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1     **Recent developments in the laboratory diagnosis of chlamydial infections**

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16

17

17 **Abstract**

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

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

31

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

34

35 **Abbreviations:**

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

## 42 1. Introduction

### 43 1.1 Historic overview and taxonomic classification

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

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

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

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

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

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

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

95

## 96 1.2 Importance of individual chlamydial pathogens

97 Most members of the family *Chlamydiaceae* represent agents of important animal and/or  
98 human diseases (Storz and Kaltenboeck, 1993b). Avian strains of *C. psittaci* cause  
99 psittacosis/ornithosis, a systemic disease of acute, protracted, chronic or subclinical  
100 manifestation occurring in psittacine birds, domestic poultry and wild fowl (Vanrompay et al.,  
101 1995). The infection is transmissible to humans, the symptoms being largely non-specific and  
102 influenza-like, but severe pneumonia, endocarditis and encephalitis are also known (Crosse,  
103 1990).

104 Enzootic abortion in sheep and goats, also called Ovine Enzootic Abortion (OEA) or  
105 Enzootic Abortion of Ewes (EAE), is caused by *C. abortus*. The disease has a major  
106 economic impact as it represents the most important cause of lamb loss in sheep in parts of  
107 Europe, North America and Africa (Longbottom and Coulter, 2003). This serious and  
108 potentially life-threatening zoonosis can also affect pregnant women after contact with  
109 lambing ewes, leading to severe febrile illness in pregnancy and loss of the foetus (Buxton,  
110 1986; Kampinga et al., 2000; Pospischil et al., 2002a).

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

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

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

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

126

### 127 *1.3 Detection of chlamydiae*

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

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

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

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

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

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



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

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

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

174

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

178

## 179 **2. Antigen detection**

### 180 *2.1. Introductory remarks*

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

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

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

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

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

223

## 224 2.2. Smears

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

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

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

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

256

### 257 2.3. Pathological specimens

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

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

266 Immunohistochemical staining procedures that utilise mAbs directed against chlamydial  
267 surface antigens, such as LPS or MOMP, are more sensitive and produce more striking results  
268 in comparison to histochemical staining. A direct immunoperoxidase method that has been  
269 developed for detecting *C. abortus* in formalin-fixed tissues has been shown to be a very rapid  
270 and sensitive test (Finlayson et al., 1985; Kunz et al., 1991; Zahn et al., 1995), however this  
271 initially requires the direct conjugation of the antibody with the enzyme horse radish  
272 peroxidase. An alternative approach, as described previously, is to use an indirect method that  
273 involves the use of a fluorescein-conjugated anti-mouse antiserum in combination with the  
274 mAb. Enhanced labelling can be achieved using the more complex streptavidin-biotin method  
275 using formalin-fixed, paraffin-embedded tissues (**Figure 1B**) (Szeredi et al., 1996; Buxton et  
276 al., 2002). This approach of immunoperoxidase staining of formalin-fixed, paraffin-embedded  
277 or cryostat sections of various tissues is commonly used for detecting chlamydial species for  
278 diagnostic purposes, as well as for epidemiological and pathogenesis studies (Juvonen et al.,  
279 1997; Tsakos et al., 2001; Buxton et al., 2002; Hotzel et al., 2004; Navarro et al., 2004;  
280 Sammin et al., 2006; Borel et al., 2006a, 2006b, 2007; Thoma et al., 1997).

281

#### 282 2.4. Immunoassays

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

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

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

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

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

326

### 327 2.5. Isolation

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

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

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

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

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



364 in confluent cover-slip cell culture monolayers gradually superseded egg culture from the  
365 1970s onwards. Several cell lines have been used to grow chlamydiae, although successful  
366 propagation is dependent on the cell line and the chlamydial species being tested. Staining of  
367 cell monolayers reveals characteristic inclusions and morphologies for the different  
368 chlamydial species, as well as their ability to infect different cell types. *Chlamydia*  
369 *trachomatis* infects many cell types but McCoy, Buffalo Green Monkey Kidney (BGMK) and  
370 HeLa 229 have been shown to be the most susceptible to infection (Croy et al., 1975; Wills et  
371 al., 1984; Barnes, 1989; Thewessen et al., 1989; Johnston and Siegel, 1992). On the other  
372 hand, for *C. pneumoniae*, which is difficult to isolate and propagate in cell culture, HL and  
373 HEp-2 cells are the preferred option (Cles and Stamm, 1990; Roblin et al., 1992). Isolation of  
374 *Chlamydia psittaci* serotype 1 (now *C. abortus*) was first reported in McCoy cells (Hobson et  
375 al., 1977; Johnson et al., 1983), the most commonly used cell type, although this species  
376 grows well in many other cell types, such as BGMK, baby hamster kidney (BHK) and the  
377 mouse fibroblast L cells. Most cell types are susceptible to *C. psittaci* infection, but the direct  
378 inoculation into cultures of BGMK, African green monkey kidney (Vero), McCoy and L cells  
379 are commonly used (Vanrompay et al., 1992). Little information is available for the remaining  
380 chlamydial species, but human colonic adenocarcinoma cells (CaCo) are considered to be the  
381 most appropriate for isolation of the problematic *Chlamydia suis* and *C. pecorum* strains  
382 (Schiller et al., 2004), whilst *C. felis* appears to grow well in McCoy cells (Wills et al., 1987).  
383 While many avian *C. psittaci*, ovine *C. abortus* and porcine *Chlamydia suis* strains can be  
384 propagated in tissue culture with relative ease, others are more difficult to grow, e.g. *C.*  
385 *psittaci* strains isolated from cattle and pigs. For *C. pecorum*, some strains, particularly those  
386 originating from the intestinal tract, are difficult to isolate in culture, either growing initially  
387 and then dying out on serial passage or not appearing to grow initially and taking several  
388 passages before the organism can be detected (Philips and Clarkson, 1995).

389 Infection of chlamydiae in cell culture can be enhanced by centrifugation and/or by  
390 chemical treatment of cultured cells, before or during infection, using for example  
391 cycloheximide (1 µg/ml), cytochalasin B (2 µg/ml), emetine (1 µg/ml), diethylaminoethyl-  
392 dextran (20 µg/ml) or 5-iodo-2-deoxyuridine (80 µg/ml) (Alexander, 1968; Kuo et al., 1972;  
393 Rota and Nichols, 1973; Sompolinsky and Richmond, 1974; Wentworth and Alexander, 1974;  
394 Ripa and Mardh, 1977; Bevan et al., 1978; Spears and Storz, 1979; Paul, 1982; Schiller et al.,  
395 2004). Cell culture media components may also influence chlamydial growth, particularly for  
396 species that are more difficult to propagate or when the infecting inoculum only contains low  
397 numbers of organisms. For example, the growth of *Chlamydia suis* in culture has been shown  
398 to be improved when using Iscove's modified Dulbecco's medium (IMDM) compared to  
399 Eagle's minimal essential medium (EMEM), yet they had no effect on the growth of selected  
400 *C. abortus*, *C. psittaci* and *C. pecorum* strains (Schiller et al., 2004).

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

406

### 407 **3. Antibody detection**

#### 408 *3.1. Introductory remarks*

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

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

422

### 423 3.1.1. Chlamydial infection in sheep and goats

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

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

439 *pecorum* infection, other than the cumbersome isolation of the organism, have made the  
440 evaluation of novel assays for the improvement of the serological diagnosis of OEA difficult.  
441 Nevertheless, several laboratory-based assays have been developed including indirect ELISAs  
442 using whole EB or extracts thereof (Cevenini et al., 1989; Markey et al., 1993; Anderson et  
443 al., 1995). Semi-quantitative methods have been proposed to distinguish between infections  
444 due to *C. abortus* and to *C. pecorum* such as an indirect immunofluorescence assay and  
445 immunoblotting (Markey et al., 1993; Jones et al., 1997). ELISAs with purified LPS have  
446 been published (Sting and Hafez, 1992; Jones et al., 1997). With the introduction of molecular  
447 techniques, more defined assays were developed based on recombinant LPS antigen (Griffiths  
448 et al., 1996), as well as on MOMP (Kaltenboeck et al., 1997a; Salti-Montesanto et al., 1997;  
449 Gut-Zangger et al., 1999; Borel et al., 2002; Hoelzle et al., 2004) and 80-90kDa POMP  
450 (Buendia et al., 2001; Longbottom et al., 2001, 2002). A major step that has facilitated the  
451 evaluation of novel assays was the implementation of large panels of documented reference  
452 sera which have been shared among laboratories (Jones et al., 1997; Longbottom et al., 2001,  
453 2002; McCauley et al., 2007; Vretou et al., 2007).

454

### 455 3.1.2. Infection in cattle

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

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

467

### 468 3.1.3. Infections in pigs

469 *Chlamydiaceae* spp. in pigs have been associated with pneumonia, polyarthritits,  
470 conjunctivitis, reproductive disorders and abortion (Busch et al., 2000). Asymptomatic  
471 infections might be prevalent and under-diagnosed. Infections are often mixed, attributed to  
472 *Chlamydia suis*, *C. abortus* and *C. pecorum* (Schiller et al., 1997). *C. psittaci* can also be a  
473 potential pathogen as was demonstrated in experimental infection of gnotobiotic piglets  
474 (Guscetti et al., 2000). In order to serologically identify the infecting chlamydial species,  
475 Hoelzle et al. (2004) expressed the entire MOMP molecule from *Chlamydia suis*, *C. abortus*  
476 and *C. pecorum* in *E. coli* and demonstrated the suitability of the recombinant antigens in  
477 ELISA assays. Though this approach was very promising it has not been further verified in  
478 animals in the field. In a recent study, the seroprevalence to chlamydiae in pigs in Italy was  
479 estimated using a MIF test that discerned antibodies against 4 chlamydial species (Di  
480 Francesco et al., 2006). An indirect ELISA with recombinant MOMP from *C. psittaci* was  
481 used to determine seropositivity in closed pig breeding farms in Belgium (Vanrompay et al.,  
482 2004). On the basis of the similarity between *Chlamydia suis* and *Chlamydia trachomatis*,  
483 detection of anti-*Chlamydia suis* antibodies in pigs by use of commercially available ELISAs  
484 based on the *Chlamydia trachomatis* MOMP VD4 peptides could be theoretically possible. In  
485 addition, MOMP-and POMP-based specific *C. abortus* ELISAs could be useful in the  
486 detection of antibodies to *C. abortus* in pigs.

487

### 488 3.1.4. Infections in birds

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

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

503

### 504 3.2. Antibodies based on elementary bodies-The microimmunofluorescence test (MIF test)

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

513

### 514 3.3. Assays based on LPS

#### 515 3.3.1. Complement Fixation Test (CFT)

516 The CFT is the first described LPS-based assay and the most commonly used in  
517 veterinary laboratories (Stamp et al., 1952). In OEA, CFT serum titres rise at the time of  
518 abortion and remain high for at least 6 weeks (Storz, 1971). A CFT titre greater than 1/32 is  
519 considered as an indication for the presence of OEA, whereas lower values can be due to  
520 subclinical enteric infections by *C. abortus* or cross-reactivity with *C. pecorum* and other  
521 bacterial LPS. (Aitken and Longbottom, 2004). The CFT is not workable with haemolytic or  
522 anti-complementary sera (Cross and Clafin, 1963). Furthermore, not all animal sera or all  
523 immunoglobulin isotypes can fix complement (Schmeer et al., 1987; Kaltenboeck et al.,  
524 1997a). Recent studies that used sera from sheep and goats with documented absence or  
525 presence of OEA reported CFT specificities between 83 and 98.1% and sensitivities between  
526 68.8% and 91.4% (Jones et al., 1997; Longbottom et al., 2001, 2002; Vretou et al., 2007). The  
527 CFT antigen used in these reports was from *C. abortus*. Interestingly, CFTs conducted with *C.*  
528 *psittaci*- and *C. abortus*-derived LPS have been reported to differ in their sensitivities, which  
529 were 96.4% and 60%, respectively (McCauley et al., 2007). In addition to the trisaccharide  
530  $\alpha\text{Kdo}(2\rightarrow8)\alpha\text{Kdo}(2\rightarrow4)\alpha\text{Kdo}$ , *C. psittaci* contains a structurally different trisaccharide with  
531 the structure  $\alpha\text{Kdo}(2\rightarrow4)\alpha\text{Kdo}(2\rightarrow4)\alpha\text{Kdo}$  and the branched tetrasaccharide  
532  $\alpha\text{Kdo}(2\rightarrow8)[\alpha\text{Kdo}(2\rightarrow4)]\alpha\text{Kdo}(2\rightarrow4)\alpha\text{Kdo}$  (Brabetz et al., 2000; Brade et al., 2000). This  
533 highlights the importance of origin and preparation of the LPS for use in CFT and other LPS-  
534 based assays. Furthermore, when testing for anti-*Chlamydiaceae* responses, assays with  
535 defined LPS composition should be the first choice. Since CFT is technically quite  
536 demanding, an automated version (Seramat) was recently developed, which correlated well  
537 with manually performed tests (Magnino et al., 2005).

538

## 539 3.3.2. Recombinant ELISA

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

555

## 556 3.3.3. Other commercial LPS-based assays

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



564 competitive ELISA have been used for the detection of antibodies in avian species (Prukner-  
565 Radovic et al., 2005). The latter was less specific when compared to LPS/LGP ELISA in  
566 detecting antibodies to the LPS/lipoglycoprotein complex, rMOMP ELISA and  
567 immunoblotting analysis on a small serum collection (Verminnen et al., 2006). The  
568 CHEKIT™ Chlamydia assay (IDEXX Laboratories, Maine, USA), an indirect ELISA, was  
569 designed for detection of *C. abortus* antibodies in ruminants. The assay has recently been  
570 reported in two studies to cross-react with sera from SPF lambs experimentally infected with  
571 conjunctival and arthritogenic subtypes of *C. pecorum*, as well as with field serum samples  
572 from sheep naturally infected with enteric and arthritogenic subtypes of *C. pecorum*,  
573 suggesting the presence of LPS in the coating antigen (Vretou et al. 2007; Wilson et al.,  
574 2008). The sensitivities and specificities of the assay tested with reference sera in these two  
575 studies were reported as 73.3/85.5% and 96.3/96.2%, respectively. This test offers the  
576 advantage of easy and speedy performance and has been used in the diagnosis of bovine  
577 chlamydiosis (Niemczuk, 2005). It might further prove useful for the serological diagnosis of  
578 avian chlamydiosis, where cross-reactivity with other chlamydial species is not an issue.

579

### 580 3.4. Assays based on the MOMP

#### 581 3.4.1. Synthetic peptides and recombinant MOMP

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

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

593 Synthetic peptides derived from VD1 and VD2 amino acid sequences were the basis of  
594 the assay Panclabort or MOMP-P (dianoSTI, Midlothian Scotland) which is no longer  
595 available. The test was the only one commercially available for anti-MOMP antibodies and  
596 was found to be 95.9% specific and 70.4% sensitive in a recent evaluation (McCauley et al.,  
597 2007).

598

#### 599 3.4.2. Competitive ELISA (cELISA)

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

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

620

### 621 3.5. Assays based on the POMP<sub>s</sub>

622 With the discovery of the multigene family encoding proteins of 90kDa in 1996, it  
623 became clear that these highly immunoreactive proteins were very complex antigens  
624 comprising epitopes present at least in the *Chlamydophila* species (Souriau et al., 1994;  
625 Longbottom et al., 1996, 1998; Vretou et al., 2003). Competitive ELISAs based on POMP-  
626 specific mAbs 181 and 192 were developed (Vretou et al., unpublished data). Initial  
627 serological screening of the original clones identified from a  $\lambda$ gt11 expression library, *p90f31*  
628 and *p91Bf99*, suggested that they were promising candidate antigens for the specific diagnosis  
629 of OEA. When a truncated fragment of *p91Bf99*, named rOMP91B expressed as GST fusion  
630 protein was tested with a panel of documented reference sera it was found more specific  
631 (98.5%) and sensitive (84.2%) than the cELISA and the CFT (Longbottom et al., 2001).  
632 Fragmentation of the whole POMP90 molecule into overlapping recombinant antigens has  
633 revealed considerable variation in the sensitivity and specificity among the different  
634 fragments. Thus, fragments rOMP90-3 and rOMP90-4 exceeded the other recombinant  
635 fragments in specificity (100%) and sensitivity (95.7%/94.3%, respectively), although  
636 fragment rOMP90-4 appeared to perform better with OEA-positive field samples  
637 (Longbottom et al., 2002). The high sensitivity and specificity of the rOMP90-4 ELISA was  
638 reconfirmed by a different study that compared this fragment to rOMP90-3, to the commercial

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

653 *Chlamydomphila abortus* ELISA (Institut Pourquier, Montpellier, France) is the only  
654 commercially available test that is based on the POMP. Recent evaluations of the assay using  
655 reference sera have reported good specificity (90-100%) and sensitivity (80-93.5%) when  
656 compared to other serological tests, including CFT, CHEKIT, cELISA, and rOMP90 tests  
657 (McCauley et al., 2007; Vretou et al., 2007; Wilson et al., 2008). When tested with field sera,  
658 however, the test was less sensitive, identifying up to half of the sera detected by the other  
659 assays, possibly because of the selected high cut-off of the test or the choice of antigen  
660 fragment used in the test (Vretou et al., 2007; Wilson et al., 2008). The Pourquier-ELISA  
661 performed equally well with the “in house” rPOMP90-3 ELISA when tested with reference  
662 sera and compared to CFT, MOMP-P, rOMP90-3 and rOPM90-4 assays (McCauley et al.,  
663 2007).

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

670

### 671 3.6. *The prospect of serological testing*

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

681

## 682 **4. DNA amplification methods**

### 683 4.1. *Conventional PCR*

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

688 Many different PCR protocols have been suggested in the literature. **Table 1** summarises  
689 some of the assays that may be relevant for veterinary diagnostic laboratories. The majority of  
690 published conventional PCR methods are based on targets in the ribosomal RNA operon  
691 (Messmer et al., 1997; Everett and Andersen, 1999) or the *ompA* gene (Kaltenboeck et al.,  
692 1997b; Yoshida et al., 1998). The latter encodes MOMP and harbours four VDs, each of  
693 which is flanked by a conserved region. While genus- and species-specific antigenic  
694 determinants are encoded by the conserved regions, serovar-specific segments are located on  
695 the variable domains, mainly VD2 and VD4. This heterogeneous primary structure makes the  
696 *ompA* gene an ideal target for diagnostic PCR, as well as for intra-species differentiation  
697 assays.

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

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

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

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

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

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

730

#### 731 4.2. Real-time PCR

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

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

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

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



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

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

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

784 The number of real-time PCR protocols developed for individual chlamydial species is  
785 steadily increasing. Using discriminatory target segments in the *ompA* gene, Pantchev et al.  
786 (2008) presented separate assays for *C. psittaci* and *C. abortus*, which could be useful in the  
787 clinical diagnosis of psittacosis and enzootic abortion, as well as epidemiological surveys.

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

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

796

## 797 **5. DNA microarray technology**

### 798 *5.1. Identification of chlamydial species*

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

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

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

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

829 Hybridisation probes for *Chlamydiaceae* spp. were designed on the basis of a multiple  
830 sequence alignment, from which a highly discriminatory segment in domain I of the 23S  
831 rRNA gene was identified. As a result of several rounds of optimisation and refinement, the  
832 present version of the chip carries 28 species-specific probes (for all nine chlamydial species),  
833 3 genus-specific probes for *Chlamydia* and *Chlamydophila*, respectively, 5 probes identifying  
834 the closest relatives *Simkania negevensis* and *Waddlia chondrophila*, as well as 4  
835 hybridisation controls (consensus probes) and a staining control (biotinylated  
836 oligonucleotide).

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

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

847

#### 848 5.2. Genotyping by microarray

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

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

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

875

## 876 **6. The quest for an alternative reference standard**

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

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

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

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

904

## 905 **7. Conclusions**

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

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

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

926

#### 927 **Conflict of interest statement**

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

932

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937

938

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- 1437
- 1438

1438 **Figure captions**

1439

1440 Figure 1. Examples of staining techniques used to diagnose *C. abortus* induced abortion in  
1441 sheep. Similar techniques can be applied to other chlamydial species and tissue samples. **A.**  
1442 Smear prepared from ovine placental membrane taken following abortion. The chlamydial  
1443 EBs, which are stained pinkish-red, appear singly and in clumps, amongst the green cellular  
1444 background material. (The area represented in the box with the solid outline is an enlarged  
1445 view of that with the dashed outline). **B.** Formalin-fixed, paraffin-embedded tissue section  
1446 prepared from the placenta of an aborted foetus and stained by the enhanced  
1447 immunoperoxidase method using anti-LPS mAb 13/5 (Buxton et al., 1996). Image shows a  
1448 clear demarcation between the basement membrane, where many chlamydial inclusions (CI)  
1449 are observed and stained brown, and the damaged, sloughing chorionic epithelial (CE) cell  
1450 layer, where released chlamydial antigen appears as more diffuse brown staining. A clear  
1451 inflammatory cell infiltrate (I) and arteritis (A) can also be observed. (This image appears  
1452 courtesy of Dr David Buxton, Moredun Research Institute, Edinburgh, UK). **C.** Giemsa  
1453 staining of *C. abortus*-infected McCoy cell monolayers following recovery from infected  
1454 placental material. **D.** Detection of *C. abortus*, recovered from placental tissue and isolated in  
1455 McCoy cells, by immunofluorescence using anti-MOMP mAb 4/11 (McCafferty et al., 1995).

1456

1457 Figure 2. Original results of the rELISA (rLPS ELISA). Group 1, serum numbers 1-67 from  
1458 OEA-infected flocks, 29 CFT positive; Group 2, sera 68-133 from OEA-free flocks, 1 CFT  
1459 positive; Group 3, sera 134-192 from 4 flocks with enteric *C. abortus* infection, 0 CFT  
1460 positive; Group 4, sera 193-210 from a flock with enteric *C. pecorum* infection, 0 CFT  
1461 positive; Group 5, sera 211-257 from animals with unknown OEA status, 20 CFT positive;  
1462 Group 6, sera 259-289 from a flock infected with arthritogenic *C. pecorum*, 7 CFT positive;

1463 Group 7, sera 290-297 from SPF lambs infected with various *C. pecorum* subtypes, 4 CFT  
1464 positive. The cut-off of the test is shown as a horizontal line.

1465

1466 Figure 3. Seroreactivity of synthetic MOMP VS1-VS4 peptides. Fifteen decapeptides  
1467 overlapping by two amino acids spanning the four variable domains of the MOMP were  
1468 tested with sera from 7 *C. abortus* –infected pregnant ewes [1], 5 SPF lambs infected with  
1469 enteric *C. pecorum* and [2] and 5 SPF lambs infected with arthritogenic *C. pecorum* [3]. VS1:  
1470 GTAAANYKTPTDRP; VS2: KGSSIAADQL VS3: TAFPLPLTAGTDQATD; VS4:  
1471 EATALDTSNKFA.

1472

1473 Figure 4 Illustration of multiple DNA tests being conducted on a clinical sample. Faeces from  
1474 a psittacine bird (ID: 07G764, marked on agarose gels with an asterisk) was subjected to  
1475 DNA extraction using the High Pure PCR Template Preparation Kit (Roche). One µl of the  
1476 extract was examined by the following tests: **A.** PCR targeting 16-23S intergenic spacer  
1477 region of *Chlamydiaceae* according to Everett and Andersen (1999), amplicon size approx.  
1478 600 bp. **B.** PCR targeting the *ompA* gene of *Chlamydiaceae* according to Kaltenboeck et al.  
1479 (1997b), amplicon size 590 bp. **C.** Reamplification of the product from **B** using *Chlamydia*  
1480 *psittaci*-specific primers in a nested PCR according to Sachse and Hotzel (2003), amplicon  
1481 size 395 bp. **D.** Real-time PCR targeting the *ompA* gene of *C. psittaci* (duplicate run)  
1482 according to Pantchev et al. (2008), Ct=27.9. **E.** AT microarray test for identification of  
1483 chlamydial species targeting the 23S rRNA gene according to Sachse et al. (2005); left: array  
1484 image, right: barplot showing specific hybridisation signals for genus *Chlamydomphila* and  
1485 species *C.psittaci*. **F.** AT microarray test for *ompA* genotyping of *C. psittaci* according to  
1486 Sachse et al. (2008); left: array image, right: barplot showing specific hybridisation signals of  
1487 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: CTGGCTCATCATGCAAAAAGG

*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

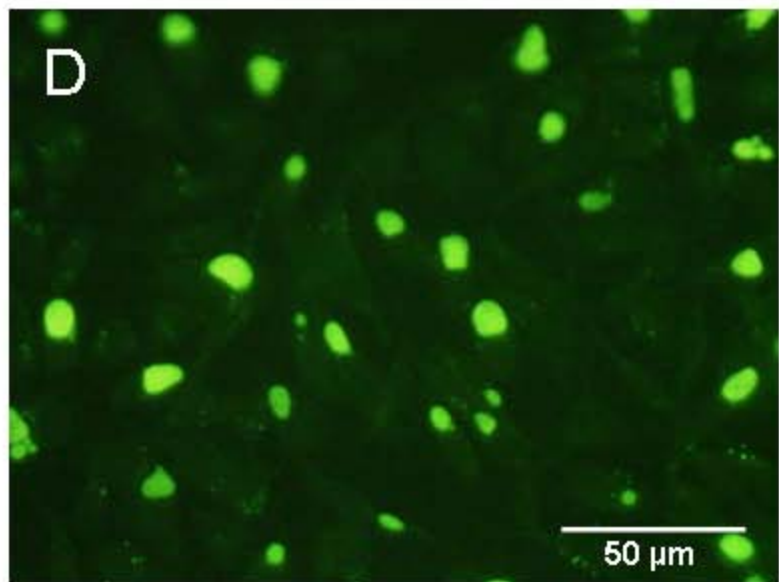
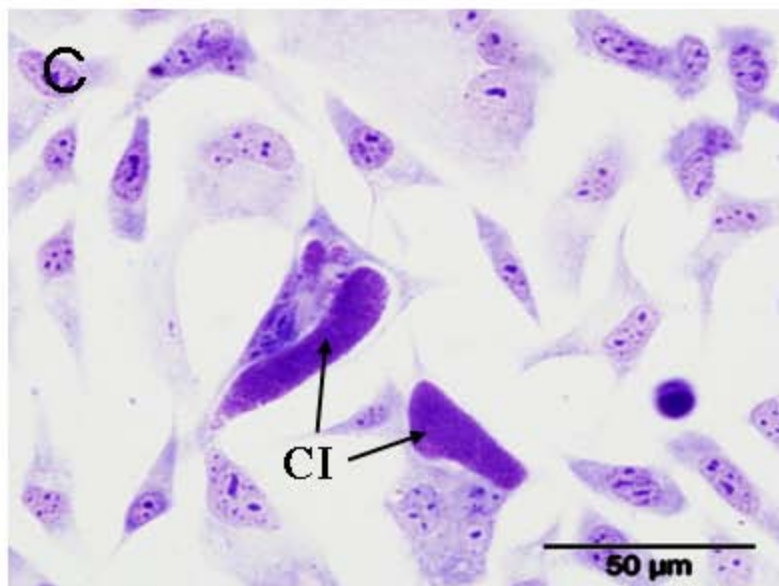
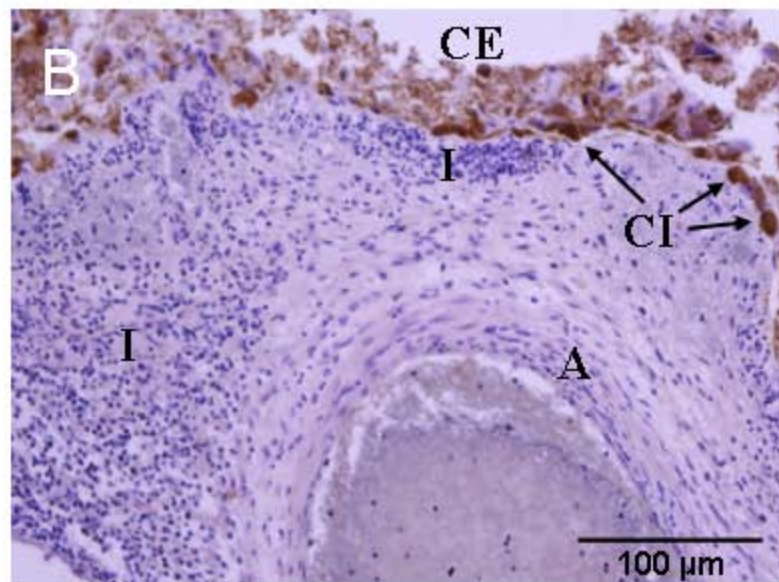
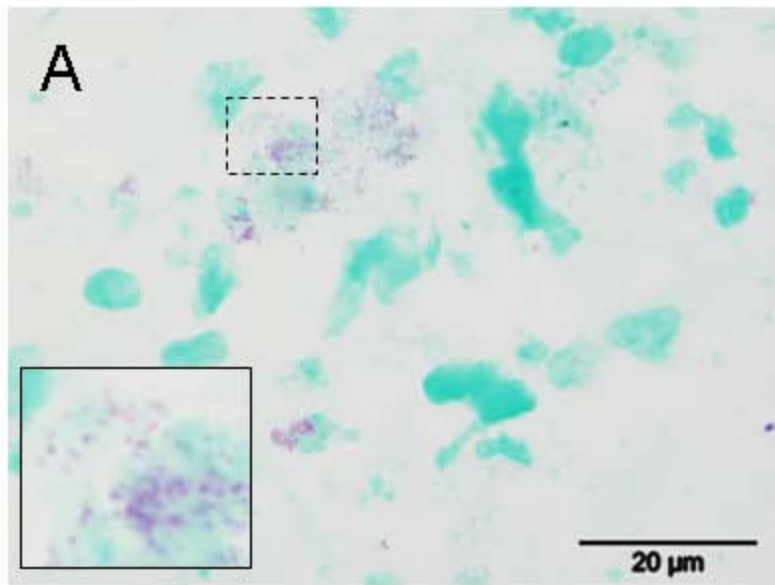
Specificity	Primers, probe and sequences (5'- 3')	Amplicon size (bp)	Target gene	Reference
<i>Chlamydiaceae</i>	TQF: GAAAAGAACCCTTGTTAAGGGAG  TQR: CTAACTCCCTGGCTCATCATG  FAM-CAAAAGGCACGCCGTCAAC-TAMRA	129-132	23S rRNA	Everett et al., 1999b
<i>Chlamydiaceae</i>	Ch23S-F: CTGAAACCAGTAGCTTATAAG CGGT  Ch23S-R: ACCTCGCCGTTTAACTTAACTCC  Ch23S-p: FAM-CTCATCATGCAAAAGGCACGCCG-TAMRA	111	23S rRNA	Ehricht et al., 2006
<i>Chlamydiaceae</i>	CHL23SUP: GGGGTTGTAGGGTYGAGRAIAWRRGATC  CHL23DN: GAGAGTGGTCTCCCCAGATTCARACTA  CHL23LCR: LCRed640-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)

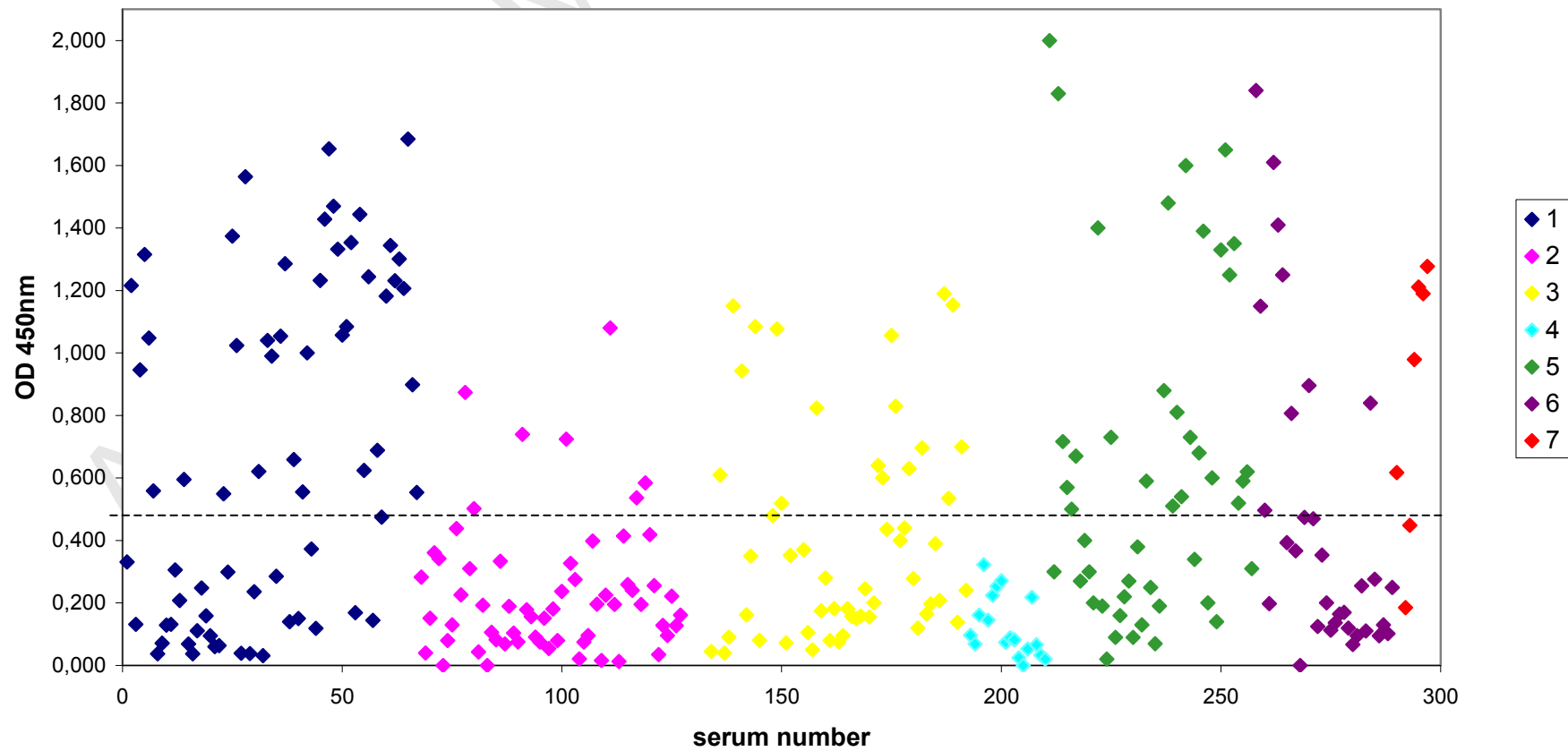
<i>C. psittaci</i>	F1-incA-Cpsi: GCCATCATGCTTGTTTCGTTT R1-incA-Cpsi: CGGCGTGCCACTTGAGA Cpsi-incA-NM: FAM-TCATTGTCATTATGGTGATTCAGGA-NFQ	74	<i>incA</i>	Menard et al., 2006  (MGB™ probe)
<i>C. abortus</i>	CpaOMP1-F: GCAACTGACACTAAGTCGGCTACA CpaOMP1-R: ACAAGCATGTTCAATCGATAAGAGA CpaOMP1-S: FAM-TAAATACCACGAATGGCAAGT-TGGTTTAGCG-TAMRA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

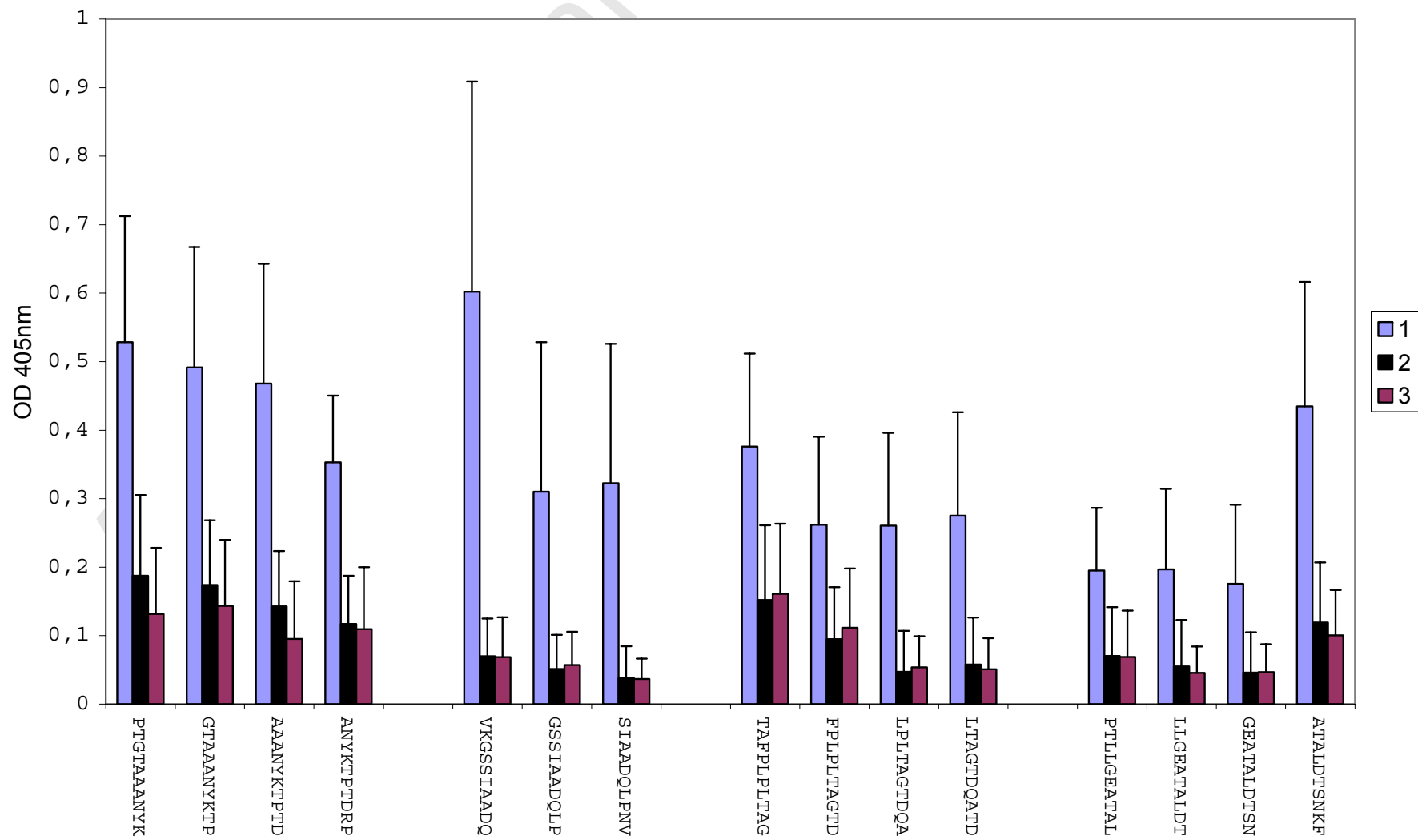
\* based on four-species classification of the family *Chlamydiaceae*

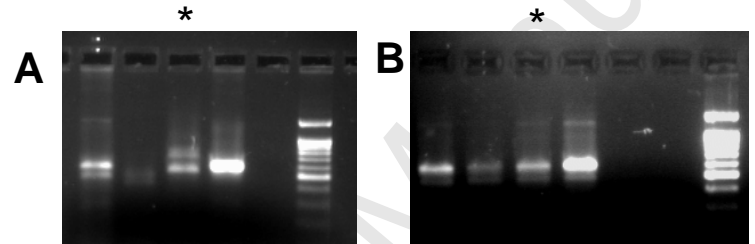
\*\* includes primer sets for genotype-specific amplification

\*\*\* 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)

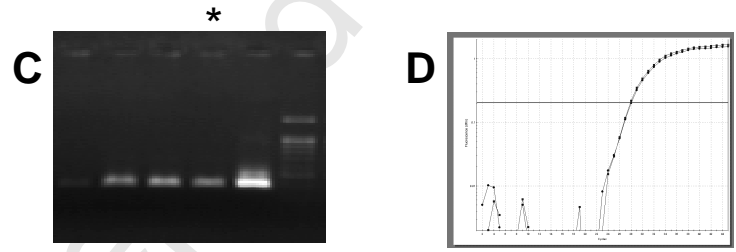




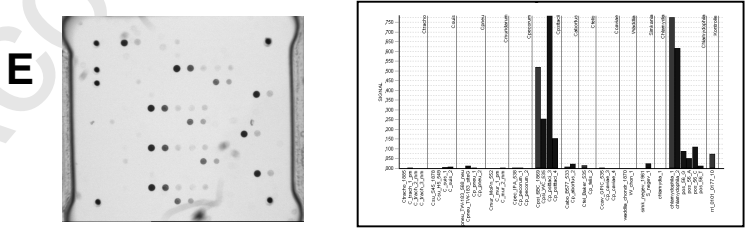




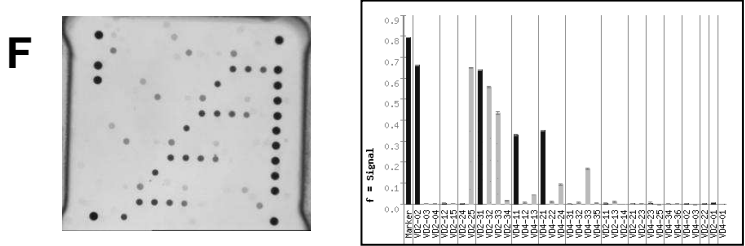
*Chlamydiaceae*



*C. psittaci*



*Chlamydophila psittaci*



Genotype A