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

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ABSTRACT

The avian and human pathogen *Chlamydophila* (*C.*) *psittaci* represents a genetically heterogeneous species. To facilitate epidemiological surveys, more rapid yet highly specific molecular tests are needed. Currently used typing methods, i.e. serotyping and PCR-RFLP, have only limited sensitivity and are incapable of covering the wide spectrum of naturally occurring types of *C. psittaci* strains. In the present study, a new DNA microarray assay based on the ArrayTube® (AT) technology was used to genotype *C. psittaci* in 98 isolates and 23 clinical tissue samples. The present array carries 35 oligonucleotide probes derived from variable domains 2 and 4 of the *omp*A gene. The assay proved highly sensitive, allowing correct genotyping of DNA from 2 inclusion-forming units.

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

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1. Introduction

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

The infection in birds also lacks specific symptoms. Instead, respiratory signs, mucopurulent nasal and ocular discharge, dyspnoea, apathy, as well as wasting and cachexia may be observed (Gerlach, 1999; Grimes, 1994;

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Vanrompay et al., 1995). The course and severity of disease are dependent on virulence properties of the particular *C. psittaci* strain, the immune status of the host, as well as the transmission route and infectious dose. Besides, latent infection seems to be widespread, which leaves the birds largely symptomless, but creates a permanent reservoir of the pathogen. Chronicity as a general feature of chlamydial infections (Hammerschlag, 2000) was also suggested to play a role in both birds and humans (Grimes and Wyrick, 1991).

According to the recently revised taxonomy of the family *Chlamydiaceae* (Everett et al., 1999), the new species *C. psittaci* was designed to retain only avian-like strains. Nevertheless, it remains a relatively heterogeneous taxon in terms of host range, virulence and also genome sequence variation. On the basis of immunofluorescent reaction with a panel of specific monoclonal antibodies (mAbs) recognising epitopes of the major outer membrane protein (MOMP), the avian strains of *C. psittaci* were classified into serovars A, B, C, D, E, and F, with some degree of host specificity ascribed to each serovar (Andersen, 1991; Vanrompay et al., 1993; Andersen, 1997). Subsequently, Sayada et al. (1995) suggested the introduction of genotypes defined by PCR amplification of the *omp*A gene combined with restriction enzyme analysis, i.e. restriction fragment length polymorphism (PCR-RFLP). Vanrompay et al. (1997) compared the data from serotyping and PCR-RFLP and concluded that each serovar could be assigned to an equivalent genotype. Thus, nine different genotypes have been generally accepted to date, seven of which are thought to predominantly occur in a particular order or class of *Aves* and two in non-avian hosts, i.e. genotype A in psittacine birds, B in pigeons, C in ducks and geese, D in turkeys, E in pigeons, ducks and others, F in parakeets, WC in cattle, and M56 in rodents. In addition, Geens et al. (2005) suggested the introduction of genotype E/B to represent a group of isolates from ducks, turkeys and pigeons. Most of the avian genotypes have also been identified sporadically in isolates from cases of zoonotic transmission to humans, particularly A, B and E/B (Heddema et al., 2006; Vanrompay et al., 2007; Gaede et al., 2008).

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

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

2. Materials and methods

2.1. Strains and diagnostic samples

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

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

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

2.2. DNA microarray design

The present array was described elsewhere (Sachse et al., 2008). Briefly, 35 oligonucleotide probes of 22–30 nt derived from VD2 and VD4 of the *C. psittaci omp*A gene were spotted onto modified glass chips and assembled into ATs. Biotinylated probes were added to monitor the

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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[a])	Delta MS[b]	*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	

x: Inconclusive result.
[a] Matching score (see Section 2).
[b] Arithmetic difference between best and second best match.

staining reaction, mark the corners of the array and facilitate normalisation of signal intensities. Each genotype probe was spotted 4-fold, the controls 15-fold, thus bringing the total number of spots on the array to 155.

2.3. DNA extraction

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

2.4. Biotinylation PCR

Target DNA was amplified and biotinylated by duplex PCR using two pairs of primers covering all VDs of the *ompA* genes. Primer pair VD1-f (5′-ACT ACG GAG ATT ATG TTT TCG ATC GTG T-3′) and VD2-r (5′-Biotin-CGT GCA CCY ACG CTC CAA GA-3′) gives rise to a 418-bp product which includes VD1 and VD2, whereas primers 201CHOMP (5′-GGI GCW GMI TTC CAA TAY GCI CAR TC-3′) and ompA-rev (5′-Biotin-TCC TTA GAA TCT GAA TTG AGC-3′) define a product of 570 bp covering VD3 and VD4. The following PCR cycling conditions were used: initial denaturation at 96 °C for 60 s, 40 cycles of 94 °C for 30 s, 50 °C for 60 s, 72 °C for 30 s, and final elongation at 72 °C for 240 s.

Each 20-μl reaction contained 10 pmol of the first and 20 pmol of the second primer pair, as well as 1 mM each of dNTP mix, 1 U *Taq* DNA polymerase (Fermentas, St. Leon-Roth, Germany), 2 μl 10 × PCR buffer (Fermentas), 1 μl 50 mM $MgCl_2$, and 1 μl of the DNA template.

2.5. DNA microarray hybridisation

The AT vessel was conditioned by washing with 500 μl each of deionised water and hybridisation buffer (0.25 M Na_2HPO_4 + 1 mM EDTA + 4.5% SDS in 1 × SSC, pH 7.25) at 50 °C for 5 min. All incubations were carried out upon slight shaking (550 rpm) on a heatable horizontal tube shaker (Thermomixer comfort, Eppendorf, Cologne, Germany). For denaturation, 1 μl of the 5′-biotinylated PCR product was diluted with 99 μl hybridisation buffer in a separate tube, heated at 95 °C for 5 min and put on ice for 30 s. After transfer into the AT, hybridisation was allowed to proceed at 50 °C for 60 min. The supernatant was then discarded, and the array was washed consecutively with 500 μl 2 × SSC/0.01% Triton X-100 (50 °C, 5 min), 500 μl 2 × SSC (50 °C, 5 min), 500 μl 0.2 × SSC (50 °C, 5 min), and 500 μl 0.2 × SSC (35 °C, 5 min). Vacant binding sites of the microarray were blocked by incubation with a 2% solution of Blocking Reagent (Roche) in hybridisation buffer at 30 °C for 15 min. Subsequently, the AT was incubated with

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

No.	Sample ID	Host	RFLP analysis	AT Genotyping (MS[a])	Delta MS[b]	*omp*A 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		*C. caviae*
44	98-3318/1	Guinea pig	x	No signal		*C. caviae*
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		*C. abortus*
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.
[a] Matching score (see Section 2).
[b] 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[a])	Delta MS[b]
1	06-1440/7	Swan, cloacal swab	B (4.42)[c]	1.93
2	06-1440/1	Turtle dove	E (5.42)[c]	0.45
3	06-1384/54 Ec 83	Turtle-dove, cloacal swab	E (4.23)[c,d]	1.09
4	M3	Pigeon	E (4.51)[c]	1.81
5	M4	Pigeon	E (5.15)[c]	1.39
6	M7	Pigeon	B (5.87)[c]	0.88
7	05-6688-T2	Chicken, cloacal swab	EB-E30[d] (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[e]	
25	07-525_2cc	Sheep, vaginal swab	No signal[e]	

[a] Matching score (see Section 2).
[b] Arithmetic difference between best and second best match.
[c] Confirmed by RFLP analysis.
[d] Confirmed by sequencing of *omp*A gene.
[e] Identified as *C. abortus* using the AT microarray test for chlamydial species identification (Sachse et al., 2005).

100 μl of a 1:5000 dilution (0.5 μg/ml) of streptavidin-peroxidase polymer (Sigma–Aldrich, Taufkirchen, Germany) at 30 °C for 15 min followed by three wash steps, i.e. 500 μl 2 × SSC/0.01% Triton X-100 (30 °C, 5 min), 500 μl 2 × SSC (20 °C, 5 min), 500 μl 0.2 × SSC (20 °C, 5 min). Finally, 100 μl of the peroxidase substrate Seramun Grün (Seramun Diagnostica, Heidesee, Germany) were added. Hybridisation signals were measured 5 min later using an ATR-01 transmission reader (Clondiag Chip Technologies, Jena, Germany) and processed using the Iconoclust software, version 2.3 (Clondiag) in combination with the Partisan Array LIMS system which provided the specific algorithms for this assay.

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

$$NI = 1 - \left(\frac{M}{BG}\right)$$

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

2.6. Processing of microarray hybridisation patterns using the pattern match algorithm

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

2.7. PCR-RFLP genotyping

The *omp*A locus was amplified from extracted DNA by PCR using primers CTU (5′-ATG AAA AAA CTC TTG AAA TCG G-3′)/CTL [5′-CAAGAT TTTCTA GA(T/C) TTCAT-(C/T)TTGTT-3′], and the product was subjected to restriction enzyme digestion using AluI and/or MboII as described by Sayada et al. (1995). Following agarose gel electrophoresis, cleavage patterns were compared with those of the reference strains for genotypes A, B, C, D, E, and F.

2.8. Sequencing of the ompA gene

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

Products were electrophoresed in 1% agarose gels, the specific bands of approximately 1200 bp were excised with a scalpel, and DNA was extracted using the

innuPREP Gel Extraction Kit (Analytik Jena, Jena, Germany). DNA sequencing was carried out by cycle sequencing using the BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Darmstadt, Germany) according to the instructions of the manufacturer. The following primers were used: CTU, VD1-f, VD2-r (not biotinylated), CHLAGEN-1 (5′-CGG CTG CAT TCA ACT TGG-3′), 201CHOMP, and ompA-rev (not biotinylated). Nucleotide sequences were determined on an ABI Prism 310 Genetic Analyzer (Applied Biosystems).

2.9. ompA sequence alignment

To determine the genotype from *omp*A sequencing data, the sequence of a sample was added to a multiple *omp*A sequence alignment of *C. psittaci* strains representing all currently recognised genotypes, including the recently suggested subgroups, and representatives of six new provisional genotypes denoted hereafter with an asterisk (Sachse et al., 2008). The following strains and sequences were included: strain VS1 (genotype A-VS1, GenBank acc. no. AF269281.1), strain 6BC (A-6BC, X56980.1), strain 84-55 (A-8455, Y16561.1), strain CP3 (B, AF269265.1), strain GR9 (C, L25436.1), strain NJ1 (D-NJ1, AF269266.1), strain 9N (D-9N, EF375557.1), strain CPMN (E, X12647.1), strain WS/RT/E30 (EB-E30, AY762613.1), strain 06-859/1 (EB-859, EU159263.1), strain KKCP-1 (EB-KKCP, AB284062.1), strain VS225 (F, AF269259.1), strain M56 (M56, AF269268.1), strain WC (WC, AF269269.1), strain 1 V (1V*, EF028916.1), strain 6N (6N*, EF197820.1), strain Mat116 (Mat116*, AB284058.1), strain R54 (R54*, AJ243525.1), strain Daruma-1981 (YP84*, AB284065.1), strain CPX0308 (CPX0308*, AB284064.1).

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

2.10. Serotyping

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

3. Results

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

The specificity of the genotyping assay was checked by examining representatives of all *Chlamydiaceae* species other than *C. psittaci*. No specific duplex PCR products were seen and no hybridisation signals were detected with these species (data not shown). Among the present panel of samples, there were also two isolates that proved to be *C. caviae* and another isolate and two swabs that contained *C. abortus*, but all of them were negative in the AT genotyping assay (see Tables 1a, 1b and 2).

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

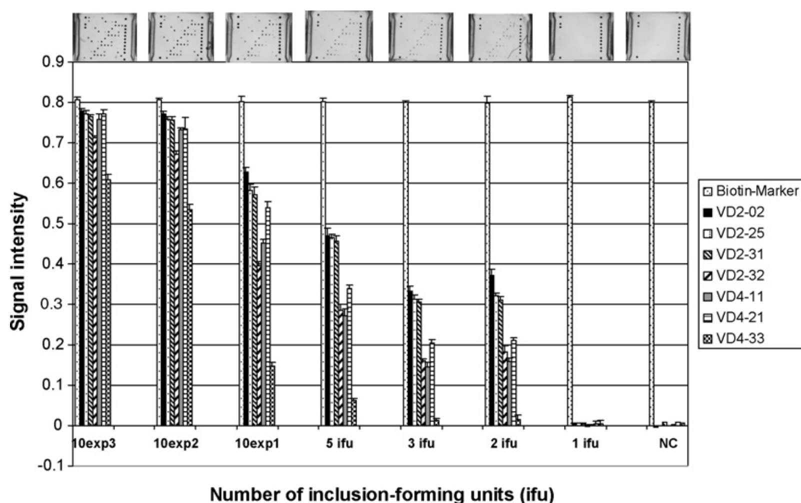

Fig. 1. Illustration of the sensitivity of the AT genotyping assay. A dilution series of *C. psittaci* strain DC11 (genotype A-VS1) was DNA extracted, amplified by biotinylation PCR and subjected to ArrayTube hybridisation as described in Section 2. The number of inclusion-forming units contained in each sample is given on the abscissa. Signal intensities of all perfect-match probes for genotype A and its subgroups, as well as the internal staining control (biotin marker) are shown.

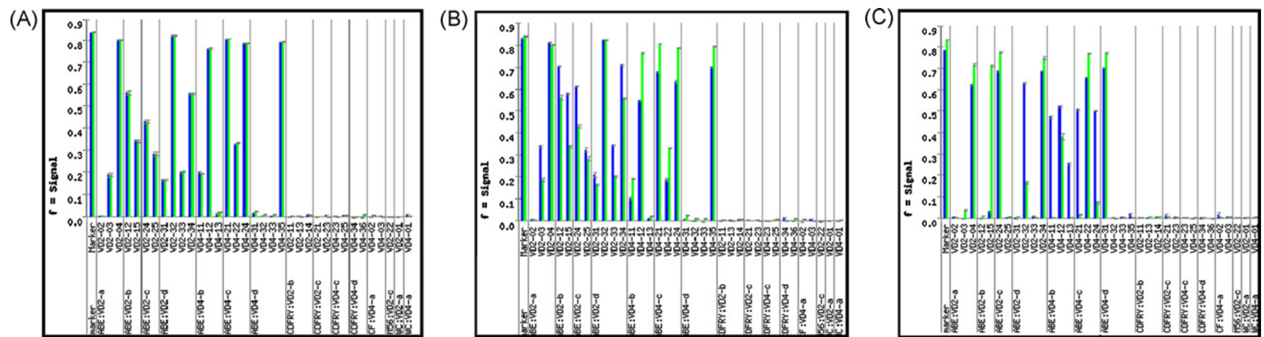

Fig. 2. Genotype identification from AT hybridisation patterns using the pattern matching program. The signal bars of the sample examined (blue) and those of the best matching reference strain (green) were superimposed. (A) Ideally matching patterns: human isolate 06-1683 (exp. ID 4609), best match: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

The identity of the genotype present in a given sample can be determined manually from the hybridisation pattern by assigning each signal to the respective probe. In principle, the probes yielding the most intense signals are pivotal because they represent perfect matches with the target under stringent hybridisation conditions. The specificity of all probes was given in the match table (Sachse et al., 2008). This manual assignment, however, can be realised only with a limited number of samples. For large series of experiments, we developed a pattern match algorithm, which is based on comparison of the measured sample hybridisation pattern with a set of 13 reference strain patterns. The degree of similarity between sample and reference patterns is expressed as the matching score (MS). The lower its value the higher is the similarity to the hybridisation pattern of the genotype reference strain, with the ideal match yielding MS = 0. Based on the experience from the present study, any scores higher than 10 were regarded as inconclusive. An important parameter indicating the reliability of a given genotyping result is the "Delta MS", which represents the arithmetic difference between best and second best match. A Delta MS value of 0.4 and higher was regarded as reflecting a sufficient degree of distinction between best and second best matches. In the case of values between 0.2 and 0.39, manual assignment according to the match table was additionally conducted, which led to confirmation of the calculated findings in each instance. Delta MS values below 0.2 were regarded as ambiguous, requiring *omp*A sequencing as an independent test. Fig. 2 illustrates the pattern match approach for three samples having different matching scores.

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

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

To demonstrate the possibility of direct AT genotyping from clinical material, 25 samples (including two specificity controls) submitted to the national reference laboratories in Germany and France were examined. The results in Table 2 show that *C. psittaci* DNA from diverse specimens, such as faeces, swabs and bronchalveolar lavage was successfully typed using the present assay. Two

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[a]
AT vs. serotyping vs. RFLP	34	31	3[b]
AT vs. RFLP	98	83	15[c]
AT vs. *omp*A sequencing	26	26	0

[a] Serological reaction inconclusive, but AT positive (nos. 86-10833, 94-2306), AT findings confirmed by *omp*A sequence.
[b] Inconclusive RFLP pattern (nos. 86-3875, 86-10833, 94-2306).
[c] 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 *omp*A sequence).

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groups of samples originated from different outbreaks of psittacosis in Germany in 2006, i.e. nos. 8–9 and 10–16, respectively. Interestingly, two different subtypes of genotype A were identified in each outbreak, thus indicating co-infection with two different strains.

4. Discussion

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

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

The *C. psittaci* strains included in the present study have been collected over a period of more than 10 years in three different European countries and are merely reflecting the inventory of the strain collections of the authors. Consequently, genotyping results shown in Tables 1a and 1b cannot be used to unveil epidemiological interrelations. Nevertheless, the predominance of genotype A (45 strains, 86.5%) among 52 samples from birds of the order *Psittaciformes* is in accordance with the general notion and literature data (Andersen, 2005). Besides, genotype B was found in 4 strains (7.7%) and E in 1 strain (1.9%). In addition, 5 isolates from unknown avian species, likely also psittacines, were identified as genotype A.

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

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

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

Although the choice of samples genotyped in the present study is probably not representative of the natural diversity encountered in *C. psittaci* strains, the fact that all strains could be assigned to an already described *omp*A genotype indicates a certain degree of stability, despite the well-documented high variability of the *omp*A genomic locus.

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

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Conflict of interest

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

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