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Abstract

Some *Mycoplasma* species are well known avian pathogens and are of importance in poultry breeder flocks due to their pathogenic potential for embryos. Mycoplasmas are regularly detected in birds of prey and a strain of *Mycoplasma lipofaciens* that was isolated from an egg of a Northern Goshawk (*Accipiter gentilis*) was examined for its pathogenicity in specific pathogen-free chicken embryos since birds of prey eggs were not available for this purpose. The strain was found to be pathogenic, causing a high mortality as well as dwarfing, curled toes and infiltrations of heterophils in the liver, kidney, intestine and chorioallantoic membrane.

Introduction

Mycoplasma lipofaciens was isolated from the infraorbital sinus of a healthy chicken and described as a new species in 1983 (Bradbury *et al.*, 1983). Since then it appears to have been reported only from the chicken, turkey and duck (Benčina *et al*., 1987). Recently, Lierz *et al.* (2007) isolated *M. lipofaciens* from a freshly laid, infertile egg of an imprinted 4 year old Northern Goshawk (*Accipiter genitilis*) suggesting that this mycoplasma may have a wider range of avian hosts.

Vertical transmission of certain *Mycoplasma* spp. in poultry is well known, and can cause pathological lesions or even death of the embryo, depending on the pathogenicity of the strain (Bradbury & McCarthy, 1983; Lockaby *et al*, 1999). For example, *M. gallisepticum* infection can result in poorly developed embryos with liver necrosis (Lin & Kleven, 1982; Glisson & Kleven, 1984, 1985). *M. synoviae* can give rise to hepatic and splenic swelling, curled toes, oedema and discoloration (Lockaby *et al*, 1999). Microscopically, infiltrates of heterophils, lymphocytes and macrophages, variably associated with necrosis in viscera and musculoskeletal tissue, have been described. Some strains of *M. iowae* are able to cause high embryo mortality with dwarfing and congestion, while others cause oedema or mustard-coloured liver and spleen (Bradbury & McCarthy, 1983). Infiltrations with heterophilic granulocytes and mononuclear cells were detected in the chorioallantoic membrane (CAM), in some areas even with necrosis. Periportal proliferations of heterophilic granulocytes were seen in the liver with a diffuse mononuclear infiltration. Heterophilic granulocytic proliferation was also found in kidney and lung (Mirsalimi *et al*, 1989).

Birds of prey are commonly bred for commercial purposes and species conservation projects but little is known about the importance of mycoplasma infections, about vertical transmission of the organisms or their impact on breeding success. Therefore it was thought useful to assess the pathogenicity of the *M. lipofaciens* raptor strain ML64 for avian embryos. As fertile raptor eggs are not available for such purposes, chicken embryos were used as a model

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(Lockaby *et al*., 1999). This report describes some preliminary findings.

Materials and Methods

Preparation of bacterial suspension. The fifth single colony subculture from strain ML64 which had been used for the immunobinding assay and DNA-sequencing (GenBank Accession No: DQ653410) for identifying it as *M. lipofaciens* (Lierz *et al*., 2007) was cultured (24 h, 37°C, 5% CO_2) in mycoplasma broth described by Bradbury (1998) but without thallium acetate. The number of colony-forming units (CFU) was determined (Albers & Fletcher, 1982) and the suspension diluted with sterile mycoplasma broth to the required dosage (see below) immediately prior to inoculation.

Pathogenicity test in chicken embryos. Pathogenicity of the isolate was estimated by inoculating the yolk sac of specific pathogen free (SPF) chick embryos (Valo®, Lohmann, Cuxhaven, Germany) using a 0.8 x 40 mm (21 gauge) needle. Three groups of 10 embryonated SPF eggs were infected with *M. lipofaciens* strain ML64 at day 7 of incubation at one of three doses (group 1: 2 x 10^6; group 2: 2 x 10^4; group 3: 2 x 10^2 CFU within 0.1ml broth). A negative control group of six embryonated eggs was inoculated with 0.1 ml sterile mycoplasma broth. All eggs were incubated at 37.8°C within the same incubator and candled daily. In cases of embryonic death, each embryo was subjected to necropsy and a sample of vitelline membrane was used to inoculate mycoplasma broth for re-isolation of the pathogen. Directly after inoculation and after incubation at 37°C in an atmosphere with 5% CO_2 for 5 days, the broth was subcultured (0.2 ml) onto solid mycoplasma agar media (Bradbury, 1998). The solid media were observed for the occurrence of mycoplasma colonies for up to ten days.

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Isolates were identified using an immunobinding assay (IBA) according to Kotani & McGarrity (1985), using antiserum against *M. lipofaciens.*

Egg yolk, amniotic and allantoic fluids were streaked on blood (5% sheep erythrocytes) and Gassner agar (Oxoid, Germany) and incubated at 37°C for 2 days in aerobic and anaerobic conditions to exclude bacterial contamination. Finally the CAM and the embryo were fixed in 10% neutral buffered formaldehyde-solution, processed according to standard histological methods, sectioned at 5µm, stained with haematoxylin and eosin and examined for morphological and inflammatory changes. Surviving embryos were chill-killed at day 19 of incubation, and the sampling procedure described above was applied. Embryonic death within 24 hours after inoculation was considered non-specific and only the bacterial investigations were carried out on these samples.

Statistical methods. Survival rates were analyzed using a chi-square test. The Kaplan-Meier method was used to construct survival curves, which were then compared by the log-rank test, with correction for multiple comparisons. A p value of <0.05 was considered significant.

Results

None of the six embryos from the control group died but nine embryos in group 1, all 10 embryos in group 2 and six embryos in group 3 (Table 1) died within 11 days after infection. The survival rate was significantly reduced in group 1 ($p=0.001$), group 2 ($p<0.001$) and group 3 ($p=0.035$) compared to the control group. The time of embryonic death was dependent on the infectious dose of *M. lipofaciens*. Groups 1 and 2 did not differ significantly from one another but had a significantly lower survival rate than group 3 ($p<0.01$). Most embryos died between day 5 and 7 post inoculation (p.i.). Mycoplasmas, identified as *M. lipofaciens* using the IBA,

were isolated from all infected embryos but from none of the negative control group. The mycoplasma broths that were inoculated with vitelline membrane and then plated onto agar without further incubation demonstrated a heavy growth of mycoplasma colonies indicating multiplication of the pathogen within the embryo. No other bacterial contamination was detected in any case.

Four embryos died within 24 h of inoculation and were not included in the pathological examination. All embryos that died exhibited a reddish discoloration (Figure 1), especially of the feet. Four of the embryos (two each of group 1 and 2) that died on day 5 or 6 p.i. demonstrated severe oedema. The two embryos from group 3 that died on day 7 p.i. displayed severe hepatomegaly with discoloration. Two embryos of group 3 that died on day 11 p.i., and the surviving embryo of group 1 demonstrated dwarfing and curled toes (Figure 2). The surviving four embryos of group 3 and all embryos of the control group displayed no macroscopic lesions.

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The histopathological findings with regard to the different groups are provided in Table 2. Microscopic examination demonstrated pathological changes in 25 of the 26 embryos infected and examined. One embryo of group 3 did not demonstrate any pathological change. The main findings were severe oedema with infiltrates of heterophils in liver, intestine, kidney and CAM as well as necrosis in liver, intestine and CAM. The heart did not demonstrate any pathological alterations. None of the embryos of the control group showed any histopathological alterations.

Discussion

Little is known about the occurrence and the pathogenicity of *Mycoplasma* spp. in raptors, especially about the importance of vertical transmission of these bacteria. The isolation of *M. lipofaciens* strain ML64 from an egg of a Northern Goshawk was the first evidence of vertical transmission of mycoplasmas in birds of prey (Lierz *et al*., 2007). The isolation was from an

unfertile egg but nothing is known about the pathogenicity of this isolate and its possible impact on raptor breeding. However, in the absence of fertile eggs of birds of prey for experimental purposes, we used infection of SPF-chicken embryos as a model.

In our study *M. lipofaciens* strain ML64 resulted in 100% mortality after inoculation of 2 x 10^4 CFU and 60% mortality after infection with 2 x 10^2 CFU. It might have been useful to include a known embryo pathogenic *Mycoplasma* species in our experiment for comparative purposes. Lockaby *et al*., (1999) described the 50%- lethal dose for different *M. synoviae* strains in chicken embryos as between 4.48 x 10^3 and 3.1 x 10^6 colour changing units (CCU). Bradbury & McCarthy (1983) described embryo mortality rates of between 10% and 33% 2- 7 days p.i. when using different *M. iowae* strains. The majority of embryos died between day 19 and 21 days of incubation (12-14 day p.i.) which is later than in the present study. Overall the mortality caused by *M. lipofaciens* strain ML64 in SPF chicken embryos was greater than that described in the literature for *M. synoviae* and *M. iowae* and also occurred earlier. The macroscopic lesions demonstrated by the infected chicken embryos (discolouration, oedema, dwarfing and curled toes) were comparable to those caused by other pathogenic *Mycoplasma* spp. (Bradbury & McCarthy, 1983; Lockaby *et al*., 1999) and seem to be typical for such infections (Lin & Kleven, 1982; Glisson & Kleven, 1984; 1985). In addition the histopathological changes (tissue oedema, infiltrations with heterophils and necrosis in the liver, CAM and intestine) detected in the infected embryos were consistent with previous reports (Mirsalimi *et al.*, 1989, Lockaby *et al.*, 1999). The changes within the CAM are of particular importance since they might lead to enlarged organs and oedema. Taking all this into account *M. lipofaciens* strain ML64 appeared to be highly pathogenic for SPF chicken embryos although this experiment should ideally be repeated and attempts should also be made to obtain more isolates from birds of prey eggs.

Lockaby *et al*. (1999) underlined the importance of infecting chicken embryos to determine the virulence of *Mycoplasma* strains as a model that could be used for other animals. However, strain virulence may not cross avian taxonomic boundaries. Therefore the actual

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pathogenicity of *M. lipofaciens* strain ML64 for raptors cannot be determined with absolute certainty from tests in galliforms. However, until the pathogenicity can be determined using raptor embryos, the high pathogenicity of this *Mycoplasma* isolate for chicken embryos can be used to indicate that this organism may also be a potential pathogen for raptor embryos.

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Table 1. *Number of SPF chicken embryos inoculated with M. lipofaciens strain ML64 that died post infection*

Group	Dose (CFU)	Days post infection						Total
		1	2	5	6	7	11	
1[a]	2×10^6	1	3	4	0	1	0	9
2	2×10^4	2	0	2	3	3	0	10
3	2×10^2	1	0	0	1	2	2	6

[a]10 embryos per group; control group contained six embryos; there were no deaths in the control group

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Table 2. *Number of SPF chicken embryos inoculated with M. lipofaciens strain ML64 showing various histopathological changes*

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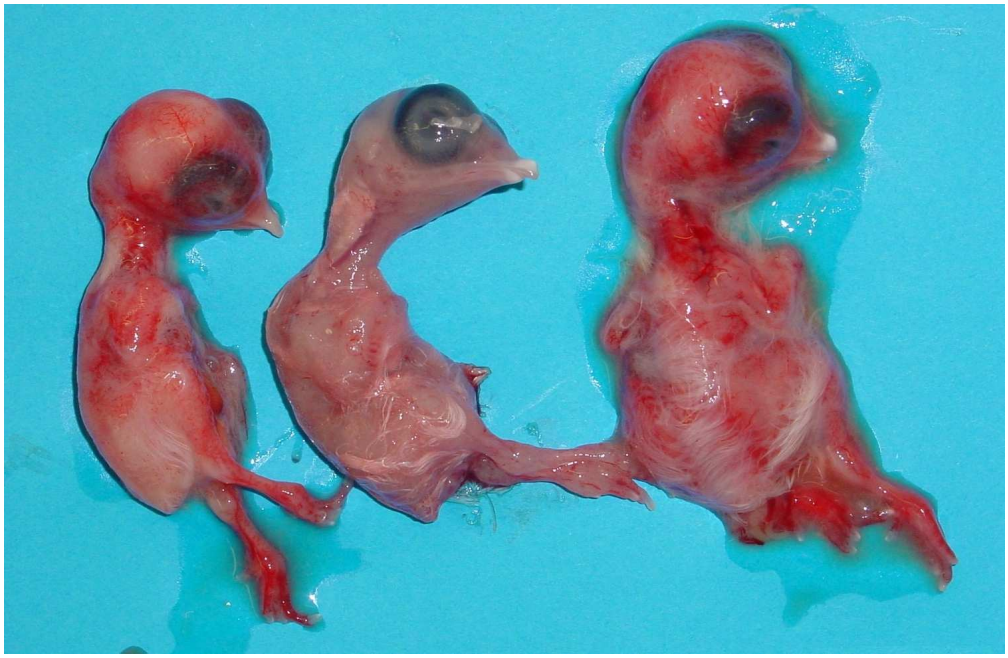
		Histopathological change							
Group	Dose (CFU)	A	B	C	D	E	F	G	H
1	2×10^6	9	9	6	6	2	0	0	0
2	2×10^4	8	8	3	0	1	0	0	0
3	2×10^2	4	4	2	2	4	2	2	1

[a]10 embryos per group; control group contained six embryos; there were no histopathological changes in the control group

A = severe tissue oedema
B = infiltrations with heterophils (liver, kidney, intestine, CAM)
C = multifocal necrosis in liver
D = multifocal necrosis in intestinal villi
E = necrosis on CAM
F = hyperaemia in liver
G = hyperaemia in kidney
H = no histopathological changes

Figure 1. Left and right: Discolouration and oedema in SPF-chicken embryos 7 days after inoculation (14th day of incubation) with 2 x 10^2 CFU of M. lipofaciens strain ML64. A control SPF chicken embryo of the same age is in the centre for comparison.

Figure 2. Right: Dwarfed SPF chicken embryo with curled toes, 12 days after inoculation (19th day of incubation) with 2 x 10^6 CFU of M. lipofaciens strain ML64. Left: SPF chicken embryo of the control group at same age for comparison.



Left and right: Discolouration and oedema in SPF-chicken embryos 7 days after inoculation (14th day of incubation) with 2 x 102 CFU of M. lipofaciens strain ML64. A control SPF chicken embryo of the same age is in the centre for comparison.

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