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Original article

Experimental evidence of indirect transmission of *Mycoplasma synoviae*

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Abstract – The aim of the study was to analyse experimental transmission of *Mycoplasma synoviae*, an avian pathogen. Three experiments using specific pathogen-free day-old chicks placed in isolators were conducted. In the first experiment, the birds were introduced in an isolator previously contaminated with a *M. synoviae* broth culture. After 34 days, these birds were eliminated and, for the second trial, the chicks were introduced in the same isolator without disinfecting. In the third assay, the chicks were placed in an isolator containing a mixture of food, feathers and dust collected less than an hour earlier from a *M. synoviae* infected laying hen flock. In the second and third experiments in order to exacerbate the *M. synoviae* infection, the birds were inoculated with infectious bronchitis (IB) virus. The presence of *M. synoviae* in the environment and in tracheal swabs was monitored by culture, a multiplex PCR (mPCR) detecting *M. synoviae* and Mycoplasma 16S rDNA and a multiplex RT-PCR (mRT-PCR) detecting the *M. synoviae* mRNA coding for a membrane protein and Mycoplasma 16S rRNA. In in vitro experimental conditions, *M. synoviae* mRNA and 16S rRNA were detected up to 20 min and 23 h respectively after mycoplasma death. In the first assay, the first infected bird was detected on the 13th day. In the second trial, culturable *M. synoviae* or viable *M. synoviae* were detected in the isolator for 3 or 4 to 5 days respectively after depopulation of the birds of the first assay whereas the first culture positive tracheal swabs were detected on the 33rd day, after IB inoculation. In the third experiment, the first infected birds were detected on the 54th day. Thus, the different assays showed that *M. synoviae* contaminated material (dust, feathers and food) can infect chicks, sometimes after remarkably long silent periods.

***Mycoplasma synoviae* / chicken / environment / transmission**

1. INTRODUCTION

Avian mycoplasmoses are frequently reported infectious diseases in poultry flocks. *Mycoplasma synoviae* is responsible for infectious synovitis and causes economic losses because of decreased egg production, growth and hatchability rates, and downgrading of carcasses at slaughter due to airsacculitis and arthritis lesions [12].

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Transmission of mycoplasmas may be vertical, through the eggs, or horizontal either by direct contact between clinically affected or unaffected carriers and susceptible birds or by indirect contact via people, wild animals or maybe contaminated equipment. Such indirect transmission is rather unexpected for wall-less bacteria, which are supposed to be sensitive to osmotic shock, heating or chemical treatments. However, *M. synoviae* may persist on feathers up to 2 or 3 days at room temperature [3] and its high dissemination capacity has been demonstrated [16].

Three experimental studies were conducted in order to analyse *M. synoviae* horizontal transmission via a contaminated environment. In the second and third experiment, the *M. synoviae* infection was exacerbated by inoculation of birds with infectious bronchitis (IB) virus. In order to monitor the presence of dead or viable cells of *M. synoviae* in chicken tracheas and in their environment, a multiplex PCR (mPCR) and a multiplex RT-PCR (mRT-PC) were developed and their specificities and sensitivities were estimated. The delay of detection of the different targets (DNA, 16S rRNA or mRNA) after mycoplasma cell death was evaluated.

2. MATERIALS AND METHODS

2.1. Bacterial strains and media

A collection of 56 reference or field strains including 18 *M. synoviae* strains (the reference strain WVU 1853 and 17 field strains isolated from 1986 to 2000 from chickens or turkeys) and 38 species other than *M. synoviae* (Mollicutes species including 16 avian mycoplasma species, and non Mollicutes species) were used to test the PCR specificity (Tab. I).

For the first assay, the field strain *M. synoviae* 86122 isolated in 1986 from a chicken air sac strain was used. For determination of the detection period of mRNA and 16S rRNA after mycoplasma death, the *M. synoviae* strain K1415, kindly given by S.H. Kleven (University of Georgia, USA) was used. The numbers of colony forming units (CFU) were determined according to Rodwell and Whitcomb [20].

M. synoviae was cultivated on FM4 media [6]. Transport Medium (TM) for mycoplasmas was 2% buffered peptone water (AES, Combourg, France) containing glycerine (Merck Eurolab, Briare Le Canal, France, 1.2% v/v), bacterial growth inhibitor (Penicillin G, Sigma-Aldrich, Saint-Quentin-Fallavier, France, 1 000 units per mL of medium) and thallium acetate (Merck Eurolab, 1.9 mM).

For the second and third assay, the infectious bronchitis (IB) virus Mass 41 strain [7] was used. The titre of the suspension was determined in embryonated chicken eggs according to the method of Reed and Muench [25].

2.2. Experimental designs

2.2.1. Artificial contamination of the isolator

In the first experiment, fifteen one-day-old specific pathogen free (SPF) chicks obtained from the experimental poultry unit of AFSSA-Ploufragan (France) were reared in one isolator. Prior to contamination, 15 g of food and 10 mL of drinking water were collected and one drag-swab (Sodibox, La-Forêt-Fouesnant, France), previously humidified with 5 mL of TM was rubbed in the isolator. The food sample and the environmental drag swab were placed in 20 mL of TM before culture, mPCR and mRT-PCR and the drinking water sample was filtered and the membrane placed in FM4 medium was used for culture, mPCR and mRT-PCR. Then, the environment of the isolator was contaminated by spreading 40 mL of a culture of the *M. synoviae* 86122 strain (1.3×10^9 CFU/m^3) on the floor of the isolator. On days 13, 27 and 34 after the first assay started (D13, D27 and D34), tracheal

Table I. Bacterial species used in the mPCR specificity test.

Bacterial species	Main host	Source	Number tested	Multiplex PCR results	
				M. synoviae	Mollicutes
M. synoviae	Poultry	R[a] - WVU1853[b]	1	+	+
	Poultry	Field strains	17	+	+
M. gallisepticum	Poultry	R - ATCC19610	1	–	+
M. meleagridis	Poultry	R - ATCC25294	1	–	+
M. iowae	Poultry	R - ATCC33552	1	–	+
M. lipofaciens	Poultry	R - ATCC35105	1	–	+
M. gallopavonis	Poultry	R - ATCC33551	1	–	+
M. gallinarum	Poultry	R - ATCC19708	1	–	+
M. glycophilum	Poultry	R - ATCC35277	1	–	+
M. gallinaceum	Poultry	R - ATCC33550	1	–	+
M. columbinasale	Poultry	R - ATCC33549	1	–	+
M. columborale	Poultry	R - ATCC29258	1	–	+
M. columbinum	Poultry	R - ATCC29257	1	–	+
M. iners	Poultry	R - ATCC15969	1	–	+
M. anseris	Poultry	R - ATCC49234	1	–	+
M. anatis	Poultry	R - ATCC25524	1	–	+
M. cloacale	Poultry	R - ATCC35276	1	–	+
M. pullorum	Poultry	R - ATCC33553	1	–	+
M. hyopneumoniae	Pig	R - ATCC25934	1	–	+
M. flocculare	Pig	R - ATCC27399	1	–	+
M. hyorhinis	Pig	R - ATCC17981	1	–	+
M. hyosynoviae	Pig	R - ATCC25591	1	–	+
M. bovis	Bovine	R - ATCC25523	1	–	+
M. arginini	Cattle	R - ATCC23838	1	–	+
M. orale	Human	R - ATCC23714	1	–	+
M. pneumoniae	Human	R - ATCC15531	1	–	+
Acholeplasma laidlawii	Various	R - ATCC23206	1	–	+
Acholeplasma axanthum	Various	R - ATCC25176	1	–	+
Spiroplasma citri	Plant	R - ATCC27556	1	–	+
Ureaplasma urealyticum	Human	R - ATCC27618	1	–	–
Campylobacter coli	Various	Field strain	1	–	–
Mannheimia haemolytica	Various	Field strain	1	–	–
Haemophilus paragallinarum	Poultry	R - ATCC29545	1	–	–
Streptococcus suis	Pig	R - ATCC43765	1	–	–
Pasteurella multocida	Various	Field strain	1	–	–
Actinobacillus	Pig	R - ATCC27088	1	–	–
Clostridium innocuum	Various	R - ATCC14501	1	–	–
Clostridium ramosum	Various	R - ATCC25582	1	–	–
Escherichia coli	Various	R - ATCC25902	1	–	–
Salmonella typhi	Various	R - ATCC167	1	–	–

[a] R: Reference strain.
[b] ATCC: American Type Culture Collection, Rockville, USA.

swabs were collected from all chicks, placed in 2 mL of TM and analysed by culture and mPCR. The birds were sacrificed on D34.

2.2.2. Contamination of the isolator with experimentally infected chickens

In the second experiment, fourteen one-day-old SPF chicks were put in the isolator that had previously contained during 34 days the 15 chickens of the first experiment. The birds were introduced less than five minutes after depopulation of *M. synoviae* infected (see below) chickens. On day 21 (D21), they were inoculated intranasally with Infectious Bronchitis virus Mass 41 strain (10^3 Embryo Infectious Doses (EID_{50}) per bird). Tracheal swabs collected on days D12, D18 and D27 from all chicks were analysed by mycoplasma culture and mPCR. On day D33, the chickens were sacrificed and autopsied. Tracheas and air sac samples were analysed by culture and mPCR.

In order to monitor *M. synoviae* in the isolator environment, for the first seven days following the elimination of the chickens of the first assay, three food samples, three feather samples, three water samples and one drag-swab previously rubbed in the isolator were analysed by culture, mPCR and mRT-PCR. *M. synoviae* cells were cultivated from environmental samples as previously described [16].

2.2.3. Contamination of the isolator with naturally contaminated dust, feathers and food

In the third experiment, another isolator was contaminated by scattering 672 g/m^3 of a mixture of dust, feathers and food that had been collected less than an hour earlier from a *M. synoviae* infected commercial laying hen flock (previous culture and PCR analysis of twenty tracheal swabs and serological tests had revealed that all analysed hens from the flock harboured *M. synoviae* and were serologically *M. synoviae* positive). Before contamination of the isolator, six samples of mixture of dust and feathers and three samples of food were collected for culture, mPCR and mRT-PCR in order to confirm the presence of *M. synoviae*. Then fifteen one-day-old SPF chicks were introduced into the isolator. Three hours later, the presence of darkling beetles was detected and Baycidal and Solfac products (Bayer, Puteaux, France) were used to kill the insects. On day 57 (D57), the chickens were inoculated intranasally with IB virus Mass 41 as previously described. Tracheal swabs were collected from all chicks on days D12, D26, D33, D40, D47, D54 and D63 and analysed by culture and mPCR. On day D69 the chickens were sacrificed and autopsied. Tracheas, spleens and air sac samples were analysed by culture or mPCR.

2.3. mPCR: sample preparation and processing

DNA extracts from environmental samples, tracheal swabs, tracheas and air sacs were prepared for PCR [10, 16].

The mPCR was developed to enable simultaneous detection of DNA of *M. synoviae* species and the genera *Mycoplasma, Acholeplasma* and *Spiroplasma* genera, which will be referred to as "Mollicutes" hereafter. A 422 base pair (bp) PCR product specific for *M. synoviae* was obtained with MSpcl5 and MSpcl4 primers (Tab. II) defined on the *pcl*42-56 gene coding for a membrane protein, described by ben Abdelmoumen et al. [2]. The second primer set (GPO3 and MGSO) based on 16S rDNA was used to detect most species of Mollicutes [23, 24] (but not *Ureaplasma* see below) with a better sensitivity than the species specific PCR. The mPCR mixture contained PCR buffer (67 mM Tris-HCl, 16 mM $(NH_4)_2SO_4$, 0.01% Tween 20, 2.5 mM $MgCl_2$ (pH 8.8)), a 600 µM concentration of each deoxyribonucleoside triphosphate (dNTP) (Pharmacia Biotech, Orsay, France), 700 nM of MSpcl5 and MSpcl4 primers, 150 nM of GPO3 and MGSO primers (Oligo-express, Paris, France), 3 units of *Taq* DNA polymerase (Eurobio,

Table II. Primers used to construct the PCR internal positive control and for mPCR.

Primer	Sequence	PCR product (size)
CI-5	5'-**TCATTCAGCAGCGCCAGCTGGTTC**CATGCATGTCGAGCGGAATTTAGC-3'	IPC (645 bp)
CI-6	5'-**GCTTGAGTCTCCATTAACTTGTTG**TATCTACGCATTCCACCGCTTCAC-3'	
MSpcl5	5'-**TCATTCAGCAGCGCCAGCTGGTTC**-3'	*M. synoviae* (422 bp)
MSpcl4	5'-**GCTTGAGTCTCCATTAACTTGTTG**-3'	
GPO3	5'-GGGAGCAAACAGGATTAGATACCCT-3'	Mollicutes (270 bp)
MGSO	5'-TGCACCATCTGTCACTCTGTTAACCTC-3'	

Les Ulis, France), 10 fg of Internal Positive Control (IPC, see below) and 5 µL of the DNA template. Amplification was performed in a Perkin-Elmer Cetus GeneAmp PCR system 9600 (Perkin Elmer, Courtaboeuf, France). The reaction procedure consisted of 40 cycles of denaturation at 94 °C for 30 s, primer annealing at 65 °C for 30 s, and extension at 72 °C for 12 s.

The amplified products were separated in a 2% agarose gel in TBE buffer (90 mM Tris, 90 mM borate, 2.5 mM EDTA (pH 8)) for 1 h at a constant voltage of 110 V. Amplified products were detected by UV transillumination with ethidium bromide staining. A Smart Ladder (Eurogentec, Angers, France) was used as a molecular size standard.

2.4. mRT-PCR: sample preparation and processing

The mRT-PCR allowed detection in the environment of viable or recently dead cells belonging to the *M. synoviae* species or to the Mollicutes genera. Extraction of total RNA from 900 µL of initial suspension of environmental samples was as previously described [17]. The RNA extracts were kept at 4 °C and controlled by PCR to confirm the absence of DNA.

The 16S rRNA fragment of *Mycoplasma, Acholeplasma* or *Spiroplasma* genera was reverse transcribed into cDNA with the MGSO primer and the *M. synovia*e specific mRNA fragment was reverse transcribed with the MSpcl4 primer. The Reverse Transcription (RT) mixture contained buffer (67 mM Tris-HCl, 16.6 mM $(NH_4)_2SO_4$, 1 mM $MnCl_2$, pH 8.8), 600 µM concentration of each deoxyribonucleoside triphosphate (Pharmacia Biotech), 750 nM of MGSO primer, 3 150 nM of MSpcl4 primer (Oligo-express), 15 units of *Tth* DNA polymerase (Eurobio), and 10 µL of the RNA template in a total volume of 20 µL, under a drop of mineral oil. RT was performed at 65 °C for 15 min. Then microtubes were placed in ice. The whole RT mixture was mixed with PCR mixture containing buffer (67 mM Tris-HCl, 16.6 mM $(NH_4)_2SO_4$, 0.75 mM EGTA, 5% glycerol, 0.02% Tween 20, 2.5 mM $MgCl_2$, pH 8.8), 150 nM of GPO3 primer, 700 nM of MSpcl5 primer and 1 fg of IPC in a total volume of 100 µL. Amplification was performed in a Perkin-Elmer Cetus GeneAmp PCR system 9600 (Perkin Elmer). The reaction procedure consisted of 40 cycles of denaturation at 94 °C for 30 s, primer annealing at 65 °C for 30 s, and extension at 72 °C for 12 s.

The amplified products were separated by electrophoresis as previously described. In the results section, a positive mPCR result means that both *M. synoviae* and Mollicute specific fragments were amplified.

2.5. Sensitivity of the mPCR and RT-PCR tests

The sensitivities of the mPCR and mRT-PCR methods were evaluated using ten fold dilutions of titrated *M. synoviae* cultures in exponential growth phase.

2.6. Detection of mRNA and 16S rRNA after mycoplasma death

The detection period of mRNA and 16S rRNA after mycoplasma death was evaluated after death due to osmotic shock of *M. synoviae* strain K1415. One millilitre of *M. synoviae* culture containing 6.6×10^6 CFU was mixed with 99 mL of TE buffer (10 mM Tris-HCl, 1mM EDTA (pH 8.0)). A control of the non cultivability of the suspension was performed by inoculating this suspension in FM4 agar or liquid media. The dead mycoplasma suspension was placed at room temperature. Immediately after osmotic shock and 1, 2, 3, 4, 5, 6, 7, 9, 10, 12, 14, 16, 18, 20, 25, 30 min and 1, 2, 3, 4, 5, 6, 21, 22, 23, 24, 26, 27, 29, 30, 45 and 69 h after, DNA and total RNA were extracted from two 50 µL samples. All DNA and RNA samples were analysed by mPCR and mRT-PCR.

2.7. Construction of the PCR Internal Positive Control

In order to check for PCR inhibitors, an Internal Positive Control (IPC) was constructed by PCR [21]. The primers CI-5 and CI-6 (Oligo Express) possessed 5' overlapping ends identical to the primers used in the *M. synoviae* PCR reaction, whereas their 3' ends were complementary to a 16S rDNA sequence of *M. synoviae* (Tab. II). Thus the IPC was a 16S rDNA fragment of 645 bp from *M. synoviae* generated by PCR. The PCR mixture contained the PCR buffer (67 mM Tris-HCl, 16 mM $(NH_4)_2SO_4$, 0.01% Tween 20, 2.5 mM $MgCl_2$ (pH 8.8)), a 500 µM concentration of each dNTP (Pharmacia Biotech), 400 nM of CI-5 and CI-6 primers, 1 unit of *Taq* DNA polymerase (Eurobio), and 5 µL of a cell lysate of pure culture of *M. synoviae* WVU 1853 strain. Amplification was performed in a Perkin-Elmer Cetus GeneAmp PCR system 9600 (Perkin Elmer). The reaction procedure consisted of 40 cycles of denaturation at 94 °C for 30 s, primer annealing at 65 °C for 30 s, and extension at 72 °C for 10 s. The PCR product was purified using a commercially available kit (Life Technologies, Cergy Pontoise, France) and stored in double-distilled water at –20 °C. DNA concentration was determined spectrophotometrically.

3. RESULTS

3.1. Specificity and sensitivity of the mPCR and sensitivity of the mRT-PCR

The PCR assay enabled amplification of three fragments: the 645 bp IPC, the 422 bp fragment of the *pcl*42-56 gene of *M. synoviae* and the 270 bp fragment of Mollicute 16S rDNA gene. The three fragments were obtained with each of the 18 *M. synoviae* strains tested. The *M. synoviae* specific fragment was not obtained with any of the other species tested. The 270 bp fragment was obtained only with the species belonging to *Mycoplasma*, *Acholeplasma* or *Spiroplasma* (but not *Ureaplasma*) genera. For all other genera tested (including *Ureaplasma)*, only the band corresponding to the IPC was obtained.

mPCR and mRT-PCR were performed on serial dilutions of DNA and RNA extracts obtained from *M. synoviae* cultures. In our conditions, the detection limit of PCR was 1.65 CFU/assay for the *M. synoviae* specific product and 0.165 CFU/assay for the product shared between all species of *Mycoplasma*, *Acholeplasma* or *Spiroplasma*. The RT-PCR detection threshold was 6.6 CFU/assay for the *M. synoviae* protein specific product and 0.66 CFU/assay for the Mollicutes 16S rRNA product.

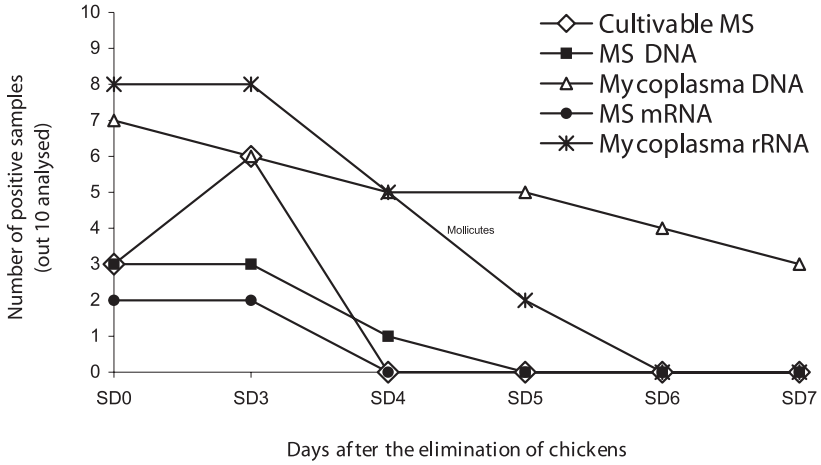

Figure 1. Detection of *M. synoviae* (MS) cultivable cells, DNA and mRNA and Mollicutes DNA and rRNA in the isolator environment after elimination of chickens infected during the first assay, as determined by culture, mPCR and mRT-PCR (experiment 2).

3.2. Detection of mRNA and 16S rRNA after mycoplasma death

M. synoviae mRNA and 16S rRNA could be detected up to 20 min and 23 h respectively after mycoplasma death. All samples collected up to 69 h (last sampling time) after mycoplasma death were positive by mPCR.

3.3. Contamination experiments

3.3.1. Artificially contaminated isolator

On D0, before isolator contamination, *M. synoviae* could not be detected by culture, mPCR and mRT-PCR in environmental samples. On D13, *M. synoviae* was detected in one chicken tracheal swab by culture and mPCR. On D27 and D34, all tracheal swabs analysed were positive by culture and mPCR. On D34, all chickens had *M. synoviae* specific antibodies according to the slide agglutination test (data not shown).

3.3.2. Contamination of the isolator with experimentally infected chickens

In the second experiment, *M. synoviae* could be isolated from only 3 feather samples on D0 and D3 and from 2 food samples on D3. *M. synoviae* DNA was detected on 3 water samples on D0, 1 feather and 2 water samples on D3 and 1 feather sample on D4. Mollicutes DNA was detected on all water samples from D0 to D7. Up to D5, most feather samples contained Mollicutes DNA whereas the only positive food sample was collected on D6. Drag-swab contained Mollicutes DNA only on D0. *M. synoviae* mRNA was detected only in one water sample and the drag-swab collected on D0, and in 2 feather samples on D3. Eight samples contained Mollicutes rRNA on D0 and on D3, and 5 (food, feather or water) and 2 (water) samples were positive on D4 and D5 respectively (Fig. 1). On D12, D18 and on D27 (6 days after avian infectious bronchitis virus inoculation), *M. synoviae* could not be detected by culture or by PCR on tracheal swabs. On D33 (12 days after virus

inoculation), culture was positive for ten tracheas and ten air sacs, from a total of 14 infected birds. All 14 tracheas and 12 air sacs were positive according to mPCR. Autopsy revealed that all chickens had severe airsacculitis lesions.

3.3.3. Contamination of the isolator with naturally contaminated dust, feathers and food

In the third experiment, on D0, before introduction of contaminated fomites, no *M. synoviae* could be detected by culture, mPCR and mRT-PCR in the isolator. Analysis of the fomites collected on the farm gave the following results; one food sample out of three enabled isolation of *M. synoviae*. Other samples were negative according to culture. Mollicutes DNA and rRNA could be amplified from six samples (1/3 food sample, and 5/6 feather and dust mixes) but no *M. synoviae* specific product could be obtained. The many darkling beetles (*Alphitobius diaperinus*) present in these dust, feathers and food samples were destroyed after treatment with the disinfestant.

On D12, 26, 33, 40 and 47, according to culture, none of the 15 chicken tracheal swabs contained *M. synoviae* cells. However, on D12, and D26, mPCR detected Mollicutes DNA in one and two swabs, respectively. On D33, nine swabs contained *M. synoviae* DNA among which 5 contained Mollicutes DNA.

On D54, culture indicated that two chickens were colonised and *M. synoviae* and Mollicutes DNA fragments were detected on the same two tracheal swabs. On day 63 (6 days after inoculation of the IB virus), some birds exhibited tracheal rales and/or lameness. *M. synoviae* was isolated from seven tracheal swabs and all fifteen tracheal swabs were positive according to mPCR. On D69, according to culture and mPCR performed on tracheas, all the chickens were infected by *M. synoviae*. *M. synoviae* could also be detected from 12 air sacs and 12 spleens. Autopsy revealed that all chickens had severe airsacculitis lesions.

4. DISCUSSION

Three assays were performed to study *M. synoviae* environmental contamination of specific pathogen free chicks. In order to monitor the presence of *M. synoviae* or Mollicutes cells in environmental samples and in tracheal swabs, molecular tools were first developed.

The mPCR assay used in this study was based on the amplification of two gene fragments, one coding for a *M. synoviae* membrane protein (accession No. U66315) [2] and the other for Mollicutes 16S rRNA [23]. The results were specific for *M. synoviae* and *Mycoplasma*, *Spiroplasma* and *Acholeplasma* genera respectively. In fact, although the *M. synoviae* primers were selected in a gene coding for a membrane protein possibly subject to antigenic variations among *M. synoviae* isolates [19], amplification was positive for the 18 tested strains. Furthermore, the mPCR test contained an IPC to detect false negative results due to polymerase inhibitors and could be used, in most cases, directly on bacteria contaminated samples without DNA purification, except when the presence of inhibitors necessitated phenol-chloroform DNA extraction (data not shown).

The aim of the mRT-PCR was to enable the detection of viable *M. synoviae* in environmental samples [1, 5, 11], because isolation of *M. synoviae* from such samples is made difficult by contaminating bacteria. The test, based on *M. synoviae* protein mRNA and Mollicutes 16S rRNA reverse-transcription, was used directly on environmental samples after RNA extraction and purification. Stability of mRNA is limited [22] and its detection is correlated with the presence of metabolically active or very recently dead bacteria. Indeed, under our laboratory conditions, *M. synoviae* mRNA could be detected up to 20 min after mycoplasma death. The simultaneous detection

of Mollicutes rRNA increased the sensitivity of the test and allowed the detection of Mycoplasma dead up to 23 h earlier. The difficulty of the assay was to maintain the samples in cold conditions and to begin the analysis within a very short time in order to keep viable mycoplasmas in environmental samples.

Environmental samples collected in the two isolators before the introduction of animals or contaminating materials did not contain Mollicutes 16S rRNA. Therefore, for the first and second assays, positive mPCR or mRT-PCR results with only the product shared by *Mycoplasma*, *Acholeplasma* and *Spiroplasma* genera were probably due to the presence of *M. synoviae* because the introduction into the isolator of Mollicutes other than *M. synoviae* was unlikely. On several occasions, the absence of the *M. synoviae* specific product could be due to higher sensitivities of mPCR or mRT-PCR to detect *Mycoplasma*, *Spiroplasma* and *Acholeplasma* genera than *M. synoviae* species. This observation, coupled with the differences of targets for mRT-PCR, could also explain that Mollicutes DNA or rRNA could be detected longer than *M. synoviae* DNA or mRNA after elimination of infected chickens. Furthermore, the results also confirmed that mycoplasma DNA was much more stable than mycoplasma rRNA and that DNA detection was not predictive of the viability of bacterial pathogens [9].

Cultivable *M. synoviae* could be detected in the isolator after elimination of infected chickens, up to 3 days according to culture, in accord with the results of Christensen et al. [3] and viable cells were found up to 4–5 days as suggested by mRT-PCR.

The infectivity of the contaminated environment or fomites was clearly demonstrated as in each trial; the chicks, originating from SPF breeders, actually became *M. synoviae* infected. First positive tracheal swabs were obtained on day D13, D33 or D54. For the first and the third assay, only one and two positive birds were detected first respectively, then all other birds were *M. synoviae* culture positive, as usually observed under field conditions [12]. For the second and third assays, the delays between the introduction of birds in a contaminated environment and the detection of infection according to culture was surprisingly long (33 and 54 days respectively) compared to the delays before observation of clinical signs or lesions in the case of intra-tracheal (4 days) or aerosol inoculation (17–21 days) of SPF birds [12]. Such a long delay could probably result from a low infectious dose in fomites compared to relatively high titres of cultures used for challenge. Moreover such a long period before the development of mycoplasma clinical infection may be encountered in field conditions. Indeed according to Ley [14] *M. gallisepticum* flocks with a probable low level of infection, develop clinical infections near the onset of egg production in response to stressors. Similar *M. synoviae* extended incubation periods may also exist in the field. Problems associated with such a long latent infection are the determination of the origin of contamination and early detection of infection during this apparently silent period. It is emphasised that during the third trial, on D33, a few tracheal swabs gave a *M. synoviae* (but not Mollicutes) positive PCR result whereas the cultures were negative. The reason why only the species specific product was obtained in the absence of a Mollicute product is unknown. However the fact that on D12, D26 and D33, a few tracheal swabs yielded positive PCR signals might indicate that these birds had dead cells in their tracheas. Another possibility that needs further exploration is that birds could harbour viable but non cultivable cells. In fact different observations concerning samples from which no mycoplasma could be isolated but which were obviously infectious have been reported [13,18] and in vivo bioassays are recommended when difficulties in isolating mycoplasmas are experienced [15]. This phenomenon seems reminiscent of the finding of Dallo and Baseman [4] which showed that intracellular *M. genitalium* could no longer be cultivated in artificial

media. Indeed *M. gallisepticum* [27] and possibly also *M. synoviae* [26], have the capacity to enter eucaryotic cells and could be submitted to such biological alteration. Additional studies are necessary to explore this hypothesis.

Despite the lack of only IB inoculated controls, the results underlined the fact that the pathogenicity of *M. synoviae* seemed exacerbated when chickens were infected with avian infectious bronchitis virus, a rather common respiratory pathogen, since, according to the experience of the authors, infections due to *M. synoviae* alone or avian infectious bronchitis virus alone do not generate airsacculitis lesions. Finally, our results corroborate the observations of Hopkins and Yoder [8] showing that infectious bronchitis vaccine virus increases the *M. synoviae* capacity to induce airsacculitis in chickens.

In conclusion, the different assays showed that *M. synoviae* contaminated material (dust, feathers and food) can infect chicks, resulting in a delayed infection. Therefore, very strict hygiene rules must be respected by farmers and farm visitors and biosecurity measures (cleaning, disinfection and emptiness) must be rigorously applied. This study also demonstrated that the control of surface contamination by the use of mycoplasma culture or RT-PCR could be used to validate the efficiency of biosecurity measures before the introduction of new birds on the premises.

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