

## Accepted Manuscript

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

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PII: S0378-1135(08)00060-6  
DOI: doi:10.1016/j.vetmic.2008.02.004  
Reference: VETMIC 3958

To appear in: *VETMIC*

Received date: 20-11-2007  
Revised date: 6-2-2008  
Accepted date: 8-2-2008

Please cite this article as: Berčič, R.L., Slavec, B., Lavrič, M., Narat, M., Zorman-Rojs, O., Dovč, P., Benčina, D., A survey of avian *Mycoplasma* species for neuraminidase enzymatic activity, *Veterinary Microbiology* (2007), doi:10.1016/j.vetmic.2008.02.004

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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- $\alpha$ -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

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

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

78

## 79 2. Materials and methods

80

### 81 2.1 *Mycoplasma* species, their strains and growth media

82 Five reference strains of *M. gallisepticum* characterized in previous studies and  
83 used in this study are listed in Table 1 (Levisohn et al., 1986; Benčina et al.,  
84 1994; Avakian et al., 1991; Winner et al., 2000). Reference strains of *M.*  
85 *synoviae* also used in previous investigations are shown in Table 2 (Avakian et  
86 al. 1992, Benčina et al., 1999, 2001, 2005). One isolate of the type strain of *M.*  
87 *synoviae* (WVU1853), one isolate of *M. meleagridis*, and reference serovars (I,  
88 J, K, N, Q, and R represented by strains 695, DJA, DK-CPA, FMN, L3-10B and  
89 DRA-O, respectively) were provided by Dr. Janet M. Bradbury. The type

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

93 A modified Frey's media (agar and broth) containing 10% porcine serum,  
94 0,1% NAD and 0,1% cysteine hydrochloride were used for *M. synoviae* and  
95 other AM spp. However, media with 10% horse serum (but without NAD) were  
96 also used to grow AM spp., except *M. synoviae*. Cultures were grown at 37-  
97 38°C and were usually harvested in a late log-phase growth. Colony forming  
98 units (CFU) were determined by standard procedures (Rodwell and Whitcomb,  
99 1983).

100

## 101 *2.2 Preparation of mycoplasma samples examined for neuraminidase enzymatic* 102 *activity (NEAC)*

103 *Mycoplasma* cells were harvested by centrifugation (usually 27000 x g,  
104 20min). Their pellets were washed in sterile phosphate buffered saline (PBS, pH  
105 7,4) using centrifugation. Samples were then assayed for NEAC or were kept  
106 frozen (at -20°C) and were thawed when the NEAC test was done.

107

## 108 *2.3 Determination of NEAC*

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

112 per ml and were kept frozen as 1ml aliquots at -20°C. Samples with AM spp.  
113 cells (20 – 30 µL of cell-suspension) were assayed in small microfuge tubes with  
114 caps or in wells of the microtiter plates (see Supplementary Figs. 1 and 2).  
115 Mycoplasma samples (~ 30 µL of suspension) were prepared (in PBS pH 5, with  
116 10 mM CaCl<sub>2</sub>) in wells of 96-well plastic microtiter plates. Usually, the  
117 suspension examined represented about 100-fold concentration of mycoplasma  
118 cells in comparison with their concentration in appropriate broth culture. In the  
119 case of *M. synoviae* and *M. gallisepticum* their samples tested contained  
120 approximately 10<sup>9</sup> CFU. Samples of AM species being negative at such  
121 concentration were then tested at higher density. For mycoplasma samples with  
122 higher NEAC, titration using double dilutions was done.

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

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

129

### 130 3. Results

131

#### 132 3.1 NEAC varies in cultures of *M. gallisepticum*

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

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

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

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

151

### 152 3.2 NEAC in *Mycoplasma synoviae* strains

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



155 the type strain WVU 1853 obtained from different *Mycoplasma* culture  
156 collections revealed strong NEAC. Comparison showed that samples of WVU  
157 1853 containing about  $10^8$  CFU had NEAC comparable to that of 1  $\mu$ g of  
158 neuraminidase of *C. perfringens*. High levels of this activity were detected also  
159 in *M. synoviae* strains F102AS, K1723, K1968, K2426D, as well as in closely  
160 related field isolates ULB02/P4, ULB02/T6 and ULB02/0V6 (Table 2). Notably,  
161 some cultures derived from a field *M. synoviae* isolate ULB9122 (Benčina et al.,  
162 2005) revealed strong NEAC, whereas some cultures of its higher passages had  
163 no detectable NEAC (Table 2). Cultures derived from the *M. synoviae* strain  
164 ULB 925 (Benčina et al., 1999) lacked detectable NEAC (Table 2). The NEAC  
165 assay reproducibility was good for all *M. synoviae* samples tested. Indeed, their  
166 NEAC was not reduced notably if their aliquots kept in refrigerator were  
167 retested after several days or if they underwent a few freezing – thawing cycles.

168

### 169 3.3 NEAC in *M. meleagridis* and *M. iowae*

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

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

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

179

180 *3.4 NEAC in other recognized avian Mycoplasma spp.*

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

185 Notably, cultures of *Mycoplasma corogypsi* (type strain BV1 isolated  
186 from a foot pad abscess of a black vulture in the USA) had extremely potent  
187 NEAC. Its broth cultures had detectable NEAC even when assayed at a dilution  
188 of 1:128. A potent NEAC was associated with washed *M. corogypsi* cells. Its  
189 pelleted cells gave the positive NEAC reaction also when they were diluted 1 :  
190 20000. However, at this dilution the positive reaction appeared after overnight  
191 incubation.

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

198

## 199 4. Discussion

200

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

207 Notably, more invasive strains of *M. synoviae*, like WVU 1853 and K  
208 1968, showed in this study potent NEAC (see Table 2 and Lockaby et al., 1998).  
209 Significant differences of NEAC among *M. synoviae* strains were recently  
210 reported also by May and coworkers (2007). They also noticed that higher  
211 NEAC, for instance in WVU1853, is associated with the capability of *M.*  
212 *synoviae* strain to cause clinical disease (May et al., 2007). Several lines of  
213 evidence show that the *nanH* gene (MS0199) encodes neuraminidase (sialidase)  
214 of about 110 kDa which exhibits NEAC (Vasconcelos et al., 2005, Berčić et al.,  
215 2007, May et al., 2007; and data of this study). Nevertheless, further studies are  
216 needed to confirm that neuraminidase is responsible for pathologic processes or  
217 apoptosis as has been suggested for the *M. alligatoris* sialidase (Hunt and Brown,  
218 2007).

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

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

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

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

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

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

245

## 246 5. Conclusions

247

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

254

## 255 Acknowledgements

256

257 This research was supported by the grant from Slovenian Research  
258 Agency (ARRS). We thank Dr J. M. Bradbury for the *Mycoplasma* cultures.  
259 Technical help of S. Salmič and I. Beretič is acknowledged.

260

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262

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

Strain	Culture/clone	Passage	NEAC <sup>a</sup>	Note <sup>b</sup>	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 <sub>LOW</sub> 1	20	strong	pathogenic	Levisohn et al., 1986;
R <sub>LOW</sub>	R <sub>LOW</sub> 2	20	strong	invasive	Winner et al., 2000

R(K781)	R <sub>HIGH</sub> 1	165	weak	apathogenic	Levisohn et al., 1986;
R <sub>HIGH</sub>	R <sub>HIGH</sub> 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

392

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

394 • Strong NEAC, positive reaction within 30-60 minutes. (100-fold concentrated cells)

395 • Moderate NEAC, positive reaction within 4 hours. (100-fold concentrated cells)

396 • Weak NEAC, positive reaction of 1000-fold concentrated cells later than in 4 hours.

397 <sup>b</sup>NA, not assayed for invasiveness or pathogenicity.

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

402

Strain	Culture/Clone	NEAC	Reference
WVU1853	S	strong <sup>a</sup>	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 <sup>b</sup>	
ULB 925	KF	negative	Narat et al., 1998
	KF6	negative	Benčina et al., 1999, 2001
	KF9	negative	Benčina et al., 1999, 2001

403

404 <sup>a</sup> Strong NEAC – samples (30µl) representing ~ 100-fold concentrated *M. synoviae* broth culture gave

405 positive NEAC within 1 hour.

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

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**Table 3: Neuraminidase activity in other avian *Mycoplasma* species<sup>a</sup>**

Species	Strain <sup>b</sup>	Usual host <sup>c</sup>	NEAC <sup>d</sup>	
<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 <sup>d</sup>	Jordan et al., 1982
<i>M. sturni</i>	UCMF	European starling	negative	

414  
 415 <sup>a</sup> Note, data for *M. gallisepticum* and *M. synoviae* are in Tables 1 and 2, whereas for *M. meleagridis*  
 416 and *M. iowae* see section 3.4.

417 <sup>b</sup> Type strains were assayed. In the case of *M. cloacale*, *M. gallinarum* and *M. pullorum* also field  
 418 isolates were tested.

419 <sup>c</sup> Usual hosts or hosts from which the strain was isolated e.g. *M. corogypsi* from a black vulture  
 420 (Panangala et al., 1993).

421 <sup>d</sup> Pelleted cells were examined for NEAC. Moderate NEAC, positive reaction at a dilution of 1:40 in  
422 2 hours; weak NEAC – positive reaction appeared after overnight incubation. One culture of *M.*  
423 *pullorum* (CKK) was (weakly) positive, whereas another culture of this strain and field isolates of *M.*  
424 *pullorum* were negative.

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426

427 Supplementary material:

428

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

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

435

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

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

440

441 **Fig. 3: Mycoplasmal proteins exhibiting neuraminidase enzymatic activity.**

442 Lane 1, *M. corogypsi* and lane 2, pre-stained molecular weight marker (PageRuler™,  
443 Fermentas). Proteins were separated by SDS-PAGE in PhastGel Gradient 8-25 (GE  
444 Healthcare, Sweden) and transferred to the Immobilon-P membrane by diffusion  
445 blotting (45°C for 30 min). Following incubation in 0,5% PBS-Tween 20 (30 min), the  
446 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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