Evidence for *Chlamydia pneumoniae* of non-human origin

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This paper describes the characterization and taxonomic status of N16, a chlamydial isolate from the respiratory tract of a horse. N16 contains plasmid DNA, has normal elementary body morphology and its inclusions do not stain with iodine. Its major outer-membrane protein (MOMP) gene was completely sequenced and compared with the MOMP genes of *Chlamydia pneumoniae*, *C. psittaci*, *C. trachomatis* and *C. pecorum*. This analysis revealed that N16 is closely related to the TWAR strain of *C. pneumoniae* (94.5% and 94.4% DNA homology with TWAR isolates IOL-207 and AR-39 respectively). By comparison, N16 shows between 72.1% and 73.7% DNA homology with *C. psittaci* strains, 70.9% and 71.1% homology with *C. pecorum* strains LW613 and 1710S and 69.2% homology with *C. trachomatis* serotype E. The MOMP gene of N16 shares 93.8% DNA homology with the MOMP gene of a chlamydial isolate KC from the conjunctiva of a koala. Monoclonal antibodies raised to *C. pneumoniae* IOL-207 and shown to be *C. pneumoniae*-specific confirmed that N16 was more closely related to *C. pneumoniae* than to *C. psittaci*. Thus DNA homology and monoclonal antibody data both suggest that horse chlamydiae, as exemplified by N16, form a new second strain of *C. pneumoniae*. This species is probably more widespread and diverse than the current literature would suggest.

Introduction

Chlamydiae are obligate intracellular bacteria that infect many mammalian and avian species, including man. Currently there are four species recognized: *Chlamydia pneumoniae*, *C. psittaci*, *C. pecorum* and *C. trachomatis*.

C. pneumoniae is a common respiratory pathogen of humans responsible for some outbreaks of community-acquired pneumonia (Grayston *et al.*, 1989*a*). It was characterized as a new species (Grayston *et al.*, 1989*b*) when shown to share 10% or less DNA homology with the other *Chlamydia* species. In addition, *C. pneumoniae* was shown to possess species-specific antigens that were recognized by monoclonal antibodies (mAbs). The infectious forms (elementary bodies; EBs) were said to have unique pear-shaped morphology. However it is now

known that not all TWAR isolates have pear-shaped EBs (Carter *et al.*, 1991; Popov *et al.*, 1991). All isolates recognized in 1989 were from humans and they comprised a single strain that was called TWAR. None of these isolates contained plasmid DNA.

Relatively little is known about genetic variation amongst TWAR isolates. Studies of the major outer-membrane protein (MOMP) gene, some portions of which exhibit a high level of variation in other chlamydiae, indicate that TWAR isolates are genetically similar. Thus a study of variable domain IV of the TWAR MOMP gene (Gaydos *et al.*, 1992) found that all of the 13 isolates tested were identical, and the complete MOMP gene of two TWAR isolates, IOL-207 and AR-39, showed over 99% DNA sequence homology (Carter *et al.*, 1991; Melgosa *et al.*, 1991).

In contrast, *C. psittaci* is a diverse species which includes many pathogens of mammals and birds and, until recently, chlamydial isolates of non-human origin were usually considered to be *C. psittaci*. However it is now realized that some chlamydiae that infect ruminants are not closely related to other *C. psittaci* strains, and it has been proposed that they be classified as a separate species called *C. pecorum* (Fukushi & Hirai, 1992). *C. trachomatis* is primarily a pathogen of humans although a strain which infects mice (mouse pneumonitis) was

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Abbreviations: EB, elementary body; FAT, fluorescent antibody test; mAb, monoclonal antibody; MOMP, major outer-membrane protein; PCR, polymerase chain reaction.

The GenBank accession numbers for the MOMP gene sequences determined in this work are L04982 (N16), L04980 (N352) and X61096 (Fpn/pring).

described by Nigg (1942). This strain is not closely related to other *C. trachomatis* strains although it is included in this species as it stains with iodine, the classic test used to distinguish *C. trachomatis* from *C. psittaci*. More recently a chlamydial isolate from pigs has also been described as *C. trachomatis* (Kaltenboeck *et al.*, 1992).

In this paper we present evidence which suggests that N16, a chlamydial isolate recovered from the respiratory tract of a horse, belongs to the species *C. pneumoniae*. N16 was one of 15 isolates obtained during a survey of chlamydial infection in 300 horses (Mair & Wills, 1992). When this isolate was investigated previously in our laboratory (Wills *et al.*, 1990) it was classified as *C. psittaci* because inclusions did not stain with iodine. N16 was also shown to have normal EB morphology and to contain a 7.5 kb plasmid. It therefore did not conform to the species description of *C. pneumoniae* (Grayston *et al.*, 1989b). However a comparison of the DNA sequence of the MOMP genes, and the reaction of mAbs to N16 and other isolates of *C. pneumoniae* and *C. psittaci*, now suggest N16 is a member of the species *C. pneumoniae*.

Methods

Strains. The following chlamydiae were used in this study: equine isolate N16 (Wills *et al.*, 1990); *C. pneumoniae* IOL-207 (Dwyer *et al.*, 1972); avian *C. psittaci* N352 isolated from a domestic duck (Richmond *et al.*, 1982); feline *C. psittaci* Fpn/pring (Wills *et al.*, 1984); avian *C. psittaci* 6BC (Morgan & Wiseman, 1946); guinea-pig *C. psittaci* GPIC (Murray, 1964); *C. trachomatis* serotype E/T181 (Sompolinsky & Richmond, 1974). All were cultured in McCoy cell monolayers by standard methods (Richmond *et al.*, 1985).

DNA extraction, polymerase chain reaction (PCR) and DNA sequence determination. Chlamydial EBs were harvested from McCoy cell monolayers infected with 10 inclusion-forming units per cell and

concentrated by centrifugation (12000 g, 30 min). The EBs were resuspended in 1 ml water containing 250 µg proteinase K, incubated for 60 min at 56 °C and then boiled for 15 min. Ten microlitres of this extract contained sufficient chlamydial DNA for a single PCR reaction. Each PCR reaction contained 0.5 mM-dATP, 0.5 mM-dCTP, 0.5 mM-dGTP and 0.5 mM-TTP, 1 unit *Taq* DNA polymerase (Boehringer Mannheim), 10 mM-Tris/HCl pH 8.8, 4 mM-MgCl₂, 50 mM-KCl, 0.1% Triton X-100 and 0.2 µM of each oligonucleotide primer pair. DNA was amplified by 30 cycles of 94 °C for 2 min, 48 °C for 1 min and 72 °C for 1 min. These conditions proved adequate for most combinations of primer pairs using *C. pneumoniae* or *C. psittaci* template DNA. The same oligonucleotide primers were used for PCR and DNA sequence determination (Table 1). PCR products were cloned directly into a PCR1000 vector (*Invitrogen* Corp.) and inserts were then sequenced by the standard dideoxynucleotide chain-termination method (Sequenase, USB). All DNA sequences were determined in both directions, and from a minimum of three clones, to negate misincorporation errors produced by *Taq* polymerase.

Sequence analysis. The following MOMP gene sequences were used for analysis: N16 (GenBank accession no. L04982); N352 (GenBank accession no. L04980); Fpn/pring (GenBank accession no. X61096); IOL-207 (GenBank accession no. M64064; Carter *et al.*, 1991); *C. pneumoniae* AR-39 (GenBank accession no. M69230; Melgosa *et al.*, 1991); GPIC (Zhang *et al.*, 1989); ovine *C. psittaci* EAE/S26 (GenBank accession no. X51859; Herring *et al.*, 1989); *C. trachomatis* L2 (GenBank accession no. M14738; Stephens *et al.*, 1986); KC, LW613 and 1710S (Kaltenboeck *et al.*, 1993). KC was isolated from the conjunctiva of a koala (Timms *et al.*, 1988). LW613 and 1710S are both *C. pecorum*; LW613 was isolated from a cow and 1710S from a pig (Perez-Martinez & Storz, 1985). (These three sequences were not complete as the first 132 bases and the last 39 bases were not determined; they were therefore 999 bases long. The sequences were kindly provided by Dr B. Kaltenboeck, Veterinaermedizinische Universitaet Wien, A-1030 Vienna, Austria.) DNA and amino acid sequences were analysed using the PCGENE suite of programs (Intelli-Genetics Inc.). A dendrogram (Fig. 2) was constructed with the CLUSTAL program, which uses the method of unweighted pair group maximum averages (Sokal & Sneath, 1963).

Fluorescent antibody test (FAT). Chlamydiae were cultured in McCoy cell monolayers grown on 10 mm glass coverslips. After incubation for 48 h, monolayers were fixed for 10 min in either acetone or ethanol. Hybridoma supernatants containing mAbs (200 µl) were added undiluted to the coverslips and incubated for 20 min at 37 °C. After washing in PBS (A) (Dulbecco's phosphate-buffered saline solution A), fluorescein-isothiocyanate-labelled anti-mouse immunoglobulins (Dako F261) diluted 1/50 in PBS(A) were added. After incubation at 37 °C for 20 min the coverslips were washed in PBS(A) and mounted in 50% (v/v) glycerol in PBS(A). The coverslips were examined with ×25 and ×63 oil immersion objectives by epi-illumination.

mAbs. *C. pneumoniae*-specific mAbs were raised to IOL-207 by standard methods (Harlow & Lane, 1988). The antibodies were screened by FAT using whole inclusion antigens from *C. pneumoniae* IOL-207, *C. psittaci* 6BC, Fpn/pring and GPIC and *C. trachomatis* serotype E/T181. Only mAbs that gave bright inclusion fluorescence with IOL-207 but did not react to the other strains were designated *C. pneumoniae*-specific.

Results

The complete sequence of the MOMP gene of N16 was determined (Fig. 1) as were the complete DNA sequences of the MOMP genes of *C. psittaci* strains N352 (avian)

Table 1. Oligonucleotide primers used in PCR amplifications and sequencing reactions

Primer	Sequence 5'–3'	Position on N16 sequence*
sp1	CAGGATATCTTGTCTGGC	–103 to –86
p2	CTGATAACAAGGTGAGGA	1206 to 1189
p3	TTAAACATTTGGGATCG	375 to 391
p4	TCCATAAAGCTCCACG	599 to 583
p5	TGAGCAATGCGGATAG	908 to 893
p6	CGTGGAGCTTTATGGGA	583 to 599
p7	AGGTGCTTCAGGAGATC	126 to 142
p8	CGATCCCAAATGTTTAA	375 to 391
p9	CCAAGTAGCGCAAGGATC	166 to 148
p10†	AGGCTGTTTTCACTTG	–154 to –139
p11†	AGGTTCTGATAGCGGGACAA	1256 to 1237
p12	TGGCTGAGCAATGCGG	912 to 897

* A minus sign denotes upstream of the start codon.

† Primers p10 and p11 were designed to hybridize to *C. psittaci* MOMP genes and the positions stated are for the N352 MOMP gene sequence.

Fig. 1. DNA and deduced amino acid sequences of the MOMP gene of N16 are aligned with the sequences of *C. pneumoniae* IOL-207 (Carter *et al.*, 1991). Identical nucleotides are shown as dashes and identical amino acids as dots. The cysteine residues are shown boxed and the variable domains are underlined.

and Fpn/pring (feline). (Sequences of N352 and Fpn/pring are not shown but have been deposited in GenBank: see Methods for accession numbers.)

DNA and deduced amino acid sequences from all three strains exhibited salient features characteristic of chlamydial MOMP described previously by Stephens *et al.* (1987), Herring *et al.* (1989), Zhang *et al.* (1989), Carter *et al.* (1991) and Melgosa *et al.* (1991). The distribution of cysteine residues found in N16 was identical to those previously described for *C. pneumoniae* IOL-207 and AR-39 (Carter *et al.*, 1991; Melgosa *et al.*, 1991) and included cysteine residues at positions 226 and 342 (Fig. 1) which have not been found in *C. psittaci* strains. A unique cysteine residue was found in Fpn/pring at position 120. The putative leader sequence of N16 was 22 amino acids long and identical to that of TWAR isolates IOL-207 and AR-39, whereas the

putative leader sequences of Fpn/pring and N352 were 23 amino acids long and identical to those described previously for *C. psittaci* strains.

When the DNA sequence of the MOMP gene of N16 was aligned with that of TWAR IOL-207 (Fig. 1) a high degree of homology was evident, with only 64 bases different (94.5% homology). The amino acid sequence homology was also very high, with only 18 differences between the two strains (94.9% amino acid homology), the majority occurring within the four variable domains. Six amino acid differences occurred within domain IV, which is the largest of the variable domains and is the proposed site of species and subspecies antigenic determinants (Baehr *et al.*, 1988). This is in stark contrast to the lack of variation found in variable domain IV within TWAR isolates (Gaydos *et al.*, 1992).

The percentage homology between N16, N352 (avian),

Table 2. Percentage DNA sequence homology between chlamydial MOMP genes

	KC	AR-39	IOL-207	N352	Fpn/pring	GPIC	EAE/S26	LW613	1710S	Ct L2
N16	93.8	94.4	94.5	73.7	73.7	72.4	72.1	70.9	71.1	69.2
KC*		97.8	98.0	73.7	73.0	71.7	72.0	72.2	71.8	68.6
AR-39			99.7	74.9	74.1	73.2	73.0	71.8	71.8	69.1
IOL-207				74.8	74.0	73.1	72.9	72.0	72.0	69.1
N352					81.5	80.8	85.6	71.0	71.5	69.3
Fpn/pring						82.3	83.4	71.8	70.8	70.1
GPIC							82.2	72.5	74.0	70.1
EAE/S26								70.2	69.0	70.5
LW613*									87.8	68.8
1710S*										68.0

* Percentage homology values for KC, LW613 and 1710S are based on partial MOMP gene sequences.

Table 3. Reaction of mAbs to chlamydial strains by FAT

Chlamydial strain	mAb			
	A1	A2	A3	A4
N16	+	+	-	-
IOL-207	+	+	+	+
TW183	+	+	+	+
6BC	-	-	-	-
Fpn/pring	-	-	-	-
GPIC	-	-	-	-
E/T181	-	-	-	-

Fpn/pring (feline) and other chlamydial MOMP genes was determined (Table 2). The results show that N16 has a much higher homology to *C. pneumoniae* isolates IOL-207 and AR-39 (94.5% and 94.4% respectively) than to any of the *C. psittaci*, *C. pecorum* or *C. trachomatis* strains. In addition N16 has a high degree of homology to the koala isolate KC (93.8%). It is evident that N16, KC, IOL-207 and AR-39 form a distinct group sharing over 93% DNA homology. This group is quite distinct from *C. psittaci* or *C. pecorum* strains.

Studies using mAbs were consistent with the DNA homology results. mAbs A1-A4 raised against *C. pneumoniae* strain IOL-207 were shown to be *C. pneumoniae*-specific by testing them against *C. pneumoniae* isolates IOL-207 and TW-183 (reactivity against TW-183 was kindly assessed by Dr O. Caul, Public Health Laboratory Service, Bristol, UK, and Dr J. Treharne, Institute of Ophthalmology, London, UK), and strains of *C. psittaci* and *C. trachomatis* (Table 3). When N16 was tested against the four mAbs, A1 and A2 were reactive but A3 and A4 were not. This suggests that N16 and TWAR share some antigens that are recognized by A1 and A2 which are *C. pneumoniae*-specific. A3 and

A4 however recognize antigens that are apparently specific for the TWAR strain. None of the four mAbs reacted to chlamydial antigens in Western blotting experiments.

Discussion

Our results suggest that chlamydial isolate N16 is a member of the species *C. pneumoniae* and not *C. psittaci* as originally thought (Wills *et al.*, 1990). The relationship of N16 to other chlamydiae, based on MOMP gene homology, is illustrated in a dendrogram (Fig. 2). This indicates that although N16 is on the *C. pneumoniae* branch it is quite distinct from the human TWAR strain.

Fig. 2. Dendrogram to illustrate the relationships of chlamydiae based on the similarity of DNA sequences of the MOMP genes. The dendrogram was constructed using partial DNA sequences; the first 132 bases and the last 39 bases of each MOMP gene sequence were not used. This was done to enable all of the sequences to be aligned with the data available for chlamydial isolates KC, LW613 and 1710S.

The results obtained with *C. pneumoniae*-specific mAbs support our DNA homology comparisons. Other differences between N16 and TWAR are that N16 contains a plasmid and has normal EB morphology (Wills *et al.*, 1990) whereas TWAR EBs were originally described as pear-shaped (Grayston *et al.*, 1989b). However not all TWAR isolates have abnormal EB morphology (Carter *et al.*, 1991; Popov *et al.*, 1991) and the presence or absence of a plasmid is not a reliable indicator of species.

Studies on the plasmid of N16 (pCpnE) showed that it is similar in size (7.5 kb) and organization to the plasmids found in *C. psittaci* and *C. trachomatis* but that it has a unique restriction profile (Lusher *et al.*, 1989). Preliminary DNA homology studies of the origin of replication and open reading frame 1 of pCpnE show that this plasmid is less closely related to the plasmids of *C. psittaci* strains N352, Fpn/pring and GPIC than these three plasmids are to each other (Yates *et al.*, 1992).

The koala conjunctival isolate KC also appears to be *C. pneumoniae* and is, by MOMP DNA homology studies, more closely related to TWAR than N16 (Fig. 2). Kaltenboeck *et al.* (1993), in a study of the relationships of chlamydiae by homology of partial MOMP gene sequences, described this isolate as being *C. pneumoniae*-like. The KC isolate was obtained from the koala conjunctiva (Timms *et al.*, 1988) and it appears to be quite different from chlamydial isolates obtained from the koala urogenital tract (Girjee *et al.*, 1988).

The fact that *C. pneumoniae*-like organisms have now been recovered from three mammalian species that are not themselves closely related indicates that *C. pneumoniae* may be a more diverse and widespread species than the current literature suggests. It seems probable that *C. pneumoniae* of other mammals will be discovered. It is also possible that other *C. pneumoniae* isolates already exist in laboratory strain collections but, like N16 and KC, have been wrongly described as *C. psittaci*.

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