NEW MICROBIOLOGICA, **33**, 163-166, 2010

Chlamydophila felis: plasmid detection in Italian isolates

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SUMMARY

Plasmids have been detected in the majority of strains in the genus *Chlamydia* and in many *Chlamydophila* species. Previous studies showed that FP Pring and FP Cello *Chlamydophila felis* strains have an extrachromosomial plasmid, whereas the FP Baker strain does not. Azuma *et al*. recently sequenced the entire genomic DNA sequence of the Japanese *Cp. felis* strain Fe/C-56 and described a 7,552 base pair circular plasmid. In the present study a highly conserved plasmid gene was detected in 11 Italian *Cp. felis* isolates, showing 100% nucleotide identity with the plasmid gene of Fe/C-56 *Cp. felis* strain.

KEY WORDS: Chlamydophila felis, Plasmid, PCR

Received October 9, 2009 *Accepted* February 3, 2010

Plasmids have been detected in the majority of strains in the genus *Chlamydia* and in many *Chlamydophila* species, e.g. the equine strain N16 of *Clamydophila pneumoniae*, several strains of *Chlamydophila psittaci* (Lovett *et al*., 1980) and some strains of *Chlamydophila pecorum* and *Chlamydophila caviae*. On the contrary, extrachromosomal DNA elements have not been identified in *Chlamydophila abortus* or human *Cp. pneumoniae* isolates.

With regard to the presence of plasmid in *Chlamydophila felis* strains, previous studies showed that FP Pring and FP Cello strains have an extrachromosomial plasmid (Lusher *et al*., 1989), whereas the FP Baker strain does not (Everett *et al*., 1999). Azuma *et al*. (2006) recently sequenced the entire genomic DNA sequence of the Japanese *Cp. felis* strain Fe/C-56 and described a 7,552 base pair (bp) circular plasmid (pCfe1), with eight genes *pCF01-pCF08*.

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In Italy, biomolecular surveys have shown *Cp. felis* DNA in both household and stray cats with and without clinical signs of conjunctivitis (Rampazzo *et al*., 2003; Di Francesco *et al*., 2003, 2004; Di Martino *et al*., 2007). To our knowledge, no study has been performed to evaluate the presence of plasmid sequences in *Cp. felis* Italian isolates.

The aim of the present study was to evaluate the presence of plasmid sequences in 11 Italian *Cp. felis* isolates.

Cp. felis isolates were taken from conjunctival swabs of cats with clinical signs of conjunctivitis between 2003 and 2007 and molecularly characterised by *omp1*, *omp2* and *groEL* genes sequencing (Di Francesco *et al*., 2005). In the present study, these isolates were propagated in LLC-MK2 (a continuous cell line derived from *Rhesus* monkey kidney tissue) cell monolayers. DNA was extracted from the infected cultures using a commercially available kit (DNeasy Blood & Tissue Kit, Qiagen) and used as a template for a PCR which amplified a 1,025 bp fragment of the *pCF03 Cp. felis* plasmid gene.

The following primers were designed from the plasmid pCfe1 sequence of *Cp. felis* Fe/C-56 strain (GenBank NC_007900.1): primer forward pCF-F

5'-CACACTAGGGAGACAATTTCCA-3', primer reverse pCF-R 5'-GACCACTATCCCTGAGATCCGA-3'. Each DNA preparation (5 µl) was added to the PCR mixture (final volume 50 µl) containing final concentrations of 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 200 µM (each) deoxynucleoside triphosphates (dATP, dTTP, dGTP, and dCTP), 20 pmol of each primer, and 1.25 U of *Taq* DNA polymerase (Qiagen).

Cycling conditions were as follows: 5 min of denaturation at 95°C; 35 cycles each consisting of denaturation at 94°C for 1 min, annealing at 56°C for 1 min and extension at 72°C for 1 min. A final elongation step of 10 min at 72 °C completed the reaction. The amplified products were visualized after electrophoresis in 1% agarose gel by ethidium bromide staining. As control, DNA from uninfected LLC-MK2 cells was checked under the PCR conditions above described.

All isolates showed the expected PCR product. Uninfected cells were PCR-negative. Amplicons were purified using a commercially available kit (High PCR Product Purification Kit, Roche), directly sequenced (Bio-Fab Research Rome, Italy) and compared with both each other and the same region of *Cp. felis* Fe/C-56. Comparison by CLUSTALW (Thompson *et al.*, 1994) showed 100% identity between the nucleotide sequences. Since previous molecular analysis of the *omp1*, *omp2* and *groEL* genes of these Italian *Cp. felis* isolates showed a total nucleotide identity (Di Francesco *et al.*, 2005), only one *Cp. felis* isolate (FEIS-49) was selected to determine the complete plasmid sequence. Eleven primer pairs were de-

TABLE 1 - *Oligonucleotide primers used for PCR.*

Primer	*Primer sequence*	*Gene location*[a]	*Amplicon size (bp)*
pl1F	5'-GACTTTGCAACTTTAGGTGGTA-3'	19-40	1232
pl1R	5'-GAAAGTCACCGGTCATTCTTCT-3'	1250-1229	
pl2F	5'-CAGCGCTCTGGCTTATCTGAAA-3'	929-950	1182
pl2R	5'-GTTTACCGAAGCAACCTGACAC-3'	2110-2089	
pl3F	5'-AGAAGAATGACCGGTGACTTTC-3'	1229-1250	1232
pl3R	5'-TGGAAATTGTCTCCCTAGTGTG-3'	2460-2439	
pl4F	5'-GTGTCAGGTTGCTTCGGTAAAC-3'	2089-2110	1374
pl4R	5'-GACCACTATCCCTGAGATCCGA-3'	3462-3441	
pl5F	5'-CACACTAGGGAGACAATTTCCA-3'	2439-2460	1204
pl5R	5'-ACCATGCTTCTTTCTGAACCGA-3'	3642-3621	
pl6F	5'-TCGGATCTCAGGGATAGTGGTC-3'	3441-3462	1142
pl6R	5'-CCCTTGGATCCTTTCGTGTTTA-3'	4582-4561	
pl7F	5'-TCGGTTCAGAAAGAAGCATGGT-3'	3621-3642	1240
pl7R	5'-GGCGTAGAGTCAGTTCCAATTA-3'	4860-4839	
pl8F	5'-TAAACACGAAAGGATCCAAGGG-3'	4561-4582	1240
pl8R	5'-GGTTGAGCAGGTTCAGTATAAC-3'	5800-5789	
pl9F	5'-TAATTGGAACTGACTCTACGCC-3'	4839-4860	1212
pl9R	5'-ATTTGGGCAAGGTTACTACCGA-3'	6050-6029	
pl10F	5'-GTTATACTGAACCTGCTCAACC-3'	5779-5800	1212
pl10R	5'-GGAGAACATGTTGCTTCTGTAT-3'	6990-6969	
pl11F	5'-TCGGTAGTAACCTTGCCCAAAT-3'	6029-6050	1522
pl11R	5'-TCTCTCGGAGTTTTCAGGGTTT-3'	7550-7529	

[a]Reference sequence GenBank Accession No. NC_007900.1.

signed (Table 1). The PCR mixture and the cycling conditions were performed as described above except that the annealing temperature was 55°C for pl1F/R and pl10F/R primers, 57°C for pl6F/R primers, 58°C for pl2F/R and pl4F/R primers. For each amplicon purified both DNA strands were sequenced and assembled by Bioedit software, generating a 7,500 bp nucleotide sequence.

Comparison of the plasmid gene of *Cp. felis* FEIS-49 with the same gene of *Cp. felis* Fe/C-56 showed 100% identity.

In the present study a highly conserved plasmid gene was observed in 11 *Cp. felis* isolates. In our previous study (Donati *et al*., 2009), antibodies to a recombinant plasmid-encoded pgp3 protein from *Cp. psittaci* were showed in forty-four (97.8%) out of 45 sera collected from cats with conjunctivitis and positive by microimmunofluorescence test to *Cp. felis*. The detection of the plasmid in all *Cp. felis* isolates tested confirms the high frequency of plasmid carriage in field *Cp. felis* isolates, in accordance with the observations of Harley *et al*. (2009) who identified plasmid-positive *Cp. felis* isolates in 99.5% of 564 clinical cases in the UK.

The molecular identity between FEIS-49 and Fe/C-56 plasmids is not surprising, given that molecular characterization of chlamydial plasmids showed that they are highly conserved inter and intraspecies. In their nucleotide sequence, chlamydial plasmids are more similar than the corresponding chromosomal DNA. All chlamydial plasmids have four 22-bp tandem repeats in the intergenic region between ORF8 and ORF1 (Thomas *et al*., 1997). Identical plasmid repeat sequences have been shown in avian *Cp. psittaci* isolates (Hugall *et al*., 1989), in *Cp. felis* isolates carrying a plasmid and in *Cp. caviae* isolates (Lusher *et al.*, 1991). Conserved sequences have also been identified in the plasmid of the equine strain N16 of *Cp. pneumoniae* and the type strain E58 of *Cp. pecorum* (Hugall *et al*., 1989; Everett *et al*., 1999). In human *C. trachomatis* isolates, the repeats are identical but show two nucleotide substitutions from the conserved *Cp. psittaci* repeat sequence (Thomas *et al*., 1997).

Among different isolates of the same chlamydial species, the similarity is very high, e. g. all plasmids from human *C. trachomatis* isolates show less than 1% nucleotide sequence variation. With regard to *Cp. felis*, Lusher *et al*. (1989) suggested that the plasmid is highly conserved within feline chlamydial strains, showing by Southern blot hybridization analysis the close similarity of the plasmids recovered from two feline chlamydial strains isolated in the USA and in the UK, respectively. Although the existence of rare plasmid-free chlamydial strains (Farencena *et al*., 1997) shows that plasmid is not essential for chlamydial growth and replication, the high degree of plasmid conservation through chlamydial evolution suggests a role for plasmid both in *in vivo* infectivity and in the control of virulence, as confirmed in a recent study on plasmid-less and plasmid-positive *C. trachomatis* strains (Carlson *et al*., 2008).

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