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Mycoplasma pulmonis in *Rattus norvegicus*: isolation and microbial diversity

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ABSTRACT

The presence of *Mycoplasma pulmonis* is very common in domestic and laboratory rats. *Mycoplasma* is the smallest bacterial cell species that was been discovered thus far. It can survive without oxygen, comes in various shapes, and lacks a cell wall. It is penicillin resistant and can be parasitic or saprotrophic. Depending on where the bacteria is located, genitalia or in the upper respiratory system, it will cause different disorders and diseases. The presence of these bacteria can cause many problems in the health of the person or animal. It poses large problems in laboratory settings and any impending experiments. This article observes the diversity of the microbiota in the throat and nose of rats, as well as the change in the ratio of *M. pulmonis* to the other microbiota at different levels of health; healthy, sick, and dead. The results of this study conclude that there is not a significant difference between the mean ratios of *M. pulmonis* to other bacteria along the decline in health of the rat.

Keywords: *Isolation, microbial diversity, Murine Respiratory Disease, Mycoplasma pulmonis, Rattus norvegicus*

INTRODUCTION

Respiratory diseases are a major health problem for people in the United States, resulting in the fifth most common cause of death (Yancey et al. 2001). Respiratory and chronic obstructive pulmonary diseases include: chronic bronchitis, chronic asthma, emphysema, and Mycoplasma Respiratory Disease. The disease is the result from an infection of *Mycoplasma pneumoniae* in humans. This infection is most commonly seen in children, and young adults (Yancey et al. 2001). Similar forms that cause similar effects can be found in other animals, such as murine animals. Mice and rats infected with *Mycoplasma pulmonis* develop Murine Respiratory Disease or MRM (Davis et al. 1985).

Mycoplasma pulmonis is part of the *Mycoplasmatacea* bacterial family which are pleomorphic bacteria and lack cell walls (McAuliffe et al. 2006). This species of bacteria is the smallest bacterial cell species discovered thus far (0.2-0.3 μm) (Madigan et al. 2017). This species of bacteria possess a very limited genome and thus little is known about its virulence mechanism and methods. *Mycoplasmas* are thought to have undergone reductive evolution resulting in its small size (McAuliffe et al. 2006). This is thought because it is a type of obligate intercellular parasite. *Mycoplasmas* lack many genes including those for cell wall synthesis, the production of all 20 amino acids, genes encoding enzymes of the citric acid cycle, and many other biosynthetic genes. The cells are capable of surviving because they are able to acquire essential resources from the host in vivo (McAuliffe et al. 2006).

Commonly affected species include mice, rats, guinea pigs, and hamsters. Transmission occurs by direct contact, aerosol, and through transplant. Typical sites for colonization are in the middle ear and the nasopharynx. Clinical signs of MRM are weight loss, ruffled coat, loud breathing, hunched posture, porphyrin, lethargy, and reproductive effects. Reproductive effects include infertility, pup infection, low birthweight, abortions, or fetal death (Charles River Laboratories 2009). The severity of this disease causes a large problem, especially in laboratory environments. Approximately 60% of all barrier-maintained murine animals are known to be infected with *M. pulmonis* (van Kuppeveld et al. 1992). The animal may be infected for months before antibodies may begin to develop. As a result the infection can decimate the host quite rapidly. Subjects that do survive the acute phase, approximately seven days post infection, develop many of the conditions previously stated (Davis et al. 1985). These conditions can only be treated symptomatically and once a subject is infected the *M. pulmonis* cannot be eradicated. There are multiple methods in preventing and purging infected populations including immunization, and the use of enzyme-linked immunosorbent assay, also known as ELISA. However, ELISA is not practiced in populations that are in the process of being studied because it requires the elimination of infected animals (Cassell et al. 1981).

There is no cure for MRM, but there are treatments that can be used to suppress the chronic symptoms. The treatment drug used in this descriptive analysis is enrofloxacin. The purpose of this study is to observe the microbiota of the nose and throat as well as the ratio of *M. pulmonis* to the rest of the microbiota over the progression of health of *Rattus norvegicus*.

MATERIALS AND METHODS

Rattus norvegicus, commonly known as the Norway rat, is used as the primary testing subject for this

experiment. These rats are optimally maintained at temperatures between 21±2°C, and at a relative humidity of 60%±10% (van Kuppeveld et al. 1992). A total of sixteen feeder rats, which are most often used as feed for other animals, were purchased from multiple Wichita, KS Scales and Tails pet store locations. Each rat was then placed in isolation. Every 24 hours the rat's health were observed along with their food and water intakes. All of the subjects were treated with antimicrobial water. The first round of treatment was for seven days. The second round lasted fourteen days and began two days after the first round ended. The antimicrobial water was a solution of $2.7E^{-3}$M enrofloxacin. Three rats were treated with a solution of 22.7 mg/mL enrofloxacin subcutaneously. During this time a series of tests were done to aid in the determination of the type of bacteria that was making the rats sick. In this small test, one live and one dead rat were used. All media used for this testing and analysis was manufactured by Carolina Biological Supply. The throat of the rats were swabbed using a sterile swab dipped in sterile Tryptic Soy Broth (TSB). The sample was then streaked on a sterile Tryptic Soy Agar (TSA) plate and incubated at 37°C for 24 hours. A hemolysis test and a Glucose test were then ran. The hemolysis test was performed by taking a sample of the microbes grown on the TSA plates and streaking the sample on a Muller Hinton Agar plate containing 5% sheep's blood. The glucose test took samples of the microbes grown on the TSA plates and homogenized them in previously sterile glucose broth containing phenol red. Both tests were incubated at 37°C for 24 hours. The results from this side study proved that there were bacteria present in both the live and dead rats that are capable of fermenting glucose and bacteria that is hemolysing in the live rat.

Microbial diversity

In order to observe the microbial diversity in the nose and throats of the rats a series of sampling and gram staining was completed. The remaining live rats are swabbed for microbes every two days for 10 days. The procedure of the swab was performed the same way as in the side study. The nose of the rat is also swabbed using the same procedure. After 24 hours of incubation a sample was taken from the growth and gram stained. The gram positive and gram negative bacteria were then counted and the diversity was compared.

Ratio and Isolation

A complex media was made to observe the microbiotic ratio. This media consisted of egg yolk and nutrient agar outlined by Atlas (1995). The isolating media contained 0.208 IU/L of penicillin. Samples were taken from the throat of all subjects. The swab sample was diluted 100 fold then 100μL of the diluted solution was used to inoculate two plates, one of egg yolk agar without penicillin and one of egg yolk agar with penicillin. The plates were then incubated at 37°C for 24 hours. The number of colonies present were then counted on each type of plate and the number of colonies were compared to each other to calculate a ratio. From the calculated ratio a One-Way ANOVA was used to analyze the differences in means.

RESULTS

The subjects were treated with an antimicrobial water containing enrofloxacin for one week at the beginning of containment. After being removed from the treatment for 24 hours the symptoms reappeared, and two subjects died. The remaining subjects were then given the treatment for another two weeks. Overall 37% of the subjects died due to symptoms caused by the *M. pulmonis*.

The bacteria *M. pulmonis* was isolated and confirmed in 100% of the rats in the study. The ratio of *M. pulmonis* to the rest of the throat microbiota showed a correlation between the ratio and the health status of the rat. There are three groups of health; healthy, sick, and dead. The healthy group consisted of rats who showed symptoms less than or equal to 10% of the duration of captivity. Specifically, less than eight days during the 77 observational duration. The sick rats were those who presented with symptoms eight or more days during the observational period. The rats in the dead category consisted of rats that died during the observational period. This was determined by the number of days each individual presented unhealthy symptoms. These symptoms included ruffled coat, loud breathing, hunched posture, porphyrin, and lethargy.

As can be seen in figure 1, the healthy rat group the average ratio of other bacteria to *M. pulmonis* was 3.65:1 with a standard deviation of 1.14. The ratio of the sick group was 3.16:1 with a standard deviation of 1.83, and the ratio of the dead group was 1.78:1 with a standard deviation of 1.20.

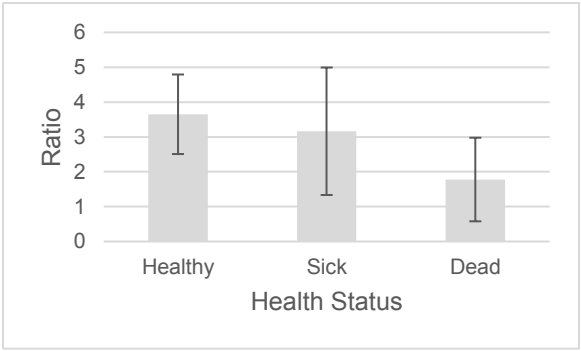

Figure 1: Ratio of *M. pulmonis* to other microbiota in the throat of the rat. Sample sizes are as follows: Healthy (*N*=*3*), Sick (*N*=*7*), Dead (*N*=*6*).

A One-Way ANOVA was used to test for differences in ratios. The α-value was set to 0.05, the resultant p-value was 0.16. It can be concluded that there is no significant difference between the means.

Microbial Diversity

The diversity study observed the phenotypic qualities of the microbiota in the throats of the rats using the gram staining method. Each rat was swabbed five times from the nose and throat. Each swab was used to innaculate one plate of TSB agar. In total 15 plates were innaculated for both the nose samples in the healthy group, 35 total plates in the sick group, and 6 total plates in the dead group. The same number of plates were used to innaculate the throat samples. From this the innaculated plates were incubated for 24 hours then a random colony was selected form each plate and gram stained. The frequencies of each type of general bacteria was recorded and the average frequencies of each type of cell in each group calculated. This can be seen in Table 1. This table shows only the types of bacteria that were observed, it is not a complete list of all bacteria that can be found in the throat of the rat. *M. pulmonis* is a gram negative cocci bacteria. As it can be seen in the table, gram negative bacteria were seen among all the groups. This does not conclude that *M. pulmonis* was what was seen every time a gram negative cocci was seen.

Table 1: The average frequencies of bacteria observed in the nose and throat of rats. The values are the frequency per plate observed.

Type of Cell	Nose			Throat		
	Healthy n=15	Sick n=35	Dead n=6	Healthy n=15	Sick n=35	Dead n=6
(+) cocci	0.27	0.69	0.17	0.33	0.57	0.50
(+) cocci chained	0.00	0.03	0.00	0.07	0.34	0.33
(-) cocci	0.07	0.11	0.17	0.13	0.03	0.33
(-) cocci chained	0.00	0.00	0.17	0.00	0.00	0.00
(+) rod	0.47	0.34	0.33	0.20	0.34	0.00
(+) rod chained	0.07	0.03	0.00	0.07	0.00	0.00
(-) rod	0.20	0.00	0.17	0.00	0.03	0.00
(-) rod chained	0.00	0.00	0.17	0.00	0.00	0.00
(+) coccobacilli	0.07	0.00	0.00	0.00	0.00	0.00

DISCUSSION

The study observed the diversity of the microbiota in the throat and nose of rats, and observed the change in the ratio of *M. pulmonis* to the other microbiota at different levels of health in the rat. A One-Way ANOVA was run in order to observe the difference in means. The p-value was 0.16 which is greater than the 0.05 α-value. Based on these results it can be concluded that there is no significant difference between the means. Meaning that the ratio of *M. pulmonis* to other bacteria does not significantly change as the health of the rat changes. Research does suggest that gender is a major factor in the severity of the disease. Males with MRM are consistently more severe clinical disease and have a higher mortality rate than females. The study observed more fatal shock-like syndrome in males and more of a chronic wasting syndrome in females (Yancey et al. 2001). In my observational study of this 40% of the females that died presented with a fatal shock-like symptom then death as opposed to the chronic wasting syndrome. The one male that died showed chronic wasting syndrome, and had more severe symptoms than the female rats. To obtain more conclusive results a much larger sample size is needed with a more even number of males and females.

Another area that I would do differently is in the diversity of the microbiome. More samples could have been taken to give a much larger view of what is present. To have a thorough understanding of the entire microbiome all of the colonies that were present should have been gram stained. The microbial diversity should have also been done under the same conditions as the ratio to *M. pulmonis* study, this would have allowed for more streamlined results. Though TSB is another broad spectrum media, like nutrient agar, it is highly likely that there are certain types of bacteria that are not capable of growing on TSB that can grow on the egg yolk nutrient agar.

Despite these various shortcomings the goal to see if there is a change in microbiota with the change in the rat's health was achieved. This descriptive study can help to understand how *M. pulmonis* evolves over time in chronic cases. These implications can also be considered in other *Mycoplasma* causing diseases like *Mycoplasma pneumonia* in humans as this bacteria acts in humans in a very similar way as *M. pulmonis* does in murine animals.

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