Restriction pattern of the major outer-membrane protein gene provides evidence for a homogeneous invasive group among ruminant isolates of *Chlamydia psittaci*

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Thirty-six ruminant isolates of *Chlamydia psittaci*, previously classified as invasive or non-invasive in a mouse model of virulence, were compared by analysing *AluI* restriction patterns of the major outer-membrane protein (MOMP) gene after DNA amplification by the polymerase chain reaction. The 24 invasive isolates, although from various origins, all belonged to serotype 1 and represented a strictly homogeneous group sharing a specific MOMP-gene restriction pattern that was not observed in the non-invasive strains. On the other hand, the 12 non-invasive strains, although all belonging to serotype 2, constituted a heterogeneous group with eight distinct MOMP-gene restriction patterns. However, all eight patterns shared a 180 bp fragment or the corresponding restricted fragments of 110 and 70 bp. MOMP-gene restriction patterns also clearly distinguished the ruminant strains from an avian *C. psittaci* isolate, a *C. pneumoniae* isolate and two *C. trachomatis* isolates which were studied for comparison. The homogeneous character of the invasive *C. psittaci* strains argues strongly for their genetic relatedness. Our results illustrate the usefulness of the MOMP-gene restriction mapping in typing chlamydiae.

Introduction

The genus *Chlamydia* is divided into three species, *Chlamydia trachomatis*, *C. psittaci* and *C. pneumoniae* (Moulder *et al.*, 1984; Grayston *et al.*, 1989). Of these, *C. trachomatis* and *C. pneumoniae* are well-characterized species. However, *C. psittaci* represents a heterogeneous group including isolates of both mammalian and avian origin. Several attempts have been made at differentiating members of the species *C. psittaci*. DNA–DNA reassociation studies (Kingsbury & Weiss, 1968; Cox *et al.*, 1988; Fukushi & Hirai, 1989) indicate substantial differences within the *C. psittaci* group and suggest the existence of at least four separate genospecies. Isolates studied included those of avian origin and ovine abortion strains, feline pneumonitis isolates, isolates from ovine polyarthritis and cattle infections, and a guinea pig inclusion conjunctivitis isolate. Other genotypic studies, as well as phenotypic data, basically agree with this classification and provide an enhanced discrimination of

epidemiological interest. Genotypic characterization has included plasmid analysis (McClenaghan *et al.*, 1988), restriction endonuclease analysis of total DNA or of specific DNA regions (McClenaghan *et al.*, 1984; Campbell *et al.*, 1987; Timms *et al.*, 1988; Girges *et al.*, 1988; Fukushi & Hirai, 1989; Andersen, 1991a) and sequencing of the major outer-membrane protein (MOMP) gene (Pickett *et al.*, 1988; Herring *et al.*, 1989; Zhang *et al.*, 1989). Phenotypic data were obtained by biotyping (Spears & Storz, 1979; Allan & Pearce, 1983), polypeptide profile analysis (McClenaghan *et al.*, 1991) and serotyping (Schachter *et al.*, 1974, 1975; Eb & Orfila, 1982; Perez-Martinez & Storz, 1985; Eb *et al.*, 1986; Fukushi *et al.*, 1987; Fukushi & Hirai, 1988; Andersen & Van Deusen, 1988; Andersen, 1991b).

In ruminants, *C. psittaci* is associated with a wide range of infections, including abortion, epididymitis, pneumonia, conjunctivitis, polyarthritis, encephalitis, enteritis and clinically inapparent, intestinal infection. Abortions due to chlamydial infection have major economic implications in ruminant breeding. In order to establish the role of chlamydiae isolated from the faeces

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Abbreviation: MOMP, major outer-membrane protein.

of healthy ruminants in the epidemiology of abortive chlamydiosis, we previously reported a murine model to study virulence. This model (Rodolakis *et al.*, 1989) distinguishes two categories of strains: invasive strains, most often isolated from symptomatic infections, induce splenomegaly and splenic infection following subcutaneous inoculation in the mouse footpad, whereas non-invasive strains, mostly of intestinal origin, neither induce a change in spleen weight nor a splenic infection. This difference of invasiveness in the mouse model is associated with dissimilar protein patterns as obtained by PAGE (Buzoni-Gatel *et al.*, 1989).

Recently, the polymerase chain reaction (PCR) has been applied to the detection and differentiation of the three *Chlamydia* species by using primers specific for the MOMP gene (Dutilh *et al.*, 1989; Holland *et al.*, 1990). Furthermore, preliminary communications have reported intra-species characterization of *C. trachomatis* (Frost *et al.*, 1990) and *C. psittaci* (Herring *et al.*, 1990) by

AluI restriction mapping of the PCR-amplified MOMP gene.

In this paper, we report on the *AluI* restriction mapping of the MOMP gene to study 36 ruminant *C. psittaci* isolates previously characterized in the murine model for virulence. An avian *C. psittaci*, a *C. pneumoniae* isolate and two *C. trachomatis* isolates were also studied for comparison.

Methods

Chlamydial strains. We studied 36 ruminant isolates of *C. psittaci* including the invasive and non-invasive reference strains AB7 and iB1, respectively. Among them, 24 strains were invasive in our mouse model and 12 were non-invasive. One avian *C. psittaci*, a *C. pneumoniae* isolate and two *C. trachomatis* isolates were included for comparison. The origins of each isolate, together with other relevant information, are summarized in Table 1.

Chlamydial strains were grown in mycoplasma-free McCoy cells in plastic flasks (150 ml); 4 and 12 flasks were used for invasive and non-

Table 1. Characteristics of the chlamydial strains used in this study

Strain* and associated disease	Geographical origin	Invasive-ness for mice†	Serotype‡	MOMP-gene restriction pattern
<i>C. psittaci</i>				
Ovine abortion				
AB7	France	+	1	A
AB4	France	+	1	A
AB6	France	+	1	A
AB8	France	+	1	A
AB9	France	+	1	A
AB10	France	—	2	B
AB13	France	+	1	A
AB15	France	+	1	A
A22 ^a	Scotland	+	1	A
S26/3 ^a	Scotland	+	1	A
H574 ^a	Scotland	+	1	A
Q18 ^b	France	+	1	A
PS22 ^c	USA	+	1	A
Bovine abortion				
AV1	France	+	1	A
Caprine abortion				
AC1	France	+	1	A
AC2	France	+	1	A
AC3	France	+	1	A
Thermo-sensitive mutant				
1B	Mutant of AB7	+	1	A
1H	Mutant of AB7	+	1	A
Ovine arthritis				
1H77 ^a	Scotland	+	1	A
LW-679 ^d	USA	—	2	B
Ovine conjunctivitis				
OC1	France	+	1	A
824 ^a	Scotland	—	2	C
Ovine pneumonia				
109-75 ^e	France?	+	1	A
Ovine epididymitis				
VB1	France	+	1	A
Intestinal (faeces of healthy sheep)				
iB1	France	—	2	D
iB3	France	—	2	E
iB4	France	—	2	F
iB5	France	—	2	G
R69 ^f	Northern Ireland	—	2	H
W73 ^f	Northern Ireland	—	2	H
MO-907 ^d	USA	+	1	A
Intestinal (faeces of healthy goats)				
iC1	France	+	1	A
iC2	France	—	2	I
iC3	France	—	2	I
iC4	France	—	2	I
Pigeon systemic infection				
Loth ^g	Netherlands		11	J
<i>C. trachomatis</i>				
Mouse Biovar				
MoPn ATCC	USA			K
VR 123				
LGV Biovar				
L2/434/Bu ^h	USA			L
<i>C. pneumoniae</i>				
IOL-207 ⁱ	Iran			M

* Sources of strains were as follows: *a*, I. D. Aitken, Moredun Research Institute, Edinburgh, Scotland, UK; *b*, P. Giroud, Institut Pasteur, Paris, France; *c*, J. Schachter, University of California, San Francisco, USA; *d*, J. Storz (Spears & Storz, 1979); *e*, P. Russo, Laboratoire de Pathologie des Petits Ruminants Sophia Antipolis, France; *f*, M. S. McNulty Veterinary Research Laboratories, Belfast, Northern Ireland, UK; *g*, F. Dekking, Hygiene Instituut, Amsterdam, The Netherlands; *h*, S. P. Wang, University of Washington, Seattle, USA; *i*, J. D. Threharne, Institute of Ophthalmology, London, UK.

† Rodolakis *et al.* (1989).

‡ Determined as in Eb *et al.* (1986).

invasive strains, respectively. Cell monolayers were infected with about 10^7 plaque forming units (p.f.u.) (Banks *et al.*, 1970) of yolk-sac-propagated chlamydiae and incubated at 37 °C; 7 d after inoculation the chlamydia-containing medium was gently collected and replaced with fresh medium. At 2 weeks, cells were detached from the plastic, disrupted by vigorous shaking with glass-beads and harvested with the medium. After both harvests, chlamydiae were immediately pelleted at 15000 *g* for 2 h at 4 °C, suspended in 1.5 ml phosphate/glutamine/sucrose buffer, pH 7.6 (Madeley, 1977) to an approximate concentration of 10^8 p.f.u. ml⁻¹ and stored at -70 °C.

PCR analysis of the MOMP gene. DNA was obtained by lysis of 1 µl of the chlamydial preparation in 250 µl of buffer containing 50 mM-KCl, 10 mM-Tris/HCl (pH 8.3), 2.5 mM-MgCl₂, 0.45% Nonidet P-40, 0.45% Tween 20 and 60 µg proteinase K ml⁻¹. Positive displacement pipettes were used to minimize sample carry-over and samples were prepared in an externally vented hood used only for this purpose. The DNA preparation was incubated at 56 °C for 60 min and proteinase K was heat-inactivated at 95 °C for 10 min. A pair of oligonucleotide primers for the MOMP gene, CTU/CTL [CTU, 5'-AT-GAAAAAAGCTCTTGAAATCGG-3'; CTL, 5'-CAAGATTTTCTA-GA(T/C)TTCAT(C/T)TTGTT-3'] was chosen from the highly conserved regions of the published DNA sequences for the MOMP gene of *C. trachomatis* (Stephens *et al.*, 1987; Yuan *et al.*, 1988) and *C. psittaci* (Pickett *et al.*, 1988; Herring *et al.*, 1989; Zhang *et al.*, 1989) strains. This allowed amplification of a 1 kbp fragment. Oligonucleotides were synthesized by the phosphoramidite method on a DNA synthesizer (Applied Biosystems). A 25 µl vol. of the DNA preparation was subjected to PCR in a 100 µl final reaction mixture containing: 10 mM-Tris/HCl, pH 8.3; 200 µM (each) dATP, dCTP, dGTP, dTTP; 200 µM (each) CTU and CTL; 2.5 mM-MgCl₂; 50 mM-KCl; and 2.5 units of *Thermus aquaticus* DNA polymerase (Perkin-Elmer/Cetus) overlaid with 100 µl of mineral oil. In order to improve specificity, DNA was added last after heating the amplification mixture at 80 °C (Mullis, 1990). Samples were subjected to 30 cycles of amplification in a DNA Thermal Cycler (Perkin-Elmer/Cetus). Cycling conditions were as follows: denaturation, 1 min at 94 °C; primer annealing, 1 min at 48 °C; and primer extension, 2 min at 72 °C. Three negative controls were systematically included in each series: a non-infected McCoy cell preparation; lysis buffer with proteinase K alone; and water. To assess the amplification, a 5 µl vol. of the PCR reaction was subjected to electrophoresis on a 1.2% agarose gel stained with ethidium bromide and photographed under UV illumination. DNA molecular size marker III (Boehringer) was used. Restriction endonuclease digestion with *AluI* (Boehringer) was performed on 5 µl aliquots of the amplified samples and the products were analysed by electrophoresis on 8% (w/v) polyacrylamide gels, stained and photographed as above. DNA molecular mass marker V (Boehringer) was used as size marker.

Results

For all the 40 *Chlamydia* strains studied, the PCR reaction produced an amplified DNA fragment of the expected size. No difference in size was seen on the agarose gels whatever the strain (Fig. 1). No PCR products were obtained with the non-infected cells.

Digestion of the MOMP gene by *AluI* generated 13 different restriction patterns (A to M) (Fig. 2, Table 1). Analysis of *AluI* fragments in the 410 to 64 bp size-range sufficed to differentiate the strains studied and fragments of smaller sizes were not considered. A summary of the fragment sizes obtained is given in Table 2.

Table 2. Sizes of the restriction fragments of the PCR-amplified *Chlamydia* MOMP genes

Values given are those deduced from our experimental data. For *C. pneumoniae* IOL-207, *C. trachomatis* L2/434/Bu and *C. psittaci* S26/3 strains, experimental values are in agreement with the published MOMP-gene sequences for these strains.

Strains	Restriction fragment sizes (bp)
<i>C. psittaci</i>	
Invasive strains	198, 171, 159, 84, 82, 81, 78, 75
LW-679, AB10	350, 270, 180, 135
824	290, 250, 180, 135, 125
iB1	290, 250, 135, 125, 110, 70
iB3	293, 250, 180, 160, 135
iB5	290, 180, 155, 125, 115, 84
iB4	275, 230, 145, 120, 110, 70
iC4, iC3, iC2	290, 198, 180, 150, 95, 90, 75
R69, W73	293, 150, 125, 110, 82, 70
Loth	410, 290, 110, 100, 84
<i>C. trachomatis</i>	
MoPn	270, 250, 132, 110, 67, 64
L2/434/Bu	227, 225, 132, 102, 96
<i>C. pneumoniae</i>	
IOL-207	239, 204, 162, 141, 129, 97, 81

All the 24 invasive *C. psittaci* isolates gave the same pattern (pattern A). The 12 non-invasive *C. psittaci* isolates exhibited eight distinct patterns (B to I). However, all these eight patterns shared a common fragment of 180 bp (patterns B, C, E, G and I) or the corresponding restricted fragments of 110 and 70 bp (patterns D, F and H) which are not found in the outer patterns. Patterns C and D (*C. psittaci* strains 824 and iB1) were differentiated by this restriction site only, in the 180 bp fragment. The avian *C. psittaci* isolate, the *C. pneumoniae* isolate and the two *C. trachomatis* isolates each gave a unique pattern (J to M, respectively).

Discussion

Serotyping of *C. psittaci* isolates provides a valuable tool for epidemiological studies but it requires access to specific reagents of restricted availability. Genotypic approaches, which are easy to perform without any specific reagents, are clearly needed for characterization of *C. psittaci* isolates. In the native DNA analyses previously reported for *C. psittaci* typing, cell cultures with or without chlamydial purification are necessary, both being costly and time-consuming. The PCR procedure allows direct MOMP-gene analysis without the need for cell culture and purification. Non-viable elementary bodies at a concentration of 10^3 ml⁻¹ are sufficient to obtain directly a signal after ethidium bromide staining, without preliminary DNA extraction being required (data not shown). MOMP-gene *AluI* restriction patterns are obtained within 2 d without the need for a probing system.

Fig. 1. Electrophoretic analysis of the PCR products after amplification of the MOMP gene. Molecular size marker III (1), non-infected McCoy cell preparation (2), lysis buffer alone (3), *C. pneumoniae* strain IOL-207 (4), *C. trachomatis* strain L2/434/Bu (5), *C. psittaci* strains Loth (6), iB4 (7), iB1 (8), LW-679 (9), S26/3 (10) and AB7 (11).

Fig. 2. MOMP-gene *AluI* restriction patterns. Molecular size marker V (1), *C. pneumoniae* strain IOL-207 (2), *C. trachomatis* strains L2/434/Bu (3) and MoPn ATCC VR123 (4), *C. psittaci* strains Loth (5), R69 (6), iC4 (7), iB4 (8), iB5 (9), iB3 (10), iB1 (11), 824 (12), LW-679 (13), AB7 (14), S26/3 (15), A22 (16), 1H77 (17) and VB1 (18). Arrows correspond to the 180 bp fragment or the corresponding 110 and 70 bp restricted fragments found in all the non-invasive ruminant strains.

Among the strains we studied, MOMP-gene *AluI* restriction mapping clearly distinguishes the *C. pneumoniae* isolate, the two *C. trachomatis* isolates one from the other and the avian *C. psittaci* isolate from the ruminant *C. psittaci* isolates (Table 1). Fragments obtained with *C. pneumoniae* IOL-207, *C. trachomatis* serovar L2 and *C. psittaci* S26/3 strains were in accordance with the published MOMP-gene sequences of these strains (Stephens *et al.*, 1987; Herring *et al.*, 1989; Carter *et al.*, 1991). The avian strain *C. psittaci* Loth gave fragments identical to those predicted from the published *C. psittaci*

Cal 10 MOMP-gene sequence (Zhang *et al.*, 1989). On the other hand, fragments obtained with the *C. psittaci* A22 strain did not correspond to the sequence data reported by Pickett *et al.* (1988) for the A22/M strain. In actual fact, the fragments were identical to those of *C. psittaci* S26/3. These data are in agreement with those of Herring *et al.* (1989) which suggested that the A22/M strain differed from the original A22 isolate and might correspond to a contaminant strain of avian origin.

The most striking feature of the present study is the genetic relatedness observed among the ruminant *C.*

psittaci isolates previously classified as invasive in the murine model of virulence. Indeed, invasive strains all belong to a homogeneous group which exhibits the MOMP-gene restriction pattern A. It is noteworthy that these invasive strains shared an identical pattern whatever the host, the associated disease or the geographical origin (France, Scotland, UK, or the USA). McClenaghan *et al.* (1984) reported the homogeneity of ovine abortion strains by restriction endonuclease analysis of total DNA; three of their strains (A22, H574 and S26/3) were included in our series. In contrast, the non-invasive strains form a heterogeneous group which exhibits eight different MOMP-gene restriction patterns (B to I). However, among these eight patterns, there is a common 180 bp fragment or the corresponding restricted fragments of 110 and 70 bp which are specific for the non-invasive strains. Furthermore, among the ruminant strain patterns, any fragment longer than 198 bp is characteristic of non-invasiveness.

A strict correspondence is observed between the MOMP-gene restriction pattern of the invasive strains and serotype 1. On the contrary, non-invasive strains which all belonged to serotype 2 exhibit eight distinct MOMP-gene restriction patterns. MOMP-gene sequences in *C. psittaci* isolates are generally conserved except for four symmetrically spaced variable domains which precisely correspond to dominant immunological determinants (Zhang *et al.*, 1989). It has been shown that the MOMP bears epitopes implicated in the serovar specificity of *C. psittaci* serovar 1 and 2 strains (Baghian *et al.*, 1990). Our data suggest the possibility of some degree of heterogeneity in serotype 2 immunologic determinants. Furthermore, differences in infectivity of avian *C. psittaci* isolates have been reported to correlate with differences in MOMP molecular mass and antigenicity (Winsor & Grimes, 1988).

The above findings clearly demonstrate the usefulness of MOMP-gene restriction mapping in typing chlamydiae. It could also provide a valuable tool to easily detect strain contamination (Herring *et al.*, 1989).

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