

POULTRY INFECTED WITH *Mycoplasma gallisepticum* OR *Mycoplasma synoviae* PRODUCE ANTIBODIES TO THEIR CYSTEINE PROTEASE CYS P

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Received May 15, 2013; accepted June 20, 2013.
Delo je prispelo 15. maja 2013, sprejeto 20. junija 2013.

Poultry infected with Mycoplasma gallisepticum and Mycoplasma synoviae produce antibodies to their cysteine protease CysP

Major poultry pathogens, *Mycoplasma gallisepticum* and *Mycoplasma synoviae* possess cysteine protease CysP, which *in vitro* cleaves chicken immunoglobulin G (IgG) into Fab (antigen-binding fragment) and Fc (crystallisable region fragment). We used recombinant CysP of *M. synoviae* (rCysP) in enzyme immunoassays to detect antibodies to CysP in sera, synovial fluids, and in washings of respiratory tract and oviducts of chickens and turkeys experimentally or naturally infected with *M. gallisepticum* or *M. synoviae*. In poultry infected with *M. synoviae*, 70.4 % of samples contained antibodies reacting with rCysP. In birds infected with *M. gallisepticum* we detected CysP antibodies in 63.1 % of samples. Our data demonstrate that CysP is immunogenic for chickens and turkeys and indicate that *M. gallisepticum* and *M. synoviae* infecting chickens and turkeys synthesise CysP *in vivo*. This is the first study demonstrating that proteases of any *Mycoplasma* species can induce production of specific antibodies in the natural host.

Key words: poultry / infections / immunology / immunogenicity / cysteine protease / *Mycoplasma synoviae* / *Mycoplasma gallisepticum*

1 INTRODUCTION

Among *Mycoplasma* species infecting poultry, *Mycoplasma gallisepticum* and *Mycoplasma synoviae* are the most pathogenic species (Ley 2008; Kleven and Ferguson-Noel, 2008). Although *M. gallisepticum* and *M. synoviae* belong to different phylogenetic groups, they share hosts, tissue tropism, transmission ways, as well as

Perutnina ob okužbi z bakterijo Mycoplasma gallisepticum ali Mycoplasma synoviae proizvede specifična protitelesa proti njuni cisteinski proteazi CysP

Mycoplasma gallisepticum in *Mycoplasma synoviae* sta pomembna patogena mikroorganizma pri perutnini. Sintetizirata cisteinsko proteazo CysP, ki *in vitro* razgrajuje kokošje imunoglobuline G na Fab in Fc fragment. Za dokazovanje protiteles proti CysP z encimsko imunskimi testi v serumih, sinovialnih tekočinah in v izpirkih dihalnih traktov in jajcevodov ptic naravno ali eksperimentalno okuženih z *M. synoviae* ali *M. gallisepticum*, smo uporabili rekombinanten protein CysP (rCysP). Pri perutnini okuženi z *M. synoviae* je protitelesa proti CysP vsebovalo 70,4 % vzorcev, pri perutnini okuženi z *M. gallisepticum* pa smo protitelesa proti CysP dokazali pri 63,1 % vzorcih. Pridobljeni podatki potrjujejo, da je CysP za perutnino imunogen protein in nakazuje, da *M. gallisepticum* in *M. synoviae* ob okužbi perutnine sintetizirata CysP *in vivo*. Z našo študijo prvič dokazujemo, da lahko mikoplazemska proteaza inducira nastanek specifičnih protiteles v naravnem gostitelju.

Ključne besede: perutnina / okužbe / imunologija / imunogenost / cisteinska proteaza / mikoplazme / *Mycoplasma synoviae* / *Mycoplasma gallisepticum*

a number of genes that have been transferred horizontally (Noormohammadi *et al.*, 1998; Vasconcelos *et al.*, 2005; Berčič *et al.*, 2011; Cizelj *et al.*, 2011). Among the transferred genes are those encoding proteins being very important in interactions with poultry hosts. *M. gallisepticum* and *M. synoviae* have multigene families encoding VlhA haemagglutinins, which play important role in binding to host cell receptors and colonization of host

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tissues (Noormohammadi *et al.*, 1998; 2000; Benčina 2002). VlhA are immunodominant lipoproteins inducing an early and strong antibody response in poultry (Markham *et al.*, 1992; Narat *et al.*, 1998; Noormohammadi *et al.*, 1999). Chickens infected with *M. synoviae* at first produced antibodies to the MSPB lipoprotein, which represents the N-terminal part of VlhA (Narat *et al.*, 1998; Noormohammadi *et al.*, 1998; 1999). Moreover, MSPB induced synthesis of nitric oxide (NO), IL 1 β and IL 6 in chicken peripheral blood leukocytes including macrophages (Lavrič *et al.*, 2007). Recent studies in our laboratories demonstrated that two proteins, shared by *M. gallisepticum* and *M. synoviae*, can modify chicken proteins, including chicken IgG. *M. synoviae* NanH neuraminidase desialylated chicken IgG and tracheal mucus glycoproteins *in vitro* (Berčič *et al.*, 2011). Moreover, *M. gallisepticum* and *M. synoviae* cleaved chicken IgG into Fab and Fc *in vitro* (Cizelj *et al.*, 2011). Their cysteine protease CysP was involved in this process and recombinant CysP (rCysP) of *M. synoviae* also cleaved chicken IgG into Fab and Fc (Cizelj *et al.*, 2011) *in vitro*. Since, CysP has potential to cleave chicken proteins also *in vivo*, it may be damaging to host during infection. Therefore, we were interested whether infected poultry hosts raise an antibody response against CysP. Indirectly, antibodies to CysP would indicate that *M. synoviae* and *M. gallisepticum* populations synthesize CysP *in vivo*, too.

The aim of this study was to determine whether poultry infected with *M. synoviae* or *M. gallisepticum* produce antibodies to CysP. For this purpose, rCysP produced in our previous study (Cizelj *et al.*, 2011) was used as a specific antigen with defined structure and a number of samples from infected chickens and turkeys were examined for antibodies to rCysP.

2 MATERIALS AND METHODS

2.1 MYCOPLASMA ANTIGENS, RECOMBINANT CYS, RECOMBINANT MSPB, PURIFIED PROTEINS AND SYNTHETIC PEPTIDES

M. synoviae WVU 1853 and *M. gallisepticum* S6 LP strains were used to prepare their whole-cell antigens (Benčina *et al.*, 1994; 2005). Their cells were grown in a modified Frey's broth containing 10 % porcine serum and harvested as described (Berčič *et al.*, 2008a; Dušanić *et al.*, 2009). Recombinant CysP (rCysP) was produced as described in detail in our previous study (Cizelj *et al.*, 2011). Briefly, we introduced eight point mutations replacing eight TGA codons with TGG using site-directed PCR-mediated mutagenesis and added C-terminal His tag in the *cysP* gene of *M. synoviae* ULB 925. The

purified rCysP protein has molecular mass of approximately 67 kDa (Cizelj *et al.*, 2011). A synthetic peptide ¹⁰²DIDEHAIDYVSNS representing immunogenic part of the amino acid sequence of *M. synoviae* and *M. gallisepticum* CysP and synthetic peptide, ⁴⁶⁴DKRVNPKD-DRGHLEQD, representing partial amino sequence of the NanH neuraminidase (Cizelj *et al.*, 2011; Berčič *et al.*, 2011) were purchased from GenWay (San Diego, USA). Recombinant MSPB (rMSPB) containing 190 amino acids of the *M. synoviae* strain ULB 925 lipoprotein MSPB, the N-terminal part of the haemagglutinin VlhA, was from our previous study (Benčina *et al.*, 2001).

M. gallisepticum (S6 strain) haemagglutinin (previously termed pMGA 1.1) (Markham *et al.*, 1992; 1993) was immuno-affinity purified using mAb 6A10 immobilized to CNBr-sepharose (Sigma) as a specific ligand (Benčina *et al.*, 1994; Milošević-Berlič *et al.*, 2000). The purified pMGA (Mw ~ 67 kDa; pI ~ 5.5) reacted in immunoblots with mAbs 71 to pMGA (Markham *et al.*, 1992), but not with mAb 3 to DnaK (Berčič *et al.*, 2008a) which have in S6 strain similar Mr and pI (Demina *et al.*, 2009).

2.2 ENZYME IMMUNOASSAYS USED TO DETECT SPECIFIC ANTIBODIES

Dot-immunobinding assay (DIBA) was used to detect specific antibodies in a variety of samples from poultry infected by *M. synoviae* or *M. gallisepticum*. Appropriate antigens were applied as separate dots (2 μ l) on strips of the Immobilon P membrane (Millipore) (Benčina *et al.*, 2005). Optimal concentrations of antigens were previously determined by DIBA using reference antibodies to *M. synoviae*, *M. gallisepticum*, rCysP, rMSPB, pMGA 1.1 and to synthetic peptides representing partial CysP and NanH (Benčina *et al.*, 1994; 2001; 2005; Berčič *et al.*, 2008a; 2011; Cizelj *et al.*, 2011). So, pelleted mycoplasma cells were diluted in PBS (pH 7.4) 1:2000; proteins (rMSPB, rCysP, pMGA) at concentrations of ~ 5–10 μ g/ml and synthetic peptides at a concentration of ~ 10 μ g/ml.

DIBA was conducted as a three-step assay. The strips bearing five different antigens (five separate dots, see Fig. 1 and 2) were blocked for 1 h in PBS containing 0.5 % Tween 20 and then incubated for 1 h with a sample examined for antibodies or in serum of SPF chicken as negative control. Following washing (3 \times 15 min) in PBS containing 0.05 % Tween 20 (PBST) strips were incubated for 50 min in PBS containing mAbs CH31 (Sigma) to chicken Igs light chain (see Cizelj *et al.*, 2011). After washing in PBST (3 \times 15 min) strips were incubated in goat antibodies (to mouse IgG) labeled with horseradish

peroxidase (HRP, A4416, Sigma) for 45 min. After washing (2 × 15 min in PBST and 15 min in PBS) reactions were visualized by the chromogen substrate TrueBlue (KPL, USA).

Assays to detect specific IgM or IgA antibodies were done similarly, but on the second step, mAbs M1 to chicken IgM or mAbs A62 to chicken IgA (Benčina *et al.*, 2005) were used instead of mAbs CH31.

Most samples were assayed with the two-step DIBA as well. After the first step, performed as described above, strips were incubated in rabbit IgG (to chicken IgG) conjugated with HRP (A9046, Sigma) for 45 min. Following washings, reactions were visualized by TrueBlue. They were evaluated arbitrarily by at least two investigators. In all enzyme immunoassays sera of specific pathogen-free (SPF) chickens were used as negative controls (Benčina *et al.*, 2005). Positive controls were chicken IgG to CysP and NanH peptides (Berčič *et al.*, 2011; Cizelj *et al.*, 2011) and antisera to rMSPB or pMGA (Benčina *et al.*, 2001; Milošević Berlič *et al.*, 2000).

Immunoblotting was used to confirm that samples giving positive DIBA reactions with rCysP react with this protein (~ 67 kDa). rCysP (5 µg/ml) was electrophoresed and transferred to the ImmobilonP membrane using the PhastSystem (Pharmacia, LKB, Sweden) and PhastGel Gradient (8–25) gels (Cizelj *et al.*, 2011). Incubations with antibodies and reaction visualization were done as described for DIBA.

The indirect immunoperoxidase assays (IIPA) were used to confirm that samples assayed by DIBA and immunoblotting contain antibodies to *M. synoviae* (WVU 1853) or *M. gallisepticum* (S6 or R). Antigens were their intact colonies *in situ* (on agar blocks) and IIPA was conducted as described elsewhere (Benčina and Bradbury, 1991).

2.3 SAMPLE COLLECTION, STORAGE AND EXAMINATIONS

All samples examined for antibodies to CysP in this study have been collected in our previous experimental studies and examinations of field chicken and turkey flocks for infections with avian *Mycoplasma* species (see References in Tables 1, 2 and 3). Samples (sera, synovial fluids, mucus from respiratory tract, oviductal washings, exudates from turkey's sinuses) were kept frozen (–20 °C). In DIBA and immunoblotting sera were used diluted 1:100, but some of them were used also at a dilution of 1:1000 or even higher.

Synovial fluids from swollen tibiotarsal-tarsometatarsal (hock) joints, were also assayed diluted 1:100. The same dilution was used for tracheal mucus, whereas res-

Table 1: Antibodies to CysP in chickens experimentally infected with *M. synoviae*
Preglednica 1: Prisotnost protiteles proti CysP pri piščančih eksperimentalno okuženimi z *M. synoviae*

Group	Inoculation		Antibodies in						References		
	Age	Strain	Way ^b	CFU	Serum		Synovial fluid			Respiratory tract	
					rCysP	peptide	rCysP	peptide		rCysP	peptide
A	4 weeks	F10-2AS	o/n and i/a	~ 10 ⁴	13/15*	3/15*	12/14*	1/10	4/10	0/10	Benčina <i>et al.</i> , 2001
B	4 weeks	JAH0-1	i/a	~ 10 ⁵	6/12	1/12	20/30*	2/30*	NT ^d	NT	Benčina <i>et al.</i> , 1999
C	5 weeks	AAY-4	i/a	~ 10 ⁷	7/11	0/11	8/10*	1/10*	NT	NT	Narat <i>et al.</i> , 1998
D	~ 1 year	K1723	i/v and i/ov	INACT ^e	4/4	1/4	NT	NT	NT	NT	Benčina <i>et al.</i> , 2005
E	~ 1 year	ULB 02/T6	i/n	~ 10 ⁸	9/12*	0/12*	NT	NT	2/2	0/2	Slavec <i>et al.</i> , 2011

^a Detected by DIBA and for selected samples confirmed by immunoblotting using rCysP. All samples contained antibodies to rMSPB (Benčina *et al.*, 2001)

^b o/n, ocular; i/a, intraarticular (hock joint); i/v, intravenous; i/ov, into oviduct; i/n, intranasal

^c Number of positive/number of samples tested

^d NT, not tested

^e *M. synoviae* cells were heat-inactivated and chickens were immunized also with rMSPB (Benčina *et al.*, 2005)

^f Two sera taken 8 days pi and one serum taken 18 days pi were negative.

* Difference between frequency of antibodies to rCysP and its peptide is significant (P < 0.01).

piratory tract (tracheas, choanal cleft) and oviduct washings were tested considering dilution degree used when they were collected. For such samples total Igs concentrations were first determined by DIBA using mAbs CH31, and then, an appropriate dilution was used in additional assays detecting antibodies to CysP.

2.4 STATISTICAL ANALYSIS

Significance of differences between frequencies of antibodies to CysP and to CysP peptide in appropriate samples was determined using two-tailed Fisher's exact test. P values < 0.01 were considered significant.

3 RESULTS

3.1 EXPERIMENTAL *M. SYNOVIAE* INFECTIONS INDUCED IN CHICKENS ANTIBODIES TO CYS P

Infections with four different *M. synoviae* strains, using different numbers of CFU and ways of inoculation,

induced antibodies to CysP in all four groups of chickens (Table 1). Moreover, antibodies to rCysP were also detected in sera of chickens inoculated with the heat-inactivated *M. synoviae* K1723. Systemic antibodies to rCysP were detected in 39/54 (72.2 %) of sera, whereas less than 10 % (5/54) of sera contained antibodies to CysP peptide. In chicken groups A, B and C injected with *M. synoviae* intraarticularly, 75.5 % (40/54) of chickens revealed antibodies to rCysP in their synovial fluids, while only 8 % of those fluids had antibodies to CysP peptide. This difference is highly statistically significant ($P < 0.001$). Chickens of group A inoculated with *M. synoviae* F10-2AS also oculonasally, had antibodies to rCysP in the upper respiratory tract (choanal cleft) already 8 days post infection. Such antibodies were detected in their synovial fluids and sera 12 days pi (Fig. 1A and Table 1) as well. Antibodies to rCysP present in joints and sera were of the IgM class too and this was found also for antibodies reacting with MSPB (data not shown). In all, antibodies to rCysP were detected in more than 70 % (70.8 %) of samples tested, whereas those to CysP peptide were found in 8.5 % of samples.

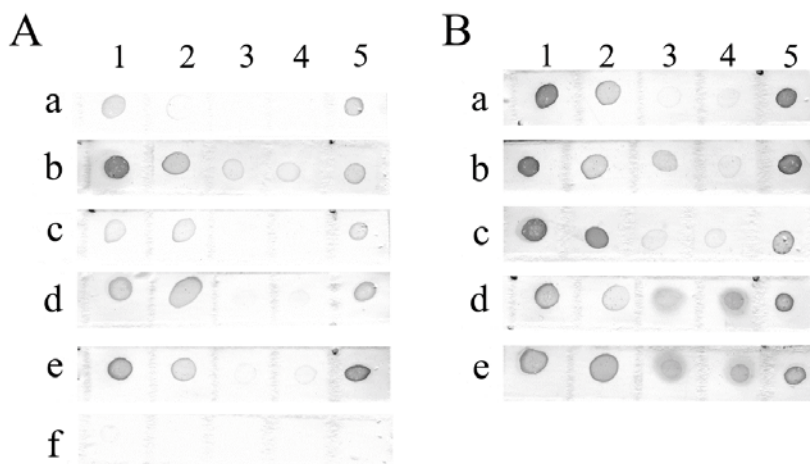


Figure 1 (A, B): Antibodies to CysP and MSPB detected by DIBA in *M. synoviae* infected chickens. Antigens on strips are: 1, *M. synoviae* WVU 1853 cells; 2, rCysP; 3, CysP peptide; 4, NanH peptide and 5, rMSPB. Strips with antigens were incubated with chicken sera (diluted 1:100) for 1 h and then with rabbit IgG (to chicken IgG) HRP conjugate for 45 min. Reactions were visualized by TrueBlue.

Panel A, reactions with sera of chickens infected experimentally with F10-2AS. Strips a and b, sera of chicken No 1 (taken 8 and 12 days pi); strips c and d, sera of chicken No 3 (8 and 12 days pi); strip e, serum of chicken No 4 (12 days pi) and strip f, serum of a SPF chicken. Panel B, reactions with sera of naturally infected chickens. Strips a, b and c, sera of chickens from group A; strips d and e, sera of chickens from group B (Table 1). Note, *M. synoviae* was isolated from tracheas of those naturally infected chickens.

Slika 1 (A, B): Detekcija protiteles proti CysP in MSPB v vzorcih piščancev okuženimi z *M. synoviae* z DIBA testom. Na trakove naneseni antigeni so: 1, celice *M. synoviae* WVU 1853; 2, rCysP; 3, peptid CysP; 4, peptid NanH in 5, rMSPB. Slika A, reakcija s serumi piščancev eksperimentalno okuženih z F10-2AS. Trakova a in b, serum piščanca št. 1 (odvzet 8 in 12 dan pi); trakova c in d, serum piščanca št. 3 (odvzet 8 in 12 dan pi); trak e, serum piščanca št. 4 (12 dan pi) in trak f, serum SPF piščanca. Slika B, reakcija s serumi naravno okuženih piščancev. Trakovi a, b in c, serumi piščancev iz skupine A; trakova d in e, serumi piščancev iz skupine B (Preglednica 1). Upoštevajte, da je bila iz trahej naravno okuženih piščancev izolirana bakterija *M. synoviae*.

3.2 ANTIBODIES TO CYS P IN *M. SYNOVIAE* NATURALLY INFECTED CHICKENS AND TURKEYS

In chickens with the natural *M. synoviae* infection two thirds of sera (28/42) revealed also antibodies to CysP (Fig. 1B and Table 2). Sera from adult hens with the positive *M. synoviae* culture (tracheas and oviducts), reacted with rCysP also at dilution of 1:1000, one serum even at a dilution of 1:8000. Almost a half of positive sera (13/28) contained antibodies reacting with the CysP peptide. The percentage of synovial fluids containing antibodies to rCysP (68.8 %) was rather similar to that of sera (66.7 %). Synovial fluids contained antibodies to the CysP peptide (10/64), but they were detected less frequently than in sera. Antibodies to rCysP were also found in tracheal washings (11/14) and oviduct washings (13/22). As could be expected, antibodies to the CysP peptide were found in those washings less frequently than antibodies to rCysP (Table 2). Differences are statistically significant for synovial fluids (for chicken breeds PPR, ITAL and JA) and respiratory tract samples ($P < 0.01$).

Synovial fluids collected from swollen hock joints of young (4–8 weeks old) or adult chickens, contained high levels of antibodies to *M. synoviae*, particularly to its MSPB. Most synovial fluids contained antibodies to CysP of all three classes, IgA, IgG and IgM (detail not shown in Table 2).

In three flocks of turkeys with relatively strong antibody response to *M. synoviae*, almost 80 % of sera (23/30) contained IgG antibodies to rCysP. Moreover, almost 37 % of sera contained antibodies reacting with the CysP peptide (Table 2). Antibodies to rCysP were also found in synovial fluids of some turkeys with acute infectious synovitis due to *M. synoviae*. Overall, in poultry with natural *M. synoviae* infection, 127 samples out of 181 (70.2 %) samples tested contained antibodies to rCysP.

3.3 INFECTIONS WITH *M. GALLISEPTICUM* INDUCED ANTIBODIES TO CYS P IN CHICKENS AND TURKEYS

Adult chickens infected intratracheally with the pathogenic *M. gallisepticum* strain S6 LP raised antibodies to CysP (Table 3). More than 75 % of sera examined (25/33) contained antibodies reacting with rCysP (Fig. 2). Such antibodies were detected also in tracheal (14/19) and in oviduct washings (6/8). Not surprisingly, antibodies to the CysP peptide were less frequent in sera (4/33), tracheal (2/20) and oviduct washing (1/8). Antibodies to rCysP were detected in sera of most in-

Table 2: Antibodies to CysP in chickens and turkeys naturally infected with *M. synoviae*
Preglednica 2: Prisotnost protiteles proti CysP pri piščančih in puranih naravno okuženimi z *M. synoviae*

Birds	Breed ^b	<i>M. synoviae</i> ^c	Antibodies in										References
			Serum		Synovial fluid		Respiratory tract		Oviduct		peptide	peptide	
			rCysP	peptide	rCysP	peptide	rCysP	peptide	rCysP	peptide			
Chickens	PPR	ULB 02/T6	22/29 ^d	8/23 ^d	15/20*	5/20*	11/14*	1/14*	7/12	1/12	Slavec <i>et al.</i> , 2011		
Chickens	ITAL	NA	NT	NT	14/20*	2/20*	NT ^e	NT	NT	NT	This study		
Chickens	JA	ULB 991	7/11	4/11	9/14*	1/14*	NT	NT	NT	NT	Benčina <i>et al.</i> , 1999		
Chickens	PX	ULB 9122	5/8	1/8	6/10	2/10	NT	NT	6/10	1/10	Berčič <i>et al.</i> , 2011		
Turkeys	PIV	PAA2	23/30*	11/30*	2/3	0/3	NT	NT	NT	NT	Zorman Rojs <i>et al.</i> , 2000		
Turkeys	PIV	PAA2	23/30*	11/30*	2/3	0/3	NT	NT	NT	NT	Zorman Rojs <i>et al.</i> , 2000		

^a Detected by DIBA and for selected samples confirmed by immunoblotting using rCysP as an antigen. All samples contained antibodies to rMSPB (Benčina *et al.*, 2001). *M. synoviae* was isolated from flocks of all those breeds.

^b Flocks kept on farms in Slovenia, except ITAL, kept on farms in northern Italy.

^c *M. synoviae* strains isolated. Their cysP sequences have been determined (Cizelj *et al.*, 2011) except for strain(s) causing infection in Italy (NA – cysP sequence not available)

^d Number of positive/number of samples tested

^e NT, not tested

* Difference between frequency of antibodies to rCysP and its peptide is significant ($P < 0.01$).

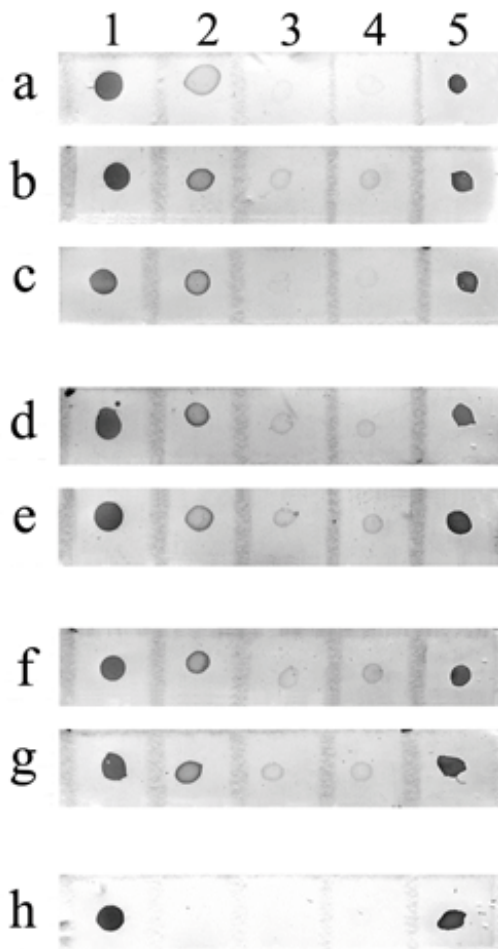


Figure 2: Antibodies to CysP and pMGA detected by DIBA in chickens inoculated with *M. gallisepticum* (S6 LP). Antigens on strips are: 1, *M. gallisepticum* (S6 LP) cells; 2, rCysP; 3, CysP peptide; 4, NanH peptide and 5, pMGA. Strips a, b and c, sera of three chickens (3 weeks pi); strips d and e, sera of two chickens (6 weeks pi) and strips f and g, sera of two chickens (4 months pi). Strip h was incubated with chicken antiserum to pMGA.

Slika 2: Detekcija protiteles proti CysP in pMGA z DIBA testom v vzorcih piščancev, ki smo jim inokulirali *M. gallisepticum* (S6 LP). Na trakove naneseni antigeni so: 1, celice *M. gallisepticum* (S6 LP); 2, rCysP; 3, peptid CysP; 4, peptid NanH in 5, pMGA. Trakov a, b in c, serumi treh piščancev (3 tedne pi); trakova d in e, seruma dveh piščancev (6 tednov pi) in trakova f in g, seruma dveh piščancev (4 mesece pi). Trak h smo inkubirali v pMGA specifičnem kokošjem antiserumu.

oculated chickens already 2 weeks pi and also 4 months after infection. Tracheas of contact-exposed chickens became colonized with *M. gallisepticum* 2 weeks after co-habitation with chickens inoculated with S6 LP. After additional 7 days, most of contact-exposed chickens had serum IgM antibodies to the haemagglutinin pMGA, as

well as to rCysP (not shown in Table 3). Serum and respiratory tract antibodies to rCysP were detected significantly more frequently than antibodies to CysP peptide (Fisher's exact test, $P < 0.0001$). Samples from naturally infected chickens revealed also IgG antibodies to CysP, and they were detectable in sera, respiratory tract and oviduct (Table 3).

Experimental infection of turkeys with *M. gallisepticum* (K4421A) induced antibodies to CysP in their tracheas already 10 days pi (Table 3). Such antibodies were detected in turkeys with the natural *M. gallisepticum* infection as well. Antibodies to CysP were found in sera and in exudates taken from swollen infraorbital sinuses. In all, in poultry infected with *M. gallisepticum*, 70 samples out of 115 (60.9 %) samples examined contained antibodies to CysP.

3.4 ANTIBODIES TO CYS P IN PHEASANTS AND PEAFOWL INFECTED WITH *M. GALLISEPTICUM*

We also examined sera of pheasants and peafowl in which infections with well characterized *M. gallisepticum* strains ULB 971 and ULB 992 caused severe disease with a considerable mortality (Benčina *et al.*, 2003). Pheasant sera contained IgG antibodies that reacted with both, rCysP and CysP peptide. Sera of peafowl contained antibodies reacting with rCysP, as well as with CysP peptide (not shown in Table 3).

4 DISCUSSION

M. gallisepticum and *M. synoviae* cleave *in vitro* chicken serum proteins. Among them is IgG, which is cleaved by the CysP protease into Fab and Fc fragments (Cizelj *et al.*, 2011). Considering potential damage caused by CysP to host's proteins, it was of interest to see whether avian hosts infected by *M. synoviae* or *M. gallisepticum* raise antibodies to their CysP. Our investigation demonstrates that most of the birds infected by either of the two *Mycoplasma spp.* produced antibodies to CysP (Tables 1–3). This suggests that *M. synoviae* and *M. gallisepticum* produce CysP also *in vivo*, what apparently triggers host's immune response and the production of specific antibodies. Experimental infections of chickens with *M. synoviae* strains indicated an early antibody response to CysP. Chickens infected with *M. synoviae* F10-2AS had CysP antibodies, including specific IgM, in sera, synovial fluids and upper respiratory tract samples 8 and 12 days pi. In comparison to the antibody response to MSPB, which occurs approximately one week after in-

Table 3: Antibodies to CysP in chickens and turkeys infected with *M. gallisepticum*
Preglednica 3: Prisotnost protiteles proti CysP pri piščančih in puranih okuženimi z *M. gallisepticum*

Birds	Infection	Strain ^b	Antibodies in				References		
			Serum		Respiratory tract			Oviduct	
			rCysP	peptide	rCysP	peptide		rCysP	peptide
Chickens	Experimental	S6 LP	25/33 ^{c*}	4/33 ^{c*}	14/19 [*]	2/20 [*]	6/8	1/8	Benčina <i>et al.</i> , 1994
Chickens	Natural	ULB 931	6/10	1/10	5/10	1/10	4/6	0/6	Benčina <i>et al.</i> , 1994
Turkeys	Experimental	K4421/A ^b	NT	NT	5/13	0/13	NT	NT	Kleven <i>et al.</i> , 2004
Turkeys	Natural ^d	ULB 02/S1	3/8	0/8	2/8	0/8	NT	NT	This study

^a Antibodies detected by DIBA. All samples contained antibodies to *M. gallisepticum* detected by the indirect immunoperoxidase assay and immunoblotting (Benčina and Bradbury, 1991; Benčina *et al.*, 1994).

^b Strain(s) isolated or used for experimental infections. Chickens were infected with S6 LP oculonasally and intratracheally (108 CFU). Infection of turkeys with K4421/A (aerosol, ~ 1011 CCU) was done at the University of Georgia, GA, USA. Samples of tracheal washings (10 days post infection) were donated by S. H. Kleven.

^c Number of positive/number of samples tested, NT – not tested.

^d An outbreak of infectious sinusitis on farms of young turkeys (~ 10 weeks old) in Slovenia. Respiratory tract samples were exudates from acutely swollen infraorbital sinuses of turkeys.

^{*} Difference between frequency of antibodies to rCysP and its peptide is significant ($P < 0.01$).

fection (Narat *et al.*, 1998; Noormohammadi *et al.*, 1999) response to CysP was not delayed. An early antibody response to CysP was observed also in chickens experimentally infected with the strain S6 LP of *M. gallisepticum* (Benčina *et al.*, 1994). Interestingly, contact-exposed chickens, whose upper tracheas became colonized with S6 LP in the second week of exposure, had systemic IgM to CysP after 3 weeks of exposure. This suggests that the colonizing *M. gallisepticum* expressed CysP in conditions simulating natural infection of chickens with *M. gallisepticum*. In addition, CysP antibodies were present in the upper respiratory tract, along with antibodies to the haemagglutinin pMGA (not shown). Chickens had antibodies to CysP in serum, trachea and oviduct also at the end of the experiment, more than four months pi.

Results shown in Table 2 indicate that CysP is immunogenic for poultry with natural *M. synoviae* infection. Antibodies to CysP were found in sera, synovial fluids, respiratory tract and oviducts. In most groups antibodies to the CysP peptide were detected too, but less frequently than those to rCysP. This could be expected, because an epitope represented by the synthetic CysP peptide is only one of more than 20 potential epitopes (antigenic sequences) predicted for the rCysP protein sequence (Larsen *et al.*, 2006). Infections with *M. synoviae* strains ULB 02/T6 and PAA2 with shorter CysP protein (572 amino acids) induced antibodies to CysP at a similar frequency as strains with longer CysP (ULB 9122, ULB 991) containing 585 amino acids (Cizelj *et al.*, 2011). Turkey flocks presented in Table 2 were co-infected with *Ornithobacterium rhinotracheale* (Zorman-Rojs *et al.*, 2000). Co-infection did not influence the turkey's antibody response to *M. synoviae* or to its CysP protein, since almost 80 % of sera contained antibodies to CysP (Table 2). Natural infections of chickens and turkeys with *M. gallisepticum* induced the production of antibodies to its CysP protease (Table 3). This seems to be true also for pheasants and peafowl in which infections with *M. gallisepticum* can be fatal (Benčina *et al.*, 2003). *M. gallisepticum* induced in poultry antibodies to rCysP less frequently than *M. synoviae*, but it should be considered that the target antigen used represents CysP of ULB 925 *M. synoviae* (Cizelj *et al.*, 2011).

The NanH neuraminidase (sialidase), also encoded by a horizontally transferred gene, is associated with invasiveness and virulence of *M. synoviae* and *M. gallisepticum* strains (May *et al.*, 2007; Berčič *et al.*, 2008b). Polymorphic sequences in the *M. synoviae* strains sialidase proteins indicate diversifying selection (May and Brown, 2009). Notably, polymorphisms in the CysP protease sequences among *M. synoviae* strains also suggest CysP diversifying selection (Cizelj *et al.*, 2011 and unpublished analyses). This study suggests that CysP might be under

“pressure” of the antibody response, because most of the infected birds produced specific antibodies to CysP (Tables 1–3). At present, this is less clear for the diversifying selection of the NanH neuraminidase by antibodies to NanH. This study indicates that, poultry infected with *M. synoviae*, raised antibodies to peptide representing NanH (see Figs. 1B and 2). Antibodies to this peptide were detected even more frequently than antibodies to CysP peptide, but the difference was not statistically significant. Interestingly, CysP and NanH can modify chicken IgG and target the same region of its heavy chain, albeit with distinct activities. CysP cleaves the heavy chain of IgG in CH2 domain and generates Fab and Fc (Cizelj *et al.*, 2011). On the other hand, NanH cleaves sialic acid from a (SA α (2-6)gal) moiety positioned in the N-glycosylated site of CH2 domain (amino acid N₃₀₈) of the IgG heavy chain (Berčič *et al.*, 2011). The activity of CysP is independent of that of NanH, because *M. synoviae* strains cultures lacking NanH and neuraminidase activity (Berčič *et al.*, 2011) cleaved chicken IgG (Cizelj *et al.*, 2011).

Our previous analyses suggest that *M. synoviae* grown *in vitro* can release CysP into growth medium (Cizelj, 2011). If this can happen *in vivo*, CysP protease would cleave host's proteins including IgG. We observed that in some synovial fluid samples from joints colonized by *M. synoviae* (group A in Table 1), a considerable part of IgG was fragmented into Fab and Fc (Benčina *et al.*, unpublished data). Antibodies to CysP were present, but it is possible that CysP cleaved IgG before antibodies to CysP appeared in synovial fluids. The same synovial fluid samples contained also IgM antibodies reacting with chicken IgG Fc (domains CH3-CH4). Such autoantibodies, termed rheumatoid factor, are frequently present in sera of chickens with infectious synovitis due to *M. synoviae* (Sells, 1976; Porter and Gooderham, 1966). It is possible that NanH and CysP activities contribute to the induction of autoantibodies to Fc, but further research is needed to confirm this hypothesis.

This study demonstrates that CysP is among cross-reactive antigens shared by *M. gallisepticum* and *M. synoviae*. It is possible that their ~ 70-kDa proteins sharing antigenic determinants were CysP (Bradley *et al.*, 1988). However, they synthesize a number of other proteins of similar molecular mass and some of them can be cross-reactive, too (Berčič *et al.*, 2008a; Benčina *et al.*, unpublished data).

In conclusion, this is the first study demonstrating that hosts can raise antibodies to proteases of *Mycoplasma* species. Birds infected by *M. gallisepticum* or *M. synoviae* produced antibodies to CysP, which is otherwise capable to cause proteolysis of their proteins, including IgG. Whereas antibodies to CysP seem to be incapable

to eliminate *M. synoviae* from the respiratory tract, additional investigations are needed to see whether they can diminish damage caused by the CysP proteolytic potential.

5 ACKNOWLEDGEMENTS

This work was supported by Slovenian Research Agency (ARRS) (grant P4-0220). We thank Ana Jakopič and Irena Beretič for technical assistance. For donation of materials from *M. gallisepticum* infected chickens and turkeys, we thank Drs Stanley H. Kleven and Anton Svetlin.

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