The Efficacy of Quadruple Therapy for Eliminating Helicobacter Infections in the Sand Rat (Psammomys obesus)

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Citation

M Johnson, T Mekonnen. *The Efficacy of Quadruple Therapy for Eliminating Helicobacter Infections in the Sand Rat (Psammomys obesus)*. The Internet Journal of Veterinary Medicine. 2014 Volume 11 Number 1.

Abstract

Although *Helicobacter spp* have been viewed as organisms of low pathogenicity, many studies have demonstrated the potential of these animal pathogens to cause severe disease in immunocompromised and inbred rodent strains4,9,12,13,14, 15,19,,24,25,30,31,32,33,34,35,39,40. According to our knowledge, this is the first time a natural *Helicobacter* was identified and speciated in the sand rat (*Psammomys obesus*) model. This investigation also evaluated the effect of an optimized dose and dosing schedules of a quadruple therapy composed of metronidazole, amoxicillin, clarithromycin, and omeprazole treating *Helicobacter rodentium* identified in this colony. After seven days of treatment, it was discovered 25 out of 27 (92.56 %) positive animals receiving the quadruple therapy were found to be negative via fecal polymerase chain reaction (PCR). This was again verified 14, 28, and 42 days after completion of treatment. Only one animal death was noted during the treatment period solidifying this regimen as a viable option for treating sand rats of the species *obesus*. This study also demonstrates success in a reduced treatment schedule in comparison to extended schedules (10 to 14 days) that can be quite debilitating in many rodent species, as can be deduced from the results seen with these antimicrobial agents.

INTRODUCTION

Numerous pathogenic microbes can interfere with animal research, and infection of rodent colonies with members of the family Helicobacteriaceae is an increasing concern for the research community. Naturally acquired Helicobacter infections have been reported in all commonly used laboratory rodents and many other domestic and wild species.4,15,27,42 The Helicobacter genus now includes at least 26 formally known species since 1982 when Marshall first described Helicobacter pylori.7,16,29 with many other species now becoming characterized.2,15,34,36,42 Many gastric, hepatic, and intestinal Helicobacter spp. have been isolated from a variety of mammals including rodents.4,26,34,35,42

Helicobacter spp. are gram-negative bacteria that vary in their morphology, growth requirements, biochemical profiles, antibiotic susceptibility, and sequence of conserved 16S rRNA genes.9 Most Helicobacter species are long, narrow, corkscrew or slightly curved rods in morphology with bipolar flagella. The most well-known Helicobacter spp. is H. pylori. Although it does not naturally infect rodents, H. pylori is frequently mentioned in literature due to its common use in research models of gastric ulcers and gastritis. Common induced models of Helicobacter pylori associated ulceration/gastritis include mice, rats, swine, guinea pigs, and the Mongolian gerbil. Rhesus macaques (Macaca mulatta), Japanese macaques (Macaca fuscata), pig-tailed macaques (Macaca nemenstrina), canines, and felids serve as both induced and natural models of disease.42 Several Helicobacter spp. capable of naturally infecting rodents (H. hepaticus, H. bilis, H. muridarum) are urease-positive, that is, capable of converting urea to ammonia. Other Helicobacter spp. (for example H. ganmani, H. rodentium, H. trogontum, H. typhlonius, and others) are urease-negative in the host animal.30 Production of urease allows the microorganisms to survive in the very acidic gastric environment. Interestingly, a recent study has shown that for H. hepaticus, urease is not required for intestinal colonization but promotes hepatic inflammation in male A/JCr mice.16,17,19,34 Most rodent Helicobacter species are urease-negative and thus preferentially colonize the intestine, although in some cases they may translocate to the liver and biliary system, stomach, or other tissues.15,27

A study of mice derived from 34 commercial and academic

institutions in Canada, Europe, Asia, Australia, and the United States showed that 88% of these institutions had mouse colonies infected with 1 or more Helicobacter spp. Approximately 59% of these mice were infected with Helicobacter hepaticus; however, monoinfections with other species also were encountered.36 Interestingly, all gastric and enterohepatic Helicobacter infections appear to persist for the life of the host, with chronic infections commonly associated with subclinical disease in immunocompetent hosts.4,9,12,13,14,15,19,25,30,31,32,33,34,35,39,40 Similar to other Helicobacter spp., Helicobacter pylori is a chronic pathogen but of the human gastric mucosa, infecting approximately half the world's population.19,24,35 Approximately 10 to 15% of infected individuals actually develop disease, which may range from acute gastric inflammation to duodenal and gastric ulcers, gastric adenocarcinoma, and mucosal-associated lymphoid tissue (MALT) lymphoma.8,23,28,41

Polymerase chain reaction (PCR)-based techniques, mainly genus-specific PCR(16 S ribosomal subunit) and quantitative PCR, typically are used for identification and detection of most Helicobacter spp. Molecular detection of Helicobacter DNA by using PCR is rapid and sensitive to the early phases of infection.5,20,28 Further enhanced sensitivity can be achieved by utilizing nested primers in conjuction with PCR.20,28,41 One of the most important features of the PCR assay is that it can be performed on fecal pellets as a noninvasive/nonlethal technique. This is an especially useful aspect when assessing the status of valuable rodents, such as rare rodent species, transgenic, and knockout species.

Infection of laboratory rodents with Helicobacter spp. is an important problem for many laboratory animal facilities as many species can acquire and transmit infection. Most Helicobacter infections are subclinical, however such infections have the potential to interfere with in vivo studies and confound results. 9,10,25,35,41 Coinfection with other Helicobacter spp. has resulted in diarrhea in some rodent species and strains.10,30,31,35 Routine screening for Helicobacter species will ensure healthy study animals and reduction in variability. Polymerase chain reaction analysis of fecal material utilizing the appropriate primers has been proven to be a successful method of detection of Helicobacter infection in mice and rats.5,11,19,41

If detected through screening methods, a pathogen such as Helicobacter must be eradicated from the model without harm to the animal. Clostridium difficile has been demonstrated to overgrow in Mongolian gerbils upon short term antibiotic treatment for Helicobacter pylori infections.6,7 An antibiotic treatment must be utilized to encompass the correct antibiotic, dose, duration, and route to prevent growth inhibition of normal intestinal flora. Such inhibition may result in overgrowth and toxin production of commensal bacteria such as Clostridium difficile. Enterotoxin production in the GI tract many times results in life-threatening disease and is a common sequella of overpopulation of C. difficile.6,7 The utilization of clarithromycin has been shown to be effective in the reduction of Helicobacter pylori in gastric infections of Mongolian gerbils, and its combination with omeprazole has shown much higher efficacy.8 A quadruple therapy consisting of amoxicillin, clarithromycin, metronidazole, and omeprazole applied over a course of 8 weeks in medicated food has been successful in the eradication of Helicobacter bilis and H. hepaticus from infected mice and may offer potential for the treatment of other helicobacters in species such as the sand rat.26

The sand rat or the fat sand rat (Psammomys obesus) is a diurnal terrestrial rodent in the subfamily Gerbilinae, family Muridae. Sand rats are an important model in the study of cutaneous leishmaniasis due to the common location (Northern Africa and the Arabian Peninsula) of sandflies, Leishmania, and infected native colonies of sand rats.37 The animal is quite unique in the fact it develops mild to moderate obesity, hyperglycemia, and the complications of diabetes such as cataracts, pancreatic atrophy, cardiovascular abnormalities, impaired renal function, and ketoacidosis by dietary induction.1,18,22 There are very limited breeding colonies of these animals in the United States, and are known for their relatively docile nature. These animals have also been studied extensively for their remarked renal conservation of water. These rodents also naturally develop otic cholesteatoma, spondylosis, and intervertebral disk disease.1 The sand rat’s diet is naturally composed of halophyte plants that survive the extreme heat and semi-arid conditions of their normal desert habitat.1

The objectives of this study were to estimate the working effective dose regimen of quadruple therapy to eliminate Helicobacter spp. from a sand rat gavage model and directly identify the implicating helicobacter(s) of such infections. The hypothesis that a quadruple therapy could be utilized with success in a sand rat for eliminating Helicobacter

infections was tested optimizing the treatment dose and schedule. The reliability of an experiment that uses an in vivo model system depends on understanding and controlling all variables that can influence the experimental outcome. Seemingly unimportant subclinical infections of rodent colonies are important to our factility and the rest of the scientific community because such infections can introduce harmful variables.4,9,14,16,17,25,41 Therefore, the ultimate goal of this study is to eliminate such a variable of future studies and better maintain disease-free animals.

MATERIALS AND METHODS

Animals and husbandry

Walter Reed Army Institute of Research (WRAIR) currently maintains one of the very few sand rat breeding colonies in the United States. Currently sand rats are not raised at any commercial rodent breeding facilities in the United States. Typically the rodents are shipped from the Hebrew University–Hadassah Medical Center for research purposes. This breeding colony originated from the colony at Wake Forest University (Winston Salem, NC), which was established from the colony stock of the Hebrew University–Hadassah Medical Center. These animals were subsequently maintained and bred at WRAIR, an AAALAC accredited facility for approximately four years. As a part of a health monitoring and surveillance program, serum samples were quarterly screened of the following organisms: cilia-associated respiratory bacillus, hamster polyoma virus (SV40), lymphocytic choriomeningitis virus, mouse adenovirus, pneumonia virus of mice, rat coronavirus–sialodacroadenitis virus, reovirus, Sendai virus, Toolan H1 virus, Kilham rat virus, Theiler mouse encephalomyelitis virus. Quartlerly fecal screening samples were submitted for Helicobacter spp. Endoparasites and ectoparasites were screened for during quarterly sentinel pathologic analysis. All sentinels to date have been negative for all tested agents, but the colony has always been historically positive for Helicobacter.

To ensure each sand rat was initially infected with a Helicobacter spp., and cross contamination was minimized, animals were individually housed with fresh bedding to isolate and collect fresh fecal material from each study animal. Animals were cared for in accordance with Animal Welfare Regulations and The Guide for the Care and Use of Laboratory Animals, and all procedures were performed in accordance with an Institutional Animal and Use Committee approved protocol. Cages were changed and sanitized at least once weekly. Environmental temperatures were maintained between 20 to 26.1 degrees Celsius. Relative humidity standards were kept between 30-70%.

All animals were individually housed in solid-bottom, polycarbonate ventilated caging with stainless steel wire-bar cage lids, each cage receiving a minimum of 10-15 HEPA-filtered air changes per minute. Approximately 200 g of aspen bedding was placed in each cage and sterilized before use. All sterile bedding and cage changes were perfomed with sterile caging under a class II type A2 biological safety cabinet. Sand rats were provided a specially formulated non-diabetogenic diet (5L09) manufactured by Purina TM (Richmond, IN). Sterile water was provided ad libetum via cage suspended bottles. Light cycles were maintained at 12h:12h (day:night), and ventilation conditions of the macro environment was 10-15 air changes per hour. Animals were individually housed throughout the experiment. This control along with frequent and routine glove changes, were employed to prevent cross contamination and minimize reinfection through coprophagy. Appropriate precautions and safety measures were adopted for ABSL-2 work in this study. Equal numbers of male and female adult sand rats between 40-60 weeks of age weighing 170-275g were utilized. Animals were observed at least daily and moribund animals in the study were euthanized with CO2 in accordance with the “2007 AVMA Guidelines on Euthanasia” followed by thoracotomy.

AntiHelicobacter therapy

Initially 40 healthy sand rats (20 males and 20 females) were tested positive and verified by PCR techniques for Helicobacter spp. in fecal material obtained from each rodent. Positive sand rats in the treatment group (28) were orally gavaged 1.8 mg amoxicillin, 0.3 mg clarithromycin, 0.6 mg metronidazole, and 0.012 mg omeprazole (0.5 mL) per day of treatment.26 All of the antibiotic preparations were formulated by Wedgewood TM pharmacy (Swedesboro, NJ) and administered orally using a flexible 3.0 inch 16-gauge silicone-tipped Teflon gavage needle daily for 7 days. Treatment control animals (8) were given an equivalent volume of sterile distilled water per os utilizing the same methods. Four animals served as gavage stress controls. An empty gavage needle was inserted and removed in each of these animals in a similar fashion and humanely euthanized at days 2, 3, 5, and 7 of the study.

Sample collection

The day before fecal material was to be collected, a complete sterile bedding change was implemented to ensure the correct timeliness of sampling occurred. If fecal samples could not be collected due to nonproduction, any samples thereafter for that time point would be excluded. Fecal material (3-4 pellets per sample) was then obtained from each treated and control animal in 1.5 mL microfuge tubes and tested for the presence of Helicobacter spp. at a diagnostic testing laboratory (IDEXX/RADIL, Columbia, MO). If fecal material was found to test positive, the species of Helicobacter was elucidated via PCR at the testing laboratory. In addition to verifying all sand rats were initially positive for Helicobacter spp., the feces were also tested a day, two weeks, and four weeks after antibiotic termination for the presence and species of Helicobacter. The time period between infectivity and shedding in a majority (approximately 88%) of mice was approximately 14 days experimentally.11 In this study a 28 day period was chosen after the last day of treatment to finalize collection and testing of fecal material. If fresh fecal material could not be collected the set day of collection, those samples were not included in the study. Due to the fact sand rats are highly susceptible to environmental stress18,22 and therapies similar in nature have been found to cause enterotoxemia due to Clostridial organisms,6,7,8 animals were weighed and monitored daily.

Statistical analysis

Statistical comparison of groups to assess treatment efficacy was performed utilizing a chi square test to compare the difference in the treatment and untreated groups. A p value of less than 0.05 was considered significant. Comparison between male and female groups was also assessed utilizing the Fisher's Exact Test. Again a p value less than 0.05 was considered to be significant.

RESULTS

All sand rats utilized in the study were initially determined to naturally carry Helicobacter rodentium. All of the animals tolerated the gavage procedures well for the entire seven days as the saline controls demonstrated no weight loss during the study, and the majority actually demonstrated significant weight gain. Gavage stress controls also maintained similar weight status. However, weight loss (7-10%) was noted in a majority of the treatment animals during the initial seven days of treatment. One animal had died during the course of therapy with this treatment, however it was not fully elucidated what role C. dificile may have played after pathological analysis, as no gross lesions were found. 25 of 27 fecal samples collected (92.56%) demonstrated no detectable Helicobacter spp. DNA present. Two samples showed clearance of Helicobacter rodentium, but PCR detection of Helicobacter hepaticus and Helicobacter typhlonius was surprisingly discovered in both animals.

After two weeks and four weeks beyond the last day of therapy, all treatment samples found previously to be negative remained negative for Helicobacter spp., and all control samples were found to remain positive. A Chi square analysis was done to compare the treatment and distilled water groups at both time periods, and a statistical difference was found between the two groups with a significance level less than 0.001. A Fisher's exact test was utilized to determine if there was an intersex difference between the treatment groups. It was found there was no significant difference ($p<0.05$) between the treatment group of each sex.

DISCUSSION

Helicobacter rodentium was initially thought to solely colonize the cecum however PCR studies have demonstrated the assortment of infected sites is more expansive than previously thought.22 This has been the first documentation of multiple Helicobacter spp. in the sand rat. In addition three species of Helicobacter have been found to colonize the sand rat GI tract when one predominant species was initially hypothesized to exist. However the two other species were found only after treatment cessation. It is possible the predominant species of Helicobacter (rodentium) overcrowds other minor species and the minor species are not detected pretherapy in PCR due to limited or dwarfed probe signal. There could exist gastrointestinal site competition between the two Helicobacter spp. and H. hepaticus and/or H. typhlonius could be more resistant to drug therapy. Physical mechanisms may also be involved based on preferred environment of each Helicobacter species, preventing exposure to effective drug interaction. Although one animal had died during the course of therapy, no overt enterocolitis or typhlitis was noted upon gross examination or histology. Utilizing a combination of

amoxicillin and metronidazole in similar therapies, deaths also usually commence around day 7 post treatment whereas this animal died on day 6 of treatment. Clostridium dificile infection was also not proven on anaerobic culture and exotoxin testing was not performed in this study, therefore it could not be ruled out completely.32 Anecdotally, fecal material changed coloration to from dark brown to a lighter brown presumably due to the eradication of normal flora denuded from the gastrointestinal tract and raw digestion was impacted during the course of therapy. Further studies identifying and enumerating the growth of gastrointestinal microflora such as Clostridium dificile and the presence of exotoxin production would be useful for assessing micobiota populations before and after therapy.

PCR utilizing specific primers has been found to be more sensitive in the detection of murine Helicobacter spp. in the intestine when compared to electron microscopy, bacterial culture, or histologic examination with the use of a silver (Steiners) stain. 5,12,20,28 However, it must be noted that the PCR technique utilized for the detection of Helicobacter spp. DNA does not address the viability of the organism detected or their ability to elicit infection.21 Utilizing fecal PCR for detection, although highly sensitive, may lead to the conclusion of a later bacterial clearance timeline during treatment. This limitation may be due to residual genetic material in the GI tract, eliciting a positive PCR response although no whole organisms are present or delayed antibiotic absorption from earlier treatment. This can be crucial when developing treatment regimens with toxic drugs in a particular animal model where every extra day of therapy might be harmful to the animal patient. Another disadvantage to PCR is that the process can also be readily inhibited by a number of elements in fecal material including hemoglobin, humic material, and stercobilin along with other bile pigments leading to false negatives.20

Based on the experiences gained in this study, the compounding of amoxicillin, clarithromycin, metronidazole, and omeprazole is effective in the eradication of Helicobacter rodentium in a sand rat model. Although other methods of eradication such as rederivation and cross fostering are effective at eliminating Helicobacter sp., elimination is dependent on the time and labor constraints, stage of animal development, and access to specific pathogen free animals.3,23,38 Cross-fostering is also very dependent foster litter acceptance. When considering the cost of the treatment, minimal animal loss, tolerance of the procedure, time considerations with dosing, and the risk of reoccurrence of infection, the use of gavaging a quad therapy is an acceptable means to eliminate Helicobacter rodentium infections. Although the gavage method can be quite laborious in large animal populations, this technique better ensures dose compliance and thus treatment efficacy. These facts can outweigh time/labor factors especially when dosing rare and valuable rodent species.

This work was performed at Walter Reed Army Institute of Research, Naval Medical Research Center, Silver Spring, MD, USA 20912-7500, and was funded by the Walter Reed Army Institute of Research. There is no objection to its presentation or publication. The opinions and

assertions contained herein are the private ones of the author and are not to be construed as such or reflecting the views of the Department of Defense. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals

and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication, 2011 edition.

ACKNOWLEDGEMENTS

I would like to thank Edmund A. Asafo-Adjei, Cary Honnold, for their assistance in pathological analysis and Luis Lugo, and Kevin Nemelka for their personal advice offered in this study.

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