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Research Article

Detection, Isolation and Identification of *M. hyorhinis* Isolates and Comparison of their Antimicrobial Susceptibility, Immunogenicity and Virulence

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Abstract

Mycoplasma hyorhinis (M. hyorhinis) field strains were isolated from the lungs of pig with pneumonic lesions, collected from Chinese slaughterhouses. The samples were designated as 11051207, 10090701, 11081111, 10072902, 100928, 11022402, 11042105 and 10071603 and subjected to antimicrobial susceptibility testing (AST) against 12 antimicrobial agents using broth micro dilution technique. Most of the *M. hyorhinis* strains were found to be sensitive to macrolide, norfloxacin and tetracycline. Tiamulin was found to have the highest activity with a minimum inhibitory concentration (MIC) of less than 0.06 for all the strains. However, strain 10090701 and 11042105 were not susceptible to gentamicin and kanamycin monosulfate (MIC>32). The level of IgG in the serum of vaccinated mice was quantified by an indirect enzyme linked immunosorbent assay (ELISA) to detect the strain with highest antigenicity. The strains 10090701, 100928, 11081111 and 10072902, especially the strain 10090701, induced higher titer of IgG antibodies than the others. The ability to induce mortality in chicken embryos after experimental inoculation with pure cultures of *M. hyorhinis* was done to determine the most virulent isolate. The most virulent strain was observed to be 11051207 that caused 100% mortality followed by 100928 and 11081111. The most immunogenic and virulent strains would subsequently be used in future studies on vaccine development and to inoculate swine and assess their ability to achieve *M. hyorhinis*-induced pathogenesis.

Key Words: *Mycoplasma hyorhinis*; Isolation; Antimicrobial susceptibility; Immunogenicity; Virulence

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Introduction

Mycoplasmas are widespread in nature as pathogens or commensal organisms that are economically important in both plants and animals. Generally, mycoplasmas are perceived as being impotent, however, different authors have cited the seriousness of these pathogens terming them as the 'next generation' pathogens due to their adaptation to new hosts or niches [1]. Pigs are the natural hosts of *Mycoplasma hyorhinis* (*M. hyorhinis*), demonstrated as the causative agent of arthritis, chronic persistent polyserositis and otitis media in piglets three to ten weeks of age [2-5] and has often been reported as a secondary pathogen in porcine pneumonias [6-10]. However, the pathogen has recently been reported to infect human and having a role in oncogenesis [11, 12]. Mycoplasmas are also major contaminants of eukaryotic cell lines, tissue cultures, therapeutic and research reagents [13].

The intracellular habitat and the antigenic variation of the mycoplasma enable the organism to evade the immune system, establish a chronic infection and drug resistant [14-16]. This antigenic variation was found to be due to the variation in the bacterial lipoproteins [17]. The complexity of the virulence factors in mycoplasma genome, makes their detection laborious [18]. However, the accurate detection of *M. hyorhinis* has significantly increased with the development of molecular biology techniques such as nested-PCR [10, 19]. At the genomic level, great heterogeneity has been reported among mycoplasma isolates from different herds using the molecular typing procedures available [7, 18, 20, 21]. It is possible to find different strains in the same animal in a farm, which could differ in virulence or cause interferences and cross reactions [21]. In comparison to other porcine respiratory pathogens *M. hyopneumoniae*, *M. flocculare*, the *M. hyorhinis* genome vary in some components concerned with metabolism and evasion of the host's immune system, which might contribute to its growth aggressiveness [9]. Isolation of *M. hyorhinis* is therefore, important for the diagnostic purposes or epidemiological studies [4, 22]. This study investigated varying aspects related to *M. hyorhinis* sensitivity to antibiotics, immuno-

genicity and virulence to understand the complex mechanisms involved in its pathogenesis.

Materials and Methods

Detection, Isolation and Identification

Fresh lung tissues with clinical signs and manifestation of gross lesions were collected from Nanjing Xiao Lin Wei and Tian Huang slaughterhouses. Samples were taken from the infected lobes and DNA was extracted with TIANamp Bacteria DNA Kit, Tiangen Biotech (Beijing) Co., Ltd. following manufacturer's instructions. Positive and negative controls were also involved in the study. For the amplification of DNA, PCR assay was carried out in a thermal cycler using 25 µL reaction mixture containing 2.5 µL 10×PCR buffer (Mg2+ Free), 1.5 µL $MgCl_2$ (25 mM), 2 µL dNTPs, 0.5 µL of each forward and reverse primer, 5 µL DNA, 0.2 µL Taq (5 U/µL), and 13.8 µL of DNAase-free deionized water under specific conditions (Table 1).

For isolation, a modified Friis media (KM2) was prepared according to previous methods [23]. The lungs were cut into small pieces, homogenized in KM2, 10-fold diluted, incubated in a 5% CO_2 humidified incubator at 37 ºC and observed daily for color change to yellow following the reduction in pH [4, 24]. About 10% (v/v) of the inoculum was sub-cultured in broth for 4-11 days and the growth of the bacteria was observed. Another sample was streaked on a solid KM2 agar using a sterile wire loop and plates incubated at 5% CO_2 humidified incubator at 37 ºC to isolate a single colony with a typical 'fried egg' appearance that would then be sub-cultured in KM2 broth by picking a single colony with a Pasteur pipette and dropping it into fresh broth. The isolates were cloned at least thrice before they were further examined [22].

Table 1. Oligonucleotide primers and amplification conditions for the nested PCR assay for *M. hyorhinis*

Mycoplasma species and target gene	nPCR, DNA Amplification conditions	Primer sequence(5′-3′)	Fragment size/bp
M. hyorhinis P37 gene	Outer: 95°C, 5min.; 94 °C, 60s; 56 °C,60s; 72 °C, 90s 30cycles	GCATCTATTTTCGCCAATAGC AGCTAGAGTTTCATCATT ACC	627
	Inner: 95°C, 5min.; 94 °C, 60s; 50 °C,60s; 72 °C, 90s 30cycles	GTAGTCAAGCAAGAGGATGT GCTGGAGTTATTATACCAGGA	346

nPCR= nested-PCR

In Vitro assessment of Antimicrobial Profiles of the Isolates against 12 Antimicrobial Agents

The antimicrobial agents used in this study were purchased from different Chinese companies listed as Ningxia Qi Yuan, North China Pharmaceutical, Benxi, Zhejiang Guobang and Shandong Shengli. Preparation of stock solutions of the antibiotics was done by weighing the antimicrobial agents according to their potency (Table 2). The stock of the antimicrobials was aliquoted to 3200 µg/mL and stored at -70°C. Actively growing broth culture was thawed on the day of the assay and added to 180 µL of appropriate broth medium for each antibiotic to be tested. 20 µL of diluted culture of the respective strain with an inoculum density of 10^5 CCU/mL was added into each well [25, 26]. There were two controls; the broth control without mycoplasma as sterility control and the mycoplasma strain inoculated into the broth as growth control [25]. The plate was then secured with a vinyl seal and incubated at 37 °C atmospheric conditions of 5% CO_2 and examined once daily until growth was noted in the mycoplasma control wells. Minimum inhibitory concentration (MIC) was noted as the lowest concentration of antimicrobial agent at which the bacterial growth was absolutely inhibited. Initial MIC was noted immediately the inoculum control lacking the antimicrobials turned from red to yellow. Final MIC was noted when there were no further changes in color for two consecutive days. The tests were repeated twice and the highest concentration was taken as the MIC if there was a difference.

Immunogenicity Testing in Mice and Determination of Antibody Titers by Indirect-ELISA

The bacterial cultures were centrifuged and washed thrice in phosphate buffered saline (PBS) and inactivated with formalin. DNA was extracted using TIANamp Bacteria DNA Kit, Tiangen Biotech (Beijing) Co., Ltd. following manufacturer's instructions. The real time PCR mix was prepared as

Table 2. Antimicrobial agents weighed according to their potency

Antibiotic	Potency	Weight in 10 mL (mg)
Tilmicosin	97.5%	32.8
Tiamulin	98%	32.7
Azithromycin	95%	33.7
Tyrosine	10%	320
Vibramycin	98%	32.7
Oxytetracycline	95%	33.7
chlortetracycline	97.6%	32.8
Tetracycline	98%	32.7
Clindamycin	98%	32.7
Norfloxacin	98.7%	32.4
Gentamicin	70.3%	45.6
Kanamycin monosulfate	77.4%	41.3

follows; p37 FQR 1 µL, p37FQF 1 µL, probe 0.51 µL, Taq premix 12.51 µL, ROX dye 0.5 µL, ddH_2O 8.5 µL and DNA template 1 µL. M. hyorhinis forward and reverse primers were;
P3-F AGAAGGTTCTTTTGCTTGAACACA;
P4-R TGCTTCCATCTTTTCATTTGCTT;
TaqMan probe; FAM-ATCAGCAACAAAACCTT-MGB.

For quantification of *M. hyorhinis* by real time PCR, the amplification was performed using a 96-well thermal cycler using 1 cycle at 95 ºC for 10 min denaturation, 40 cycles at 95 ºC for 15 s, annealing at 53ºC for 1 min elongation and extension at 72°C for 30 s (7500 Fast Real-Time PCR System, Applied Biosystems, Foster City, CA, USA). Quality controls included on each plate consisted of one well negative control containing the reaction mix and a pure culture of M. hyorhinis as a positive control. Analysis of the amplification and standard curves was performed using the manufacturer's software. The reactions were done in triplicates to eliminate discordant results, and the average of the three samples was assumed to be the concentration of the original DNA template (Table 3).

The immunological studies were then performed in inbred male *BALB/c* mice and the concentration of the strains determined by real time PCR was equalized by diluting with PBS. 7 mice for every strain and 7 control mice were randomly distributed into eight groups and housed in plastic cages in the animal house and fed *ad libitum*. Initial vaccination with Freund's complete adjuvant and the *M. hyorhinis* antigens emulsified in the ratio of 1:1 (v/v) was done. The initial immunization was carried out and each mouse received 200 µL of the vaccine, 100 µL intramuscular injections in each hind leg. The second vaccination was done on two weeks later with *M. hyorhinis* strains emulsified with Freund's incomplete adjuvant. Weekly blood samples were taken for quantification of IgG antibody levels by an indirect-ELISA assay to detect and compare the antibody responses to different *M. hyorhinis* strains, with whole *M. hyorhinis* proteins as the coating antigen. Flat bottomed 96-well ELISA plates were coated overnight with 10 µg/ml of the antigen (100 µL/well) at 4 ºC. After blocking with 3% bovine serum albumin, each well was incubated for 30 minutes at 37 °C with 100 µL of serum diluted in PBS containing 1% bovine serum albumin at 1:100, followed by 100 µL horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG at a dilution of 1:10000. After washing, the substrate containing 3, 3', 5, 5'-tetramethylbenzidine and H_2O_2-urea was added and the plates were incubated at 37 °C for 15 minutes, then H_2SO_4 was added to stop the reaction, and the absorbance was measured at the dual wavelengths of OD 450 nm and OD 630 nm by a computer assisted micro-plate reader.

Virulence Screening of the Isolates in Chicken Embryos by Mortality Index

Pathogenicity of each isolate was estimated by inoculating the yolk sac [27, 28] of 6-7 days old specific pathogen free (SPF) chick embryos (Nanjing Tianbang Bio-Industry Co., Ltd, Nanjing, China). The eggs were disinfected with iodine-alcohol solution applied gently with a swab and then carefully drilling a small hole of the shell membrane for the needle of 1.0 mL syringe with of 4 mm x 5 gauges in size to deliver the culture into the eggs after which, the holes were sealed with melted paraffin wax. A total of 45 embryos were infected with 0.3 mL of different strains of *M. hyorhinis* at a concentration of 10^7 CCU/mL. A negative control group of 10 embryonated eggs was inoculated with 0.3 mL sterile mycoplasma broth. 10 eggs were incubated without any inoculation to serve as viability controls. All eggs were stored within the same humidified incubator at 37°C and candled daily to observe the growth and development of the embryos, which could be easily seen due to the movements in response to the light and the well-defined blood vessels. Embryo mortality was observed after inoculation status. Embryonic death within 24 hours after inoculation would be considered non-specific and would only be evaluated for bacterial contamination. The surviving embryos were slaughtered 1 day before hatching. Re-isolation of *M. hyorhinis* was done from the heart tissue and

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Table 3. Quantification of formalin inactivated antigens from different isolates by real time PCR

M. hyorhinis strains	Average cDNA/mL
10090701	5.0×10^6
100928	2.35×10^7
11081111	1.82×10^8
11022402	8.57×10^7
11042105	2.03×10^9
10072902	2.39×10^8
11051207	9.83×10^8

yolk [27]. The tissues were cut into small pieces and continuously pipetted in 200 µl KM2, mixed well and the supernatant inoculated in KM2 medium at a ratio of 1:5, incubated and observed for change in color. DNA was extracted from the grown cultures for nested PCR detection.

Results

Different *M. hyorhinis* field strains isolated were designated as 11051207, 10090701, 11081111, 10072902, 100928, 11022402, 11042105 and 10071603. The size of the amplified DNA fragment of 346 bp for P37 gene from M. hyorhinis genome in 10 µL of the reaction mixture on a 1% agarose gel was visualized (Fig. 1). The cultured isolates were observed under inverted microscope to identify the typical fried egg colonies (Fig. 2). Among the antimicrobial agents used in this experiment, most of the *M. hyorhinis* strains tested in this study were found to be sensitive to tiamulin, tilmicosin, clindamycin, tylosin, azithromycin, norfloxacin, chlortetracycline, tetracycline, oxytetracycline and vibramycin. However, strain 10090701 and 11042105 were not susceptible to gentamicin and kanamycin monosulfate (MIC>32). Strain 100928 was susceptible to kanamycin monosulfate (MIC=4) but less susceptible to gentamicin (MIC>32) while strain 11022402 was susceptible to gentamicin (MIC=8) but less susceptible to kanamycin monosulfate (MIC>32). Of all these antimicrobials, tiamulin was found to have the highest activity with an MIC of less than 0.06 for all the strains (Table 4).

Inactivated *M. hyorhinis* vaccines in this experiment are demonstrated to trigger long-term IgG antibodies response for extended periods of time. After vaccination, *M. hyorhinis*-specific antibodies in the group immunized with strain 10090701 significantly increased after the second inoculation. The level of the response in this group was obviously higher than that of mice immunized with other strains ($P < 0.01$). The mice of the groups immunized with strains 100928, 11081111 and 10072902 also produced significant serum antibodies ($P < 0.01$, compared to control), but the titer became significant after five weeks. The antibodies of mice in the group of strain 11022402 only increased notably after 8 weeks ($P < 0.01$, compared to control), and the duration period was very short. The strains of 11042105 and 11051207 could not stimulate the humoral immune response effectively at this concentration ($P > 0.05$, compared to control) (Fig. 3). The pathogenicity testing in chicken embryos show that the highest mortality was noted on the third day and reduced after the fifth day. No mortality was observed during the last two days before necropsy. All the embryos from the control group survived to the time of necropsy as well as those challenged with the mycoplasma media alone. All embryos that died from the inoculated groups before necropsy, exhibited a dark or reddish discoloration while some

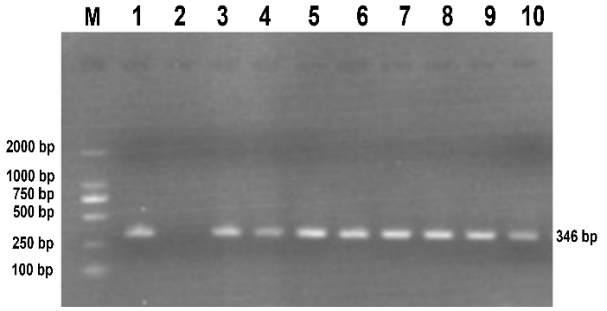

Fig. 1. Gel electrophoresis of nested-PCR for 8 different strains identified to be *M. hyorhinis*. P37 gene was used as the target gene to be amplified. M: DNA marker 2000 bp. 1. Positive control (*M. hyorhinis* DNA); 2. Negative control (ddH_2O); 3-10 are strains of 11051207, 10090701, 11081111, 10072902, 100928, 11022402, 11042105 and 10071603 respectively.

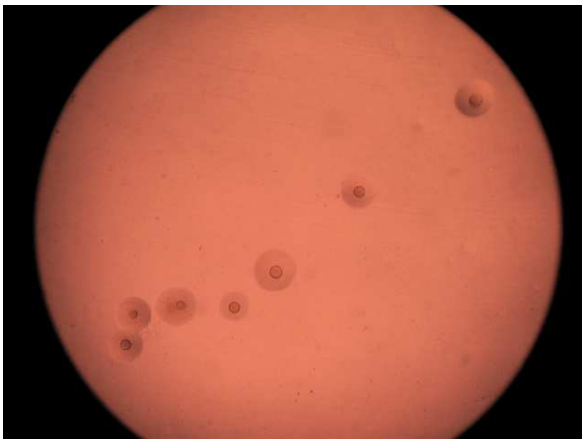

Fig. 2. Colony morphology of *M. hyorhinis* strain 11022402 colonies with a 'fried egg' appearance (4 x).

Table 4. MICs of 8 field isolates of *M. hyorhinis* against 12-antimicrobials determined through serial broth micro-dilution technique.

	MICs for *M. hyorhinis* Field Isolates (µg/mL)											
Strain/drug	**Tet**	**Oxyte**	**Chlort**	**Vibra**	**Tylo**	**Tiam**	**Tilm**	**Azithr**	**Clind**	**Norfl**	**Gent**	**Kana**
11051207	1	<0.06	2	4	0.5	<0.06	2	8	0.5	0.25	2	<0.06
10090701	2	1	2	<0.06	0.125	<0.06	<0.06	<0.06	<0.06	2	>32	>32
11081111	0.25	1	1	<0.06	0.125	<0.06	0.5	0.25	<0.06	0.25	8	0.5
10072902	0.25	0.25	0.5	<0.06	0.125	<0.06	0.125	<0.06	<0.06	2	4	2
100928	2	8	4	0.25	0.25	<0.06	<0.06	0.125	<0.06	4	>32	4
11022402	1	4	2	0.125	0.25	<0.06	<0.06	0.125	0.125	4	8	>32
11042105	4	2	4	0.5	0.25	<0.06	<0.06	0.125	<0.06	4	>32	>32
10071603	2	4	1	0.125	<0.06	<0.06	<0.06	<0.06	<0.06	2	8	16

Field Strains (n=8), Tet = Tetracycline, Oxyte = Oxytetracycline, Chlor = Chlorotetracyline, Vibra = Vibramycin, Tylo = Tylosin, Tiam = Tiamulin, Tilm = Tilmicosin, Azithr = Azithromycin, Clind = Clindamycin, Norfl = Norfloxacine, Gent = Gentamycin, Kana = Kanamycin.

Table 5. Number of infected embryos for each strain and the corresponding percentage mortality

Strain	**Total Number of infected eggs**	**Number of Dead embryos**	**%Mortality**
11051207	10	10	100
100928	10	9	90
11081111	10	8	80
11022402	5	1	20
11042105	5	0	0
10071603	5	3	60
KM2control	10	0	0
Viability control	10	0	0

demonstrated hemorrhage. In general, at 10^7 CCU/mL, *M. hyorhinis* strain 11051207 caused a complete death (100%), within the first week post inoculation followed by strains 100928 and 1108111 that induced 90% and 80% death respectively within the same time. Strain 10071603 caused 60% mortality followed by strain 11022402 with 20% mortality. Strain 11042105 did not induce any death and all the embryos survived to necropsy (Table 5). Mycoplasma broths that were inoculated with suspensions from the yolk and heart from the infected embryos demonstrated a heavy growth of mycoplasma identified as *M. hyorhinis* by PCR, indicating multiplication of the pathogen within the embryo. The cultures and DNA samples from the control groups inoculated with KM2 were negative. Mycoplasmas, identified as *M. hyorhinis* by PCR were re-isolated from the heart and yolk of all infected embryos.

Discussion

Resistance to antimicrobial agents such as tetracyclines, macrolides and fluoroquinolones has increased in recent years partly due to the overuse and misuse of antibiotics both in animals and human. This has been reported for mycoplasma species which have been seen to be changing in the susceptibility [29, 30]. Accurate knowledge of the *in vitro* susceptibility patterns at a local level could aid in recommending empirical treatment of the resistant strains or where diagnosis has failed [31]. Fluoroquinolones such as the norfloxacin used in this study are sensitive to *M. hyorhini*s but they are restricted to be used in veterinary species due to their importance in human medicine and concerns about their use has been raised for fear of development of resistance [21]. Similar to this study, low MIC for tilmicosin, tetracycline, and tylosin has been reported [25, 32]. On the contrary, lower MIC for amino-glycosides has previously been reported [25], [33] although mycoplasma strains that are insensitive to Kanamycin have recently been described [29]. Susceptibility to macrolides is still warranted and tiamulin is seen to have the highest level of activity [25, 32, 34]. Tetracycline is recommended for treating porcine mycoplasma infections due to their low MIC and affordability [26, 35, 36].

Development of effective vaccines in the swine industry could limit the economic losses as a result of *M. hyorhinis* infection [37, 38]. IgG antibody is easy to detect and is also the major immunoglobulin circulating in the blood and offers a prolonged resistance to infections by recognizing antigens exposed to [38] and was therefore used as

an index to compare the immunogenicity of different strains. The strains with high immunogenicity would be useful for developing inactivated vaccines in future research. In the present experiment, serum antibody titer was detected using the whole protein of *M. hyorhinis* as the coating antigen. Although it is an ordinary choice, it should also be noted that the detection by whole protein might be influenced by some nonspecific proteins present in the media that might bind to *M. hyorhinis* and produce antibodies [39].

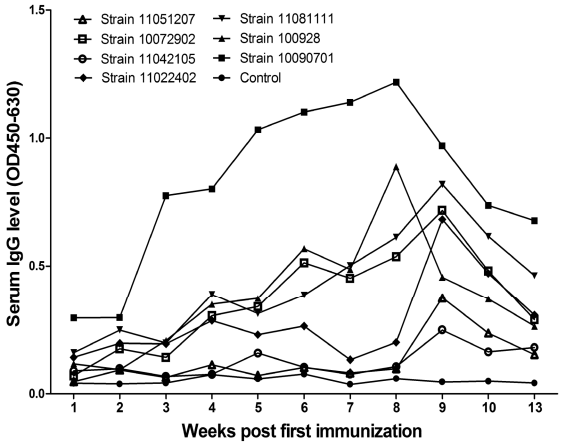

Fig. 3. Estimated marginal means of IgG antibodies response in BALB/c mice immunized with various *M. hyorhinis* strains, analyzed individually by ELISA. The data are expressed as the mean. The statistical analysis was performed using repeated measures ANOVA

Embryonated chicken eggs have previously been used as models for the study of viral and bacterial pathology [40-42] including *M. hyorhinis* [43] due to the fact that they are available, economic, convenient in size, relatively free from extraneous contamination and latent infection, and they do not produce antibodies against the inoculums [44]. The use of chicken embryos is seen to be useful in assessing and comparing the pathogenicity of different strains of *M. hyorhinis* since the cells of an embryo rapidly divide and undergo differentiation, and the developing embryo becomes sensitive to infectious processes. As a result, pathogenesis is marked as mortality from the infection, which can be assessed in a short duration of time. *M. hyorhinis* is demonstrated to be pathogenic in chicken embryos by inducing an average of 68.9% mortality, indicating that this organism may also be a potential pathogen for species other than swine.

Mycoplasmas are seen as inconspicuous predators that have more often than not concealed their etiological significance due to their ubiquity and understated pathogenicity. Despite the absence of a cell wall and a small genome, they are able to cause chronic infections in diverse hosts. Their virulence was screened by their ability to induce mortality in chicken embryos. Laboratory models are a useful tool in the studies of infectious diseases. Strain virulence however, may not cross the genetic or taxonomic boundaries and therefore, the pathogenicity tests of *M. hyorhinis* strains in chicken embryos cannot be absolutely ascertained to induce pathogenicity in swine and flaws may exist during extrapolation of such results. The pathogenesis of the isolates should therefore, be reconfirmed in the host animals, swine models.

Conclusion

This study reports that the most virulent strains are 11051207, 100928 and 11081111 respectively. Among them, strains 100928 and 11081111 were also observed to be highly immunogenic in the in mice models. The isolates could be used in future, to study the molecular pathogenesis of the bacteria and to develop vaccines against M. hyorhinis infection.

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