlamydial species for diagnostic purposes, as well as for epidemiological and pathogenesis studies (Juvonen et al., 1997; Tsakos et al., 2001; Buxton et al., 2002; Hotzel et al., 2004; Navarro et al., 2004; Sammin et al., 2006; Borel et al., 2006a, 2006b, 2007; Thoma et al., 1997).

2.4. Immunoassays

Most of the commercially available antigen detection tests that have been developed over the last 25 years are used primarily and extensively for the detection of *Chlamydia trachomatis* infections in human clinical specimens, although many in theory should also be suitable for detecting chlamydial infections in animals because they are based on the family-specific LPS antigen. These immunoassays include direct fluorescent antibody (DFA) tests (for example IMAGEN, Celltech; Chlamydia-Direct IF, BioMerieux; Vet-IF, Cell Labs),

plate-based ELISAs (Chlamydiazyme, Abbott; IDEIA, Dako; IDEIA PCE, Dako; Pathfinder, Kallestad; Chlamydia-EIA, Pharmacia) and solid-phase ELISAs (Clearview Chlamydia MF, Unipath; Surecell, Kodak). One of the main advantages of using immunoassays over cell culture for diagnosing infection, other than the shorter time it takes to complete the test, is that they are not dependent on viability, detecting both viable and non-viable EBs, as well as soluble LPS antigen in secretions. The tests have been shown to vary considerably in terms of sensitivity and specificity depending on the type of samples tested, with the lowest sensitivities generally observed when there is a low prevalence of infection and thus low numbers of detectable organisms. The DFA tests have been reported as having low sensitivity when detecting chlamydial infection in koalas, although it has been suggested that this may be due to a lack of experience in identifying individual EBs from smear and swab samples by fluorescence microscopy, where the same amplification effect that is achieved with other immunoassays, such as ELISAs, is absent (Wood and Timms, 1992). There are also concerns with the specificity of some of these tests, particularly the early commercial immunoassays, as the antibodies used in them have been reported to cross-react with other bacterial species, such as *Acinetobacter calcoaceticus*, *Escherichia coli*, group B streptococci, *Staphylococcus aureus* and *Klebsiella pneumoniae*, in human clinical samples (Taylor-Robinson et al., 1987; Demaio et al., 1991). This was mainly due to the test being based on polyclonal antiserum, however subsequent immunoassays have improved specificity by using mAbs and advanced blocking procedures.

An evaluation of immunoassays for detecting *C. psittaci* in turkey specimens found the IDEIA to be particularly insensitive (Vanrompay et al., 1994), whilst studies on *C. abortus* and *Chlamydia suis*-infected samples showed that specificity was the major problem with IDEIA (Thomas et al., 1990; Sachse et al., 2003). In a comparison of nine antigen detection kits for the detection of chlamydial urogenital infections in koalas, the DFA tests were shown

to be the least sensitive, while the Clearview test was the most sensitive (91%) followed by the IDEIA (88%) and Surecell (73%) kits, in comparison to cell culture (36%) (Wood and Timms, 1992). The low sensitivity of culture highlights the loss in viability of organisms that can occur during transportation because of inadequate preservation and cooling. In contrast, the Clearview test was found to lack sensitivity and specificity in its ability to detect *C. abortus* in ovine foetal membranes (Wilsmore and Davidson, 1991), or to detect *C. psittaci* in conjunctival and cloacal samples from turkeys (Vanrompay et al., 1994).

In view of the inconsistencies with these immunoassays, probably as a result of the differing antigen loads in the different types of samples, they are rarely used for the detection of chlamydial infections in animals, with many laboratories preferring to use serological methods of detection (**Section 3**) and more specific and sensitive molecular methods of detection (**Section 4**).

2.5. Isolation

In addition to the precautions necessary to prevent loss of infectivity during the storage and transportation of clinical samples to the microbiology laboratory for analysis, as mentioned previously, care must also be taken when collecting samples to limit possible contamination with other bacteria that can interfere with the isolation of chlamydiae. The addition of antibiotics helps reduce the effects of contamination; however, care should also be taken in the choice of antibiotics used. Suitable antibiotics include streptomycin (200 µg/ml), gentamicin (50 µg/ml), vancomycin (75 µg/ml) and nystatin (25 units/ml), whereas penicillin, tetracycline and chloramphenicol inhibit chlamydial growth and should not be used.

Historically, the first method to cultivate chlamydiae outside the natural host was developed in 1935, when Burnett and Rountree succeeded in growing the agent of psittacosis in the chorioallantoic membrane of developing chicken eggs (Burnet and Rountree, 1935).

Later in 1940, Rake and co-workers discovered that lymphogranuloma venereum (LGV) could multiply in the yolk-sac cells of developing chick embryos (Rake et al., 1940). Subsequently it was shown that all other chlamydial agents could be grown in these cells (Stamp et al., 1950; Tang et al., 1957) and thereafter isolation or culture in fertile hen's eggs became routinely used in laboratories.

Yolk-sacs of 6-8 day old embryos are inoculated with 10% sample suspensions and embryos die between 4 and 14 days after infection. Smears prepared from yolk-sac membranes at the time of death, or from surviving eggs on completion of the experiment, can be stained, using a variety of procedures, such as MZN, modified Machiavello or Giemsa (Stamp et al., 1950) to reveal EBs, thus confirming chlamydial infection. Although egg culture still remains a useful technique for isolating difficult test samples, it is expensive, cumbersome, lacks reproducibility, and sensitivity to infection can be variable between chlamydial species and subtypes. However, egg culture is still used today in laboratories, particularly for the bulk growth of antigen or inoculum required for experimental challenge studies. Another consideration is that experimentation on live embryos is strictly regulated in many countries; hence the requirement for importation and scientific procedures licences, and therefore appropriate facilities, with the required biohazard containment, and expertise must be available.

The advent of cell culture meant that an alternative and more sensitive method of isolation became available and could be employed by laboratories with suitable facilities. Pioneering attempts to grow *Chlamydia psittaci* (old nomenclature), at that time still believed to be a virus, in tissue culture were performed by Bland and Canti in 1935 using the 'Maitland' tissue culture technique (Bland and Canti, 1935). From the late 1940s on, an increasing number of monolayer tissue cultures were used to culture different chlamydial species and strains (for details see Storz, 1971). As a result, the direct isolation of chlamydiae

in confluent cover-slip cell culture monolayers gradually superseded egg culture from the 1970s onwards. Several cell lines have been used to grow chlamydiae, although successful propagation is dependent on the cell line and the chlamydial species being tested. Staining of cell monolayers reveals characteristic inclusions and morphologies for the different chlamydial species, as well as their ability to infect different cell types. *Chlamydia trachomatis* infects many cell types but McCoy, Buffalo Green Monkey Kidney (BGMK) and HeLa 229 have been shown to be the most susceptible to infection (Croy et al., 1975; Wills et al., 1984; Barnes, 1989; Thewissen et al., 1989; Johnston and Siegel, 1992). On the other hand, for *C. pneumoniae*, which is difficult to isolate and propagate in cell culture, HL and HEp-2 cells are the preferred option (Cles and Stamm, 1990; Roblin et al., 1992). Isolation of *Chlamydia psittaci* serotype 1 (now *C. abortus*) was first reported in McCoy cells (Hobson et al., 1977; Johnson et al., 1983), the most commonly used cell type, although this species grows well in many other cell types, such as BGMK, baby hamster kidney (BHK) and the mouse fibroblast L cells. Most cell types are susceptible to *C. psittaci* infection, but the direct inoculation into cultures of BGMK, African green monkey kidney (Vero), McCoy and L cells are commonly used (Vanrompay et al., 1992). Little information is available for the remaining chlamydial species, but human colonic adenocarcinoma cells (CaCo) are considered to be the most appropriate for isolation of the problematic *Chlamydia suis* and *C. pecorum* strains (Schiller et al., 2004), whilst *C. felis* appears to grow well in McCoy cells (Wills et al., 1987). While many avian *C. psittaci*, ovine *C. abortus* and porcine *Chlamydia suis* strains can be propagated in tissue culture with relative ease, others are more difficult to grow, e.g. *C. psittaci* strains isolated from cattle and pigs. For *C. pecorum*, some strains, particularly those originating from the intestinal tract, are difficult to isolate in culture, either growing initially and then dying out on serial passage or not appearing to grow initially and taking several passages before the organism can be detected (Philips and Clarkson, 1995).

Infection of chlamydiae in cell culture can be enhanced by centrifugation and/or by chemical treatment of cultured cells, before or during infection, using for example cycloheximide (1 µg/ml), cytochalasin B (2 µg/ml), emetine (1 µg/ml), diethylaminoethyl-dextran (20 µg/ml) or 5-iodo-2-deoxyuridine (80 µg/ml) (Alexander, 1968; Kuo et al., 1972; Rota and Nichols, 1973; Sompolinsky and Richmond, 1974; Wentworth and Alexander, 1974; Ripa and Mardh, 1977; Bevan et al., 1978; Spears and Storz, 1979; Paul, 1982; Schiller et al., 2004). Cell culture media components may also influence chlamydial growth, particularly for species that are more difficult to propagate or when the infecting inoculum only contains low numbers of organisms. For example, the growth of *Chlamydia suis* in culture has been shown to be improved when using Iscove's modified Dulbecco's medium (IMDM) compared to Eagle's minimal essential medium (EMEM), yet they had no effect on the growth of selected *C. abortus*, *C. psittaci* and *C. pecorum* strains (Schiller et al., 2004).

Following incubation at 37°C for 2-3 days (depending on chlamydial species), inoculated cover-slip monolayers can be either fixed in methanol and stained with Giemsa (**Figure 1C**) or fixed in acetone/methanol and stained by indirect immunofluorescence (**Figure 1D**) or using the immunoperoxidase method, as described in **Section 2.3**, to demonstrate the presence of intracytoplasmic chlamydial inclusions.

3. Antibody detection

3.1. Introductory remarks

The detection of antibodies in animal chlamydial infections has multiple purposes, i.e. confirmation of clinical disease or confirmation of the presence or absence of infection, performance of epidemiological surveys to estimate the prevalence of infection, or the determination of immune status after vaccination to name some. In general, antibodies are identified following binding to inclusions, to EBs or to individual chlamydial antigens.

Detection of the bound antibody is achieved by fluorescent, i.e. indirect immunofluorescence test and MIF, or otherwise tagged secondary antibodies, i.e. indirect ELISA, or by estimating the consumption or fixation of complement, i.e. direct and indirect CFT. Infected animals are often asymptomatic, possibly shedding infectious chlamydiae from the intestine, with low or borderline levels of antibodies (Storz, 1971; Storz, 1988; Longbottom and Coulter, 2003). Furthermore, multiple chlamydial species can infect the same host, a situation often encountered in ruminants and pigs. The selection of the suitable antibody test depends therefore on the particular epidemiological situation in the infected host species.

3.1.1. Chlamydial infection in sheep and goats

The serological diagnosis of OEA has been complicated by multiple factors, the first being the latent nature of the infection itself, which remains undetected until day 90 of gestation (Buxton et al., 1990). Furthermore, ruminants are also often infected with *C. pecorum*, which causes a variety of clinical manifestations depending on the subspecies (Fukushi and Hirai, 1992; Kaltenboeck et al., 1993; Philips and Clarkson, 1995; Anderson et al., 1996). In addition, subclinical enteric infections due to *C. abortus* have been known for a long time, and *C. abortus* strains have been isolated from the faeces of sheep with no previous history of abortion but with borderline levels of antibodies (Storz, 1971; Rodolakis and Soriau, 1989; Salti-Montesanto et al., 1997; Gut-Zangger et al., 1999).

For more than 50 years, the CFT has been the most widely used assay for the serological diagnosis of OEA and the one recommended by the Office Internationale des Epizooties (OIE) (www.oie.int; Aitken and Longbottom, 2004). However, the CFT lacks specificity due to its antigen, consisting mainly of the heat-resistant LPS, which is common in all *Chlamydiaceae* species (Brade et al., 1987). Up to now, the lack of both an appropriate specific reference method for *C. abortus* and a confirmatory assay for the presence of *C.*

pecorum infection, other than the cumbersome isolation of the organism, have made the evaluation of novel assays for the improvement of the serological diagnosis of OEA difficult. Nevertheless, several laboratory-based assays have been developed including indirect ELISAs using whole EB or extracts thereof (Cevenini et al., 1989; Markey et al., 1993; Anderson et al., 1995). Semi-quantitative methods have been proposed to distinguish between infections due to *C. abortus* and to *C. pecorum* such as an indirect immunofluorescence assay and immunoblotting (Markey et al., 1993; Jones et al., 1997). ELISAs with purified LPS have been published (Sting and Hafez, 1992; Jones et al., 1997). With the introduction of molecular techniques, more defined assays were developed based on recombinant LPS antigen (Griffiths et al., 1996), as well as on MOMP (Kaltenboeck et al., 1997a; Salti-Montesanto et al., 1997; Gut-Zangger et al., 1999; Borel et al., 2002; Hoelzle et al., 2004) and 80-90kDa POMP (Buendia et al., 2001; Longbottom et al., 2001, 2002). A major step that has facilitated the evaluation of novel assays was the implementation of large panels of documented reference sera which have been shared among laboratories (Jones et al., 1997; Longbottom et al., 2001, 2002; McCauley et al., 2007; Vretou et al., 2007).

3.1.2. Infection in cattle

Bovine chlamydial abortion due to *C. abortus* is similar to OEA, but more sporadic and less common (Pospischil et al., 2002b; Borel et al., 2006b). Infections by *C. pecorum* include a series of severe clinical manifestations such as bovine encephalomyelitis and purulent endometritis among others. Cases of infection by *C. psittaci* have also been reported (Piercy et al., 1999). Recent data have demonstrated that subclinical chlamydial infections by both species, *C. abortus* and *C. pecorum*, are ubiquitous in cattle and often not detected due to low sensitivity of diagnostic techniques (Jee et al., 2004; Kaltenboeck et al., 2005). Such subclinical infections do occasionally develop into clinical disease. Specific antibodies to *C.*

abortus can then be directly detected by MOMP- or POMP-based specific assays (Kaltenboeck et al., 1997a; Niemczuk, 2005). As no specific assay for *C. pecorum* is available, the presence of these antibodies can only be shown indirectly.

3.1.3. Infections in pigs

Chlamydiaceae spp. in pigs have been associated with pneumonia, polyarthrititis, conjunctivitis, reproductive disorders and abortion (Busch et al., 2000). Asymptomatic infections might be prevalent and under-diagnosed. Infections are often mixed, attributed to *Chlamydia suis*, *C. abortus* and *C. pecorum* (Schiller et al., 1997). *C. psittaci* can also be a potential pathogen as was demonstrated in experimental infection of gnotobiotic piglets (Guscetti et al., 2000). In order to serologically identify the infecting chlamydial species, Hoelzle et al. (2004) expressed the entire MOMP molecule from *Chlamydia suis*, *C. abortus* and *C. pecorum* in *E. coli* and demonstrated the suitability of the recombinant antigens in ELISA assays. Though this approach was very promising it has not been further verified in animals in the field. In a recent study, the seroprevalence to chlamydiae in pigs in Italy was estimated using a MIF test that discerned antibodies against 4 chlamydial species (Di Francesco et al., 2006). An indirect ELISA with recombinant MOMP from *C. psittaci* was used to determine seropositivity in closed pig breeding farms in Belgium (Vanrompay et al., 2004). On the basis of the similarity between *Chlamydia suis* and *Chlamydia trachomatis*, detection of anti-*Chlamydia suis* antibodies in pigs by use of commercially available ELISAs based on the *Chlamydia trachomatis* MOMP VD4 peptides could be theoretically possible. In addition, MOMP-and POMP-based specific *C. abortus* ELISAs could be useful in the detection of antibodies to *C. abortus* in pigs.

3.1.4. Infections in birds

Avian chlamydiosis represents a major risk for zoonotic transmission to humans, as well as economic loss to the poultry industry. Clinically inapparent, latent infections may be predominant in avian species. Wild birds may act as natural reservoirs and species that migrate may contribute to the dissemination of the infection. Antibody detection tests for avian chlamydiosis are more suited to epidemiological studies than to the diagnosis of infection in individual birds (Vanrompay et al., 1995). The CFT and the MIF are currently used in routine diagnosis, as well as a latex agglutination test for the demonstration of IgM (Moore et al., 1991; Andersen, 2004). A novel ELISA based on recombinant MOMP from a serotype D strain has been developed and evaluated. The test was 100% sensitive and 100% specific and was successfully applied in cases of human psittacosis (Verminnen et al., 2006; Vanrompay et al., 2007).

In the following sections, serological assays based on EBs and different preparations of LPS, MOMP and POMP are further described and compared with emphasis on the diagnosis of OEA.

3.2. Antibodies based on elementary bodies-The microimmunofluorescence test (MIF test)

Developed as an epidemiological tool by Wang and Grayston (1970) for the serotyping of human *Chlamydia trachomatis* strains, the MIF test proved also pivotal in the classification of chlamydiae of animal origin (Eb and Orfila, 1982; Eb et al., 1986) and the serotyping of avian strains (Andersen, 1991). The test was shown to be useful in serological surveys for the assessment of co-infection by several chlamydial species (Di Francesco et al., 2006) and was even considered as a possible reference standard for detection of zoonotic infections, as commercial kits are available (Dovic et al., 2005). However, a recent study raised serious doubts on the MIF test's suitability for diagnosing human psittacosis (Verminnen et al., 2008).

3.3. Assays based on LPS

3.3.1. Complement Fixation Test (CFT)

The CFT is the first described LPS-based assay and the most commonly used in veterinary laboratories (Stamp et al., 1952). In OEA, CFT serum titres rise at the time of abortion and remain high for at least 6 weeks (Storz, 1971). A CFT titre greater than 1/32 is considered as an indication for the presence of OEA, whereas lower values can be due to subclinical enteric infections by *C. abortus* or cross-reactivity with *C. pecorum* and other bacterial LPS. (Aitken and Longbottom, 2004). The CFT is not workable with haemolytic or anti-complementary sera (Cross and Clafin, 1963). Furthermore, not all animal sera or all immunoglobulin isotypes can fix complement (Schmeer et al., 1987; Kaltenboeck et al., 1997a). Recent studies that used sera from sheep and goats with documented absence or presence of OEA reported CFT specificities between 83 and 98.1% and sensitivities between 68.8% and 91.4% (Jones et al., 1997; Longbottom et al., 2001, 2002; Vretou et al., 2007). The CFT antigen used in these reports was from *C. abortus*. Interestingly, CFTs conducted with *C. psittaci*- and *C. abortus*-derived LPS have been reported to differ in their sensitivities, which were 96.4% and 60%, respectively (McCauley et al., 2007). In addition to the trisaccharide $\alpha\text{Kdo}(2\rightarrow8)\alpha\text{Kdo}(2\rightarrow4)\alpha\text{Kdo}$, *C. psittaci* contains a structurally different trisaccharide with the structure $\alpha\text{Kdo}(2\rightarrow4)\alpha\text{Kdo}(2\rightarrow4)\alpha\text{Kdo}$ and the branched tetrasaccharide $\alpha\text{Kdo}(2\rightarrow8)[\alpha\text{Kdo}(2\rightarrow4)]\alpha\text{Kdo}(2\rightarrow4)\alpha\text{Kdo}$ (Brabetz et al., 2000; Brade et al., 2000). This highlights the importance of origin and preparation of the LPS for use in CFT and other LPS-based assays. Furthermore, when testing for anti-*Chlamydiaceae* responses, assays with defined LPS composition should be the first choice. Since CFT is technically quite demanding, an automated version (Seramat) was recently developed, which correlated well with manually performed tests (Magnino et al., 2005).

3.3.2. Recombinant ELISA

The recombinant LPS-based rELISA (medac, Hamburg, Germany) has been reported as a notable alternative to the CFT (Griffiths et al., 1996; Kaltenboeck et al., 1997a). The deacylated, BSA-conjugated antigen used in the rELISA contains both types of epitopes, the $\alpha 2 \rightarrow 4$ linked disaccharide moiety and the (2 \rightarrow 8) linked chlamydia-specific epitope (Brade et al., 1994). The assay was originally developed for human *Chlamydia trachomatis* and *C. pneumoniae* infections, but was adapted to sheep and evaluated for serological diagnosis of OEA (Griffiths et al., 1996). As anticipated, the rELISA could not differentiate between *C. abortus* and *C. pecorum* infections, but it was shown to be a more sensitive primary screening test for OEA as compared to CFT (Griffiths et al., 1996). This test identified more positive sera than CFT or eELISA in four flocks with documented enteric *C. abortus* infections. Furthermore, it was more sensitive than CFT in flocks with unknown OEA status but in which positive CFT titres were recorded, and it did recognise more positive sera in a flock known to be infected with arthritogenic *C. pecorum*. In general, the rELISA was characterised by good sensitivity and reproducibility, as well as 80.6% concordance with the CFT in 297 serum samples. The original results of this test are shown in **Figure 2**.

3.3.3. Other commercial LPS-based assays

ImmunoComb (Orgenics, Yavne, Israel) is a dot blot assay using LPS-extracted *Chlamydia trachomatis*. The test was evaluated for its suitability to detect *C. abortus*-specific antibodies and was reported to be the most sensitive, but the least specific compared to CFT, two ELISAs and immunoblotting (Jones et al., 1997). Most recently the high sensitivity of this test has been attributed to the identification of a high proportion of false-positive samples (Wilson et al., 2008). RIDASCREEN™ (Biopharm, Darmstadt, Germany), an LPS-based indirect ELISA, and *Chlamydia psittaci* AK-EIA (Roehm Pharma, Germany), an LPS-based

competitive ELISA have been used for the detection of antibodies in avian species (Prukner-Radovic et al., 2005). The latter was less specific when compared to LPS/LGP ELISA in detecting antibodies to the LPS/lipoglycoprotein complex, rMOMP ELISA and immunoblotting analysis on a small serum collection (Verminnen et al., 2006). The CHEKIT™ Chlamydia assay (IDEXX Laboratories, Maine, USA), an indirect ELISA, was designed for detection of *C. abortus* antibodies in ruminants. The assay has recently been reported in two studies to cross-react with sera from SPF lambs experimentally infected with conjunctival and arthritogenic subtypes of *C. pecorum*, as well as with field serum samples from sheep naturally infected with enteric and arthritogenic subtypes of *C. pecorum*, suggesting the presence of LPS in the coating antigen (Vretou et al. 2007; Wilson et al., 2008). The sensitivities and specificities of the assay tested with reference sera in these two studies were reported as 73.3/85.5% and 96.3/96.2%, respectively. This test offers the advantage of easy and speedy performance and has been used in the diagnosis of bovine chlamydiosis (Niemczuk, 2005). It might further prove useful for the serological diagnosis of avian chlamydiosis, where cross-reactivity with other chlamydial species is not an issue.

3.4. Assays based on the MOMP

3.4.1. Synthetic peptides and recombinant MOMP

The diversity of the variable domains VD1-4 in the *ompA* genes of *C. abortus* and *C. pecorum* strains has provided the molecular basis for the development of a specific test capable of discriminating between the two species (Kaltenboeck et al., 1993). The reactivity of synthetic decapeptides spanning the four VDs of MOMP with sera from 7 *C. abortus*-infected pregnant ewes and with sera from 10 *C. pecorum*-infected SPF lambs differed significantly ($p < 0.0001$, two-tailed t-test, **Figure 3**). However, an ELISA that was based on recombinant VD2 expressed as a GST fusion protein was found to be very specific (98.4 %),

but only 66 % sensitive when tested with a panel of 57 positive and 65 negative documented reference sera (Vretou et al., unpublished data). The enhanced seroreactivity of VD1 and VD2 compared to the other VDs was confirmed when testing sera from experimental infections with recombinant variable domains expressed as fusion proteins (Livingstone et al., 2005).

Synthetic peptides derived from VD1 and VD2 amino acid sequences were the basis of the assay Panclabort or MOMP-P (dianoSTI, Midlothian Scotland) which is no longer available. The test was the only one commercially available for anti-MOMP antibodies and was found to be 95.9% specific and 70.4% sensitive in a recent evaluation (McCauley et al., 2007).

3.4.2. Competitive ELISA (cELISA)

The test is based on the binding of specific mAbs against the MOMP VD1 or VD2 that is inhibited by the presence of serum antibodies. The inhibition depends largely on the quantity and quality (affinity) of the competing antibodies, which might recognise linear or conformational epitopes on the native MOMP (Vretou et al., 2001). The original test had two cut-offs, at 30% and at 55% inhibition; the gray zone encompassed sera originating from either enteric *C. pecorum* or enteric *C. abortus* infections (Salti-Montesanto et al., 1997; Gut-Zangger et al., 1999). A recent evaluation of the assay with documented reference sera and a cut-off at 50% inhibition reported 98.1% specificity and 77.7% sensitivity for the cELISA (Vretou et al., 2007). The test proved suitable for a large-scale study representing 76% of the Swiss sheep population, which used the pool approach and sophisticated statistics for the estimation of true prevalence. The results of the serological survey correlated well with the incidence of abortions (Borel et al., 2004). 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). Several conclusions can be drawn from that study: a) anti-MOMP titres

did not decline significantly in any group in the two-year period, thus confirming previous observations after experimentally induced chlamydial abortion (Papp et al., 1994); b) anti-MOMP titres elicited by vaccination with the live vaccine were individual and animal-specific and c) these titres compared to the titres observed in naturally infected animals. Moreover, the study highlighted the urgent need for assays capable of discriminating between infected and vaccinated animals (Borel et al., 2005; Gerber et al., 2007).

3.5. Assays based on the POMP_s

With the discovery of the multigene family encoding proteins of 90kDa in 1996, it became clear that these highly immunoreactive proteins were very complex antigens comprising epitopes present at least in the *Chlamydophila* species (Souriau et al., 1994; Longbottom et al., 1996, 1998; Vretou et al., 2003). Competitive ELISAs based on POMP-specific mAbs 181 and 192 were developed (Vretou et al., unpublished data). Initial serological screening of the original clones identified from a λ gt11 expression library, *p90f31* and *p91Bf99*, suggested that they were promising candidate antigens for the specific diagnosis of OEA. When a truncated fragment of *p91Bf99*, named rOMP91B expressed as GST fusion protein was tested with a panel of documented reference sera it was found more specific (98.5%) and sensitive (84.2%) than the cELISA and the CFT (Longbottom et al., 2001). Fragmentation of the whole POMP90 molecule into overlapping recombinant antigens has revealed considerable variation in the sensitivity and specificity among the different fragments. Thus, fragments rOMP90-3 and rOMP90-4 exceeded the other recombinant fragments in specificity (100%) and sensitivity (95.7%/94.3%, respectively), although fragment rOMP90-4 appeared to perform better with OEA-positive field samples (Longbottom et al., 2002). The high sensitivity and specificity of the rOMP90-4 ELISA was reconfirmed by a different study that compared this fragment to rOMP90-3, to the commercial

POMP 80-90 (Pourquier) test and the commercial MOMP-P test. The authors reported 98.1% sensitivity and 100% specificity for rOMP90-4 (McCauley et al., 2007). In a more recent comparison with six serological assays, including the Pourquier and CHEKIT tests, the two rOMP90 ELISAs were found to be the most sensitive and specific (Wilson et al., 2008). Overall, the rOMP90-3 ELISA performed the best, with 96.8% sensitivity and no cross-reaction with sera from animals infected with *C. pecorum* or from EAE-free flocks (100% specificity). Antibodies to the POMP family have been shown to appear earlier than anti-MOMP antibodies after experimental infection of pregnant ewes or after hyperimmunisation in mice (Vretou et al., 1996; Livingstone et al., 2005). Furthermore, the anti-POMP response was elevated and exceeded the anti-MOMP response in animals that lambed normally (Livingstone et al., 2005). This observation suggested that *C. abortus*-infected animals could be detected serologically **before** the manifestation of the disease and has important implications for the eradication of OEA. Further studies are needed to address this issue in the field.

Chlamydophila abortus ELISA (Institut Pourquier, Montpellier, France) is the only commercially available test that is based on the POMP. Recent evaluations of the assay using reference sera have reported good specificity (90-100%) and sensitivity (80-93.5%) when compared to other serological tests, including CFT, CHEKIT, cELISA, and rOMP90 tests (McCauley et al., 2007; Vretou et al., 2007; Wilson et al., 2008). When tested with field sera, however, the test was less sensitive, identifying up to half of the sera detected by the other assays, possibly because of the selected high cut-off of the test or the choice of antigen fragment used in the test (Vretou et al., 2007; Wilson et al., 2008). The Pourquier-ELISA performed equally well with the “in house” rPOMP90-3 ELISA when tested with reference sera and compared to CFT, MOMP-P, rOMP90-3 and rOPM90-4 assays (McCauley et al., 2007).

Currently, POMP-based tests with the exception of a study in cattle (Niemczuk, 2005) have not been applied to other species, though application to all *C. abortus*-infected animals is theoretically possible. The N-terminal part of the POMPs has been shown to share common epitopes with *C. psittaci* (Souriau et al., 1994; Vretou et al., 2003). The application of the POMP-based assays to avian serology and human psittacosis might therefore prove interesting.

3.6. The prospect of serological testing

Over the last few years novel assays have been developed, either "in house" or commercial, that have met the requirements for the specific serological diagnosis of *C. abortus*. Novel needs have emerged, i.e. a requirement for the specific detection of *C. pecorum* antibodies and the serological differentiation of vaccinated and infected animals. The understanding of the "enzootic" character of animal chlamydiosis however, (from Greek εν=in, ζώον=animal), that is the latency, persistence or chronic infection within the animal is far from being understood. It is possible that the novel specific assays, when applied together, might help differentiate between the different conditions and thereby contribute to our understanding of the pathogenesis of the disease.

4. DNA amplification methods

4.1. Conventional PCR

The possibilities of rapid and specific detection of chlamydiae have considerably improved since the introduction of molecular methods, particularly PCR, which permits DNA-based differentiation among individual species and direct identification from clinical specimens.

Many different PCR protocols have been suggested in the literature. **Table 1** summarises some of the assays that may be relevant for veterinary diagnostic laboratories. The majority of published conventional PCR methods are based on targets in the ribosomal RNA operon (Messmer et al., 1997; Everett and Andersen, 1999) or the *ompA* gene (Kaltenboeck et al., 1997b; Yoshida et al., 1998). The latter encodes MOMP and harbours four VDs, each of which is flanked by a conserved region. While genus- and species-specific antigenic determinants are encoded by the conserved regions, serovar-specific segments are located on the variable domains, mainly VD2 and VD4. This heterogeneous primary structure makes the *ompA* gene an ideal target for diagnostic PCR, as well as for intra-species differentiation assays.

An optimised nested PCR assay based on the elaborate primer system of Kaltenboeck et al. (1997) was modified and described in detail by Sachse and Hotzel (2003). The first round of amplification generates a *Chlamydiaceae*-specific product, which serves as template in the second round, where species-specific primers are used. This assay was found to be very robust for routine use and proved the most sensitive among several protocols. However, the fact that primers had been designed on the basis of the traditional four-species classification is now becoming a major limitation.

Hartley et al. (2001) proposed a PCR system targeting the *ompB* gene, which initially generates a family-specific product and, after restriction enzyme analysis and/or enzyme-linked oligonucleotide assay of the amplicons, can identify most of the chlamydial species according to the revised classification.

A well-validated assay for detection of *Chlamydia trachomatis*, *Chlamydia pneumoniae* and *Chlamydia psittaci* (old nomenclature), which can also be run in triplex mode, was published by Madico et al. (2000). The high sensitivity of this procedure was attained through a touchdown enzyme time release methodology featuring the use of hot start DNA

polymerase, a touchdown protocol for annealing temperatures to improve primer binding specificity, and an enzyme time release protocol to allow 60 cycles to be run for improved sensitivity.

Laroucau et al. (2007) suggested a PCR assay identifying strains of *C. psittaci* (old classification), which they used to identify chlamydiae from birds and sheep. Notably, this assay targeting the *pmp* locus was found to have higher sensitivity than other conventional PCR assays targeting the *ompA* gene or the 16S-23S intergenic spacer.

As a large number of PCR tests for chlamydiae and other organisms are currently in use, it is not always clear from the respective publication, whether the test has been properly validated. Indeed, the use of poorly validated or unvalidated test protocols may lead to the generation of invalid data due to poor performance parameters of that test. This issue has been addressed in a recent review by Apfalter et al. (2005). The authors emphasised that pre-analytical procedures, sample preparation and DNA extraction, assay design and setup, as well as interpretation and confirmation of results should be subject to validation in order to ensure high accuracy of data, high specificity and sensitivity. If considering the use of a new amplification assay from the literature, the prospective user should always check the publication for adherence to these general rules on test validation.

4.2. Real-time PCR

While conventional PCR can only confirm the presence or absence of a given pathogen, real-time PCR additionally enables the diagnostician to quantitate the amount of this agent present in the sample. The accumulation of amplified product is monitored by measurement of the fluorescent signal generated by exonuclease digestion of a specifically annealed dual-labelled fluorogenic probe (Livak et al., 1995; Heid et al., 1996). As a prominent advantage, real-time PCR does not require post-PCR sample handling, which precludes potential PCR

product carry-over contamination and results in more rapid and high-throughput assays. The procedure has a large dynamic range of target molecule detection comprising at least five orders of magnitude.

The quantitation option represents the elimination of a major drawback of conventional PCR, where logarithmic amplification is confined to a limited number of cycles only. This so-called log-linear phase, which is characterised by the logarithmic increase of amplification product yield after each cycle, is followed by a plateau where the increase in yield becomes less and less steep. While conventional PCR is usually run to the plateau (35-50 cycles) to produce high amounts of amplicon, quantitative real-time PCR is based on measurements in the log-linear phase. Fluorescence signals are generated by specific binding of dye molecules, such as SYBR Green I, to double-stranded DNA (Wittwer et al., 1997) or by labelled oligonucleotide hybridisation probes (Livak et al., 1995). As fluorescence readings of PCR in the log-linear phase can be correlated to the initial number of target gene copies, quantitation of the number of microbial cells present in the sample can be accomplished. Practically, DNA concentration (as copy number) is calculated from the number of amplification cycles necessary to generate a fluorescence signal of a given threshold intensity.

A summary of published real-time PCR assays for chlamydiae of veterinary interest is given in **Table 2**. In contrast to conventional PCR assays discussed above, most of the family-specific real-time PCR tests are targeting the 23S rRNA gene. The methodologies developed by Everett et al. (1999b), DeGraves et al. (2003a) and Ehricht et al. (2006) have been validated and used in routine testing. Detection limits were reported to be in the order of a single target copy. However, Ehricht et al. (2006) pointed out that the actual detection limit may be dependent on the integrity of target DNA. While a single target copy cloned in a plasmid was reproducibly detected, the detection limit of normally processed chromosomal DNA of *Chlamydia trachomatis* was at 56 copies. This discrepancy was attributed to strand

breaks and partial degradation during the course of DNA extraction from the clinical sample matrix, as well as the effect of steric hindrance during the enzymatic amplification reaction.

DeGraves et al. (2003b) developed a highly sophisticated real-time PCR platform for sensitive detection of chlamydiae, which is distinguished by its optimised nucleic acid extraction protocol ensuring high template yield, its step-down thermal cycling profile ensuring high product yield, and its design for the high-throughput regime of a diagnostic laboratory. This comprehensive and robust system is based on fluorescence resonance energy transfer (FRET) technology run on the LightCycler and allows detection of the pathogens both at genus and species level, according to the traditional four-species nomenclature. An interesting experiment was the inclusion of reverse transcriptase in the reaction mix, so that the concurrently isolated chlamydial RNA was converted into an additional portion of DNA template. However, the authors stated that more experiments were necessary to demonstrate the anticipated positive effect on the assay's sensitivity.

Geens et al. (2005b) also used LightCycler technology to develop a panel of real-time PCR tests for *C. psittaci*. While the species-specific assay is based on SYBR Green detection and, therefore, slightly less sensitive than fluorescent probe-based assays, the authors managed to design individual tests for the *C. psittaci* genotypes A, B, C, D, E, F, and E/B, respectively. Interestingly, some of the assays had to be designed as competitive reactions. Thus, the closely related genotypes A, B, and E could not be distinguished by individual TaqMan probes, and non-fluorescent competitor oligonucleotides had to be included along with the probes to attain the necessary specificity.

The number of real-time PCR protocols developed for individual chlamydial species is steadily increasing. Using discriminatory target segments in the *ompA* gene, Pantchev et al. (2008) presented separate assays for *C. psittaci* and *C. abortus*, which could be useful in the clinical diagnosis of psittacosis and enzootic abortion, as well as epidemiological surveys.

They stressed the difficulties in designing species-specific hybridisation probes in view of the close genetic relatedness of the two agents, which required the selection of a minor groove-binding (MGB™) probe. Furthermore, this assay includes an internal amplification control (Hoffmann et al., 2006), which acts as a sensitive indicator of amplification efficiency and the presence or absence of DNA polymerase inhibitors.

Based on *incA* gene sequence analysis of five *C. psittaci* strains, Menard et al. (2006) published a TaqMan real-time PCR protocol for detection of this species from clinical samples. They also used a MGB probe to rule out cross-reactions with *C. abortus*.

5. DNA microarray technology

5.1. Identification of chlamydial species

As diagnostic testing is being extended to an increasing number of microbial pathogens and ever more sophisticated technology becomes available, diagnosticians are facing a growing demand for new diagnostic tests with higher information content. In many instances, the mere identification of the species involved will no longer be sufficient, but information on subspecies, serotype or genotype, toxins and other virulence factors will be expected. Whenever "multi-dimensional" evidence on the microorganism present in a sample is required the limitations of PCR-based tests become obvious. Detection of subtle differences among strains, such as single-nucleotide polymorphisms and other intra-species variations, is problematic with standard amplification assays, and even in multiplex PCR only a few target regions can be examined in parallel.

DNA microarray technology opens up new possibilities that may be particularly beneficial for laboratory diagnosis of infectious diseases. Its highly parallel approach allows DNA samples to be simultaneously examined by a large number of probes, which may be derived from a polymorphic gene segment and/or from different genomic regions. In essence,

a specific microarray hybridisation test is equivalent to re-sequencing the respective genomic site. Thus, DNA microarray-based tests can attain far higher resolution than PCR.

Although microarrays have become a widely accepted tool for mRNA expression monitoring in gene transcription analysis, their use in the rapid diagnosis of bacterial and viral pathogens is only emerging. In a recent study, Sachse et al. (2005) developed a microarray assay for the detection and differentiation of *Chlamydia* spp. and *Chlamydophila* spp. The authors used the commercially available ArrayTubeTM (AT) system (Clontech Chip Technologies, Jena, Germany), which represents a less expensive system for processing low- and high-density DNA arrays. It involves spotted or *in situ* synthesised DNA chips of 3x3 mm size, which are assembled onto the bottom of 1.5-ml plastic micro-reaction tubes. In contrast to other microarray equipment, hybridisation and signal processing can be conducted in an easy and rapid fashion on standard laboratory equipment without additional devices, such as hybridisation chambers. Hybridisation signals are amplified by an enzyme-catalysed precipitation reaction. A CCD camera integrated in a light transmission reader is used to monitor DNA duplex formation by kinetic measurement of the precipitation reaction at each spot.

Hybridisation probes for *Chlamydiaceae* spp. were designed on the basis of a multiple sequence alignment, from which a highly discriminatory segment in domain I of the 23S rRNA gene was identified. As a result of several rounds of optimisation and refinement, the present version of the chip carries 28 species-specific probes (for all nine chlamydial species), 3 genus-specific probes for *Chlamydia* and *Chlamydophila*, respectively, 5 probes identifying the closest relatives *Simkania negevensis* and *Waddlia chondrophila*, as well as 4 hybridisation controls (consensus probes) and a staining control (biotinylated oligonucleotide).

Target DNA is prepared by standard extraction and consensus PCR using a biotinylated primer. The AT assay for chlamydiae provides unique species-specific hybridisation patterns for all nine species of the family *Chlamydiaceae*, which are processed by the Iconoclust software (Clondiag).

The AT test has been used for the direct detection of chlamydiae from clinical tissue in a validation study (Borel et al., 2008). As many clinical samples contain only low numbers of bacteria, sensitivity became the crucial parameter in this application. In a previous publication, the same group had been able to show that the sensitivity of the microarray assay was equivalent to that of real-time PCR (Ehricht et al., 2006), thus rendering the test suitable for use in the diagnostic lab.

5.2. Genotyping by microarray

The great potential of the AT microarray technology for rapid intraspecies characterisation has been demonstrated by the recent development of a genotyping assay for *C. psittaci* (Sachse et al., 2008). These authors conducted an extensive analysis of all available *ompA* gene sequences from *C. psittaci* strains, which revealed a remarkable genetic diversity within this species, and which is only incompletely covered by the currently used genotyping scheme comprising seven avian (A, B, C, D, E, F, E/B) and two non-avian (WC, M56) genotypes (Vanrompay et al., 1997; Geens et al., 2005a). This scheme evolved from serology and is based on *ompA* sequence variations. Restriction enzyme cleavage of the amplified *ompA* gene and, less frequently, *ompA* sequencing are being used for genotyping. However, the former is not sensitive enough for direct typing from clinical samples and the latter is not ideal for routine diagnosis. Moreover, an increasing number of recently tested strains could not be assigned to any of the established genotypes.

The conclusions from *ompA* sequence analysis were incorporated in the design of a new DNA microarray for genotyping of *C. psittaci* (Sachse et al., 2008). The AT microarray-based genotyping assay was shown to discriminate all established genotypes and identify so far untyped strains. Its high specificity, which allows detection of single-nucleotide polymorphisms, is due to the parallel approach consisting in the use of 35 hybridization probes derived from VD2 and VD4 of the *ompA* gene. This test represents a promising diagnostic tool for tracing epidemiological chains, exploring the dissemination of genotypes and identifying non-typical representatives of *C. psittaci*. As *ompA* sequence analysis had revealed that the traditional genotyping system did not adequately reflect the extent of intra-species heterogeneity, the authors suggested adjustments and extensions to the present scheme, which include the introduction of subtypes to the more heterogeneous genotypes A, E/B and D, as well as six new provisional genotypes representing so far untypable strains.

Figure 4 illustrates the examination of samples from avian chlamydiosis using several of the DNA-based assays described here.

6. The quest for an alternative reference standard

Evaluations of various DNA amplification methods by comparison with chlamydial cell culture (Thejls et al., 1994; Johnson et al., 2000; Ostergaard, 2002; Sachse et al., 2003) have led to the general conclusion of them having superior analytical sensitivity. Moreover, studies in humans showed that the use of culture alone as a reference standard results in significant underestimates of the specificity of DNA amplification tests, as many infected patients were considered falsely negative by culture (Jaschek et al., 1993).

In view of a large amount of data in favour of PCR and related techniques, the introduction of an alternative gold standard replacing cell culture has been widely discussed. In addition to being highly sensitive and specific, such a reference test should be easily

standardisable and practicable in any diagnostic laboratory. Johnson et al. (2000) showed that a PCR and a ligase chain reaction test for *Chlamydia trachomatis* can be used as a combined standard in the examination of urine and genital samples from humans. In this study, the combined results of two amplification tests were used to estimate the performance of a third assay. More recently, Martin et al. (2004) suggested the use of at least three different nucleic acid amplification tests to define the "infected-patient gold standard" and evaluate new diagnostic tests. Although this raises new questions, such as the number and kind of specimens to be collected from each patient, as well as the handling of discrepant results, the approach will clearly increase the accuracy of diagnostic testing.

With the availability of a validated microarray test, the idea of a multiple test being the gold standard in chlamydial diagnosis can be further substantiated. As the results of the validation study by Borel et al. (2008) revealed the equivalence of data from the real-time PCR assay according to Ehricht et al. (2006) and the AT test for chlamydiae, the combination of both assays to form an alternative standard has proved practicable and appears justified. Such a two-test reference standard will ensure accuracy and comparability because two different test mechanisms and two different target sequences are involved. Another important argument in favour of this standard is its applicability to detection of any species of the family *Chlamydiaceae*, since the AT test covers all currently defined chlamydiae.

7. Conclusions

A variety of assays and techniques that either directly detect antigen in tissue and swab samples or detect anti-chlamydial antibodies in blood samples are available for the diagnosis of chlamydial infection. Although these tests vary markedly in terms of sensitivity and specificity, each has an important role to play in diagnosis. Ultimately the test that is used is dependent on a number of factors, including (a) the type of sample submitted, (b) the viability

of the organisms in the specimen, which depends on the preservation and transportation of the sample, (c) a possible presumptive diagnosis based on possible presenting clinical symptoms and pathology, and (d) possible clinical history.

In terms of serological diagnosis, the authors advocate the use of the most specific of the commercially available serological tests, the *Chlamydia abortus* ELISA (Institut Pourquier), in the veterinary diagnostic laboratory. Although there is a move towards up-to-date molecular based techniques that eliminate the need for viable organisms, culture still remains invaluable for the generation of new isolates for more detailed characterisation and pathogenesis studies. However, given the well-documented advantages of PCR and other DNA amplification tests over chlamydial cell culture in terms of sensitivity, throughput and time consumption, the introduction of an alternative gold standard consisting of a combination of independent DNA tests appears to be a promising and realistic path to pursue. Thus, the authors suggest using a real-time PCR detection assay in conjunction with the ArrayTube microarray test as a reference standard for the detection of chlamydiae in clinical and tissue samples.

Conflict of interest statement

None of the authors (Konrad Sachse, Evangelia Vretou, Morag Livingstone, Nicole Borel, Andreas Pospischil, and David Longbottom) has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the paper entitled "Recent developments in the laboratory diagnosis of chlamydial infections".

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References

- Aitken, I.D., Longbottom, D., 2004. Enzootic abortion of ewes (ovine chlamydiosis). In: OIE Biological Standards Commission (Ed.), Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Mammals, Birds and Bees). Office International des Epizooties, Paris, pp. 635-641.
- Alexander, J.J., 1968. Separation of protein synthesis in meningopneumonitis agent from that in L cells by differential susceptibility to cycloheximide. J. Bacteriol. 95, 327-332.
- Andersen, A.A., 1991. Serotyping of *Chlamydia psittaci* isolates using serovar-specific monoclonal antibodies with the microimmunofluorescence test. J. Clin. Microbiol. 29, 707-711.
- Andersen, A.A., 2004. Avian chlamydiosis. In: OIE Biological Standards Commission (Ed.), Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Mammals, Birds and Bees). Office International des Epizooties, Paris, pp. 856-867.
- Anderson, I.E., Baxter, S.I.F., Dunbar, S., Rae, A.G., Philips, H.L., Clarkson, M.J., Herring, A.J., 1996. Analyses of the genomes of chlamydial isolates from ruminants and pigs support the adoption of the new species *Chlamydia pecorum*. Int. J. Syst. Bacteriol., 46, 245-251.

- Anderson, I.E., Herring, A.J., Jones, G.E., Low, J.C., Greig, A., 1995. Development and evaluation of an indirect ELISA to detect antibodies to abortion strains of *Chlamydia psittaci* in sheep sera. *Vet. Microbiol.* 43, 1-12.
- Apfalter, P., Reischl, U., Hammerschlag, M.R., 2005. In-house nucleic acid amplification assays in research: how much quality control is needed before one can rely upon the results? *J. Clin. Microbiol.* 43, 5835-5841.
- Barnes, R.C., 1989. Laboratory diagnosis of human chlamydial infections. *Clin. Microbiol. Rev.* 2, 119-136.
- Bevan, B.J., Cullen, G.A., Read, W.M.F., 1978. Isolation of *Chlamydia psittaci* from avian sources using growth in cell culture. *Avian Pathol.* 7, 203-211.
- Bland, J.O.W., Canti, R.G., 1935. The growth and development of psittacosis virus in tissue cultures. *J. Pathol. Bacteriol.* 40, 231-241.
- Borel, N., Doherr, M.G., Vretou, E., Psarrou, E., Thoma, R., Pospischil, A., 2002. [Ovine enzootic abortion: seroprevalence in Switzerland using a competitive enzyme-linked immunosorbent assay (cELISA)]. *Schweiz. Arch. Tierheilk.* 144, 474-482.
- Borel, N., Doherr, M.G., Vretou, E., Psarrou, E., Thoma, R., Pospischil, A., 2004. Seroprevalences for ovine enzootic abortion in Switzerland. *Prev. Vet. Med.* 65, 205-216.
- Borel, N., Kempf, E., Hotzel, H., Schubert, E., Torgerson, P., Slickers, P., Ehricht, R., Tasara, T., Pospischil, A., Sachse, K., 2008. Direct identification of chlamydiae from clinical samples using a DNA microarray assay - A validation study. *Mol. Cell. Probes* 22, 55-64.

- 979 Borel, N., Mukhopadhyay, S., Kaiser, C., Sullivan, E.D., Miller, R.D., Timms, P.,
 980 Summersgill, J.T., Ramirez, J.A., Pospischil, A., 2006a. Tissue MicroArray (TMA)
 981 analysis of normal and persistent *Chlamydophila pneumoniae* infection. BMC Infect.
 982 Dis. 6, 152.
- 983 Borel, N., Sachse, K., Rassbach, A., Bruckner, L., Vretou, E., Psarrou, E., Pospischil, A.,
 984 2005. Ovine enzootic abortion (OEA): antibody response in vaccinated sheep
 985 compared to naturally infected sheep. Vet. Res. Commun. 29 Suppl. 1, 151-156.
- 986 Borel, N., Summersgill, J.T., Mukhopadhyay, S., Miller, R.D., Ramirez, J.A., Pospischil, A.,
 987 2007. Evidence for persistent *Chlamydia pneumoniae* infection of human coronary
 988 atheromas. Atherosclerosis Oct 27; [Epub ahead of print].
- 989 Borel, N., Thoma, R., Spaeni, P., Weilenmann, R., Teankum, K., Brugnera, E., Zimmermann,
 990 D.R., Vaughan, L., Pospischil, A., 2006b. Chlamydia-related abortions in cattle from
 991 Graubunden, Switzerland. Vet. Pathol. 43, 702-708.
- 992 Brabetz, W., Lindner, B., Brade, H., 2000. Comparative analyses of secondary gene products
 993 of 3-deoxy-D-manno-oct-2-ulosonic acid transferases from *Chlamydiaceae* in
 994 *Escherichia coli* K-12. Eur. J. Biochem. 267, 5458-5465.
- 995 Brade, H., Brade, L., Nano, F.E., 1987. Chemical and serological investigations on the genus-
 996 specific lipopolysaccharide epitope of *Chlamydia*. Proc. Natl. Acad. Sci. U S A 84,
 997 2508-2512.
- 998 Brade, L., Brunnemann, H., Ernst, M., Fu, Y., Holst, O., Kosma, P., Naher, H., Persson, K.,
 999 Brade, H., 1994. Occurrence of antibodies against chlamydial lipopolysaccharide in
 1000 human sera as measured by ELISA using an artificial glycoconjugate antigen. FEMS
 1001 Immunol. Med. Microbiol. 8, 27-41.

- 1002 Brade, L., Rozalski, A., Kosma, P., Brade, H., 2000. A monoclonal antibody recognizing the
1003 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) trisaccharide
1004 α Kdo(2→4) α Kdo(2→4) α Kdo of *Chlamydophila psittaci* 6BC
1005 lipopolysaccharide. J. Endotoxin Res. 6, 361-368.
- 1006 Buendia, A.J., Cuello, F., Del Rio, L., Gallego, M.C., Caro, M.R., Salinas, J., 2001. Field
1007 evaluation of a new commercially available ELISA based on a recombinant antigen
1008 for diagnosing *Chlamydophila abortus* (*Chlamydia psittaci* serotype 1) infection. Vet.
1009 Microbiol. 78, 229-239.
- 1010 Burnet, F.M., Rountree, P.M., 1935. Psittacosis in the developing egg. J. Path. Bact. 40, 471-
1011 481.
- 1012 Busch, M., Thoma, R., Schiller, I., Corboz, L., Pospischil, A., 2000. Occurrence of
1013 chlamydiae in the genital tracts of sows at slaughter and their possible significance for
1014 reproductive failure. J. Vet. Med. B (Infect Dis Vet Public Health.) 47, 471-480.
- 1015 Bush, R.M., Everett, K.D., 2001. Molecular evolution of the *Chlamydiaceae*. Int. J. Syst.
1016 Evol. Microbiol. 51, 203-220.
- 1017 Buxton, D., 1986. Potential danger to pregnant women of *Chlamydia psittaci* from sheep. Vet.
1018 Rec. 18, 510-511.
- 1019 Buxton, D., Anderson, I.E., Longbottom, D., Livingstone, M., Wattegedera, S., Entrican, G.,
1020 2002. Ovine chlamydial abortion: characterization of the inflammatory immune
1021 response in placental tissues. J. Comp. Pathol. 127, 133-141.
- 1022 Buxton, D., Barlow, R.M., Finlayson, J., Anderson, I.E., Mackellar, A., 1990. Observations
1023 on the pathogenesis of *Chlamydia psittaci* infection of pregnant sheep. J. Comp.

- 1024 Pathol. 102, 221-237.
- 1025 Buxton, D., Rae, A.G., Maley, S.W., Thomson, K.M., Livingstone, M., Jones, G.E., Herring,
 1026 A.J., 1996. Pathogenesis of *Chlamydia psittaci* infection in sheep: detection of the
 1027 organism in a serial study of the lymph node. J. Comp. Pathol. 114, 221-230.
- 1028 Canfield, P.J., Love, D.N., Mearns, G., Farram, E., 1991. Evaluation of an
 1029 immunofluorescence test on direct smears of conjunctival and urogenital swabs taken
 1030 from koalas for the detection of *Chlamydia psittaci*. Aust. Vet. J. 68, 165-167.
- 1031 Cevenini, R., Moroni, A., Sambri, V., Perini, S., La Placa, M., 1989. Serological response to
 1032 chlamydial infection in sheep, studied by enzyme-linked immunosorbent assay and
 1033 immunoblotting. FEMS Microbiol. Immunol. 1, 459-464.
- 1034 Cles, L.D., Stamm, W.E., 1990. Use of HL cells for improved isolation and passage of
 1035 *Chlamydia pneumoniae*. J. Clin. Microbiol. 28, 938-940.
- 1036 Cross, R.F., Clafin, R.M., 1963. The incidence and removal of procomplementary activity in
 1037 swine serum. Am. J. Vet. Res. 24, 334-336.
- 1038 Crosse, B., 1990. Psittacosis: a clinical review. J. Infect. 21, 251-259.
- 1039 Croy, T.R., Kuo, C.C., Wang, S.P., 1975. Comparative susceptibility of eleven mammalian
 1040 cell lines to infection with trachoma organisms. J. Clin. Microbiol. 1, 434-439.
- 1041 Dagnall, G.J., Wilsmore, A.J., 1990. A simple staining method for the identification of
 1042 chlamydial elementary bodies in the fetal membranes of sheep affected by ovine
 1043 enzootic abortion. Vet. Microbiol. 21, 233-239.
- 1044 DeGraves, F.J., Gao, D., Hehnen, H.-R., Schlapp, T., Kaltenboeck, B., 2003a. Quantitative
 1045 detection of *Chlamydia psittaci* and *C. pecorum* by high-sensitivity real-time PCR

- 1046 reveals high prevalence of vaginal infection in cattle. J. Clin. Microbiol. 41, 1726-
1047 1729.
- 1048 DeGraves, F.J., Gao, D., Kaltenboeck, B., 2003b. High-sensitivity quantitative PCR platform.
1049 Biotechniques 34, 106-115
- 1050 Demaio, J., Boyd, R.S., Rensi, R., Clark, A., 1991. False-positive Chlamydiazyme results
1051 during urine sediment analysis due to bacterial urinary tract infections. J. Clin.
1052 Microbiol. 29, 1436-1438.
- 1053 Di Francesco, A., Baldelli, R., Cevenini, R., Magnino, S., Pignanelli, S., Salvatore, D.,
1054 Galuppi, R., Donati, M., 2006. Seroprevalence to chlamydiae in pigs in Italy. Vet.
1055 Rec. 159, 849-850.
- 1056 Dovc, A., Dovc, P., Kese, D., Vlahovic, K., Pavlak, M., Zorman-Rojs, O., 2005. Long-term
1057 study of Chlamydophilosis in Slovenia. Vet. Res. Commun. 29 Suppl 1, 23-36.
- 1058 Eb, F., Orfila, J., 1982. Serotyping of *Chlamydia psittaci* by the micro-immunofluorescence
1059 test: isolates of ovine origin. Infect. Immun. 37, 1289-1291.
- 1060 Eb, F., Orfila, J., Milon, A., Geral, M.F., 1986. [Epidemiologic significance of the
1061 immunofluorescence typing of *Chlamydia psittaci*]. Ann. Inst. Pasteur Microbiol.
1062 137B, 77-93.
- 1063 Ehricht, R., Slickers, P., Goellner, S., Hotzel, H., Sachse, K., 2006. Optimized DNA
1064 microarray assay allows detection and genotyping of single PCR-amplifiable target
1065 copies. Mol. Cell. Probes 20, 60-63.
- 1066 Everett, K.D., Andersen, A.A., 1999. Identification of nine species of the *Chlamydiaceae*
1067 using PCR-RFLP. Int. J. Syst. Bacteriol. 49 Pt 2, 803-813.

- 1068 Everett, K.D., Bush, R.M., Andersen, A.A., 1999a. Emended description of the order
1069 *Chlamydiales*, proposal of *Parachlamydiaceae* fam. nov. and *Simkaniaceae* fam. nov.,
1070 each containing one monotypic genus, revised taxonomy of the family
1071 *Chlamydiaceae*, including a new genus and five new species, and standards for the
1072 identification of organisms. Int. J. Syst. Bacteriol. 49 Pt 2, 415-440.
- 1073 Everett, K.D., Hornung, L.H., Andersen, A.A., 1999b. Rapid detection of the *Chlamydiaceae*
1074 and other families in the order *Chlamydiales*: Three PCR tests. J. Clin. Microbiol. 37,
1075 575-580.
- 1076 Finlayson, J., Buxton, D., Anderson, I.E., Donald, K.M., 1985. Direct immunoperoxidase
1077 method for demonstrating *Chlamydia psittaci* in tissue sections. J. Clin. Pathol. 38,
1078 712-714.
- 1079 Fukushi, H., Hirai, K., 1992. Proposal of *Chlamydia pecorum* sp. nov. for *Chlamydia* strains
1080 derived from ruminants. Int. J. Syst. Bacteriol. 42, 306-308.
- 1081 Geens, T., Desplanques, A., Van Loock, M., Bonner, B.M., Kaleta, E.F., Magnino, S.,
1082 Andersen, A.A., Everett, K.D., Vanrompay, D., 2005a. Sequencing of the
1083 *Chlamydophila psittaci* ompA gene reveals a new genotype, E/B, and the need for a
1084 rapid discriminatory genotyping method. J. Clin. Microbiol. 43, 2456-2461.
- 1085 Geens, T., Dewitte, A., Boon, N., Vanrompay, D., 2005b. Development of a *Chlamydophila*
1086 *psittaci* species-specific and genotype-specific real-time PCR. Vet. Res. 36, 787-797.
- 1087 Gerber, A., Thoma, R., Vretou, E., Psarrou, E., Kaiser, C., Doherr, M.G., Zimmermann, D.R.,
1088 Polkinghorne, A., Pospischil, A., Borel, N., 2007. Ovine Enzootic Abortion (OEA): a
1089 comparison of antibody responses in vaccinated and naturally-infected swiss sheep
1090 over a two year period. BMC Vet. Res. 3, 24.

- 1091 Griffiths, P.C., Plater, J.M., Horigan, M.W., Rose, M.P., Venables, C., Dawson, M., 1996.
1092 Serological diagnosis of ovine enzootic abortion by comparative inclusion
1093 immunofluorescence assay, recombinant lipopolysaccharide enzyme-linked
1094 immunosorbent assay, and complement fixation test. J. Clin. Microbiol. 34, 1512-
1095 1518.
- 1096 Guscetti, F., Hoop, R., Schiller, I., Corboz, L., Sydler, T., Pospischil, A., 2000. Experimental
1097 enteric infection of gnotobiotic piglets with a *Chlamydia psittaci* strain of avian origin.
1098 J. Vet. Med. B (Infect Dis Vet Public Health) 47, 561-572.
- 1099 Gut-Zangger, P., Vretou, E., Psarrou, E., Pospischil, A., Thoma, R., 1999. [Chlamydia
1100 abortion in sheep: possibilities for serological diagnosis using a competitive ELISA
1101 and insight into the epidemiologic situation in Switzerland]. Schweiz. Arch.
1102 Tierheilkd. 141, 361-366.
- 1103 Halberstaedter, L., von Prowazek, S., 1907. Über Zelleinschlüsse parasitärer Natur beim
1104 Trachom. Arbeiten aus dem Kaiserlichen Gesundheitsamte 26, 44-47.
- 1105 Hartley, J.C., Kaye, S., Stevenson, S., Bennett, J., Ridgway, G., 2001. PCR detection and
1106 molecular identification of *Chlamydiaceae* species. J. Clin. Microbiol. 39, 3072-3079.
- 1107 Heid, C.A., Stevens, J., Livak, K.J., Williams, P.M., 1996. Real-time quantitative PCR.
1108 Genome Res. 6, 968-994.
- 1109 Herring, A.J., 1993. Typing *Chlamydia psittaci* - a review of methods and recent findings. Br.
1110 Vet. J. 149, 455-475.
- 1111 Hobson, D., Johnson, F.W., Byng, R.E., 1977. The growth of the ewe abortion chlamydial
1112 agent in McCoy cell cultures. J. Comp. Pathol. 87, 155-159.

- 1113 Hoelzle, L.E., Hoelzle, K., Wittenbrink, M.M., 2004. Recombinant major outer membrane
1114 protein (MOMP) of *Chlamydophila abortus*, *Chlamydophila pecorum*, and *Chlamydia*
1115 *suis* as antigens to distinguish chlamydial species-specific antibodies in animal sera.
1116 Vet. Microbiol. 103, 85-90.
- 1117 Hoffmann, B., Depner, K., Schirrmeier, H., Beer, M., 2006. A universal heterologous internal
1118 control system for duplex real-time RT-PCR assays used in a detection system for
1119 pestiviruses. J. Virol. Methods 136, 200-209.
- 1120 Hotzel, H., Berndt, A., Melzer, F., Sachse, K., 2004. Occurrence of *Chlamydiaceae* spp. in a
1121 wild boar (*Sus scrofa* L.) population in Thuringia (Germany). Vet. Microbiol. 103,
1122 121-126.
- 1123 Jaschek, G., Gaydos, C.A., Welsh, L.E., Quinn, T.C., 1993. Direct detection of *Chlamydia*
1124 *trachomatis* in urine specimens from symptomatic and asymptomatic men by using a
1125 rapid polymerase chain reaction assay. J. Clin. Microbiol. 31, 1209-1212.
- 1126 Jee, J., Degraes, F.J., Kim, T., Kaltenboeck, B., 2004. High prevalence of natural
1127 *Chlamydophila* species infection in calves. J. Clin. Microbiol. 42, 5664-5672.
- 1128 Johnson, F.W., Clarkson, M.J., Spencer, W.N., 1983. Direct isolation of the agent of enzootic
1129 abortion of ewes (*Chlamydia psittaci*) in cell cultures. Vet. Rec. 113, 413-414.
- 1130 Johnson, R.E., Green, T.A., Schachter, J., Jones, R.B., Hook, E.W. 3rd, Black, C.M., Martin,
1131 D.H., St Louis, M.E., Stamm, W.E., 2000. Evaluation of nucleic acid amplification
1132 tests as reference tests for *Chlamydia trachomatis* infections in asymptomatic men. J.
1133 Clin. Microbiol. 38, 4382-4386.
- 1134 Johnston, S.L., Siegel, C., 1992. Comparison of Buffalo green monkey kidney cells and

- 1135 McCoy cells for the isolation of *Chlamydia trachomatis* in shell vial centrifugation
1136 culture. *Diagn. Microbiol. Infect. Dis.* 15, 355-357.
- 1137 Jones, G.E., Low, J.C., Machell, J., Armstrong, K., 1997. Comparison of five tests for the
1138 detection of antibodies against chlamydial (enzootic) abortion of ewes. *Vet. Rec.* 141,
1139 164-168.
- 1140 Juvonen, J., Laurila, A., Juvonen, T., Alakarppa, H., Surcel, H. M., Lounatmaa, K., Kuusisto,
1141 J., Saikku, P., 1997. Detection of *Chlamydia pneumoniae* in human nonrheumatic
1142 stenotic aortic valves. *J. Am. Coll. Cardiol.* 29, 1054-1059.
- 1143 Kaltenboeck, B., Heard, D., DeGraves, F.J., Schmeer, N., 1997a. Use of synthetic antigens
1144 improves detection by enzyme-linked immunosorbent assay of antibodies against
1145 abortigenic *Chlamydia psittaci* in ruminants. *J. Clin. Microbiol.* 35, 2293-2298.
- 1146 Kaltenboeck, B., Hehnen, H.R., Vaglenov, A., 2005. Bovine *Chlamydophila* spp. infection:
1147 do we underestimate the impact on fertility? *Vet. Res. Commun.* 29 Suppl 1, 1-15.
- 1148 Kaltenboeck, B., Kousoulas, K.G., Storz, J., 1993. Structures of and allelic diversity and
1149 relationships among the major outer membrane protein (*ompA*) genes of the four
1150 chlamydial species. *J. Bacteriol.* 175, 487-502.
- 1151 Kaltenboeck, B., Schmeer, N., Schneider, R., 1997b. Evidence of numerous *omp1* alleles of
1152 porcine *Chlamydia trachomatis* and novel chlamydial species obtained by PCR. *J.*
1153 *Clin. Microbiol.* 35, 1835-1841.
- 1154 Kampinga, G.A., Schroder, F.P., Visser, I.J., Anderson, J.M., Buxton, D., Moller, A.V., 2000.
1155 [Lambing ewes as a source of severe psittacosis in a pregnant woman]. *Ned. Tijdschr.*
1156 *Geneesk.* 144, 2500-2504.

- 1157 Kunz, U.S., Pospischil, A., Paccaud, M.F., 1991. Immunohistochemical detection of
1158 chlamydiae in formalin-fixed tissue sections: comparison of a monoclonal antibody
1159 with yolk derived antibodies (IgY). Zentralbl. Veterinarmed. B 38, 292-298.
- 1160 Kuo, C., Wang, S., Wentworth, B.B., Grayston, J.T., 1972. Primary isolation of TRIC
1161 organisms in HeLa 229 cells treated with DEAE-dextran. J. Infect. Dis. 125, 665-668.
- 1162 Laroucau, K., Trichereau, A., Vorimore, F., Mahe, A.M., 2007. A pmp genes-based PCR as a
1163 valuable tool for the diagnosis of avian chlamydiosis. Vet. Microbiol. 121, 150-157.
- 1164 Livak, K.J., Flood, S.J., Marmaro, J., Giusti, W., Deetz, K., 1995. Oligonucleotides with
1165 fluorescent dyes at opposite ends provide a quenched probe system useful for
1166 detecting PCR product and nucleic acid hybridization. PCR Methods Appl. 4, 357-
1167 362.
- 1168 Livingstone, M., Entrican, G., Wattegedera, S., Buxton, D., McKendrick, I. J., Longbottom,
1169 D., 2005. Antibody responses to recombinant protein fragments of the major outer
1170 membrane protein and polymorphic outer membrane protein POMP90 in
1171 *Chlamydophila abortus*-infected pregnant sheep. Clin. Diagn. Lab. Immunol. 12, 770-
1172 777.
- 1173 Longbottom, D., Coulter, L.J., 2003. Animal chlamydioses and zoonotic implications. J.
1174 Comp. Path. 128, 217-244.
- 1175 Longbottom, D., Fairley, S., Chapman, S., Psarrou, E., Vretou, E., Livingstone, M., 2002.
1176 Serological diagnosis of ovine enzootic abortion by enzyme-linked immunosorbent
1177 assay with a recombinant protein fragment of the polymorphic outer membrane
1178 protein POMP90 of *Chlamydophila abortus*. J. Clin. Microbiol. 40, 4235-4243.

- 1179 Longbottom, D., Psarrou, E., Livingstone, M., Vretou, E., 2001. Diagnosis of ovine enzootic
 1180 abortion using an indirect ELISA (rOMP91B iELISA) based on a recombinant protein
 1181 fragment of the polymorphic outer membrane protein POMP91B of *Chlamydophila*
 1182 *abortus*. FEMS Microbiol. Lett. 195, 157-161.
- 1183 Longbottom, D., Russell, M., Dunbar, S.M., Jones, G.E., Herring, A.J., 1998. Molecular
 1184 cloning and characterization of the genes coding for the highly immunogenic cluster
 1185 of 90-kilodalton envelope proteins from the *Chlamydia psittaci* subtype that causes
 1186 abortion in sheep. Infect. Immun. 66, 1317-1324.
- 1187 Longbottom, D., Russell, M., Jones, G.E., Lainson, F.A., Herring, A.J., 1996. Identification of
 1188 a multigene family coding for the 90 kDa proteins of the ovine abortion subtype of
 1189 *Chlamydia psittaci*. FEMS Microbiol. Lett. 142, 277-281.
- 1190 Lutz-Wohlgroth, L., Becker, A., Brugnera, E., Huat, Z. L., Zimmermann, D., Grimm, F.,
 1191 Haessig, M., Greub, G., Kaps, S., Spiess, B., Pospischil, A., Vaughan, L., 2006.
 1192 Chlamydiales in guinea-pigs and their zoonotic potential. J. Vet. Med. A 53, 185–193.
- 1193 Madico, G., Quinn, T.C., Boman, J., Gaydos, C.A., 2000. Touchdown enzyme time release-
 1194 PCR for detection and identification of *Chlamydia trachomatis*, *C. pneumoniae*, and
 1195 *C. psittaci* using the 16S and 16S-23S spacer rRNA genes. J. Clin. Microbiol. 38,
 1196 1085-1093.
- 1197 Magnino, S., Giovannini, S., Paoli, C., Ardenghi, P., Sambri, V., 2005. Evaluation of an
 1198 automated complement fixation test (Seramat) for the detection of chlamydial
 1199 antibodies in sheep and goat sera. Vet. Res. Commun. 29 Suppl 1, 157-161.
- 1200 Markey, B.K., McNulty, M.S., Todd, D., 1993. Comparison of serological tests for the
 1201 diagnosis of *Chlamydia psittaci* infection of sheep. Vet. Microbiol. 36, 233-252.

- 1202 Martin, D.H., Nsuami, M., Schachter, J., Hook, E.W. 3rd, Ferrero, D., Quinn, T.C., Gaydos,
1203 C., 2004. Use of multiple nucleic acid amplification tests to define the infected-patient
1204 "gold standard" in clinical trials of new diagnostic tests for *Chlamydia trachomatis*
1205 infections. J. Clin. Microbiol. 42, 4749-4758.
- 1206 McCafferty, M.C., Herring, A.J., Andersen, A.A., Jones, G.E., 1995. Electrophoretic analysis
1207 of the major outer membrane protein of *Chlamydia psittaci* reveals multimers which
1208 are recognized by protective monoclonal antibodies. Infect. Immun. 63, 2387-2389.
- 1209 McCauley, L.M., Lancaster, M.J., Young, P., Butler, K.L., Ainsworth, C.G., 2007.
1210 Comparison of ELISA and CFT assays for *Chlamydophila abortus* antibodies in ovine
1211 sera. Aust. Vet. J. 85, 325-328.
- 1212 Menard, A., Clerc, M., Subtil, A., Megraud, F., Bebear, C., de Barbeyrac, B., 2006.
1213 Development of a real-time PCR for the detection of *Chlamydia psittaci*. J. Med.
1214 Microbiol. 55, 471-473.
- 1215 Messmer, T.O., Skelton, S.K., Moroney, J.F., Daugharty, H., Fields, B.S., 1997. Application
1216 of a nested, multiplex PCR to psittacosis outbreaks. J. Clin. Microbiol. 35, 2043-2046.
1217 Erratum in: J. Clin. Microbiol. 1998, 36:1821.
- 1218 Moore, F.M., McMillan, M.C., Petrak, M.L., 1991. Comparison of culture, peroxidase-
1219 antiperoxidase reaction, and serum latex agglutination methods for diagnosis of
1220 chlamydiosis in pet birds. J. Am. Vet. Med. Assoc. 199, 71-73.
- 1221 Navarro, J.A., Garcia de la Fuente, J.N., Sanchez, J., Martinez, C.M., Buendia, A.J.,
1222 Gutierrez-Martin, C.B., Rodriguez-Ferri, E.F., Ortega, N., Salinas, J., 2004. Kinetics
1223 of infection and effects on the placenta of *Chlamydophila abortus* in experimentally
1224 infected pregnant ewes. Vet. Pathol. 41, 498-505.

- 1225 Niemczuk, K., 2005. Prevalence of antibodies against *Chlamydophila psittaci* and
1226 *Chlamydophila abortus* in cattle in Poland. A preliminary report. Bull. Vet. Inst.
1227 Pulawy 49, 293-297.
- 1228 Ostergaard, L., 2002. Microbiological aspects of the diagnosis of *Chlamydia trachomatis*.
1229 Best Pract. Res. Clin. Obstet. Gynaecol. 16, 789-799.
- 1230 Page, L.A., 1966. Revision of the family *Chlamydiaceae* Rake (*Rickettsiales*): Unification of
1231 the Psittacosis-Lymphogranuloma venereum-Trachoma group of organisms in the
1232 genus *Chlamydia* Jones, Rake and Stearns 1945. Int. J. Syst. Bacteriol. 16, 223-252.
- 1233 Page, L.A., 1968. Proposal for the recognition of two species in the genus *Chlamydia* Jones,
1234 Rake and Stearns 1945. Int. J. Syst. Bacteriol. 18, 51-66.
- 1235 Pantchev, A., Sting, R., Bauerfeind, R., Tyczka, J., Sachse, K., 2008. New real-time PCR tests
1236 for species-specific detection of *Chlamydophila psittaci* and *Chlamydophila abortus*
1237 from tissue samples. Vet. J. (in press) doi:10.1016/j.tvjl.2008.02.025
- 1238 Papp, J.R., Shewen, P.E., Gartley, C.J., 1994. Abortion and subsequent excretion of
1239 chlamydiae from the reproductive tract of sheep during estrus. Infect. Immun. 62,
1240 3786-3792.
- 1241 Paul, I.D., 1982. The growth of *Chlamydia* in McCoy cells treated with emetine. Med. Lab.
1242 Sci. 39, 15-32.
- 1243 Philips, H.L., Clarkson, M.J., 1995. Spontaneous change from overt to covert infection of
1244 *Chlamydia pecorum* in cycloheximide-treated mouse McCoy cells. Infect. Immun. 63,
1245 3729-3730.
- 1246 Piercy, D.W., Griffiths, P.C., Teale, C.J., 1999. Encephalitis related to *Chlamydia psittaci*

- 1247 infection in a 14-week-old calf. Vet Rec 144, 126-128.
- 1248 Pospischil, A., Thoma, R., Hilbe, M., Grest, P., Gebbers, J.O., 2002a. Abortion in woman
 1249 caused by caprine *Chlamydophila abortus* (*Chlamydia psittaci* serovar 1). Swiss Med.
 1250 Wkly. 132, 64-66.
- 1251 Pospischil, A., Thoma, R., Hilbe, M., Grest, P., Zimmermann, D., Gebbers, J.O., 2002b.
 1252 Abortion in humans by *Chlamydophila abortus* (*Chlamydia psittaci* serovar 1).
 1253 Schweiz. Arch. Tierheilk. 144, 463-466.
- 1254 Pospischil, A., Wood, R. L., 1987. Intestinal Chlamydia in pigs. Vet. Pathol. 24, 568-570.
- 1255 Prukner-Radovic, E., Horvatek, D., Gottstein, Z., Grozdanic, I.C., Mazija, H., 2005.
 1256 Epidemiological investigation of *Chlamydophila psittaci* in pigeons and free-living
 1257 birds in Croatia. Vet. Res. Commun. 29 Suppl. 1, 17-21.
- 1258 Rake, G., McKee, C.M., Shaffer, M.F., 1940. Agent of lymphogranuloma venereum in the
 1259 yolk sac of the developing chick embryo. Proc. Soc. Exp. Biol. Med. 43, 332-334.
- 1260 Ripa, K.T., Mardh, P.A., 1977. Cultivation of *Chlamydia trachomatis* in cycloheximide-
 1261 treated McCoy cells. J. Clin. Microbiol. 6, 328-331.
- 1262 Ritter, J., 1879. Über Pneumotyphus, eine Hausepidemie in Uster. Dtsch. Arch. klin. Med.,
 1263 25, 53.
- 1264 Roblin, P.M., Dumornay, W., Hammerschlag, M.R., 1992. Use of HEp-2 cells for improved
 1265 isolation and passage of *Chlamydia pneumoniae*. J. Clin. Microbiol. 30, 1968-1971.
- 1266 Rodolakis, A., Souriau, A., 1989. Variations in the virulence of strains of *Chlamydia psittaci*
 1267 for pregnant ewes. Vet. Rec. 125, 87-90.

- 1268 Rota, T.R., Nichols, R.L., 1973. *Chlamydia trachomatis* in cell culture. I. Comparison of
 1269 efficiencies of infection in several chemically defined media, at various pH and
 1270 temperature values, and after exposure to diethylaminoethyl-dextran. Appl. Microbiol.
 1271 26, 560-565.
- 1272 Sachse, K., Grossmann, E., Jager, C., Diller, R., Hotzel, H., 2003. Detection of *Chlamydia*
 1273 *suis* from clinical specimens: comparison of PCR, antigen ELISA, and culture. J.
 1274 Microbiol. Meth. 54, 233-238.
- 1275 Sachse, K., Hotzel, H., 2003. Detection and differentiation of chlamydiae by nested PCR.
 1276 Methods Mol. Biol. 216, 123-136.
- 1277 Sachse, K., Hotzel, H., Slickers, P., Ellinger, T., Ehricht, R., 2005. DNA microarray-based
 1278 detection and identification of *Chlamydia* and *Chlamydophila* spp. Mol. Cell. Probes
 1279 19, 41-50.
- 1280 Sachse, K., Laroucau, K., Hotzel, H., Schubert, E., Ehricht, R., Slickers, P., 2008. Genotyping
 1281 of *Chlamydophila psittaci* using a new DNA microarray assay based on sequence
 1282 analysis of *ompA* genes. BMC Microbiology 8, 63 (17Apr2008)
- 1283 Salti-Montesanto, V., Tsoli, E., Papavassiliou, P., Psarrou, E., Markey, B.K., Jones, G.E.,
 1284 Vretou, E., 1997. Diagnosis of ovine enzootic abortion, using a competitive ELISA
 1285 based on monoclonal antibodies against variable segments 1 and 2 of the major outer
 1286 membrane protein of *Chlamydia psittaci* serotype 1. Am. J. Vet. Res. 58, 228-235.
- 1287 Sammin, D.J., Markey, B.K., Quinn, P.J., McElroy, M.C., Bassett, H F., 2006. Comparison of
 1288 fetal and maternal inflammatory responses in the ovine placenta after experimental
 1289 infection with *Chlamydophila abortus*. J. Comp. Pathol. 135, 83-92.

- 1290 Schiller, I., Koesters, R., Weilenmann, R., Thoma, R., Kaltenboeck, B., Heitz, P., Pospischil,
1291 A., 1997. Mixed infections with porcine *Chlamydia trachomatis/pecorum* and
1292 infections with ruminant *Chlamydia psittaci* serovar 1 associated with abortions in
1293 swine. Vet. Microbiol. 58, 251-260.
- 1294 Schiller, I., Schifferli, A., Gysling, P., Pospischil, A., 2004. Growth characteristics of porcine
1295 chlamydial strains in different cell culture systems and comparison with ovine and
1296 avian chlamydial strains. Vet. J. 168, 74-80
- 1297 Schmeer, N., Krauss, H., Apel, J., Adami, M., Muller, H.P., Schneider, W., Perez-Martinez,
1298 J.A., Rieser, H., 1987. Analysis of caprine IgG1 and IgG2 subclass responses to
1299 *Chlamydia psittaci* infection and vaccination. Vet. Microbiol. 14, 125-135.
- 1300 Soldati, G., Lu, Z.H., Vaughan, L.H., Polkinghorne, A., Zimmermann, D.R., Huder, J.B.,
1301 Pospischil, A., 2004. Detection of mycobacteria and chlamydiae in granulomatous
1302 inflammation of reptiles: a retrospective study. Vet. Pathol. 41, 388–397.
- 1303 Sompolinsky, D., Richmond, S., 1974. Growth of *Chlamydia trachomatis* in McCoy cells
1304 treated with cytochalasin B. Appl. Microbiol. 28, 912-914.
- 1305 Souriau, A., Salinas, J., De Sa, C., Layachi, K., Rodolakis, A., 1994. Identification of
1306 subspecies- and serotype 1-specific epitopes on the 80- to 90-kilodalton protein region
1307 of *Chlamydia psittaci* that may be useful for diagnosis of chlamydial induced abortion.
1308 Am. J. Vet. Res. 55, 510-514.
- 1309 Spears, P., Storz, J., 1979. Biotyping of *Chlamydia psittaci* based on inclusion morphology
1310 and response to diethylaminoethyl-dextran and cycloheximide. Infect. Immun. 24,
1311 224-232.

- 1312 Spencer, W.N., Johnson, F.W., 1983. Simple transport medium for the isolation of *Chlamydia*
 1313 *psittaci* from clinical material. Vet. Rec. 113, 535-536.
- 1314 Stamp, J.T., McEwen, A.D., Watt, J.A., Nisbet, D I., 1950. Enzootic abortion in ewes:
 1315 transmission of the disease. Vet. Rec. 62, 251-254.
- 1316 Stamp, J.T., Watt, J.A., Cockburn, R.B., 1952. Enzootic abortion in ewes; complement
 1317 fixation test. J. Comp. Pathol. 62, 93-101.
- 1318 Sting, R., Hafez, H.M., 1992. Purification of *Chlamydia psittaci* antigen by affinity
 1319 chromatography on polymyxin B agarose for use in the enzyme-linked
 1320 immunosorbent assay (ELISA). Zentralbl .Bakteriol. 277, 436-445.
- 1321 Storz, J., 1971. Intestinal chlamydial infections of ruminants. In: *Chlamydia and Chlamydia-*
 1322 *Induced Diseases*. Charles C. Thomas, Springfield, Illinois, U.S.A., pp. 146-154.
- 1323 Storz, J., 1988. Overview of animal diseases induced by chlamydial infections. In: A.L.
 1324 Barron (Ed.), *Microbiology of Chlamydia*, CRC Press Inc., Boca Raton, FL, pp. 166-
 1325 192.
- 1326 Storz, J., Kaltenboeck, B., 1993a. The *Chlamydiales*. In: Woldehiwet, Z., Ristic, M. (Eds.),
 1327 *Rickettsial and Chlamydial Diseases of Domestic Animals*, Pergamon Press, Oxford,
 1328 UK, pp. 27-64.
- 1329 Storz, J., Kaltenboeck, B., 1993b. Diversity of Chlamydia-Induced Diseases. In: Woldehiwet,
 1330 Z., Ristic, M. (Eds.), *Rickettsial and Chlamydial Diseases of Domestic Animals*,
 1331 Pergamon Press, Oxford, UK, pp. 363-393.
- 1332 Szeredi, L., Bacsadi, A., 2002. Detection of *Chlamydothila (Chlamydia) abortus* and
 1333 *Toxoplasma gondii* in smears from cases of ovine and caprine abortion by the

- 1334 streptavidin-biotin method. J. Comp. Pathol. 127, 257-263.
- 1335 Szeredi, L., Schiller, I., Sydler, T., Guscetti, F., Heinen, E., Corboz, L., Eggenberger, E.,
 1336 Jones, G.E., Pospischil, A., 1996. Intestinal chlamydia in finishing pigs. Vet. Pathol.
 1337 33, 369-374.
- 1338 Tang, F.F., Chang, H.L., Huang, Y.T., Wang, K.C., 1957. Studies on the etiology of trachoma
 1339 with special reference to isolation of the virus in chick embryo. Chin. Med. J. 75, 429-
 1340 447.
- 1341 Taylor-Robinson, D., Thomas, B.J., Osborn, M.F., 1987. Evaluation of enzyme immunoassay
 1342 (Chlamydiazyme) for detecting *Chlamydia trachomatis* in genital tract specimens. J.
 1343 Clin. Pathol. 40, 194-199.
- 1344 Thejls, H., Gnärpe, J., Gnärpe, H., Larsson, P.G., Platz-Christensen, J.J., Ostergaard, L.,
 1345 Victor, A., 1994. Expanded gold standard in the diagnosis of *Chlamydia trachomatis*
 1346 in a low prevalence population: diagnostic efficacy of tissue culture, direct
 1347 immunofluorescence, enzyme immunoassay, PCR and serology. Genitourin. Med. 70,
 1348 300-303.
- 1349 Thewessen, E.A., Freundt, I., van Rijsoort-Vos, J.H., Stolz, E., Michel, M.F., Wagenvoort,
 1350 J.H., 1989. Comparison of HeLa 229 and McCoy cell cultures for detection of
 1351 *Chlamydia trachomatis* in clinical specimens. J. Clin. Microbiol. 27, 1399-1400.
- 1352 Thoma, R., Guscetti, F., Schiller, I., Schmeer, N., Corboz, L., Pospischil, A., 1997.
 1353 Chlamydiae in porcine abortion. Vet. Pathol. 34, 467-469.
- 1354 Thomas, R., Davison, H.C., Wilsmore, A.J., 1990. Use of the IDEIA ELISA to detect
 1355 *Chlamydia psittaci* (ovis) in material from aborted fetal membranes and milk from

- 1356 ewes affected by ovine enzootic abortion. Br. Vet. J. 146, 364-367.
- 1357 Tsakos, P., Siarkou, V., Guscetti, F., Chowdhury, H., Papaioannou, N., Vretou, E.,
 1358 Papadopoulos, O., 2001. Experimental infection of pregnant ewes with enteric and
 1359 abortion-source *Chlamydophila abortus*. Vet. Microbiol. 82, 285-291.
- 1360 Vanrompay, D., Butaye, P., Sayada, C., Ducatelle, R., Haesebrouck, F., 1997.
 1361 Characterization of avian *Chlamydia psittaci* strains using omp1 restriction mapping
 1362 and serovar-specific monoclonal antibodies. Res. Microbiol. 148, 327-333.
- 1363 Vanrompay, D., Ducatelle, R., Haesebrouck, F., 1992. Diagnosis of avian chlamydiosis:
 1364 specificity of the modified Gimenez staining on smears and comparison of the
 1365 sensitivity of isolation in eggs and three different cell cultures. Zentralbl.
 1366 Veterinarmed. B 39, 105-112.
- 1367 Vanrompay, D., Ducatelle, R., Haesebrouck, F., 1995. *Chlamydia psittaci* infections: a review
 1368 with emphasis on avian chlamydiosis. Vet. Microbiol. 45, 93-119.
- 1369 Vanrompay, D., Geens, T., Desplanques, A., Hoang, T.Q., De Vos, L., Van Loock, M.,
 1370 Huyck, E., Mirry, C., Cox, E., 2004. Immunoblotting, ELISA and culture evidence for
 1371 *Chlamydiaceae* in sows on 258 Belgian farms. Vet. Microbiol. 99, 59-66.
- 1372 Vanrompay, D., Harkinezhad, T., van de Walle, M., Beeckman, D., van Droogenbroeck, C.,
 1373 Verminnen, K., Leten, R., Martel, A., Cauwerts, K., 2007. *Chlamydophila psittaci*
 1374 transmission from pet birds to humans. Emerg. Infect. Dis. 13, 1108-1110.
- 1375 Vanrompay, D., van Nerom, A., Ducatelle, R., Haesebrouck, F., 1994. Evaluation of five
 1376 immunoassays for detection of *Chlamydia psittaci* in cloacal and conjunctival
 1377 specimens from turkeys. J. Clin. Microbiol. 32, 1470-1474.

- 1378 Verminnen, K., Duquenne, B., De Keukeleire, D., Duim, B., Pannekoek, Y., Braeckman, L.,
 1379 Vanrompay, D., 2008. Evaluation of a *Chlamydophila psittaci* diagnostic platform for
 1380 zoonotic risk assessment. J. Clin. Microbiol. 46, 281-285.
- 1381 Verminnen, K., Van Loock, M., Hafez, H.M., Ducatelle, R., Haesebrouck, F., Vanrompay, D.,
 1382 2006. Evaluation of a recombinant enzyme-linked immunosorbent assay for detecting
 1383 *Chlamydophila psittaci* antibodies in turkey sera. Vet. Res. 37, 623-632.
- 1384 Volopich, S., Benetka, V., Schwendenwein, I., Mostl, K., Sommerfeld-Stur, I., Nell, B., 2005.
 1385 Cytologic findings, and feline herpesvirus DNA and *Chlamydophila felis* antigen
 1386 detection rates in normal cats and cats with conjunctival and corneal lesions. Vet.
 1387 Ophthalmol. 8, 25-32.
- 1388 von Bomhard, W., Polkinghorne, A., Lu, Z.H., Vaughan, L., Vogtlin, A., Zimmermann, D.R.,
 1389 Spiess, B., Pospischil, A., 2003. Detection of novel chlamydiae in cats with ocular
 1390 disease. Am. J. Vet. Res. 64, 1421-1428.
- 1391 von Bomhard, W., Pospischil, A., Richter, M., Kaps, S., Polkinghorne, A., Zimmermann,
 1392 D.,R. 2002. Amoebic infections in cats with ocular disease. Vet. Rec. 150, 556.
- 1393 Vretou, E., Giannikopoulou, P., Longbottom, D., Psarrou, E., 2003. Antigenic organization of
 1394 the N-terminal part of the polymorphic outer membrane proteins 90, 91A, and 91B of
 1395 *Chlamydophila abortus*. Infect. Immun. 71, 3240-3250.
- 1396 Vretou, E., Loutrari, H., Mariani, L., Costelidou, K., Eliades, P., Conidou, G., Karamanou, S.,
 1397 Mangana, O., Siarkou, V., Papadopoulos, O., 1996. Diversity among abortion strains
 1398 of *Chlamydia psittaci* demonstrated by inclusion morphology, polypeptide profiles
 1399 and monoclonal antibodies. Vet. Microbiol. 51, 275-289.

- 1400 Vretou, E., Psarrou, E., Kaisar, M., Vlisidou, I., Salti-Montesanto, V., Longbottom, D., 2001.
 1401 Identification of protective epitopes by sequencing of the major outer membrane
 1402 protein gene of a variant strain of *Chlamydia psittaci* serotype 1 (*Chlamydophila*
 1403 *abortus*). Infect. Immun. 69, 607-612.
- 1404 Vretou, E., Radouani, F., Psarrou, E., Kritikos, I., Xylouri, E., Mangana, O., 2007. Evaluation
 1405 of two commercial assays for the detection of *Chlamydophila abortus* antibodies. Vet.
 1406 Microbiol. 123, 153-161.
- 1407 Wang, S.P., Grayston, J.T., 1970. Immunologic relationship between genital TRIC,
 1408 lymphogranuloma venereum, and related organisms in a new microtiter indirect
 1409 immunofluorescence test. Am. J. Ophthalmol. 70, 367-374.
- 1410 Wentworth, B.B., Alexander, E.R., 1974. Isolation of *Chlamydia trachomatis* by use of 5-
 1411 iodo-2-deoxyuridine-treated cells. Appl. Microbiol. 27, 912-916.
- 1412 Wills, J.M., Gruffydd-Jones, T.J., Richmond, S.J., Gaskell, R.M., Bourne, F.J., 1987. Effect of
 1413 vaccination on feline *Chlamydia psittaci* infection. Infect. Immun. 55, 2653-2657.
- 1414 Wills, P.J., Johnson, L., Thompson, R.G., 1984. Isolation of *Chlamydia* using McCoy cells
 1415 and buffalo green monkey cells. J. Clin. Pathol. 37, 120-121.
- 1416 Wilshire, A.J., Davidson, I., 1991. 'Clearview' rapid test compared with other methods to
 1417 diagnose chlamydial infection. Vet. Rec. 128, 503-504.
- 1418 Wilson, K., Livingstone, M., Longbottom, D., 2008. Comparative evaluation of eight
 1419 serological assays for diagnosing *Chlamydophila abortus* infection in sheep. Vet.
 1420 Microbiol. (this issue)
- 1421 Wittenbrink, M.M., Schoon, H.A., Bisping, W., Binder, A., 1993. Infection of the bovine

- 1422 female genital tract with *Chlamydia psittaci* as a possible cause of infertility. *Reprod.*
1423 *Dom. Anim.* 28, 129-136.
- 1424 Wittwer, C.T., Herrmann, M.G., Moss, A.A., Rasmussen, R.P., 1997. Continuous
1425 fluorescence monitoring of rapid cycle DNA amplification. *Biotechniques* 22, 130-
1426 131, 134-138.
- 1427 Wood, M.M., Timms, P., 1992. Comparison of nine antigen detection kits for diagnosis of
1428 urogenital infections due to *Chlamydia psittaci* in koalas. *J. Clin. Microbiol.* 30, 3200-
1429 3205.
- 1430 Yoshida, H., Kishi, Y., Shiga, S., Hagiwara, T., 1998. Differentiation of *Chlamydia* species
1431 by combined use of polymerase chain reaction and restriction endonuclease analysis.
1432 *Microbiol. Immunol.* 42, 411-414.
- 1433 Zahn, I., Szeredi, L., Schiller, I., Straumann Kunz, U., Burgi, E., Guscetti, F., Heinen, E.,
1434 Corboz, L., Sydler, T., Pospischil, A., 1995. [Immunohistochemical determination of
1435 *Chlamydia psittaci/pecorum* and *C. trachomatis* in the piglet gut]. *Zentralbl.*
1436 *Veterinärmed. B* 42, 266-276.
- 1437
- 1438

Figure captions

Figure 1. Examples of staining techniques used to diagnose *C. abortus* induced abortion in sheep. Similar techniques can be applied to other chlamydial species and tissue samples. **A.** Smear prepared from ovine placental membrane taken following abortion. The chlamydial EBs, which are stained pinkish-red, appear singly and in clumps, amongst the green cellular background material. (The area represented in the box with the solid outline is an enlarged view of that with the dashed outline). **B.** Formalin-fixed, paraffin-embedded tissue section prepared from the placenta of an aborted foetus and stained by the enhanced immunoperoxidase method using anti-LPS mAb 13/5 (Buxton et al., 1996). Image shows a clear demarcation between the basement membrane, where many chlamydial inclusions (CI) are observed and stained brown, and the damaged, sloughing chorionic epithelial (CE) cell layer, where released chlamydial antigen appears as more diffuse brown staining. A clear inflammatory cell infiltrate (I) and arteritis (A) can also be observed. (This image appears courtesy of Dr David Buxton, Moredun Research Institute, Edinburgh, UK). **C.** Giemsa staining of *C. abortus*-infected McCoy cell monolayers following recovery from infected placental material. **D.** Detection of *C. abortus*, recovered from placental tissue and isolated in McCoy cells, by immunofluorescence using anti-MOMP mAb 4/11 (McCafferty et al., 1995).

Figure 2. Original results of the rELISA (rLPS ELISA). Group 1, serum numbers 1-67 from OEA-infected flocks, 29 CFT positive; Group 2, sera 68-133 from OEA-free flocks, 1 CFT positive; Group 3, sera 134-192 from 4 flocks with enteric *C. abortus* infection, 0 CFT positive; Group 4, sera 193-210 from a flock with enteric *C. pecorum* infection, 0 CFT positive; Group 5, sera 211-257 from animals with unknown OEA status, 20 CFT positive; Group 6, sera 259-289 from a flock infected with arthritogenic *C. pecorum*, 7 CFT positive;

Group 7, sera 290-297 from SPF lambs infected with various *C. pecorum* subtypes, 4 CFT positive. The cut-off of the test is shown as a horizontal line.

Figure 3. Seroreactivity of synthetic MOMP VS1-VS4 peptides. Fifteen decapeptides overlapping by two amino acids spanning the four variable domains of the MOMP were tested with sera from 7 *C. abortus* –infected pregnant ewes [1], 5 SPF lambs infected with enteric *C. pecorum* and [2] and 5 SPF lambs infected with arthritogenic *C. pecorum* [3]. VS1: GTAAANYKTPTDRP; VS2: KGSSIAADQL VS3: TAFPLPLTAGTDQATD; VS4: EATALDTSNKFA.

Figure 4 Illustration of multiple DNA tests being conducted on a clinical sample. Faeces from a psittacine bird (ID: 07G764, marked on agarose gels with an asterisk) was subjected to DNA extraction using the High Pure PCR Template Preparation Kit (Roche). One µl of the extract was examined by the following tests: **A.** PCR targeting 16-23S intergenic spacer region of *Chlamydiaceae* according to Everett and Andersen (1999), amplicon size approx. 600 bp. **B.** PCR targeting the *ompA* gene of *Chlamydiaceae* according to Kaltenboeck et al. (1997b), amplicon size 590 bp. **C.** Reamplification of the product from **B** using *Chlamydia psittaci*-specific primers in a nested PCR according to Sachse and Hotzel (2003), amplicon size 395 bp. **D.** Real-time PCR targeting the *ompA* gene of *C. psittaci* (duplicate run) according to Pantchev et al. (2008), Ct=27.9. **E.** AT microarray test for identification of chlamydial species targeting the 23S rRNA gene according to Sachse et al. (2005); left: array image, right: barplot showing specific hybridisation signals for genus *Chlamydophila* and species *C. psittaci*. **F.** AT microarray test for *ompA* genotyping of *C. psittaci* according to Sachse et al. (2008); left: array image, right: barplot showing specific hybridisation signals of genotype A.

Table 1

Conventional PCR methods for chlamydiae

| Specificity | Primer and sequence (5'- 3') | Amplicon size (bp) | Target gene | Reference (Remarks) |
|----------------------|---|--------------------|----------------------|---|
| <i>Chlamydiaceae</i> | 16S-FCh: ACGGAATAATGACTTCGG 16S-RCh: TACCTGGTACGCTCAATT | 436 | 16S rRNA | Messmer et al., 1997 (nested PCR system with inner primers for <i>C. trachomatis</i> , <i>C. pneumoniae</i> and <i>C. psittaci</i>) * |
| <i>Chlamydiaceae</i> | 16SF2: CCGCCCGTCACATCATGG 23R: TACTAAGATGTTTCAGTTC | 585-600 | 16-23S spacer region | Everett and Andersen, 1999 (identification of species via REA) |
| <i>Chlamydiales</i> | cIGS-1f: CAAGGTGAGGCTGATGAC cIGS-2r: TCGCCTKTCAATGCCAAG | 352 | 16-23S spacer region | Borel et al., 2006a |
| <i>Chlamydiaceae</i> | 191CHOMP: GCI YTI TGG GAR TGY GGI TGY GCI AC CHOMP371: TTA GAA ICK GAA TTG IGC RTT IAY GTG IGC IGC | 576-597 | <i>ompA</i> | Kaltenboeck et al., 1997b; Sachse and Hotzel, 2003 (nested PCR system with inner primers for <i>C. psittaci</i> , <i>C. pecorum</i> , <i>C. trachomatis</i> and <i>C. pneumoniae</i>) * |
| <i>Chlamydiaceae</i> | cIGS-1f: CAAGGTGAGGCTGATGAC cIGS-1r: AGTGGTCTCCCCAGATTC | 750 | 16-23S spacer region | Lutz-Wohlgroth et al., 2006 |
| <i>Chlamydiaceae</i> | Ch1: ATGTCCAAACTCATCAGACGAG Ch2: CCTTCTTTAAGAGGTTTTACC | 603 | <i>omp2</i> | Hartley et al., 2001 (identification of species via REA or enzyme-linked oligonucleotide assay) |
| <i>Chlamydiaceae</i> | 16SF2: CCGCCCGTCACATCATG 23R: TACTAAGATGTTTCAGTTC | 585-600 | 16+23S rRNA | Everett and Andersen, 1999 (identification of all chlamydial species via REA) |
| <i>Chlamydiales</i> | 23SAPF2: GAACCTGAAACCA(AG)TAGC | 92 | 23S | Soldati et al., 2004 |

23APR: CTGGCTCATCATGCAAAAGG

Cp. felis 23APFeIF: CGGCGAGCGAAAGGGGATT 369 23S von Bomhard et al., 2003

23APFeIR: GGCACGCCGTCAACCATTG

| | | | | |
|---------------------------------|---|---------|----------------------------|-----------------------|
| <i>Chlamydia psittaci</i> * | CPS 100: CCCAAGGTGAGGCTGATGAC CPS 101: CAAACCGTCCTAAGACAGTTA | 111 | 16-23S spacer region | Madico et al., 2000** |
| <i>Chlamydia trachomatis</i> | CTR 70: GGCGTATTTGGGCATCCGAGTAACG CTR 71: TCAAATCCAGCGGGTATTAACCGCCT | 315 | 16S rRNA | Madico et al., 2000** |
| <i>Chlamydophila pneumoniae</i> | CPN 90: GGTCTCAACCCCATCCGTGTCGG CPN 91: TGCGGAAAGCTGTATTTCTACAGTT | 197 | 16S rRNA | Madico et al., 2000** |
| <i>Chlamydia psittaci</i> * | CpsiA CpsiB | ca. 300 | <i>pmp</i> genes | Laroucau et al., 2007 |

* based on four-species classification of the family *Chlamydiaceae*

** also used as multiplex assay for *Chlamydia trachomatis*, *C. pneumoniae* and *Chlamydia psittaci*

Table 2

Real-time PCR methods for chlamydiae

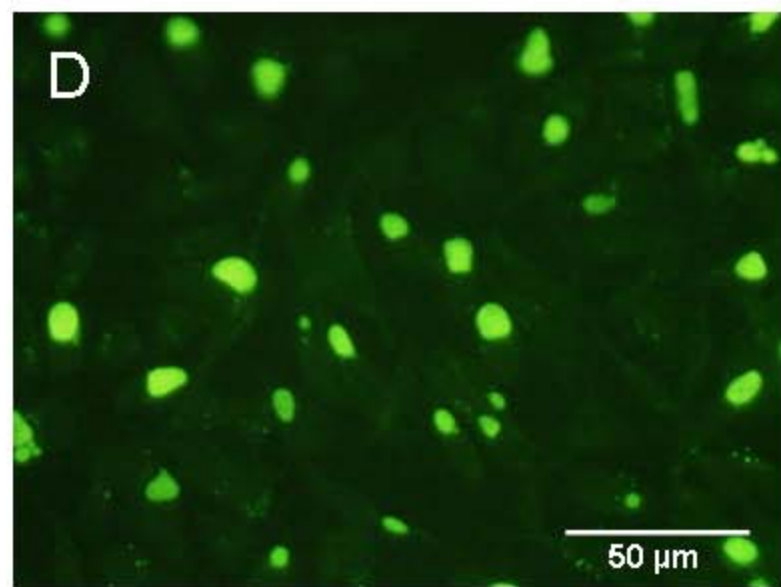
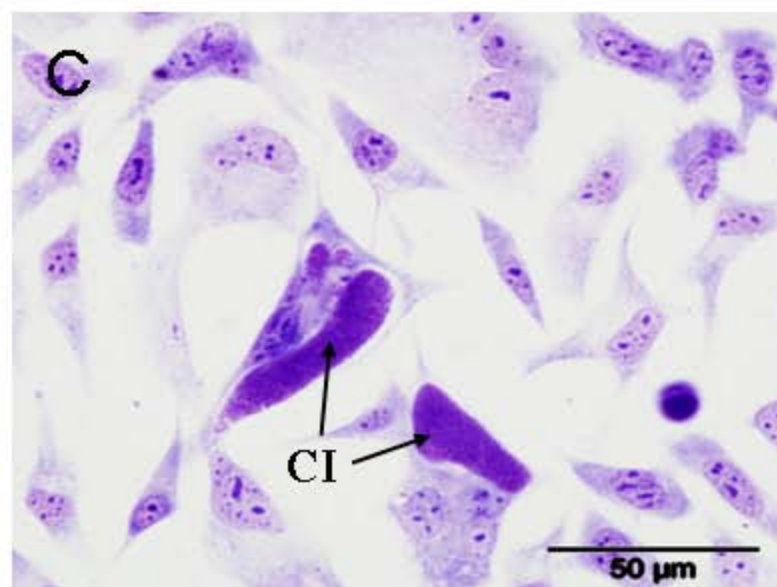
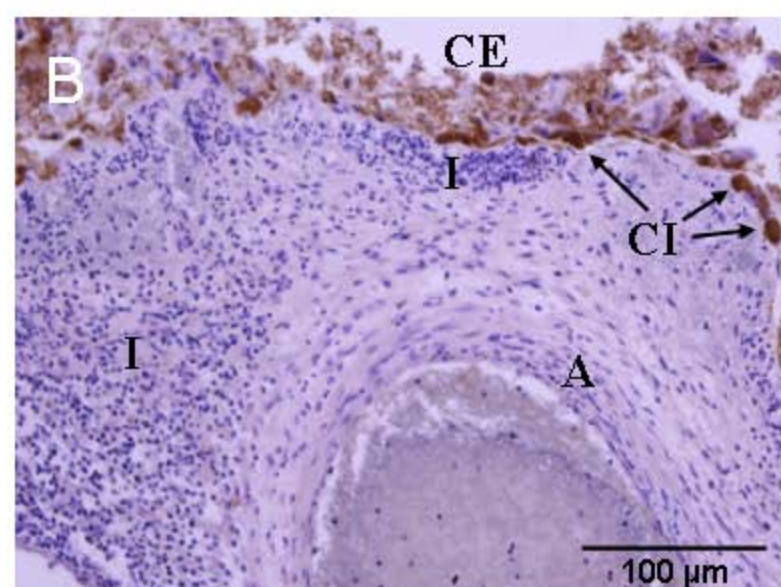
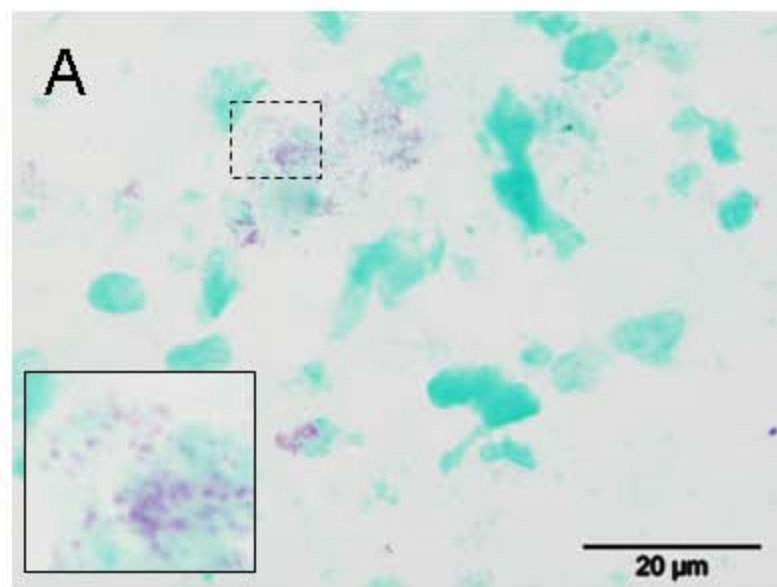
| Specificity | Primers, probe and sequences (5'- 3') | Amplicon size (bp) | Target gene | Reference |
|---|--|--------------------|-------------|--------------------------|
| <i>Chlamydiaceae</i> | TQF: GAAAAGAACCCTTGTTAAGGGAG TQR: CTAACTCCCTGGCTCATCATG FAM-CAAAAGGCACGCCGTCAAC-TAMRA | 129-132 | 23S rRNA | Everett et al., 1999b |
| <i>Chlamydiaceae</i> | Ch23S-F: CTGAAACCAGTAGCTTATAAG CGGT Ch23S-R: ACCTCGCCGTTTAACTTAACTCC Ch23S-p: FAM-CTCATCATGCAAAAGGCACGCCG-TAMRA | 111 | 23S rRNA | Ehricht et al., 2006 |
| <i>Chlamydiaceae</i> | CHL23SUP: GGGGTTGTAGGGTYGAGRAIAWRRGATC CHL23DN: GAGAGTGGTCTCCCCAGATTCTACTA CHL23LCR: LCR640-CCTGAGTAGRRCTAGACACGTGAAAC-Phosphate | 168 | 23S rRNA | DeGraves et al., 2003b |
| <i>Chlamydia psittaci</i> *, <i>C. pneumoniae</i> , <i>C. pecorum</i> | CP23FLU: ACGAAARAACAARAGACKCTAWTCGAT-6-FAM | | | |
| <i>C. pneumoniae</i> , <i>C. pecorum</i> | CPN23FLU: ACGAAAAAACAAGACGCTAATCGAT-6-FAM | | | |
| <i>Chlamydia trachomatis</i> | CTR23FLU: ACGAAAGGAGAKMAAGACYGACCTCAAC-6-FAM | | | |
| <i>C. psittaci</i> | CpPsSSfor: TTATTAAGAGCTATTGGTGGATGCC CpPsSSrev: AACGTATAATGGTAGATGATTAATCTACCG | 151 | <i>ompA</i> | Geens et al., 2005b** |
| <i>C. psittaci</i> | CppsOMP1-F: CACTATGTGGGAAGGTGCTTCA CppsOMP1-R: CTGCGCGGATGCTAATGG | 76 | <i>ompA</i> | Pantchev et al., 2008*** |
| | CppsOMP1-S: FAM-CGCTACTTGGTGTGAC-TAMRA | | | (MGB™ probe) |

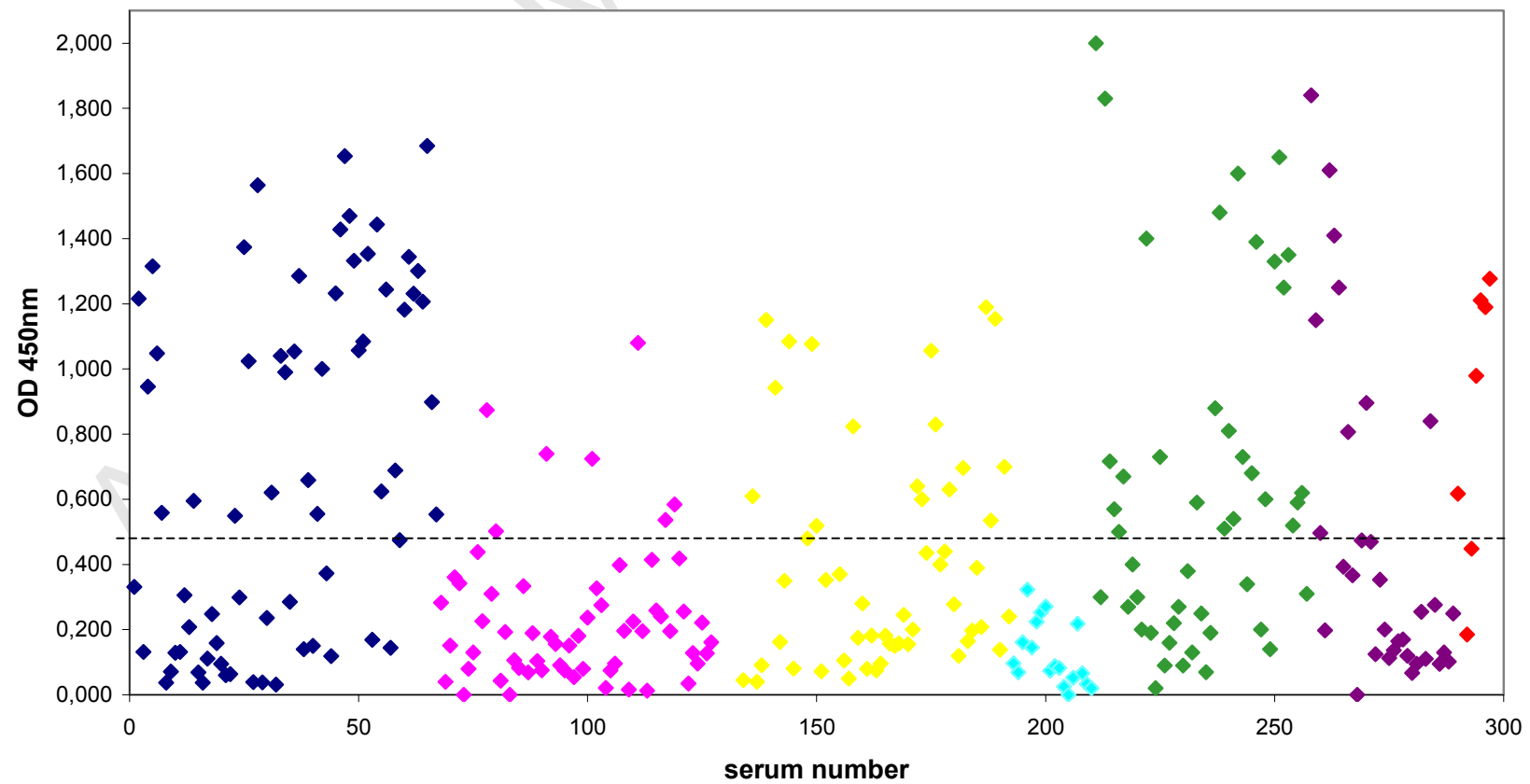
| | | | | |
|--------------------|--|----|-------------|---|
| <i>C. psittaci</i> | F1-incA-Cpsi: GCCATCATGCTTGTTTCGTTT R1-incA-Cpsi: CGGCGTGCCACTTGAGA Cpsi-incA-NM: FAM-TCATTGTCATTATGGTGATTCAGGA-NFQ | 74 | <i>incA</i> | Menard et al., 2006 (MGB™ probe) |
| <i>C. abortus</i> | CpaOMP1-F: GCAACTGACACTAAGTCGGCTACA CpaOMP1-R: ACAAGCATGTTCAATCGATAAGAGA CpaOMP1-S: FAM-TAAATACCACGAATGGCAAGT-TGGTTTAGCG-TAMRA | 82 | <i>ompA</i> | Pantchev et al., 2008*** |

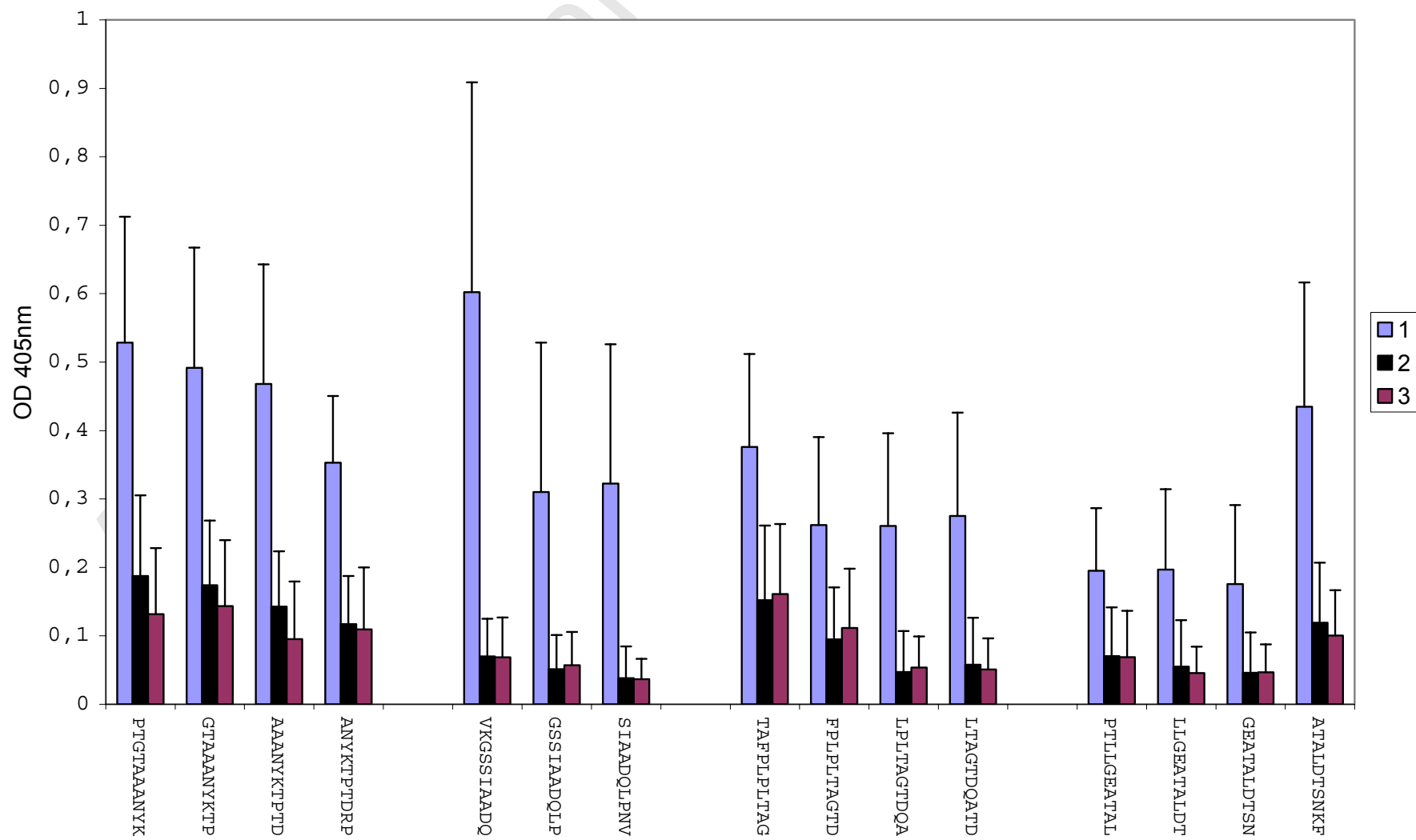
* based on four-species classification of the family *Chlamydiaceae*

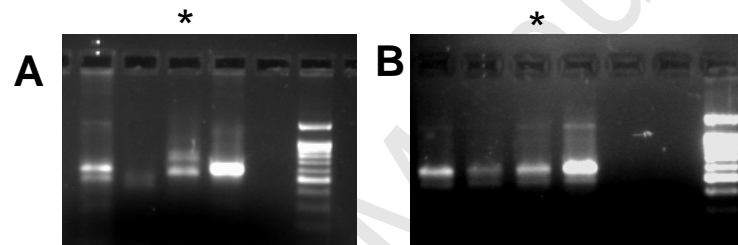
** includes primer sets for genotype-specific amplification

*** includes internal amplification control consisting of target DNA from the EGFP gene cloned into a plasmid, specific primers and a TaqMan probe (Hoffmann et al., 2006)

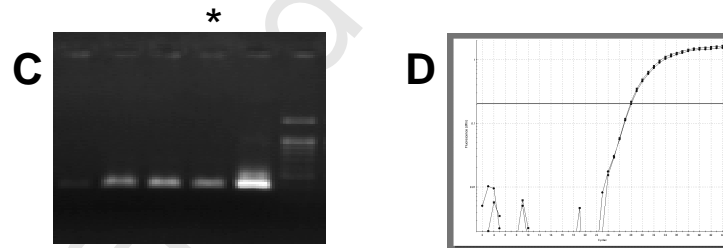




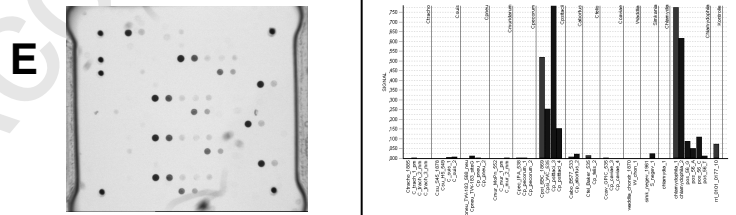




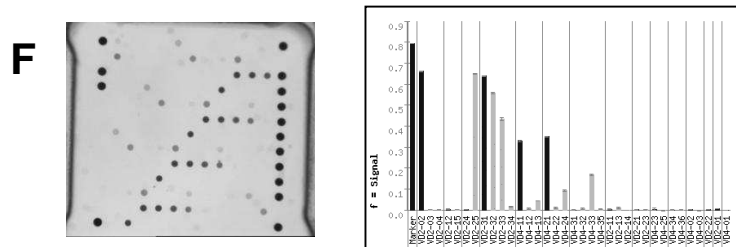
Chlamydiaceae



C. psittaci



Chlamydophila psittaci



Genotype A