# A NOVEL PROTOCOL FOR TREATMENT AND ELIMINATION OF *PASTEURELLA PNEUMOTROPICA* INFECTION FROM RODENT COLONIES WITH SUBCLINICAL LATENT INFECTION

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## ABSTRACT

*Pasteurella pneumotropica* is a Gram-negative bacterium which poses a serious challenge to laboratory animal facilities. Elimination of the pathogen from rodent colonies is laborious and requires significant changes in regular standard operating procedures. In this study we attempted to develop a treatment protocol that would be both effective and compliant with standard work conditions for the elimination of chronic subclinical infection with *P. pneumotropica*. The aim of this study was to examine whether a treatment protocol for the elimination of chronic subclinical infection with Pp could be adapted to the standard operating procedures in a specified pathogen-free (SPF) rodent colony, without radically modifying the drinking water or diet, and without disrupting the normal replacement schedules of cages and water bottles. Enrofloxacin was added to acidified water once weekly at a Concentration of 0.0125%. A comparison of the mean inhibitory results between the enrofloxacin in acidified water and autoclaved water showed its effectiveness for at least 14 days. In this study acidified water/ enrofloxacin was replaced every week for a period of two weeks. Five groups of C57BL/6JOlaHsd mice were assigned to the experiment: Group1 served as the negative control. Group 2 and 3 served as the positive controls, and groups 4, 5 and 6. were the treatment groups. Positive control and treatment groups were infected with *P. pneumotropica*, and after confirmation of infection were treated for 2 weeks with enrofloxacin in the drinking water. The mice were monitored for up to 6 weeks post-treatment. Control groups were culture positive throughout the study whereas the negative control and treated groups were culture negative.

## INTRODUCTION

The importance of maintaining animal units under defined and standardized microbiological conditions is well known, and is a major factor in achieving reproducible research with laboratory animals. Animals of high standard microbiological quality are an essential requirement of good quality research. *Pasteurella pneumotropica* also classified as an *Actinobacillus* are Gram-negative opportunistic bacteria. They are nonmotile, coccobacillary to rod-shaped organisms; generally oxidase and catalase positive and nonhemolytic, growing under aerobic, microaerophilic or anaerobic conditions (2).

*P. pneumotropica* was first described in laboratory animals by Jawitz in 1948 (3). Infections with *P. pneumotropica* are mostly latent. Pasteurellosis of rats has been described as either an upper respiratory disease with signs such as rhinitis, sinusitis, otitis media, conjunctivitis, and ophthalmitis, or as a pyogenic syndrome with subcutaneous abscessesation and/or mastitis (4). However it is currently accepted that *P. pneumotropica* is mainly a secondary pathogen. Stress due to experimentation, or immunosuppression may cause the appearance of clinical symptoms in otherwise asymptomatic animals (4).

*P. pneumotropica* can be found in conventional as well as barrier maintained colonies. Susceptible animals are mice, rats, hamsters and guinea pigs.

*P pneumotropica* can be detected in cotton rats, kangaroo rats, poultry, rabbits, dogs, cats and humans. Horizontal transmission by aerosol is the main route of infection; vertical contamination may also occur and infection can occur during birth and even under conditions of cesarean section(4).

The use of the antibiotic enrofloxacin to decontaminate rodent colonies from *P. pneumotropica* has been studied previously and was shown to be effective (5). Enrofloxacin is a synthetic 6-fluoroquinolone antibiotic. Its mechanism of action is not thoroughly understood, but it is believed to act by inhibiting bacterial DNA-gyrase (a type-II topoisomerase), thereby preventing DNA supercoiling and DNA synthesis (6). In Gram-

negative organisms, DNA gyrase is the primary target, whereas in Gram-positive bacteria topoisomerase IV was recently found to be most affected. The antibacterial spectrum of enrofloxacin includes Gram-negative and Gram-positive organisms, mycoplasma and members of the Rickettsiales family.

After oral administration, enrofloxacin is readily absorbed from the intestinal tract, and achieves high plasma concentrations within a short time. High concentrations of the antibiotic are maintained in target tissues, body fluids and secretions, and generally tissue concentrations significantly exceed those in the plasma.

In this study we attempted to develop a treatment protocol for the elimination of chronic subclinical infection with *P. pneumotropica*. The proposed protocol is envisaged to be both effective and compliant with standard work conditions.

## MATERIALS AND METHODS

**Antimicrobial activity and stability of enrofloxacin indrinking water**

*P. pneumotropica* was tested for its sensitivity by using 3 different concentrations of enrofloxacin at 0.0625%, 0.0250% and 0.0125% in acidified and autoclaved water, respectively. Two solutions of acidified and autoclaved water without enrofloxacin served as negative controls. Bottles with and without antibiotic were kept under identical conditions as commonly used in the animal unit, for 2 weeks.

Paper disks were daily dipped into the two solutions over a period of 14 days. The disks (blank paper disks measuring 6 mm diameter, Becton Dickinson Co., Sparks, MD 21152 USA) were placed on blood agar plates inoculated with *P. pneumotropica*. The calibrated inhibitory activity was measured in mm at 24 hours after placing the disks.

**Animals**

Specific pathogen free (SPF) six week- old female C57BL/6JOlaHsd mice were purchased from Harlan Laboratories, Israel. The animals were transported to the facility in filtered containers in climate-controlled vans and allowed to acclimatize for at least 3 days before experimentation.

Acidified-softened water was provided to the mice in polycarbonate water bottles covered with stainless steel lids. The water was acidified to a pH of 3.0±0.2. For the specific treatment groups, the acidified water was supplemented with enrofloxacin.

The mice were housed on autoclaved wood shavings in polysulfone cages in groups of 8-12. The mice were fed an autoclaved Koffolk 19520 commercial laboratory animal diet (Koffolk, Petach Tikqwa, Israel). Fluorescent lighting was cycled automatically to give 12 hour day / 12 hour night phases. The ambient temperature was set at 21±2$^{o}$C and the relative humidity set at 40-60%.

All the animals were maintained in the SPF unit of the Hebrew University of Jerusalem Animal Facility and housed in micro-isolator cages. Each rodent room included with sentinel mice (C57Bl/6) or rats (Sprague Dawley), respectively. Sentinels were exposed to bedding from different cages containing animals of different age groups.Each cage change was monitored on a monthly basis for subclinical infections with adventitious pathogens.

Health screening was carried out in accordance with FELASA recommendations (7). As part of the ongoing health-monitoring program mice were monitored every two months for mouse hepatitis virus (MHV), pneumonia virus of mice (PVM), Sendai virus and minute virus of mice (MVM) also intestinal and nasal bacteriology was preformed. At every six months the following agents were examined: Ectromelia virus (mousepox), *Mycoplasma pulmonis*, and Theiler's mouse encephalomyelitis virus (including GDVII).,In addition, serology was carried out on a yearly basis to detect exposure to mouse rotavirus (EDIM), lymphocytic choriomeningitis virus (LCM), polyoma virus, reovirus type 3, mouse adenovirus, CAR bacillus, Tyzzer's agent, mouse cytomegalovirus (MCMV), K virus, Hantaan virus and *Encephalitozoon cuniculi*. Endoparasite and ectoparasite examinations were performed throughout the year on sentinels (monthly) and research animals (weekly).

**Bacterial growth of *P. pneumotropica***

*P. pneumotropica* was obtained from infected mice housed in a conventional rodent unit in a separate building on the campus, and identified by characterization of biological and biochemical properties with Gram staining, oxidase and catalase analyses and the API 20NE diagnostic kit. Specimens of the bacteria were cultivated on blood and MacConkey agar incubated for 18-24h. Growth was noticed on blood agar but not on MacConkey. Suspected colonies which appeared small to medium in size with smooth, white with entire margins were confirmed as *P. pneumotropica* by API 20NE kit (bioMerieux, France).

Gram staining was preformed: The bacteria were Gram-negative pleomorphic, bipolar diplococci. On microscopy they were non-motile. Biochemical tests showed them to be oxidase and catalase positive.

API results: (nitrate) NO3 positive, (nitrogen) N2 negative, (tryptophane) TRP negative,( glucose) Glu negative,(arginine dihydrolase) ADH negative, (urease) URE delayed positive, (esculin) ESC negative, (Gelatin) GEL negative, (para-nitrophenyl-galactopyranosidase) PNPG positive, negative to: (glucose) GLU, (arabinose) ARA,(Mannose) MNE, (mannitol) MAN,(N-acetyl-glucosamine) NAG, (Maltose) MAL, (potassium gluconate) GNT, (capric acid) CAP,(adipic acid) ADI, (malate) MLT, (Citrate) CIT, (phenylacetic acid) PAC. The API number calculated was 1,220,004 giving 98.7% certainty in identification as *P. pneumotropica.*

**Antibiotic preparation and dosing method**

Enrofloxacin was added to the acidified drinking water using Baytril 10% veterinary (Bayer, Germany) . Water acidification was achieved by adding HCl to softened drinking water , pH 3.0± 0.2. The dose of enrofloxacin was set at 25.5 mg/kg (antibiotic concentration 0.0125% enrofloxacin) based on the mouse drinking approximately 5 ml per day. Water bottles were replaced weekly, and the treated groups received the antibiotic continuously for 14 days.

**Experimental groups**

Mice were randomly assigned to 6 experimental groups.

Group1: Negative control consisting of 9 mice. These mice remained uninfected and untreated.

Group 2 and 3: Positive control groups each consisting of 8 and 12 mice, respectively, were infected by nasal instillation with $5x10^7$ CFU *P. pneumotropica* in 100µl brain - heart infusion broth (BHI). These mice were monitored but not treated.

Group 4, 5 and 6: Treatment groups consisting of 12 mice infected by nasal instillation with $5x10^7$ CFU in 100µl BHI. Treatment with enrofloxacin was administered for 14 days and the mice were monitored for 6 weeks.

Mice experiencing marked acute symptoms following artificial infection with *P. pneumotropica* or showing a loss of weight of more than 10% were excluded from the study during the first week post-infection. This was justified since this study was designed to investigate the elimination of subclinical infections in mice with *P. pneumotropica.*

**Mouse inoculation**

Before artificial infection and one week after arrival at the facility, all the animals were tested and were negative for *P. pneumotropica*. For this purpose swabs were taken under isoflurane (USP, Terrell$^{tm}$, MINRAD INC. USA) anesthesia from the oropharynx. Plain sterile aluminum applicator rayon tipped swabs were used for bacterial sampling.

*P. pneumotropica* colonies were mixed into BHI broth, and incubated for 24 hours. Tenfold dilutions were prepared to reach a bacterial concentration of $5x10^8$ CFU/ml. Each mouse was anesthetized with isoflurane and infected with 100µl, equivalent to $5x10^7$ CFU *P. pneumotropica.*

**Evaluation of mice post- inoculation**

During the first week after inoculation the mice were examined daily for food and water consumption and body weight. Thereafter, mice were weighed once a week Every 14 days after infection with *P. pneumotropica,* all the mice were anesthetized with isoflurane, and oropharyneal swabs were collected. On the day of study termination., (i.e. six weeks after infection) mice were anesthetized with isoflurane, and swabs were taken followed by euthanasia by cervical dislocation. A post-mortem exam was performed; lungs, heart, liver, spleen, kidneys and intestine were sampled for histopathology. Swabs were taken from vulva, peritoneum, thoracic cavity and nasal sinuses for *P. pneumotropica* isolation.

**Statistical analysis**

The concentration of enrofloxacin and its inhibitory efficacy were tested in both autoclaved and acidified water over a period of 14 days. The inhibitory diameters of the enrofloxacin solutions were compared by evaluating the mean inhibitory diameter of each concentration during the first four days after preparation compared to the respective inhibitory diameters of the solutions on days 11 to 14. Furthermore, comparisons between the autoclaved and acidified water were also made for the each concentration of enrofloxacin.

Student's t-test was used and statistical significance was defined as $p<0.05$.

The protocol was approved by the Institutional Animal and Health Committee of the Hebrew University, Jerusalem, before commencing the study.

## RESULTS

During the first week after infection, the mice were monitored daily. Marked clinical signs of infection were noticed in a number of mice of the different experimentally infected groups. Sick mice were excluded from the study only during the first week,. The number of mice excluded from the experiment due to mortality and morbidity during the first week, is presented in Table 1.

Figures 1 and 2 show a graphic presentation of the inhibitory diameters of the three enrofloxacin concentrations in acidified and autoclaved water, over a 14-day period. No differences in the mean inhibitory diameters were detected for the three concentrations between the first four days after preparation and the last 4 days (days 11-14) after preparation for the acidified or autoclaved water. respectively. Statistical analysis of the mean inhibitory results between the enrofloxacin in acidified and autoclaved water did not show significant difference in the concentration of enrofloxacin even at 14 days, which constituted a longer period of time than used in the study.

One week after infection all surviving mice from all infected groups (groups 2, 3, 4, 5, 6) tested positive for *P. pneumotropica*. The uninfected control mice (group 1) remained negative for bacterial isolation throughout the study. Mice from groups 3, 4, 5 and 6 treated with enrofloxacin at a concentration 0.0125% in their drinking water for a period of 6 weeks, were all negative for *P. pneumotropica* at the termination of the experiment. Furthermore, during treatment none of the mice showed any clinical symptoms related to the disease or to treatment with the antibiotic. Untreated animals (group 2) remained positive for the duration of the study. *P. pneumotropica* was isolated from untreated animals at the termination of the experiment from the nasal cavity in 2 out of 6 mice, the vagina in 4 out of 6 mice, and the pharynx in all six mice.

Post mortem and histopathological examination from animals in groups 1, 2, 4 and 6 did not present significant changes, except for rare aggregations of mononuclear cells and neutrophils in the liver, accompanied by focal necrosis and apoptosis in one animal.

## DISCUSSION

*P. pneumotropica* was identified by characterization of its biological and biochemical properties with Gram staining, oxidase and catalase analyses and using the API 20NE diagnostic kit. This kit is considered to be a valuable diagnostic tool for the diagnosis of *P. pneumotropica*. The high API calculated number gave a 98.7% certainty in identification of the strain used in this study. The method of infection, also used in other studies, proved to be effective in our hands. The relatively high morbidity and mortality rate of infected mice was unexpected and probably reflected the high dose of bacteria used in this study. It is interesting to note the large variations in response of

the mice to this high dose ranging from death to severe clinical signs to subclinical infection indicating the heterogeneity of response to *P. pneumotropica*. Despite the high mortality, an adequate number of animals survived in all the infected groups with subclinical infection to carry out this study and draw conclusions regarding the efficacy of the treatment. The treatment was carried out by adding enrofloxacin to acidified drinking water. Enrofloxacin has been found to be active against many Gram-negative bacilli and cocci, including most species and strains of *Pseudomonas aeruginosa*, *Klebsiella* spp., *E. coli*, *Enterobacter*, *Campylobacter*, *Shigella*, *Salmonella*, *Aeromonas*, *Haemophilus*, *Proteus*, *Yersinia*, *Serratia*, and *Vibrio* species. Of the currently commercially available quinolones, ciprofloxacin and enrofloxacin have the lowest MIC values for the majority of these pathogens treated. Other organisms that are generally susceptible include *Brucella* sp, *Chlamydia trachomatis*, *Staphylococci* (including penicillinase-producing and methicillin-resistant strains), Mycoplasma, and *Mycobacterium* sp.

Enrofloxacin is distributed throughout the body bound to plasma proteins. Studies in dogs showed that the highest concentrations are in the bile, kidney, liver, lungs, and reproductive system. Enrofloxacin reportedly concentrates in macrophages. Therapeutic levels are also attained in bone, synovial fluid, skin, muscle, aqueous humor and pleural fluid. Low concentrations are found in the cerebrospinal fluid, and levels may only reach 6-10% of those found in the serum. Enrofloxacin is eliminated via both renal and non-renal routes. Approximately 15-50% of the drug is eliminated unchanged into the urine, by both tubular secretion and glomerular filtration. Enrofloxacin is metabolized to various metabolites, most of which are less active than the parent compound. Approximately 10-40% of circulating enrofloxacin is metabolized to ciprofloxacin in most species. These metabolites are eliminated in both the urine and feces (6).

Enrofloxacin was administered in a previous study in an attempt to eradicate *P. pneumotropica* from a contaminated mouse colony (5). The contaminated mice, were dosed with enrofloxacin in untreated drinking water at a daily dosage of 25.5 mg/kg for 2 weeks. Following one week of treatment, mice were selected randomly from each room and examined for presence of *P. pneumotropica*. This procedure was repeated two or three times until all examined mice tested negative. Furthermore, the data showed that the treated mice consistently tested negative for *P. pneumotropica* for up to 45 weeks following completion of enrofloxacin treatment, except for one room. It is possible that this recurrence reflects incomplete treatment of P. *pneumotropica*. The use of untreated drinking water may have played a role either in the reintroduction of the pathogen or its recurrence.

From the results of antibiotic inhibition and the comparison with autoclaved drinking water, it does not appear that its relatively low pH affected the activity of the antibiotic over two weeks. Ueno et al. (9) also dosed mice with enrofloxacin in the drinking water, but they used untreated water. Their data showed that the antimicrobial activity of enrofloxacin lasted only 4-5 days (9); in contrast we found its antimicrobial activity lasted for 14 days in both acidified water and autoclaved water. This novel finding enables enrofloxacin replacement in acidified or autoclaved water at a lower frequency while still retaining its beneficial antimicrobial activity. The reason for the discrepancy between the two studies is not entirely clear. It may be related to the method of activity evaluation, or the treatment (acidification or autoclave) which in some way allows the antibiotic to remain antimicrobially active for a substantially longer time. This aspect needs to be investigated further.

Other antibiotics have also been tested for their ability to eradicate *P. pneumotropica*. In a comparative study between the efficacy of enrofloxacin and tetracycline, tetracycline-treated and control groups remained consistently culture-positive throughout the study . In another study (10) a colony of nude rats were treated with the antibiotics ampicillin, spiramycin, sulfadoxine/trimethoprim, tetracycline, lincomycin and spectinomycin over a period of 30 weeks. The rats were monitored bacteriologically during the entire treatment period and for 24 weeks after treatment. The primary target, *P. pneumotropica*, was suppressed by the treatment, but reappeared after ending the medication (11). The most appropriate sampling sites from healthy animals for *P. pneumotropica* are the nose, trachea, lungs and genitals (4). In this study the method of sampling for *P. pneumotropica* from the larynx of anesthetized mice proved to be reliable and a good indicator of *P. pneumotropica* infection. The detection of *P. pneumotropica* from the vagina by sampling with vaginal swabs proved to be reliable. In a previous study *P. pneumotropica* was found to be associated with infertility abortion, metritis, and stillbirths in two congenic strains of mice (12). *P. pneumotropica* was recovered from the affected uterine tracts of these mice. Intrauterine infection by *P. pneumotropica* was documented to reach a prevalence of 60 to 70% and the vagina or prepuce is therefore the best site for isolation . The findings of this study support the premise that without making wide-ranging changes in the regular husbandry of mice it is possible to eliminate *P. pneumotropica* infection with techniques that are compatible with everyday maintenance routines. Despite the fact that the water bottles could have been changed twice weekly and still maintain effective antimicrobial activity, it was decided to replace them once a week as is the standard practice in most animal facilities.

In summary, this study demonstrated that it is possible to eliminate *P. pneumotropica* infection in C57Bl/6N mice by providing enrofloxacin in acidified-softened drinking water for 14 days without making wide-ranging changes in their regular

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## TABLES

**Table 1:** Number of mice infected with *P. pneumotropica* excluded from the study due to mortality or severe clinical sings of disease.

| Group | Original number of mice | Number of mice excluded during first week |
|---|---|---|
| 1 (negative control) | 9 | 0 |
| 2 (positive control) | 8 | 6 |
| 3 (positive control) | 12 | 8 |
| 4 (study infection group) | 12 | 7 |
| 5 (study infection group) | 12 | 5 |
| 6 (study infection group) | 12 | 8 |

**Table 2:** Inhibition diameter (mm) for *P. pneumotropica* in different concentrations of enrofloxacin over a period of 14 days.

| | Concentration of enrofloxacin in autoclaved water | | | Concentration of enrofloxacin in acidified | | |
|---|---|---|---|---|---|---|
| Day | 0.0625% | 0.0250% | 0.0125% | 0.0625% | 0.0250% | 0.0125% |
| 1 | 30 | 33 | 33 | 31 | 34 | 33 |
| 2 | 27 | 34 | 30 | 29 | 31 | 30 |
| 3 | 30 | 33 | 31 | 31 | 34 | 33 |
| 4 | 31 | 34 | 32 | 31 | 33 | 31 |
| 5 | nr | nr | nr | nr | nr | nr |
| 6 | 32 | 35 | 35 | 33 | 36 | 35 |
| 7 | 28 | 30 | 29 | 28 | 31 | 29 |
| 8 | 28 | 31 | 30 | 27 | 31 | 29 |
| 9 | 27 | 30 | 29 | 27 | 31 | 29 |
| 10 | nr | nr | nr | nr | nr | nr |
| 11 | 33 | 35 | 35 | 34 | 37 | 35 |
| 12 | 27 | 27 | 28 | 26 | 29 | 28 |
| 13 | 27 | 27 | 29 | 27 | 31 | 28 |
| 14 | 26 | 27 | 29 | 25 | 28 | 26 |

nr = no result

**Fig. 1:** The inhibitory diameters of three concentrations of enrofloxacin in acidified water for 14 days.

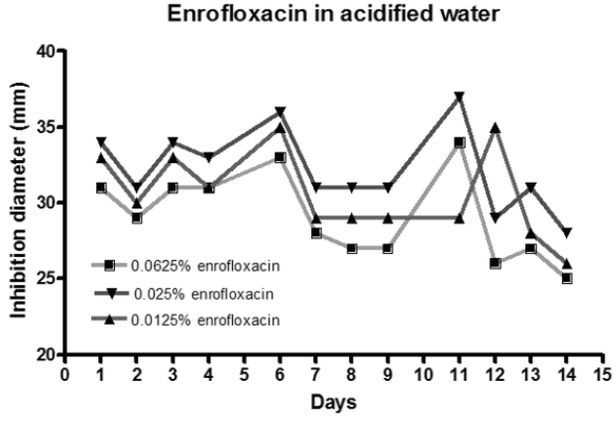


**Fig. 2:** The inhibitory diameters of three concentrations of enrofloxacin in autoclaved water for 14 days.