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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 \textit{Chlamydia} and \textit{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: \textit{Chlamydia}; \textit{Chlamydophila}; antigen detection; antibody detection; DNA amplification tests; specificity; sensitivity

Abbreviations:

\textit{C.}, \textit{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-venereal-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
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 (≤4 µm) 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; Thewessen 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-ido-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, polyarthritis, 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 chlamydioidosis 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 chlamydioidosis 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 (Dovc 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 αKdo(2→8)αKdo(2→4)αKdo, *C. psittaci* contains a structurally different trisaccharide with the structure αKdo(2→4)αKdo(2→4)αKdo and the branched tetrasaccharide αKdo(2→8)[αKdo(2→4)]αKdo(2→4)αKdo (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 cELISA 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-Radovcic 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 POMPs

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 *Chlamydia* 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 *λgt11* 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 POMPs. 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 (McCabe 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 \textit{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 \textit{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 \textit{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 \textit{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 \textit{Chlamydia trachomatis}, \textit{Chlamydia pneumoniae} and \textit{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 *omp* A 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 ArrayTube™ (AT) system (Clondiag 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 (Ehrich 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 *omp*A 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 *omp*A 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 *omp*A 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 chlamydia 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 Chlamydophila 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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This review has been written by members of the European COST Action 855 "Animal Chlamydioses and the Zoonotic Implications", who would like to acknowledge the support of COST.

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Thomas, R., Davison, H.C., Wilsmore, A.J., 1990. Use of the IDEIA ELISA to detect Chlamydia psittaci (ovis) in material from aborted fetal membranes and milk from...


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: GTAAANYKPTDRP; 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>
<thead>
<tr>
<th><strong>Conventional PCR methods for chlamydiaceae</strong></th>
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<tr>
<td><strong>Specificity</strong></td>
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<td><strong>Chlamydiaceae</strong></td>
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23APR: CTGGCTCATCATGCAAAAGG

**Cp. felis**

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<td>23APFelF</td>
<td>CGCGCGACGAAAGGGGATT</td>
<td>369</td>
<td>23S</td>
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<td>23APFelR</td>
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</table>

23APR: CTGGCTCATCATGCAAAAGG

**Chlamydia psittaci**

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<td>CPS 100</td>
<td>CCCAAGGTGAGGCTGATGAC</td>
<td>111</td>
<td>16-23S spacer region</td>
<td>Madico et al., 2000**</td>
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<tr>
<td>CPS 101</td>
<td>CAAACCGTCTAAGACAGTTA</td>
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<td></td>
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<tr>
<td>CPS 100</td>
<td>CCCAAGGTGAGGCTGATGAC</td>
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<tr>
<td>CPS 101</td>
<td>CAAACCGTCTAAGACAGTTA</td>
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**Chlamydia trachomatis**

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<tbody>
<tr>
<td>CTR 70</td>
<td>GGCCTATTTGGGCGATCGGAACG</td>
<td>315</td>
<td>16S rRNA</td>
<td>Madico et al., 2000**</td>
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<td>CTR 71</td>
<td>TCAATCCAGCGGTAATTAACGCCT</td>
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<td>CTR 71</td>
<td>TCAATCCAGCGGTAATTAACGCCT</td>
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**Chlamydophila pneumoniae**

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<tbody>
<tr>
<td>CPN 90</td>
<td>GGTCTCAACCCCCATCCGTGTCG</td>
<td>197</td>
<td>16S rRNA</td>
<td>Madico et al., 2000**</td>
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<td>CPN 91</td>
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<td>CPN 90</td>
<td>GGTCTCAACCCCCATCCGTGTCG</td>
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<td></td>
</tr>
<tr>
<td>CPN 91</td>
<td>TGCGGAAAGCTGTATTTCTACAGTT</td>
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**Chlamydia psittaci**

<table>
<thead>
<tr>
<th>Genes</th>
<th>Length</th>
<th>Function</th>
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<tr>
<td>CpsiA</td>
<td>ca. 300</td>
<td>pmp genes</td>
<td>Laroucau et al., 2007</td>
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<tr>
<td>CpsiB</td>
<td></td>
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* based on four-species classification of the family Chlamydiaceae
** also used as multiplex assay for Chlamydia trachomatis, C. pneumoniae and Chlamydia psittaci
<table>
<thead>
<tr>
<th>Specificity</th>
<th>Primers, probe and sequences (5’- 3’)</th>
<th>Amplicon size (bp)</th>
<th>Target gene</th>
<th>Reference</th>
</tr>
</thead>
</table>
| *Chlamydiaceae*               | TQF: GAAAAGAACCCTTTGTTAAGGGAG 129-132 23S rRNA  
TQR: CTTAACTCCCTGGGTCATCATG  
FAM-CAAAAAGGCACGCGGCTCAAC-TAMRA |                    |             | Everett et al., 1999b  |
|                               | Ch23S-F: CTGAAACCAGTAGCTTATAAG CGGT 111 23S rRNA  
Ch23S-R: ACCTCGCCGTTAACTAACC  
Ch23S-p: FAM-CTCATCATGCAAAGGCACGCGG-TAMRA |                    |             | Ehrlich et al., 2006    |
|                               | CHL23SUP: GGGGTGTAGGGTYGAGRAIAWRGRATC 168 23S rRNA  
CHL23DN: GAGAGTGGTCTCCCCAGATTCARACTA  
CHL23LRCR: LCRed640-CTTGAGTCTAGACAGTGAAAC-Phosphate |                    |             | DeGraves et al., 2003b  |
| *Chlamydia psittaci*, *C. pneumoniae*, *C. pecorum* | CP23FLU: ACGAAAAAACARAGACKCTAWTCGAT-6-FAM  
CPN23FLU: ACGAAAAAACARAGACKCTAATCGAT-6-FAM  
CTR23FLU: ACGAAAAAACARAGACKCTAATCGAT-6-FAM |                    |             | Geens et al., 2005b**   |
| *C. pneumoniae*, *C. pecorum* | CppsSSfor: TTATTAAGAGCTATTGGTGATTGCC 151 ompA  
CppsSSrev: AACGTATAATGGTAGATGATTAATCTACCG |                    |             | Pantchev et al., 2008*** |
| *Chlamydia trachomatis*       | CppsOMP1-F: CACTATGTTGGGAAGGTGCTTCA 76 ompA  
CppsOMP1-R: CTGCGCGGTAGGATGGTAATGG |                    |             |                          |
|                               | CppsOMP1-S: FAM-CGCTACTTGGTGAC-TAMRA (MGB™ probe) |                    |             |                          |
### C. psittaci

- **F1-incA-Cpsi**: GCCATCATGCTTGGTTTCGTT
- **incA Menard et al., 2006**
- **R1-incA-Cpsi**: CGGCGTGCCACTTGAGA
- **Cpsi-incA-NM**: FAM-TCATTGTCAATGATTGAGATCAGGATCAGGAG-NFQ (MGB™ probe)

### C. abortus

- **CpaOMP1-F**: GCAACTGACACTAAGTCGGCTACA
- **ompA Pantchev et al., 2008***
- **CpaOMP1-R**: ACAAGCATGTCTTCAATCGATAAGAGA
- **CpaOMP1-S**: FAM-TAAATACCACGAATGGCAAGT TGGTTTAGCG-TAMRA

* 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)
Figure 1: Molecular detection methods for Chlamydophila psittaci and genotyping.

A. Gel electrophoresis showing DNA bands for Chlamydiaceae. * indicates a specific band.
B. Gel electrophoresis showing DNA bands for C. psittaci. * indicates a specific band.
C. Gel electrophoresis showing DNA bands for Chlamydophila psittaci. * indicates a specific band.
D. Sensitivity analysis for Chlamydophila psittaci detection.
E. Hybridization assay for Chlamydophila psittaci genotyping.
F. Immunoblot for identification of Chlamydophila psittaci genotypes.

* indicates a specific band or marker for identification.