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► **To cite this version:**

Manuela Donati, Karine Laroucau, Elisa Storni, Costanza Mazzeo, Simone Magnino, et al.. Serological response to pgp3 protein in animal and human chlamydial infections. *Veterinary Microbiology*, 2009, 135 (1-2), pp.181. 10.1016/j.vetmic.2008.09.037 . hal-00532491

**HAL Id: hal-00532491**

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Submitted on 4 Nov 2010

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## Accepted Manuscript

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PII: S0378-1135(08)00406-9  
DOI: doi:10.1016/j.vetmic.2008.09.037  
Reference: VETMIC 4191

To appear in: *VETMIC*

Please cite this article as: Donati, M., Laroucau, K., Storni, E., Mazzeo, C., Magnino, S., Di Francesco, A., Baldelli, R., Ceglie, L., Renzi, M., Cevenini, R., Serological response to pgp3 protein in animal and human chlamydial infections, *Veterinary Microbiology* (2008), doi:10.1016/j.vetmic.2008.09.037

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## Serological response to *pgp3* protein in animal and human chlamydial infections

Manuela Donati<sup>a\*</sup>, Karine Laroucau<sup>b</sup>, Elisa Storni<sup>a</sup>, Costanza Mazzeo<sup>a</sup>, Simone Magnino<sup>c</sup>, Antonietta Di Francesco<sup>d</sup>, Raffaella Baldelli<sup>d</sup>, Letizia Ceglie<sup>e</sup>, Maria Renzi<sup>f</sup>, Roberto Cevenini<sup>a</sup>.

<sup>a</sup>Section of Microbiology, DMCSS, University of Bologna, S. Orsola Hospital, Bologna, Italy; <sup>b</sup> French Food Safety Agency, Bacterial Zoonoses Unit, LERPAZ Maison-Alfort, France; <sup>c</sup>Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna, National Reference Laboratory for Animal Chlamydioses, Pavia, Italy; <sup>d</sup>Department of Veterinary Public Health and Animal Pathology, University of Bologna, Ozzano Emilia, Bologna, Italy; <sup>e</sup>Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro, Padova, Italy; <sup>f</sup>Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna, Bologna.

\*Corresponding Author. Mailing address:

Manuela Donati, M.D.

Sezione di Microbiologia, DMCSS, Policlinico S. Orsola, Università di Bologna, Via Massarenti 9, 40138 Bologna, Italy. Phone: 39 0514290944. Fax: 39 051 307397. E-mail: [manuela.donati@unibo.it](mailto:manuela.donati@unibo.it)

### Abstract

Specific antibodies to plasmid-encoded protein *pgp3* are known to be encountered in human *Chlamydia (C.) trachomatis* infections. In order to verify whether antibodies to this protein could be developed in animals infected with plasmid-carrying chlamydial strains, 454 animal sera were examined using a home-made *pgp3* protein ELISA and Western blots (WB) of recombinant *pgp3* protein from *Chlamydophila (Cp.) psittaci*. Likewise, 50 human sera were tested by ELISA and WB of recombinant *pgp3* from *C. trachomatis*. The reactivity against *pgp3* protein was compared to the

reactivity against chlamydial elementary bodies (EBs) detected by microimmunofluorescence (MIF) test. The presence of pgp3-specific antibodies was demonstrated in most ducks and pigeons with *Cp. psittaci* infection detected by MIF, as well as in the majority of symptomatic cats and pigs infected with *Cp. felis* and *C. suis*, respectively, which reacted at high titres to *Cp. felis* and *C. suis* EBs by MIF. Moreover, most of the sera collected from patients with *C. trachomatis* culture-confirmed infection and seropositive to *C. trachomatis* by MIF, presented antibodies specific to *C. trachomatis* pgp3 recombinant protein. Therefore, pgp3 protein could be a useful marker of chlamydial infections in animals, as well as in humans.

Keywords: pgp3 protein, plasmid, ELISA, WB, MIF, chlamydial infection

## 1. Introduction

Members of the family *Chlamydiaceae* are intracellular pathogens responsible for a wide range of important medical and veterinary diseases. According to Everett et al. (1999) two genera, *Chlamydia* and *Chlamydophila*, are recognized in this family, although this classification remains controversial (Schachter et al., 2001). The presence of plasmids has been determined in the majority of strains in the genus *Chlamydia* and in many *Chlamydophila* species e.g. the equine strain N16 of *Cp. pneumoniae*, several strains of *Cp. psittaci* (Lovett et al., 1980) and some strains of *Cp. felis*, *Cp. pecorum* and *Cp. caviae*. On the contrary, extrachromosomal DNA elements have not been identified in human *Cp. pneumoniae* isolates or in isolates of *Cp. abortus*.

The existence of some plasmid-free chlamydial strains (An et al., 1992; Farencena et al., 1997), suggests that the plasmid is probably not essential for chlamydial growth and replication. On the other hand, a recent study on the molecular and biological properties of plasmidless and plasmid positive *C. trachomatis* strains supports a primary role for plasmid in *in vivo* infectivity and in the control of virulence (Carlson et al., 2008).

Many studies have described the molecular characterization of chlamydial plasmids (Joseph et al., 1986; Palmer and Falkow, 1986; Sriprakash and MacAvoy, 1987; Hatt et al., 1988; Comanducci et al., 1990) and shown that they are highly conserved. The genomic organization of *Cp. psittaci* plasmid accounts for the presence of eight major open reading frames (ORFs), as also observed in *C. trachomatis* strains (Thomas et al., 1997). All chlamydial plasmids have four 22-pb 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 (Lusher et al., 1991) 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* (sporadic bovine encephalomyelitis isolate) (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).

*C. trachomatis* ORFs can code for polypeptides greater than 10 kDa and putative functions have been shown for five of them. ORF3, which has been expressed in *E. coli*, encodes *pgp3*, a highly antigenic protein of 28 kDa (Comanducci et al., 1988). This protein is a marker of current *C. trachomatis* infection in humans (Comanducci et al., 1994) and can be identified by staining inclusions in *C. trachomatis*-infected cells.

In addition, DNA immunization with the *pgp3* gene of *C. trachomatis* was demonstrated to inhibit the spread of *C. trachomatis* infection from the lower to the upper genital tract in C3H/HeN mice (Donati et al., 2003).

In a recent study, we demonstrated a homogeneous restriction profile in the plasmid of 10 avian *Cp. psittaci* isolates (Storni et al., 2006). Moreover, in the same work, serum antibodies against recombinant *Cp. psittaci* *pgp3* protein were detected by Western blot (WB) in a patient with culture-confirmed psittacosis and in 48 *Cp. psittaci*-infected pigeons.

In the present study, the serological response to recombinant pgp3 protein of *Cp. psittaci* was evaluated by a home-made ELISA and WB in different animal species to detect the infection by plasmid-carrying strains of chlamydiae, i.e. *Cp. psittaci* infection in ducks and pigeons, *Cp. felis* infection in cats and *C. suis* infection in pigs. In addition, sera collected from humans infected by *C. trachomatis* were tested to detect the presence of antibodies to recombinant pgp3 protein of *C. trachomatis*. The serological reactivity to pgp3 protein of the different animal and human sera was compared to their reactivity in the microimmunofluorescence (MIF) test against *Cp. psittaci*, *Cp. felis*, *C. suis* and *C. trachomatis* elementary bodies (EBs).

## 2. Materials and methods

### 2.1. Serum samples

A total of 454 animal sera and 50 human sera were tested for their reactivity to pgp3 protein. Three hundred and fifty-four of the animal sera were avian: 134 sera were randomly collected from French ducks (from 4 to 14 weeks of age) presenting no specific clinical signs of *Cp. psittaci* infection and belonging to seven different flocks depending on their age and 220 sera were taken from otherwise healthy pigeons in Emilia-Romagna (Northern Italy). The remaining 100 sera consisted of 50 feline sera and 50 porcine sera. The feline sera were collected in Emilia-Romagna from 45 household cats suffering from conjunctivitis and 5 cats presenting no clinical signs of *Cp. felis* infection. Porcine sera were collected in Emilia-Romagna from 45 pigs with conjunctivitis reared in two different *C. suis* isolation-positive farms and 5 pigs without clinical signs of conjunctivitis, reared in another farm.

The 50 human sera were collected in Emilia-Romagna from *C. trachomatis* isolation-positive patients (35 men and 15 women).

The presence of antibodies specific to *pgp3* in avian, feline and porcine sera was studied by using a home-made ELISA and WB performed with recombinant *pgp3* protein of *Cp. psittaci*. The same techniques, performed with *pgp3* recombinant protein of *C. trachomatis* were used to detect antibodies to *pgp3* in human sera.

The serological reactivity to *pgp3* of the 504 sera studied was then compared to their reactivity by MIF against *Cp. psittaci* EBs (duck and pigeon sera), *Cp. felis* EBs (feline sera), *C. suis* EBs (porcine sera) and *C. trachomatis* EBs (human sera). The 134 duck sera studied by WB and ELISA had been previously tested in France by complement fixation (CF) test and a commercial ELISA test (Chekit Chlamydia ELISA, Bommeli Idexx) based on detection of chlamydial anti-LPS group-specific antibodies.

## 2.2. MIF

MIF test was performed by using purified EBs of the following strains: 6BC of *Cp. psittaci*, FEIS-M of *Cp. felis*, MS04 of *C. suis* and GO/86 of *C. trachomatis*. The EBs were purified by sucrose density-gradient ultracentrifugation using the method of Fukushi and Hirai (1988). Human sera were screened at 1:16 dilution; animal sera were tested at 1:32 dilution in phosphate-buffered saline (PBS) supplemented with 2% foetal calf serum. The test was performed according to the method of Wang and Grayston (1986). Serial twofold dilutions of sera found to be positive at cut-off dilution were made and tested to determine the antibody titre.

## 2.3. SDS-PAGE and *pgp3* Western Immunoblotting

After expression and purification of the recombinant pgp3 proteins of *Cp. psittaci* and *C. trachomatis* as previously described (Storni et al., 2006), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretic transfer were performed (Comanducci et al., 1994), and the blots were incubated overnight with sera under test diluted 1:100 in skimmed milk containing 5% w/v Tween 20/PBS pH 7.4. After washing, antigen-antibody complexes were detected, depending on the animal sera to be tested, with peroxidase-labelled rabbit anti-duck, anti-pigeon, anti-pig or anti-cat IgG (Nordic Immunological Laboratories, Tilburg, The Netherlands). When human sera were tested, antigen-antibody complexes were detected with peroxidase-labelled rabbit anti-human IgG (Dako, Glostrup, Denmark).

### 2.3. *pgp3* ELISA

IgG anti-*pgp3* specific antibody response was determined by direct ELISA. Microtitre plates were coated overnight at 4°C with 100 µl of *pgp3* at the concentration of 6 µg/ml in carbonate buffer pH 9.6, then blocked with skimmed milk 5% w/v in carbonate buffer pH 9.6. In subsequent stages, the wells received 100 µl of reagents diluted in skimmed milk containing 5% w/v Tween 20/PBS pH 7.4. All sera diluted 1:100 were assayed in duplicate. Each plate assayed included replicate dilutions of two reference sera with known activity. The plates were incubated at 37°C for 60 min., then washed three times and incubated with a 1:1000 dilution of alkaline phosphatase-conjugated goat antibody anti-duck or anti-pigeon or anti-cat or anti-pig IgG (Nordic Immunological Laboratories) or anti-human IgG (Dako), depending on the sera to be tested. After incubation for 60 min at 37°C, the plates were washed three times and the alkaline phosphatase substrate paranitrophenylphosphate was added. After incubation at room temperature for 20 min, the reaction was stopped by adding 3M NaOH and the plates were read at a wavelength of 405 nm. A positive sample was defined as a sample that yielded an OD<sub>405</sub> value of at least two standard deviations above the mean of 20

negative samples. In addition, a MIF positive control serum for each species tested was introduced in each experimental session.

Twofold dilutions from 1:100 of the MIF positive control serum and 5 positive pgp3 ELISA sera for each species were tested to compare their dilution curve.

### 3. Results

The serum reactivities to pgp3 determined by the home-made ELISA and WB in comparison to the serological reactivities to chlamydial EBs tested by MIF are reported in Table 1.

An OD of  $\geq 0.5$  was considered the cut-off value. The OD<sub>405</sub>/serum dilution curve of 5 sera in each species tested was comparable to that obtained with the MIF positive control serum.

The recombinant *Cp. psittaci* pgp3 ELISA detected IgG antibodies in 59 (78.7%) out of 75 sera collected from ducks of 8, 11, 13, 14 weeks of age living in four different flocks. The WB demonstrated reactivity to pgp3 in 56 (74.7%) sera. Sixty (80.0%) out of the 75 sera resulted positive by MIF at titres ranging from 64 to 256. On the other hand, IgG antibodies to LPS chlamydial antigen were detected by Chekit commercial ELISA in only 34 (45.3%) of the 75 sera tested. These ducks were also tested for *Cp. psittaci* excretion, where 24 (32%) of 75 coecal swabs resulted positive by PCR.

Out of the 59 sera collected in another three different flocks from very young ducks (4, 5 and 6 weeks old), in which no antibodies to *Cp. psittaci* were detected by the commercial Chekit ELISA, only 3 (5.1%) sera resulted positive both by pgp3 ELISA and WB. Ten of these 59 sera (16.9%) were positive by MIF. Most of these ducks proved negative also for *Cp. psittaci* excretion.

All 134 duck sera tested were negative by CF test.

IgG anti-pgp3 antibody response was determined by the home-made ELISA and WB in 86 (39.1%) and 84 (38.2%) of the 220 pigeon sera tested, respectively; 88 (40%) resulted positive by MIF for the presence of antibodies to *Cp. psittaci* at titres ranging from 32 to 256.

Fourty-four (97.8%) out of 45 sera collected from cats with conjunctivitis and positive by MIF to *Cp. felis* at titres ranging from 128 to 512, reacted to pgp3 both in ELISA and WB (concordance of 97.8%).

All the 45 porcine sera collected from pigs with conjunctivitis reared in *C. suis* isolation-positive farms and positive by MIF for the presence of antibodies at titres ranging from 128 to 1024, demonstrated a strong reactivity to pgp3 both by ELISA and WB (concordance of 100%).

On the contrary, the 10 sera collected from 5 cats and 5 pigs with no signs of conjunctivitis and negative by MIF to *Cp. felis* and *C. suis* respectively, were not reactive by pgp3 ELISA and WB assays.

Finally, 33 (66%) out of 50 sera collected from *C. trachomatis*-isolation positive patients reacted to *C. trachomatis* pgp3 both in ELISA and WB. Thirty-six (72%) of these 50 sera were positive by MIF for the presence of antibodies to *C. trachomatis* EBs. The remaining 14 MIF-negative sera did not react with recombinant pgp3 protein of *C. trachomatis* both in ELISA and WB.

In Table 2, the concordance and discordance between the ELISA, WB and MIF tests in duck, pigeon and human sera are reported. The data obtained by setting MIF as the reference method (gold standard) show that pgp3-ELISA and WB are less sensitive than the MIF test, but are nevertheless very specific techniques.

#### 4. Discussion

Humoral immune response to plasmid protein pgp3 in human *C. trachomatis* infections has been described (Comanducci et al., 1994; Bas et al., 2001a; Bas et al., 2001b). To verify whether animals infected by plasmid-carrying chlamydial strains could develop antibodies against pgp3 protein, 454 animal sera were tested by a home-made ELISA and WB performed with recombinant pgp3 protein of *Cp. psittaci*.

The serological response to *pgp3* was compared to the serological reactivity detected by MIF to determine whether *pgp3* could be a marker of chlamydial infections in animals as well as in humans.

The presence of antibodies against *pgp3* protein was detected in most of the duck and pigeon sera in which *Cp. psittaci* infection was documented by their reactivity to *Cp. psittaci* EBs in MIF. In addition, the commercial ELISA and the CF test previously used in France on duck sera to detect a chlamydial infection, were shown to be less specific and sensitive than our home-made ELISA and WB based on recombinant *pgp3*.

Similarly, almost all sera collected from cats with conjunctivitis and strongly reacting in MIF to *Cp. felis* presented antibodies to the *pgp3* protein.

In addition, a serological response to *pgp3* was detected in all sera from pigs reared in *C. suis* isolation-positive farms, which had also proved positive at high titres to *C. suis* by MIF.

Finally, almost all sera of patients with *C. trachomatis* culture-confirmed infection, which had been found seropositive for *C. trachomatis* by MIF were reactive with recombinant *pgp3* protein of *C. trachomatis* in ELISA and WB.

These results suggest ORF3, the only plasmid ORF found to encode an immunogenic protein in *C. trachomatis*, i.e. *pgp3*, codes similarly in *Cp. psittaci*, *Cp. felis* and *C. suis* for a protein that is highly antigenic. This is demonstrated by the finding of serum *pgp3* antibody response in the majority of animals, such as ducks, pigeons and symptomatic cats and pigs, which were seropositive for *Chlamydia* by MIF, as well as by the absence of antibodies to *pgp3* in the asymptomatic animals that were serologically negative by MIF.

The present results show that *pgp3* can be an immune response target of diagnostic importance. The newly developed ELISA could prove a valuable serological tool, especially in those infections that are caused by only one plasmid-carrying species such as in avian and feline chlamydial infections. In contrast, this ELISA may not have the same diagnostic importance in other cases, such as pig

infections caused by several, often associated, chlamydial species. Further studies are needed to evaluate the degree of cross-reactivity between the *pgp3* proteins of different chlamydial species.

Given that the MIF is a tedious assay requiring a) culture of the relevant chlamydial strains, which is restricted to specialized laboratories, b) serum dilutions, and c) trained personnel and expertise, a novel sensitive and specific ELISA will certainly be of advantage.

### **Conflict of interest statement**

None declared.

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**Table 1.**

Sera reactivities to *pgp3* detected by the home-made ELISA and WB techniques in comparison to the serological reactivities to chlamydial EBs tested by MIF

Sera	N. of ELISA positive sera/ N. of sera tested (%)	N. of WB positive sera/ N. of sera tested (%)	N. of MIF positive sera/ N. of sera tested (%)
<b>Duck*</b> (8-11-13-14 weeks)	59/75 (78.7)	56/75 (74.7)	60/75 (80.0)
<b>Duck**</b> (4-5-6 weeks)	3/59 (5.1)	3/59 (5.1)	10/59 (16.9)
<b>Pigeon</b>	86/220 (39.1)	84/220 (38.2)	88/220 (40.0)
<b>Cat</b> Conjunctivitis	44/45 (97.8)	44/45 (97.8)	45/45 (100.0)
No clinical signs of chlamydial infection	0/5 (0.0)	0/5 (0.0)	0/5 (0.0)
<b>Pig</b> Conjunctivitis	45/45 (100.0)	45/45 (100.0)	45/45 (100.0)
No clinical signs of chlamydial infection	0/5 (0.0)	0/5 (0.0)	0/5 (0.0)
<b>Human patients</b>	33/50 (66.0)	33/50 (66.0)	36/50 (72.0)

\* Positivity to Chekit commercial ELISA: 34/75 (45.3%) ducks.

\*\* Positivity to Chekit commercial ELISA: 0/75 (0.0%) ducks.

**Table 2.**

Concordance/discordance of sera between ELISA, WB and MIF tests.

		pgp3-ELISA		pgp3-WB	
		+	-	+	-
MIF duck sera	+	61	9	58	12
	-	1	63	1	63

## pgp3-ELISA

		+	-
MIF pigeon sera	+	84	4
	-	2	130

## pgp3-WB

		+	-
MIF pigeon sera	+	82	6
	-	2	130

## pgp3-ELISA

		+	-
MIF human sera	+	29	7
	-	4	10

## pgp3-WB

		+	-
MIF human sera	+	29	7
	-	4	10

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