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A survey of avian *Mycoplasma* species for neuraminidase enzymatic activity

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24 Abstract

25

26 Among 23 currently recognized avian *Mycoplasma* (AM) species only *M.*
27 *gallisepticum*, *M. synoviae*, *M. meleagridis* and *M. iowae* cause disease and loss
28 of production in chickens and/or turkeys. Because neuraminidases are
29 considered virulence factors in many pathogenic microorganisms the aim of our
30 study was to determine which AM species possess neuraminidase enzymatic
31 activity (NEAC). Small samples of AM cells were assayed for NEAC using the
32 chromogenic substrate 5-Bromo-4-chloro-3-indolyl- α -D-N-acetylneuraminic
33 acid. In the case of positive NEAC reaction the substrate gave the insoluble
34 indigoblue product what enabled simple test and easy estimation of NEAC. *M.*
35 *gallisepticum* and *M. synoviae* which share sequences of the gene encoding
36 neuraminidase (sialidase NanH) exhibited considerable levels of NEAC.
37 However, NEAC levels differed among their strains, as well as among cultures
38 of different strains. Only certain cultures of the type strain of *M. meleagridis*
39 showed NEAC, whereas among six serovars of *M. iowae* only serovar I (type
40 strain 695) showed NEAC. Weak NEAC was detectable in *M. anseris*, *M.*
41 *cloacale* and *M. pullorum*, whereas the type strain of *M. corrogypsi* (BV1)
42 showed strong NEAC. Our study provides novel informations about NEAC in
43 AM species and suggests that higher invasiveness and possibly, the pathological
44 processes might be associated with their NEAC.

45

46 Key words: avian *Mycoplasma* species, neuraminidase activity

47

48 1. Introduction

49

50 Pathogenic avian *Mycoplasma* (AM) species are important causes of
51 disease and loss of production in intensively reared poultry (Chin et al., 2003;
52 Kleven, 2003b; Ley 2003). Chicken and/or turkeys are natural hosts of twelve
53 *Mycoplasma* spp., whereas 23 recognized species could be found in birds
54 (Bradbury, 1998; Kleven, 2003a). However, only *Mycoplasma gallisepticum*,
55 *Mycoplasma synoviae*, *Mycoplasma meleagridis* (infects only turkeys) and
56 *Mycoplasma iowae* are considered to be significant poultry pathogens (Bradbury,
57 1998; Kleven, 2003a). These pathogenic AM spp. synthesize haemagglutinins
58 which are immunodominant antigens (Benčina, 2002). Their strains vary in
59 tissue tropism, invasiveness and pathogenicity, but factors contributing to such
60 differences are largely unknown.

61 Recently, Brown et al. (2004) suggested that *Mycoplasma alligatoris*
62 sialidase (neuraminidase) is associated with its high invasiveness and virulence.
63 Interestingly, *M. gallisepticum* and *M. synoviae* share sequences of genes coding
64 for putative sialidase *i.e.* neuraminidase (Papazisi et. al., 2003; Vasconcelos et
65 al., 2005). However, data about the neuraminidase enzymatic activity (NEAC)
66 in different *M. gallisepticum* strains are conflicting (Roberts, 1967; Sethi and
67 Müller, 1972; Glasgow and Hill, 1980). Indeed, it has been reported that

reference strains of *M. gallisepticum*, S6 and A5969, did not reveal detectable neuraminidase activity (Glasgow and Hill, 1980; Kahane et al., 1990). Both, *M. gallisepticum* and *M. synoviae* bind to host cell via receptors containing sialic acid residues (Razin, 1985). Therefore, their potential NEAC might be very important in interactions with their host cells and glycoproteins containing sialic acid receptors.

The main aim of this study was to assess recognized avian *Mycoplasma* spp. concerning their NEAC. Using commercially available substrate in a simple and relatively rapid test we developed a novel assay for detecting NEAC in *M. gallisepticum*, *M. synoviae* and other recognized AM spp.

2. Materials and methods

2.1 *Mycoplasma* species, their strains and growth media

Five reference strains of *M. gallisepticum* characterized in previous studies and used in this study are listed in Table 1 (Levisohn et al., 1986; Benčina et al., 1994; Avakian et al., 1991; Winner et al., 2000). Reference strains of *M. synoviae* also used in previous investigations are shown in Table 2 (Avakian et al. 1992, Benčina et al., 1999, 2001, 2005). One isolate of the type strain of *M. synoviae* (WVU1853), one isolate of *M. meleagridis*, and reference serovars (I, J, K, N, Q, and R represented by strains 695, DJA, DK-CPA, FMN, L3-10B and DRA-O, respectively) were provided by Dr. Janet M. Bradbury. The type

strains of *M. meleagridis* (17529) and *M. synoviae* (WVU 1853) were obtained also from FAO/WHO Collaborating Center for Animal Mycoplasmas, Aarhus, Denmark). Other AM species and their type strains are listed in Table 3.

A modified Frey's media (agar and broth) containing 10% porcine serum, 0,1% NAD and 0,1% cysteine hydrochloride were used for *M. synoviae* and other AM spp. However, media with 10% horse serum (but without NAD) were also used to grow AM spp., except *M. synoviae*. Cultures were grown at 37-38°C and were usually harvested in a late log-phase growth. Colony forming units (CFU) were determined by standard procedures (Rodwell and Whitcomb, 1983).

2.2 Preparation of mycoplasma samples examined for neuraminidase enzymatic activity (NEAC)

Mycoplasma cells were harvested by centrifugation (usually 27000 x g, 20min). Their pellets were washed in sterile phosphate buffered saline (PBS, pH 7,4) using centrifugation. Samples were then assayed for NEAC or were kept frozen (at -20°C) and were thawed when the NEAC test was done.

2.3 Determination of NEAC

The substrate used for NEAC assays was 5-Bromo-4-chloro-3-indolyl- α -D-N-acetylneuraminic acid sodium salt (BIN, B4666, Sigma, St. Louis, MO)). Stock dilutions of this substrate in MilliQ water contained 4 mg of the substrate

per ml and were kept frozen as 1ml aliquots at -20°C. Samples with AM spp. cells (20 – 30 µL of cell-suspension) were assayed in small microfuge tubes with caps or in wells of the microtiter plates (see Supplementary Figs. 1 and 2). Mycoplasma samples (~ 30 µL of suspension) were prepared (in PBS pH 5, with 10 mM CaCl₂) in wells of 96-well plastic microtiter plates. Usually, the suspension examined represented about 100-fold concentration of mycoplasma cells in comparison with their concentration in appropriate broth culture. In the case of *M. synoviae* and *M. gallisepticum* their samples tested contained approximately 10⁹ CFU. Samples of AM species being negative at such concentration were then tested at higher density. For mycoplasma samples with higher NEAC, titration using double dilutions was done.

The substrate BIN was added to each well with sample, usually 10µl of the solution containing 5µg of the substrate.

In most cases samples were incubated at room temperature. The positive NEAC reactions occurred as indigoblue staining. In each NEAC assay the positive control was neuraminidase of *Clostridium perfringens* (type V, N2876, Sigma), whereas the negative control was PBS.

3. Results

3.1 NEAC varies in cultures of *M. gallisepticum*

We examined NEAC in cultures of five reference *M. gallisepticum* strains which differ in infectivity, invasiveness and pathogenicity for chickens and turkeys, as well as their embryos.

All examined cultures of the type strain PG31 and strain A5969 (both at high levels of *in vitro* passages) revealed lower levels of NEAC than strain R at a low passage (Table 1). However, in contrast to a previous study using A5969 (Kahane et al., 1990), cultures of A5969 had a detectable NEAC in this study.

In strain R, only its cultures at low passages (R_{LOW} , 15-25) had high NEAC, whereas its cultures at high passages (R_{HIGH} 163-170) revealed much lower NEAC (Table 1 and Supplementary Fig. 1). Direct comparison showed that a sample of R_{LOW} containing about 5×10^8 CFU had NEAC comparable to that of 1 μg of *C. perfringens* neuraminidase (time point – 1 hour). NEAC of R_{HIGH} was about 10 – fold lower.

Different populations of the S6 strain revealed a considerable differences in NEAC, although they represented cultures at similar levels of *in vitro* passage. In comparison to the parent culture S6, its clone C1 had higher level of NEAC, whereas a sibling culture designated C 19 revealed much lower NEAC (Table 1). Similar differences were notable in clones of the vaccinal strain F (Table 1).

3.2 NEAC in *Mycoplasma synoviae* strains

Considerable differences in NEAC were observed among different *M. synoviae* strains (Table 2 and Supplementary Fig. 2). All cultures derived from

the type strain WVU 1853 obtained from different *Mycoplasma* culture collections revealed strong NEAC. Comparison showed that samples of WVU 1853 containing about 10^8 CFU had NEAC comparable to that of 1 μ g of neuraminidase of *C. perfringens*. High levels of this activity were detected also in *M. synoviae* strains F102AS, K1723, K1968, K2426D, as well as in closely related field isolates ULB02/P4, ULB02/T6 and ULB02/0V6 (Table 2). Notably, some cultures derived from a field *M. synoviae* isolate ULB9122 (Benčina et al., 2005) revealed strong NEAC, whereas some cultures of its higher passages had no detectable NEAC (Table 2). Cultures derived from the *M. synoviae* strain ULB 925 (Benčina et al., 1999) lacked detectable NEAC (Table 2). The NEAC assay reproducibility was good for all *M. synoviae* samples tested. Indeed, their NEAC was not reduced notably if their aliquots kept in refrigerator were retested after several days or if they underwent a few freezing – thawing cycles.

3.3 NEAC in *M. meleagridis* and *M. iowae*

Striking differences in neuraminidase activity were found in cultures of the type strain 17529 of *M. meleagridis*. High NEAC was detected in two cultures of 17529, whereas NEAC was absent in cultures derived from another culture of this strain (Table 3).

M. iowae is a heterogenous species with six serovars (I, J, K, N,Q, R). A weak NEAC was detected in the type strain 695 (serovar I). In other serovars, NEAC was not detected. A weak neuraminidase activity was also found in a

field isolate of *M. iowae* (ULB 953) obtained from a yolk sac membrane of a turkey embryo.

3.4 NEAC in other recognized avian *Mycoplasma* spp.

Relatively low NEAC was detected in the type strains of *M. anseris*, *M. cloacale* and *M. pullorum* (Table 3). In contrast to many *M. gallisepticum* cultures (see 3.1), detectable NEAC was not present in *M. imitans* (strains 4229 and B2/85) which is closely related to *M. gallisepticum*.

Notably, cultures of *Mycoplasma corogypsi* (type strain BV1 isolated from a foot pad abscess of a black vulture in the USA) had extremely potent NEAC. Its broth cultures had detectable NEAC even when assayed at a dilution of 1:128. A potent NEAC was associated with washed *M. corogypsi* cells. Its pelleted cells gave the positive NEAC reaction also when they were diluted 1 : 20000. However, at this dilution the positive reaction appeared after overnight incubation.

Potent NEAC of *M. corogypsi* cells enabled identification of its proteins with this activity. Following SDS-PAGE and transfer of separated proteins to the Immobilon P membrane its protein of about 110 kDa reacted with the substrate and revealed indigoblue color within 2 hours (Supplementary Fig. 3). Nevertheless, after overnight incubation also a protein of ~130 kDa (narrow band) showed indigoblue staining.

4. Discussion

The pathogenesis of diseases associated with pathogenic AM spp. is probably a multifactorial process in which invasiveness of mycoplasma may be an important factor. In both poultry pathogens, *M. gallisepticum* and *M. synoviae*, more invasive strains tend to cause more intense pathologic changes in the lower respiratory tract and/or joints (Lockaby et al., 1998; Much et al., 2003; Hinz et al., 2003).

Notably, more invasive strains of *M. synoviae*, like WVU 1853 and K 1968, showed in this study potent NEAC (see Table 2 and Lockaby et al., 1998). Significant differences of NEAC among *M. synoviae* strains were recently reported also by May and coworkers (2007). They also noticed that higher NEAC, for instance in WVU1853, is associated with the capability of *M. synoviae* strain to cause clinical disease (May et al., 2007). Several lines of evidence show that the *nanH* gene (MS0199) encodes neuraminidase (sialidase) of about 110 kDa which exhibits NEAC (Vasconcelos et al., 2005, Berčič et al., 2007, May et al., 2007; and data of this study). Nevertheless, further studies are needed to confirm that neuraminidase is responsible for pathologic processes or apoptosis as has been suggested for the *M. alligatoris* sialidase (Hunt and Brown, 2007).

A considerable differences of NEAC were observed also among strains of *M. gallisepticum*, as well as among different cultures derived from appropriate

strains. This was found also for cultures of the S6 strain which is widely used as *M. gallisepticum* antigen in serology. The fact that some S6 cultures had very low NEAC might be the explanation why Glasgow and Hill (1980) did not detect NEAC in the S6 strain. On the other hand, our results confirm suggestion that *M. gallisepticum* exhibits neuraminidase activity (Sethi and Müller, 1972).

Interestingly, in cultures of the *M. gallisepticum* strain R the levels of NEAC was high at a low passage (R_{LOW}), but weak after >160 passages (R_{HIGH}). It should be noted, that R_{LOW} is highly invasive, whereas R_{HIGH} lacks invasiveness (Winner et al., 2000; Much et al., 2003). Moreover, data from previous studies indicate that R_{LOW} is virulent for poultry, whereas R_{HIGH} is not (Levisohn et al., 1986; Much et al., 2003). In all, it seems that NEAC might be associated with higher invasiveness and virulence of R_{LOW} , but further investigations are needed to confirm this.

The last topic should be investigated also in other AM spp. which seem to possess NEAC (sections 3.3 and 3.4). Of those AM species *M. meleagridis* is a well known pathogen of turkeys (Chin et al., 2003).

Among *Mycoplasma* species examined so far, we found the highest level of NEAC in *M. corogypsi*. This species belongs to the *M. synoviae* cluster in which several species, including *M. alligatoris* exhibit strong NEAC (Brown et al., 2004; and our unpublished data). However, a number of *Mycoplasma* species still remain to be examined for NEAC. Such information would be very useful because in *M. alligatoris* its sialidase activity (i.e. NEAC) is considered

to be among the most important pathogenic factors (Brown et al., 2004; Hunt and Brown, 2007).

5. Conclusions

Major poultry pathogens *M. gallisepticum* and *M. synoviae* synthesize enzymatically active neuraminidase. Two other poultry pathogens, *M. meleagridis* and *M. iowae* also revealed NEAC, indicating that this activity might be associated with their pathogenicity. *M. corogypsi* showed very strong NEAC and is another member of the *M. synoviae* phylogenetic cluster with potent NEAC.

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Table 1: Neuraminidase enzymatic activity (NEAC) in reference *M. gallisepticum* strains

Strain	Culture/clone	Passage	NEAC ^a	Note ^b	Reference
PG 31	X-95	> 100	weak	apathogenic	Levisohn et al., 1986
A5969	SHK	> 500	weak	noninfectious	Avakian et al., 1991; Levisohn et al., 1986
R(K781)	R _{LOW} 1	20	strong	pathogenic	Levisohn et al., 1986;
R _{LOW}	R _{LOW} 2	20	strong	invasive	Winner et al., 2000

R(K781)	R _{HIGH} 1	165	weak	apathogenic	Levisohn et al., 1986;
R _{HIGH}	R _{HIGH} 2	163	weak	noninvasive	Winner et al., 2000
S6	parental	unknown	moderate	pathogenic	Benčina et al., 1994
	clone C1		strong	NA	
	clone C19		weak	NA	
F(K810)	clone7/2	25	strong	vaccinal strain	Levisohn et al., 1986
	clone9/4	>25	weak		Benčina et al., 1994

^a NEAC assayed with samples (30 µl) of 100-fold and/or 1000-fold concentrated broth cultures.

- Strong NEAC, positive reaction within 30-60 minutes. (100-fold concentrated cells)
- Moderate NEAC, positive reaction within 4 hours. (100-fold concentrated cells)
- Weak NEAC, positive reaction of 1000-fold concentrated cells later than in 4 hours.

^b NA, not assayed for invasiveness or pathogenicity.

Table 2: Neuraminidase enzymatic activity (NEAC) in *M. synoviae* strains

Strain	Culture/Clone	NEAC	Reference
WVU1853	S	strong ^a	Benčina et al., 2001
WVU1853	Aar	strong	Ramirez et al., 2006
F102 AS	C1	strong	Avakian et al., 1992
K1723	C23	strong	Noormohammadi et al., 1997
			Benčina et al., 2001, 2005
K1968	C3	strong	Lockaby et al., 1998
			Benčina et al., 2001
K2426D	AA3	strong	Avakian et al., 1992
			Benčina et al., 2001
ULB 02/P4	P4	strong	Benčina et al., 2001, 2005
ULB 02/T6	T6	strong	Berčič et al., 2007
ULB 9122	C1	strong	Benčina et al., 2001, 2005
	C2	negative ^b	
ULB 925	KF	negative	Narat et al., 1998
	KF6	negative	Benčina et al., 1999, 2001
	KF9	negative	Benčina et al., 1999, 2001

^a Strong NEAC – samples (30µl) representing ~ 100-fold concentrated *M. synoviae* broth culture gave positive NEAC within 1 hour.

^b Samples did not give positive NEAC even when they contained highly concentrated cells (1000-fold) and were incubated with BIN for 24 hours.

Table 3: Neuraminidase activity in other avian *Mycoplasma* species^a

Species	Strain ^b	Usual host ^c	NEAC ^d	
<i>M. anatis</i>	1340	Duck	negative	
<i>M. anseris</i>	1219	Goose	moderate	Bradbury et al., 1988
<i>M. buteonis</i>	BbT2g	Buteo hawk	negative	
<i>M. cloacale</i>	383	Turkey, goose	weak	Bradbury and Forrest, 1984
<i>M. columbinasale</i>	694	Pigeon	negative	
<i>M. columbinum</i>	MMP1	Pigeon	negative	
<i>M. columborale</i>	MMP4	Pigeon	negative	
<i>M. corogypsi</i>	BV1	Black vulture	very strong	Panangala et al., 1993
<i>M. falconis</i>	HT1	Saker falcon	negative	
<i>M. gallinarum</i>	PG 16	Chicken	negative	
<i>M. gallinaceum</i>	DD	Chicken	negative	
<i>M. gallopavonis</i>	WR1	Turkey	negative	
<i>M. glycyphilum</i>	486	Chicken	negative	
<i>M. gypis</i>	B1T1	Griffon vulture	negative	
<i>M. imitans</i>	4229	Duck, Goose	negative	
<i>M. iners</i>	PG 30	Chicken	negative	
<i>M. lipofaciens</i>	R171	Chicken	negative	
<i>M. pullorum</i>	CKK	Chicken	weak ^d	Jordan et al., 1982
<i>M. sturni</i>	UCMF	European starling	negative	

^a Note, data for *M. gallisepticum* and *M. synoviae* are in Tables 1 and 2, whereas for *M. meleagridis* and *M. iowae* see section 3.4.

^b Type strains were assayed. In the case of *M. cloacale*, *M. gallinarum* and *M. pullorum* also field isolates were tested.

^c Usual hosts or hosts from which the strain was isolated e.g. *M. corogypsi* from a black vulture (Panangala et al., 1993).

^d Pelleted cells were examined for NEAC. Moderate NEAC, positive reaction at a dilution of 1:40 in 2 hours; weak NEAC – positive reaction appeared after overnight incubation. One culture of *M. pullorum* (CKK) was (weakly) positive, whereas another culture of this strain and field isolates of *M. pullorum* were negative.

Supplementary material:

Fig. 1: NEAC in cultures of *M. gallisepticum* (strain R) and in cultures of *M. meleagridis* (type strain 17529).

Panel A, R strain's cultures at low passages (designated L) revealed higher NEAC than its cultures at high passages (designated H). Panel B, only *M. meleagridis* (17259) cultures originating from the culture received from Dr. J. M. Bradbury revealed NEAC (under 1 and 3).

Fig. 2: NEAC titration in 96-well microtiter plates (double dilutions).

Row A, neuraminidase of *C. perfringens* (type V, Sigma); Row B, wells 2 – 6, negative control (only buffer and BIN); Row C, *M. synoviae* WVU 1853; Row D, *M. synoviae* ULB 925, KF; Row E, *M. synoviae* K1968; Row F, *M. corogypsi* BV1.

Fig. 3: Mycoplasmal proteins exhibiting neuraminidase enzymatic activity.

Lane 1, *M. corogypsi* and lane 2, pre-stained molecular weight marker (PageRuler™, Fermentas). Proteins were separated by SDS-PAGE in PhastGel Gradient 8-25 (GE Healthcare, Sweden) and transferred to the Immobilon-P membrane by diffusion blotting (45°C for 30 min). Following incubation in 0,5% PBS-Tween 20 (30 min), the membrane was overlaid with 0,5 ml of buffer containing 250 µg of BIN substrate. The

447 indigoblue staining of about 110 kDa protein of *M.corogypsi* appeared after 2 hours
448 (the arrowhead), whereas the staining of about 135-kDa *M. corogypsi* protein (lane 1,
449 arrow) appeared after 6 hours.

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