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1 **DNA microarray-based genotyping of *Chlamydophila psittaci* strains from**
2 **culture and clinical samples**

3

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17

18

19 **Abstract**

20 The avian and human pathogen *Chlamydophila (C.) psittaci* represents a genetically
21 heterogeneous species. To facilitate epidemiological surveys, more rapid yet highly specific
22 molecular tests are needed. Currently used typing methods, i.e. serotyping and PCR-RFLP,
23 have only limited sensitivity and are incapable of covering the wide spectrum of naturally
24 occurring types of *C. psittaci* strains. In the present study, a new DNA microarray assay based
25 on the ArrayTube[®] (AT) technology was used to genotype *C. psittaci* in 98 isolates and 23
26 clinical tissue samples. The present array carries 35 oligonucleotide probes derived from

27 variable domains 2 and 4 of the *ompA* gene. The assay proved highly sensitive, allowing
28 correct genotyping of DNA from 2 inclusion-forming units.

29 The results of DNA microarray genotyping of cultured strains proved highly concordant with
30 the data from PCR-RFLP typing and serotyping. Sequencing of the *ompA* gene served as the
31 reference test to verify the accuracy of AT genotyping results. In 15 instances (15.3 %),
32 strains were successfully typed by the AT assay, while serotyping and/or PCR-RFLP
33 genotyping failed to produce unambiguous results. Eleven of these samples were *ompA*
34 sequenced to confirm the AT findings. In addition to the currently accepted nine *ompA*
35 genotypes, the microarray test was shown to recognise new provisional genotypes, such as
36 Mat116 and YP84. In conclusion, the new AT assay proved to be suitable for rapid, sensitive
37 and reproducible genotyping of *C. psittaci* strains and can be recommended for routine
38 diagnosis.

39

40 **Keywords**

41 *Chlamydophila psittaci*; *ompA* gene; serotyping; genotyping; diagnostic DNA microarray
42 test; testing of clinical samples

43

43 Introduction

44 Infection of birds with the obligate intracellular bacterium *Chlamydophila (C.) psittaci* is
45 common all over the world and has been found in about 465 avian species (Kaleta et al.,
46 2003). Outbreaks of avian chlamydiosis or psittacosis in psittacine birds and domestic poultry
47 farms can cause considerable economic damage (SCAHAW, 2002) and pose a serious risk to
48 exposed individuals (Heddema et al., 2006; Gaede et al., 2008; Vanrompay et al., 2007).
49 Transmission to humans occurs mainly via contaminated aerosol and airborne dust, and the
50 severity of disease varies from flu-like symptoms to severe febrile illness associated with
51 atypical pneumonia.

52 The infection in birds also lacks specific symptoms. Instead, respiratory signs,
53 mucopurulent nasal and ocular discharge, dyspnoea, apathy, as well as wasting and cachexia
54 may be observed (Gerlach, 1999; Grimes, 1994; Vanrompay et al., 1995). The course and
55 severity of disease are dependent on virulence properties of the particular *C. psittaci* strain,
56 the immune status of the host, as well as the transmission route and infectious dose. Besides,
57 latent infection seems to be widespread, which leaves the birds largely symptomless, but
58 creates a permanent reservoir of the pathogen. Chronicity as a general feature of chlamydial
59 infections (Hammerschlag, 2000) was also suggested to play a role in both birds and humans
60 (Grimes et al., 1991).

61 According to the recently revised taxonomy of the family *Chlamydiaceae* (Everett et al.,
62 1999), the new species *C. psittaci* was designed to retain only avian-like strains. Nevertheless,
63 it remains a relatively heterogeneous taxon in terms of host range, virulence and also genome
64 sequence variation. On the basis of immunofluorescent reaction with a panel of specific
65 monoclonal antibodies (mAbs) recognising epitopes of the major outer membrane protein
66 (MOMP), the avian strains of *C. psittaci* were classified into serovars A, B, C, D, E, and F,
67 with some degree of host specificity ascribed to each serovar (Andersen, 1991; Vanrompay et
68 al., 1993; Andersen, 1997). Subsequently, Sayada et al. (1995) suggested the introduction of

69 genotypes defined by PCR amplification of the *ompA* gene combined with restriction enzyme
70 analysis, i.e. restriction fragment length polymorphism (PCR-RFLP). Vanrompay et al.
71 (1997) compared the data from serotyping and PCR-RFLP and concluded that each serovar
72 could be assigned to an equivalent genotype. Thus, nine different genotypes have been
73 generally accepted to date, seven of which are thought to predominantly occur in a particular
74 order or class of *Aves* and two in non-avian hosts, i.e. genotype A in psittacine birds, B in
75 pigeons, C in ducks and geese, D in turkeys, E in pigeons, ducks and others, F in parakeets,
76 WC in cattle, and M56 in rodents. In addition, Geens et al. (2005) suggested the introduction
77 of genotype E/B to represent a group of isolates from ducks, turkeys and pigeons. Most of the
78 avian genotypes have also been identified sporadically in isolates from cases of zoonotic
79 transmission to humans, particularly A, B and E/B (Heddema et al., 2006; Vanrompay et al.,
80 2007; Gaede et al., 2008).

81 The fact that serotyping can be conducted only with cultured strains represents a general
82 limitation. In addition, the type-specific mAbs are not commercially available. Concerning
83 PCR-RFLP, the technique can be conducted in any diagnostic laboratory and even DNA
84 extracts from clinical samples have been examined (Vanrompay et al., 1997), but it requires
85 substantial amounts of a PCR amplicon to generate distinctive and reproducible RFLP
86 patterns. Extensive expertise of laboratory personnel is needed. For instance, since *AluI*
87 restriction patterns of genotypes A, B and E are quite similar, a second digestion with *MboII*
88 may become necessary. In addition, genotypes E/B, M56 and WC cannot be identified using
89 this method. These constraints preclude the use of PCR-RFLP for genotyping in routine
90 diagnosis. As genotype-specific sites are located in the variable domains (VDs) 2 and 4 of the
91 *ompA* gene, sequencing and subsequent comparison with type strain sequences can be used to
92 identify the genotype of *C. psittaci* strains (Geens et al., 2005; Heddema et al., 2006).
93 However, mixed chlamydial infections cannot be diagnosed using standard sequencing
94 protocols, its throughput is limited, and some diagnostic laboratories lack direct access to

95 sequencing equipment. Thus, a more rapid yet highly specific test is needed for routine typing
96 and epidemiological tracking.

97 In this situation, diagnostic DNA microarray analysis can be a powerful alternative. Its
98 high discriminatory capability results from the possibility of using more than one specific
99 probe for each genotype. The ArrayTube™(AT) platform was shown to be practicable and
100 easy to handle when used for detection and identification of all species of *Chlamydia* and
101 *Chlamydophila* (Sachse et al., 2005; Ehricht et al., 2006; Borel et al., 2007). In a recent study,
102 we developed an AT microarray for genotyping of *C. psittaci* (Sachse et al., 2008). Based on
103 comprehensive analysis of all available *ompA* sequences, we proposed subgroups for three of
104 the more heterogeneous genotypes, i.e. A-VS1, A-6BC, A-8455, EB-E30, EB-859, EB-
105 KKCP, D-NJ1, D-9N, and suggested the introduction of new provisional genotypes to cover
106 the strains that were previously non-typable, i.e. 1V, 6N, Mat116, R54, YP84, and CPX0308.
107 In the present study, we describe the use of this array in a diagnostic AT assay for rapid
108 genotyping of *C. psittaci* from cell culture and clinical tissue samples.

109

110

111 **Materials and Methods**

112 *Strains and diagnostic samples*

113 Genomic DNA of the following reference strains, each representing a particular
114 genotype, was used to generate reference patterns for the genotyping assay: VS1 (genotype
115 A), CP3 (genotype B), GR9 (genotype C), NJ1 (genotype D), CPMN (genotype E), VS225
116 (genotype F), all of which were from the chlamydia strain collection of AFSSA, Maisons-
117 Alfort, France. Strains WS/RT/E30 (genotype E/B; isolated from a duck, Germany, 2001),
118 WC (from cattle, USA, 1990) and M56 (from muskrat, USA, 1961) were kindly provided by
119 Daisy Vanrompay, University of Ghent, Belgium.

120 To determine the detection limit of the genotyping assay, strains C5/98 (genotype A-VS1;
121 isolated from a calf, Germany, 1998), DC5 (genotype A-VS1; isolated from a horse,
122 Germany, 2001), and DC11 (genotype A-VS1; isolated from a pig, Germany, 2001) were
123 grown on BGM cells, titrated and dilution series examined.

124 The field strains examined are listed in Tables 1a and 1b. Strains no. 1-66 were from
125 France, no. 67-91 from Germany, and no. 92-98 from Italy. In addition, 23 diagnostic samples
126 from confirmed cases of *C. psittaci* infection and 2 specificity control samples from *C.*
127 *abortus*-infected sheep were examined (Table 2). These samples had been submitted to the
128 national reference laboratories for psittacosis at AFSSA, Maisons-Alfort, France (no. 1-6, 17,
129 24-25) and Friedrich-Loeffler-Institut, Jena, Germany (no. 7-16, 18-23).

130

131 *DNA microarray design*

132 The present array was described elsewhere (Sachse et al., 2008). Briefly, 35
133 oligonucleotide probes of 22-30 nt derived from VD2 and VD4 of the *C. psittaci ompA* gene
134 were spotted onto modified glass chips and assembled into ATs. Biotinylated probes were
135 added to monitor the staining reaction, mark the corners of the array and facilitate
136 normalisation of signal intensities. Each genotype probe was spotted four-fold, the controls
137 15-fold, thus bringing the total number of spots on the array to 155.

138

139 *DNA extraction*

140 Clinical samples and strains grown on BGM cells were DNA extracted using the High
141 Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany) following the
142 instructions of the manufacturer.

143

144 *Biotinylation PCR*

145 Target DNA was amplified and biotinylated by duplex PCR using two pairs of primers
146 covering all VDs of the *ompA* genes. Primer pair VD1-f (5'-ACT ACG GAG ATT ATG TTT
147 TCG ATC GTG T-3') and VD2-r (5'-Biotin-CGT GCA CCY ACG CTC CAA GA-3') gives
148 rise to a 418-bp product which includes VD1 and VD2, whereas primers 201CHOMP (5'-GGI
149 GCW GMI TTC CAA TAY GCI CAR TC-3') and *ompA*-rev (5'-Biotin-TCC TTA GAA TCT
150 GAA TTG AGC-3') define a product of 570 bp covering VD3 and VD4. The following PCR
151 cycling conditions were used: initial denaturation at 96°C for 60 s, 40 cycles of 94°C for 30 s,
152 50°C for 60 s, 72°C for 30 s, and final elongation at 72°C for 240 s.

153 Each 20- μ l reaction contained 10 pmol of the first and 20 pmol of the second primer pair,
154 as well as 1 mM each of dNTP mix, 1 U *Taq* DNA polymerase (Fermentas, St. Leon-Roth,
155 Germany), 2 μ l 10xPCR buffer (Fermentas), 1 μ l 50 mM MgCl₂, and 1 μ l of the DNA
156 template.

157

158 *DNA microarray hybridisation*

159 The AT vessel was conditioned by washing with 500 μ l each of deionised water and
160 hybridisation buffer (0.25M Na₂HPO₄ + 1mM EDTA + 4.5% SDS in 1xSSC, pH=7.25) at
161 50°C for 5 min. All incubations were carried out upon slight shaking (550 rpm) on a heatable
162 horizontal tube shaker (Thermomixer comfort, Eppendorf, Cologne, Germany). For
163 denaturation, 1 μ l of the 5'-biotinylated PCR product was diluted with 99 μ l hybridisation
164 buffer in a separate tube, heated at 95°C for 5 min and put on ice for 30 s. After transfer into
165 the AT, hybridisation was allowed to proceed at 50°C for 60 min. The supernatant was then
166 discarded, and the array was washed consecutively with 500 μ l 2xSSC/0.01% Triton X-100
167 (50°C, 5 min), 500 μ l 2xSSC (50°C, 5 min), 500 μ l 0.2xSSC (50°C, 5 min), and 500 μ l
168 0.2xSSC (35°C, 5 min). Vacant binding sites of the microarray were blocked by incubation
169 with a 2% solution of Blocking Reagent (Roche) in hybridisation buffer at 30°C for 15 min.
170 Subsequently, the AT was incubated with 100 μ l of a 1:5000 dilution (0.5 μ g/ml) of

171 streptavidin-peroxidase polymer (Sigma-Aldrich, Taufkirchen, Germany) at 30°C for 15 min
172 followed by three wash steps, i.e. 500 µl 2xSSC/0.01% Triton X-100 (30°C, 5 min), 500 µl
173 2xSSC (20°C, 5 min), 500 µl 0.2xSSC (20°C, 5 min). Finally, 100 µl of the peroxidase
174 substrate Seramun Grün (Seramun Diagnostica, Heidesee, Germany) were added.
175 Hybridisation signals were measured 5 min later using an ATR-01 transmission reader
176 (Clondiag Chip Technologies, Jena, Germany) and processed using the Iconoclust software,
177 version 2.3 (Clondiag) in combination with the Partisan Array LIMS system which provided
178 the specific algorithms for this assay.

179 Normalised intensities of the spots were calculated according to the following equation:

180 $NI = 1 - (M/BG)$, with NI being the normalised intensity, M the average intensity of the
181 spot, and BG the average intensity of the local background. The values of NI cover a range
182 from 0 (no signal) to 1 (strongest signal).

183 *Processing of microarray hybridisation patterns using the Pattern Match algorithm*

184 To automatically identify the *C. psittaci* genotype from the hybridisation pattern we used
185 an algorithm comparing the measured signals of a given sample with a panel of 13 reference
186 patterns obtained with the type strains of genotypes A-F, E/B, WC, and M56, and additionally
187 the patterns of strains 6BC (A), 589 (E/B), Mat116, and YP84. The parameter expressing the
188 difference between the individual sample and the reference patterns is the matching score
189 (MS), which represents the sum of differences between all normalised signal intensities of the
190 sample pattern and the corresponding signals from reference patterns. An ideal match of two
191 patterns will yield MS=0.

192

193 *PCR-RFLP genotyping*

194 The *ompA* locus was amplified from extracted DNA by PCR using primers CTU (5'-ATG
195 AAA AAA CTC TTG AAA TCG G-3') / CTL [5'-CAAGAT TTTCTA GA(T/C) TTCAT-

196 (C/T)TTGTT-3'], and the product was subjected to restriction enzyme digestion using *AluI*
197 and/or *MboII* as described by Sayada et al. (1995). Following agarose gel electrophoresis,
198 cleavage patterns were compared with those of the reference strains for genotypes A, B, C, D,
199 E, and F.

200

201 *Sequencing of the ompA gene*

202 The complete gene was amplified by PCR using primers CTU and ompA-rev (not
203 biotinylated) using the following cycling profile: initial denaturation 96°C for 60 sec, 40
204 cycles of 96°C/15 sec, 50°C/60 sec, 72°C/60 sec, final extension 72°C for 60 sec.

205 Products were electrophoresed in 1% agarose gels, the specific bands of approximately
206 1200 bp were excised with a scalpel, and DNA was extracted using the innuPREP Gel
207 Extraction Kit (Analytik Jena, Jena, Germany). DNA sequencing was carried out by cycle
208 sequencing using the BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied
209 Biosystems, Darmstadt, Germany) according to the instructions of the manufacturer. The
210 following primers were used: CTU, VD1-f, VD2-r (not biotinylated), CHLAGEN-1 (5'-CGG
211 CTG CAT TCA ACT TGG-3'), 201CHOMP, and ompA-rev (not biotinylated). Nucleotide
212 sequences were determined on an ABI Prism 310 Genetic Analyzer (Applied Biosystems).

213

214 *ompA sequence alignment*

215 To determine the genotype from *ompA* sequencing data, the sequence of a sample was
216 added to a multiple *ompA* sequence alignment of *C. psittaci* strains representing all currently
217 recognised genotypes, including the recently suggested subgroups, and representatives of six
218 new provisional genotypes denoted hereafter with an asterisk (Sachse et al., 2008). The
219 following strains and sequences were included: strain VS1 (genotype A-VS1, GenBank
220 acc.no. AF269281.1), strain 6BC (A-6BC, X56980.1), strain 84-55 (A-8455, Y16561.1), strain
221 CP3 (B, AF269265.1), strain GR9 (C, L25436.1), strain NJ1(D-NJ1, AF269266.1), strain 9N

222 (D-9N, EF375557.1), strain CPMN (E, X12647.1), strain WS/RT/E30 (EB-E30,
223 AY762613.1), strain 06-859/1 (EB-859, EU159263.1), strain KKCP-1 (EB-KKCP,
224 AB284062.1), strain VS225 (F, AF269259.1), strain M56 (M56, AF269268.1), strain WC
225 (WC, AF269269.1), strain 1V (1V*, EF028916.1), strain 6N (6N*, EF197820.1), strain
226 Mat116 (Mat116*, AB284058.1), strain R54 (R54*, AJ243525.1), strain Daruma-1981
227 (YP84*, AB284065.1), strain CPX0308 (CPX0308*, AB284064.1).

228 The alignment was done using the Vector NTI Suite 10.0 software package (Invitrogen,
229 Paisley, UK).

230

231 *Serotyping*

232 Serotyping of the *C. psittaci* strains in Table 1a was reported in the paper of Duan et al.
233 (1999). The findings have been included here for comparison.

234

235

236 **Results**

237 A diagnostic DNA microarray was developed and used for genotyping of *C. psittaci* field
238 isolates and strains contained in clinical tissue samples. The assay is based on the AT
239 technology and involves an array carrying 35 oligonucleotide probes recognising
240 discriminatory targets in VD2 and VD4 of the *ompA* gene of *C. psittaci*. Prior to microarray
241 hybridisation, the variable portions of the *ompA* gene were amplified in 418- and 570-bp
242 segments using a duplex PCR and biotinylated reverse primers to introduce 5'-biotin labels
243 into the double-stranded amplicons.

244 The specificity of the genotyping assay was checked by examining representatives of all
245 *Chlamydiaceae* species other than *C. psittaci*. No specific duplex PCR products were seen
246 and no hybridisation signals were detected with these species (data not shown). Among the
247 present panel of samples, there were also two isolates that proved to be *C. caviae* and another

248 isolate and two swabs that contained *C. abortus*, but all of them were negative in the AT
249 genotyping assay (see Tables 1 and 2).

250 The sensitivity of the assay was evaluated by running three independent experimental
251 trials on decimal dilutions of three different *C. psittaci* strains of the same genotype and
252 subgroup, i.e. C5/98, DC5 and DC11. The lowest amount of DNA template allowing correct
253 genotyping was the equivalent of 2 inclusion-forming units (ifu). As an example, the data of
254 the dilution series involving *C. psittaci* strain DC11 (genotype A-VS1) is shown in Fig. 1.

255 The identity of the genotype present in a given sample can be determined manually from
256 the hybridisation pattern by assigning each signal to the respective probe. In principle, the
257 probes yielding the most intense signals are pivotal because they represent perfect matches
258 with the target under stringent hybridisation conditions. The specificity of all probes was
259 given in the match table (Sachse et al., 2008). This manual assignment, however, can be
260 realised only with a limited number of samples. For large series of experiments, we developed
261 a pattern match algorithm, which is based on comparison of the measured sample
262 hybridisation pattern with a set of 13 reference strain patterns. The degree of similarity
263 between sample and reference patterns is expressed as the matching score (MS). The lower its
264 value the higher is the similarity to the hybridisation pattern of the genotype reference strain,
265 with the ideal match yielding MS=0. Based on the experience from the present study, any
266 scores higher than 10 were regarded as inconclusive. An important parameter indicating the
267 reliability of a given genotyping result is the "Delta MS", which represents the arithmetic
268 difference between best and second best match. A Delta MS value of 0.4 and higher was
269 regarded as reflecting a sufficient degree of distinction between best and second best matches.
270 In the case of values between 0.2 and 0.39, manual assignment according to the match table
271 was additionally conducted, which led to confirmation of the calculated findings in each
272 instance. Delta MS values below 0.2 were regarded as ambiguous, requiring *ompA*

273 sequencing as an independent test. Figure 2 illustrates the pattern match approach for three
274 samples having different matching scores.

275 To validate the AT genotyping assay, we compared its findings with the data from
276 serotyping, PCR-RFLP and *ompA* sequencing. In the first series of experiments, 34 *C. psittaci*
277 field isolates, mainly from psittacine birds, were examined by all three tests. Table 1a shows
278 that results were identical in 31 cases. There were only 2 discordant results between AT and
279 serology, namely for isolates 86-10883 and 94-2306, which could not be serotyped for
280 belonging to the rare *ompA* genotype YP84 and the recently introduced genotype E/B,
281 respectively. PCR-RFLP also failed to type these two samples, and, additionally, sample 86-
282 3875. In contrast, the AT genotyping assay identified the genotypes of these samples
283 correctly, as has been confirmed by *ompA* sequencing. Another panel of 64 field isolates from
284 France, Germany and Italy was added to the study and examined by PCR-RFLP and the AT
285 genotyping test (Table 1b). Twenty-six of the samples were additionally sequenced at the
286 *ompA* gene locus, and unique sequences have been submitted to the GenBank database (for
287 accession numbers, see Tables 1a and b).

288 Comparison between AT genotyping and PCR-RFLP for all 98 isolates tested revealed
289 agreement in 83 cases and discordant findings in 15 instances. Eleven samples were *ompA*
290 sequenced to verify the AT findings. The discrepancies were due to RFLP yielding
291 inconclusive patterns in 12 instances, whereas the AT assay was able to identify the genotype.
292 Contradiction between RFLP and AT in 3 cases, i.e. VG65/22/3, VG65/24/3, 3A-CL395, was
293 due to the inability of RFLP to recognise genotypes E/B and Mat116. AT results were
294 confirmed by *ompA* sequencing in each of these cases. Table 3 gives a summary of
295 comparative testing conducted on field isolates.

296 To demonstrate the possibility of direct AT genotyping from clinical material, 25 samples
297 (including two specificity controls) submitted to the national reference laboratories in
298 Germany and France were examined. The results in Table 2 show that *C. psittaci* DNA from

299 diverse specimens, such as faeces, swabs and bronchalveolar lavage was successfully typed
300 using the present assay. Two groups of samples originated from different outbreaks of
301 psittacosis in Germany in 2006, i.e. nos. 8-9 and 10-16, respectively. Interestingly, two
302 different subtypes of genotype A were identified in each outbreak, thus indicating co-
303 infection with two different strains.

304

305

306 **Discussion**

307 The specificity of the AT genotyping assay is based on the selection of probe sequences
308 recognising *C. psittaci* genotype-specific targets. To assure the maximum discriminatory
309 power, we introduced stringent wash steps at elevated temperature and low-salt concentration,
310 which efficiently removed sample targets having one or more mismatches. In these
311 conditions, the hybridisation temperature could be lowered to 50°C to ensure maximum yield
312 of duplex formation on the array and high sensitivity of the assay. In a previous study, we
313 demonstrated the assay's ability to discriminate single-nucleotide mismatches (Sachse et al.,
314 2008). It is, therefore, not surprising that samples from other chlamydial species failed to
315 produce hybridisation signals. In the light these findings, the present AT assay can also be
316 regarded as a confirmatory test for the species *C. psittaci*.

317 The fact that the presence of 2 ifu in a dilution was sufficient to generate a hybridisation
318 pattern and identify the genotype (Fig. 1) demonstrates the sensitivity of the assay and renders
319 it suitable for direct typing of clinical tissue samples, thus superseding the need for culturing
320 the respective strains in a routine diagnostic setting.

321 The *C. psittaci* strains included in the present study have been collected over a period of
322 more than 10 years in three different European countries and are merely reflecting the
323 inventory of the strain collections of the authors. Consequently, genotyping results shown in
324 Tables 1a and 1b cannot be used to unveil epidemiological interrelations. Nevertheless, the

325 predominance of genotype A (45 strains, 86.5 %) among 52 samples from birds of the order
326 *Psittaciformes* is in accordance with the general notion and literature data (Andersen, 2005).
327 Besides, genotype B was found in 4 strains (7.7 %) and E in 1 strain (1.9 %). In addition, 5
328 isolates from unknown avian species, likely also psittacines, were identified as genotype A.

329 The identification of strains belonging to the recently proposed provisional genotypes
330 YP84 and Mat116 (samples 86-10883 and 3A-CL395) indicates that, although rarely
331 encountered, these isolates may indeed be representatives of separate *ompA* genotypes, and
332 more strains of these types are probably occurring in nature.

333 Regarding host ranges, the isolates from *Columbiformes* (n=16) harboured three different
334 genotypes, i.e. B (n=8), E, (n=6), and A (n=2). The most frequently encountered type among
335 samples from *Anseriformes* (n=12) was E/B (n=10, 83.3 %), alongside single isolates of types
336 A and C. This seems to confirm suggestions from the literature that ducks are the preferred
337 host of E/B strains (Geens et al., 2005).

338 Furthermore, there were four bovine (17A-CL94, 24A-CL199, C5/98 and DC15), two
339 ovine (42A-CL273, C19/98) and one porcine (30A-CL207) isolates among the samples
340 examined. The assignment of these strains to the species *C. psittaci* is not only based on
341 *ompA* sequences and AT genotyping patterns of the present study, but is further supported by
342 partial 23S rRNA sequences and results of the AT species identification test (data not shown).
343 Interestingly, these non-avian isolates were all assigned to one of the classical avian
344 genotypes A, B or C, rather than WC or M56. It is unclear whether these are autochthonous
345 strains from non-avian hosts or whether they were originally transmitted from birds to the
346 mammals. In any case, these findings indicate that non-avian domestic animals deserve more
347 attention as possible reservoirs of *C. psittaci*, and detection of more strains from these sources
348 can be expected.

349 Although the choice of samples genotyped in the present study is probably not
350 representative of the natural diversity encountered in *C. psittaci* strains, the fact that all strains

351 could be assigned to an already described *ompA* genotype indicates a certain degree of
352 stability, despite the well-documented high variability of the *ompA* genomic locus.

353 In conclusion, it seems to be an intriguing prospect to combine the previously described
354 23S rDNA array for identification of chlamydial species (Sachse et al., 2005) with the present
355 microarray and add further panels of oligonucleotide probes that allow genotyping of other
356 chlamydiae. This would create a highly informative diagnostic tool enabling simultaneous
357 identification of species and genotypes in a single working day.

358

359 **Conflict of interest statement**

360 None of the authors (Konrad Sachse, Karine Laroucau, Fabien Vorimore, Simone Magnino,
361 Jens Feige, Wolfgang Müller, Steffen Kube, Helmut Hotzel, Evelyn Schubert, Peter Slickers,
362 and Ralf Ehrlich) has a financial or personal relationship with other people or organisations
363 that could inappropriately influence or bias the paper entitled "DNA microarray-based
364 genotyping of *Chlamydomphila psittaci* strains from culture and clinical samples".

365

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372 Implications".

373

374

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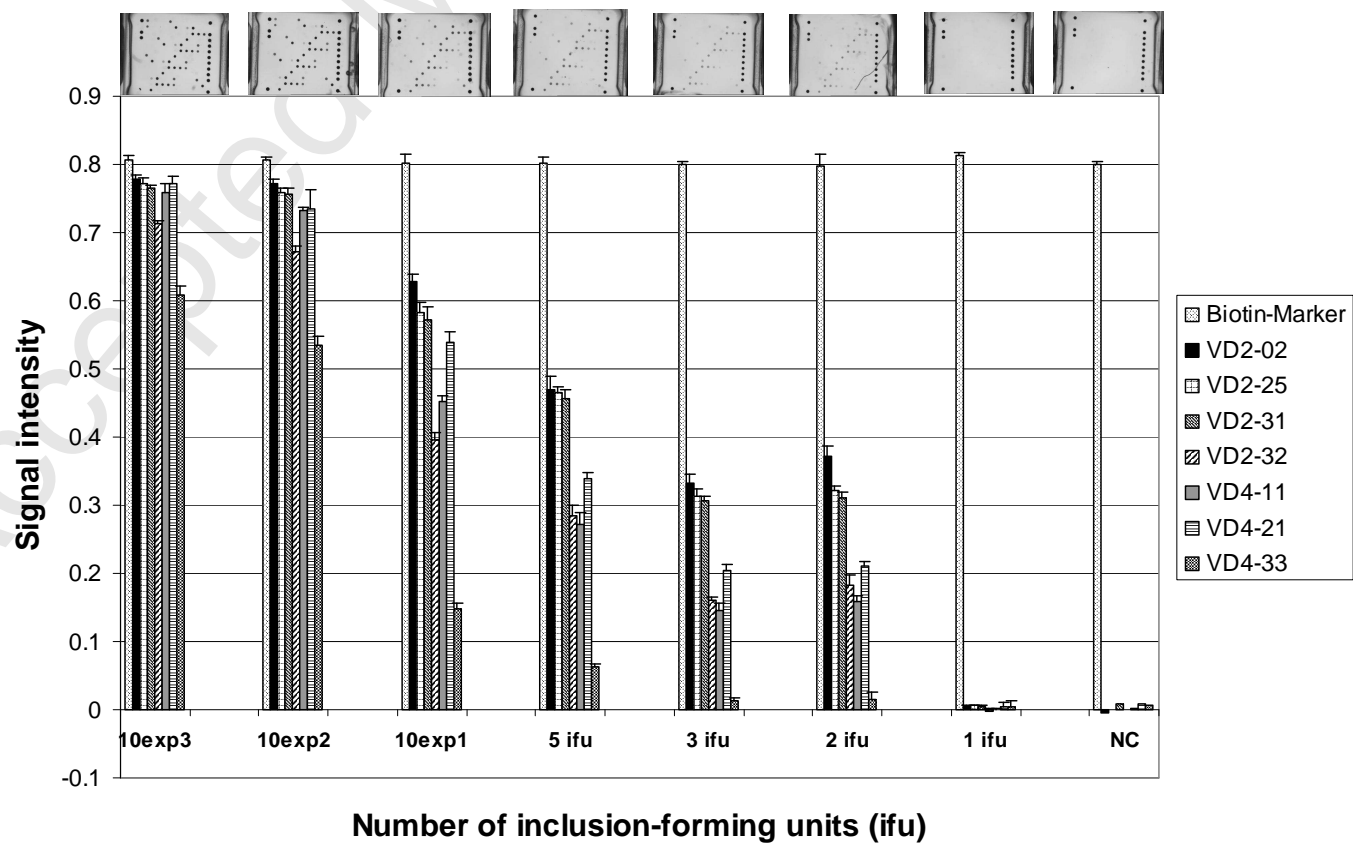
445 **Figure captions**

446

447 **Fig. 1** Illustration of the sensitivity of the AT genotyping assay. A dilution series of *C.*
448 *psittaci* strain DC11 (genotype A-VS1) was DNA extracted, amplified by biotinylation PCR
449 and subjected to ArrayTube hybridisation as described in Materials and Methods. The number
450 of inclusion-forming units contained in each sample is given on the abscissa. Signal
451 intensities of all perfect-match probes for genotype A and its subgroups, as well as the
452 internal staining control (biotin marker) are shown.

453

454 **Fig. 2** Genotype identification from AT hybridisation patterns using the pattern matching
455 program. The signal bars of the sample examined (blue) and those of the best matching
456 reference strain (green) were superimposed. **A.** Ideally matching patterns: human isolate 06-
457 1683 (exp. ID 4609), best match: genotype EB-859, MS=0.63, second best match: EB-E30,
458 MS=4.81, Delta MS=4.18. **B.** Well-matching patterns: duck isolate 06-871/2 (exp. ID 4610),
459 best match: genotype EB-859, MS=2.53, second best match: EB-E30, MS=4.53, Delta
460 MS=2.0. **C.** Moderately matching patterns: pigeon isolate 05-4036 (exp. ID 5336), best
461 match: genotype E, MS=5.03, second best match: EB-E30, MS=5.80, Delta MS=0.77.



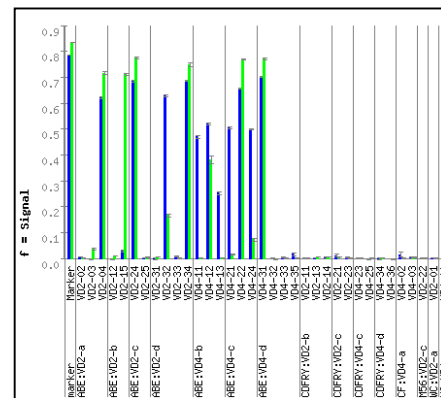
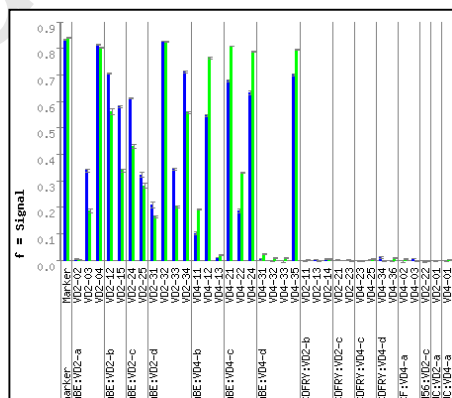
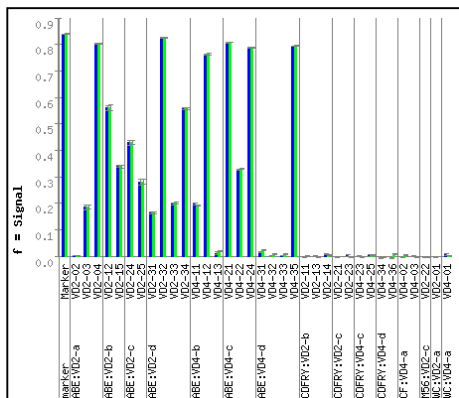


Table 1a: Results of AT genotyping of *Chlamydophila psittaci* isolates and comparison with serotyping using mAbs and RFLP analysis

No.	Sample ID	Host	mAb typing	RFLP analysis	AT Genotyping (MS*)	Delta MS**	ompA sequencing
1	84-0576	parrot	A	A	A-VS1 (1.85)	1.50	
2	84-8471	parrot	A	A	A-VS1 (1.44)	2.39	
3	84-9462	unknown	A	A	A-VS1 (2.94)	1.74	
4	85-11073	parrot	A	A	A-6BC (6.04)	0.93	
5	86-3026	canary	A	A	A-VS1 (1.44)	1.84	
6	86-3389	parakeet	A	A	A-VS1 (1.38)	2.12	
7	86-3875	parrot	A	x	A-6BC (7.49)	0.90	
8	86-10703	unknown	A	A	A-VS1 (5.38)	0.29	
9	86-10883	parrot	x	x	YP84 (1.31)	3.66	YP84 (EU682090)
10	86-14356	parrot	A	A	A-VS1 (1.13)	1.90	
11	87-13654	unknown	A	A	A-VS1 (1.42)	1.39	
12	88-2014	parakeet	A	A	A-6BC (5.02)	1.05	
13	88-5558	parrot	A	A	A-VS1 (1.20)	1.98	
14	88-5821	parakeet	A	A	A-VS1 (1.90)	2.21	
15	88-8795	parakeet	A	A	A-VS1 (1.04)	2.34	
16	89-2930	parakeet	A	A	A-VS1 (1.90)	2.22	
17	89-3753/2	budgerigar	A	A	A-VS1 (1.16)	1.95	
18	89-13210	pigeon	E	E	E (6.45)	0.13	E (EU682085)
19	90-00057	parakeet	A	A	A-VS1 (1.99)	1.85	
20	90-00475	parrot	A	A	A-VS1 (3.46)	1.92	
21	90-10445	parrot	A	A	A-6BC (4.54)	1.13	
22	90-11404	parrot	A	A	A-VS1 (3.66)	0.06	A
23	90-12617	pigeon	B	B	B (7.27)	0.28	
24	90-12937	parrot	A	A	A-VS1 (3.88)	0.30	
25	91-5983	parakeet	E	E	E (4.42)	1.42	
26	91-6047	parrot	A	A	A-VS1 (3.62)	0.34	
27	91-6568	dove	B	B	B (5.35)	1.03	
28	91-12516	pigeon	B	B	B (4.22)	1.95	
29	94-1133	parakeet	A	A	A-VS1 (2.79)	1.21	
30	94-2306	duck	x	x	EB-859 (1.42)	3.73	E/B
31	94-10673	parakeet	A	A	A-VS1 (1.59)	2.00	
32	95-1334	parrot	A	A	A-VS1 (3.28)	0.71	
33	95-7496	parrot	A	A	A-6BC (5.52)	1.04	
34	96-12451	parrot	A	A	A-6BC (4.32)	0.76	

Table 1b: Results of AT genotyping of *Chlamydophila psittaci* isolates and comparison with RFLP analysis

No.	Sample ID	Host	RFLP analysis	AT Genotyping (MS*)	Delta MS**	<i>ompA</i> sequencing (GenBank acc. no.)
35	84-8344	pigeon	A	A-6BC (7.74)	0.75	
36	85-12098	parrot	A	A-6BC (5.68)	0.79	
37	86-00191	parrot	A	A-VS1 (1.24)	2.11	
38	88-0544	parakeet	A	A-VS1 (1.57)	2.28	
39	97-3176	parrot	A	A-VS1 (2.45)	1.48	
40	97-4777	parakeet	A	A-VS1 (1.32)	2.41	
41	97-4779	parakeet	A	A-VS1 (1.76)	2.28	
42	97-6475	parakeet	A	A-VS1 (2.57)	1.49	
43	98-3196/1	guinea pig	x	no signal		<i>C. caviae</i>
44	98-3318/1	guinea pig	x	no signal		<i>C. caviae</i>
45	98-7627	parrot	A	A-6BC (5.26)	0.78	
46	99-182	parrot	A	A-6BC (6.84)	0.79	
47	99-8157	parrot	A	A-6BC (6.61)	0.75	
48	99-923	parakeet	A	A-6BC (8.93)	0.90	
49	00-3593	parrot	x	A-VS1 (4.88)	0.28	
50	02-8282	parakeet	A	A-VS1 (3.83)	0.75	
51	03-3353/1	exotic bird	A	A-VS1 (2.65)	1.53	
52	05-941	budgerigar	A	A-VS1 (3.67)	0.93	
53	05-949	budgerigar	A	A-6BC (4.40)	0.84	
54	05-4036	pigeon	E	E (5.03)	0.77	
55	05-4098	parrot	A	A-6BC (4.89)	0.45	
56	05-4325/8	duck	x	EB-859 (0.81)	3.89	
57	05-4461/2	duck	C	C (3.19)	1.40	C
58	06-1683	human	x	EB-859 (0.63)	4.18	EB
59	06-372	parakeet	A	A-VS1 (3.10)	0.96	
60	06-852	exotic bird	A	A-VS1 (3.02)	1.30	
61	06-859/1	duck	x	EB-859 (0.92)	5.40	EB (EU159263)
62	06-871/2	duck	x	EB-859 (2.53)	2.00	EB
63	06-881	duck	x	EB-859 (9.90)	2.35	
64	06-885/14	duck	x	EB-859 (7.12)	2.42	EB
65	06-886/9	duck	x	EB-859 (8.24)	2.08	EB
66	06-889/3	duck	x	EB-859 (8.01)	2.34	EB
67	3A-CL395	budgerigar	B	Mat116 (0.84)	2.19	Mat116 (EU682087)
68	4A-CL24	budgerigar	A	A-VS1 (5.49)	0.67	
69	5A-CL229	pigeon	B	B (6.34)	0.58	
70	12A-CL330	budgerigar	B	B (5.68)	0.76	
71	15A-CL72/1	budgerigar	B	B (6.95)	0.49	
72	16A-CL72/2	budgerigar	B	B (6.60)	0.54	
73	17A-CL94	cattle	B	B (6.75)	0.44	
74	21A-CL181	budgerigar	A	A-VS1 (2.10)	1.00	
75	23A-CL191	pheasant	A	A-VS1 (1.58)	1.63	
76	24A-CL199	cattle	A	A-VS1 (1.43)	1.84	
77	30A-CL207	pig	A	A-6BC (7.00)	0.86	
78	36A-CL219	pigeon	A	A-VS1 (1.44)	1.79	

79	41A-CL259	budgerigar	B	B (6.55)	0.59	
80	42A-CL273	sheep	x	no signal		<i>C. abortus</i>
81	C1/97	sheep	C	C (2.78)	3.37	C (EU682086)
82	C5/98	calf	A	A-VS1 (1.22)	1.52	A (EU682088)
83	C19/98	sheep	A	A-VS1 (1.06)	2.23	A
84	DC15	cattle	A	A-VS1 (3.02)	1.35	A
85	DC28	parrot	A	A-VS1 (1.50)	1.13	A
86	DC29	parrot	A	A-VS1 (1.58)	1.17	A
87	DC32	pigeon	B	B (4.53)	1.56	B
88	VG 65/8/4	chicken	A	A-VS1 (1.57)	1.85	A (EU019088)
89	VG 65/22/3	duck	E	EB-E30 (2.14)	1.94	E/B (EU019091)
90	VG 65/23/3	duck	A	A-VS1 (4.79)	0.22	A (EU019089)
91	VG 65/24/3	duck	E	EB-E30 (1.73)	2.31	E/B (EU019092)
92	7339/4	pigeon	E	E (5.48)	0.37	
93	7339/5	pigeon	E	E (5.51)	0.76	
94	7341/4	pigeon	E	E (5.38)	0.57	
95	7341/5	pigeon	E	E (5.18)	0.58	
96	2806/43	pigeon	B	B (4.39)	1.24	B
97	2806/48	pigeon	B	B (4.98)	0.50	B (EU682089)
98	2437/2	pigeon	B	B (4.21)	0.97	B

x Inconclusive result

* Matching score (see Materials and Methods)

** Arithmetic difference between best and second best match

Table 2: Results of direct AT genotyping of *Chlamydophila psittaci* strains from clinical samples

No.	Sample ID	Origin	AT Genotyping (MS*)	delta MS**
1	06-1440/7	swan, cloacal swab	B (4.42) ^a	1.93
2	06-1440/1	turtle dove	E (5.42) ^a	0.45
3	06-1384/54 Ec 83	turtle-dove, cloacal swab	E (4.23) ^{a,b}	1.09
4	M3	pigeon	E (4.51) ^a	1.81
5	M4	pigeon	E (5.15) ^a	1.39
6	M7	pigeon	B (5.87) ^a	0.88
7	05-6688-T2	chicken, cloacal swab	EB-E30 ^b (6.32) (EU019093)	1.13
8	06-G399	psittacine flock, faeces	A-VS1 (4.90)	0.20
9	06-G400	psittacine flock, faeces	A-6BC (4.84)	0.51
10	06-G427	budgerigar, faeces	A-6BC (8.81)	0.92
11	06-G428	budgerigar, faeces	A-VS1 (2.81)	1.66
12	06-G429	cockatiel, faeces	A-VS1 (3.13)	1.19
13	06-G430	psittacine flock, faeces	A-6BC (8.95)	0.79
14	06-G432	psittacine flock, faeces	A-VS1 (4.06)	1.23
15	06-G436	psittacine flock, faeces	A-VS1 (4.15)	0.93
16	06-G437	psittacine flock, faeces	A-VS1 (4.11)	0.43
17	06-1440/35	pigeon, cloacal swab	B (4.58)	1.25
18	07-G156	pig, conjunctival swab	A-6BC (6.79)	0.78
19	07-G237	psittacine flock, faeces	A-6BC (7.57)	0.79
20	07-G109	human, bronchoalveolar lavage	A-VS1 (5.11)	0.74
21	07-G096	calf, brain tissue	A-6BC (5.82)	0.12
22	07-G753	psittacine flock, faeces	A-VS1 (3.83)	0.33
23	07-G764	psittacine flock, faeces	A-VS1 (2.24)	0.97
24	07-525_1cc	sheep, vaginal swab	no signal ^c	
25	07-525_2cc	sheep, vaginal swab	no signal ^c	

* and ** see Legend to Table 1

^a confirmed by RFLP analysis

^b confirmed by sequencing of *ompA* gene

^c identified as *C. abortus* using the AT microarray test for chlamydial species identification (Sachse et al., 2005)

Table 3: Comparison of test results for field isolates of *C. psittaci*

Tests	Total (n)	Concordant results	Discordant results
AT vs. serotyping	34	32	2*
AT vs. serotyping vs. RFLP	34	31	3**
AT vs. RFLP	98	83	15***
AT vs. <i>ompA</i> sequencing	26	26	0

* serological reaction inconclusive, but AT positive (no. 86-10833, 94-2306), AT findings confirmed by *ompA* sequence

** inconclusive RFLP pattern (no. 86-3875, 86-10833, 94-2306)

*** samples 86-3875, 86-10833^{seq}, 94-2306^{seq}, 00-3593, 05-4325/8, 06-1683^{seq}, 06-859/1^{seq}, 06-871/2^{seq}, 06-881, 06-885/14^{seq}, 06-886/9^{seq}, 06-889/3^{seq}, 3A-CL395^{seq}, VG65/22/3^{seq}, VG65/24/3^{seq} (^{seq} AT findings confirmed by *ompA* sequence)