UNDERSTANDING THE EFFECTS OF IMPORTATION ON COMMENSAL ENTERIC BACTERIAL DIVERSITY AND ANTIBIOTIC RESISTANCE OF THE TOKAY GECKO (*GEKKO GECKO*)

by

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(Under the Direction of Sonia M. Hernandez)

ABSTRACT

Wildlife traded for the pet market, particularly the stressful transportation conditions, has the potential to influence the rate of antimicrobial resistance development. Using the Tokay gecko (*Gekko gecko*) as a model for the pet trade, I described the culturable lactose fermenting *Enterobacteriaceae*, the antimicrobial resistance patterns, and how overcrowding conditions impact the composition and antimicrobial resistance. The diversity of genera cultured from the individually housed geckos decreased after geckos were combined. There was an increase in *Salmonella* sp. prevalence observed between individual and combined groups. Several of the antimicrobial resistance patterns were consistent with the presence of a beta lactamase, which have important clinical consequences. Another concerning trend was the presence of intermediate resistance, because low-level resistance can act as the foundation for clinically relevant resistance to develop. The data demonstrates a need for future investigations to determine the mechanisms conferring resistance and their potential to disseminate.

INDEX WORDS: *Enterobacteriaceae,* pet trade, antimicrobial resistance, reptile,

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DEDICATION

I would like to dedicate this manuscript to my parents, Donna and Albert Robertson, whom without their inspiration and support I would not be the person I am today.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

There is ample evidence supporting the pet trade's ability to introduce pathogens, microorganisms capable of causing disease (Stephenson 2003; Karesh et al. 2005). There have been several examples where the commercial pet trade has been implicated in ecological and public health disasters including: the introduction of non-native species like the red-eared slider (Rödder et al. 2009), the introduction of zoonotic pathogens like the 2003 Monkeypox outbreak (Stephenson 2003), or the release of pathogens into a naïve population such as the chyrid fungus into amphibian populations (Weldon et al. 2004). The pet trade also has the potential to introduce non-pathogenic bacteria which could potentially be resistant to antibiotics. Wildlife used in the pet trade harbor commensal organisms, which are microbes usually associated with the host and do not typically cause disease. Most commensal organisms of reptiles are non-pathogenic; however, they can cause opportunistic infections in immunocompromised individuals, such as children or the elderly. The tokay geckos in this study were destined for the pet trade and are often purchased by households with children. Treatment of opportunistic infections or reptile-associated salmonellosis can be hindered if the organism expresses resistance against the antibiotic used for treatment.

The majority of wild caught animals imported into the United States originated from Southeast Asia (Smith et al. 2009). Southeast Asia is a region of the world currently experiencing an increase in prevalence of resistance to quinolones and extended spectrum

β-lactamases (Okeke et al. 2005; Hawser et al. 2009). Importing pets from regions of the world where the antimicrobial resistance of *Enterobacteriaceae* is high has the potential to introduce and establish novel antimicrobial resistance genes in the local bacteria populations or potentially cause serious clinical infections. A study of zoo animals demonstrated that animals in captivity, like pets, have the potential to act as reservoirs for antimicrobial resistant bacteria and clinically important resistance genes (Barten 1993; Ahmed et al. 2007). The nature of bacteria allows for horizontal transfer of genes to other species and genera, which allows for the dissemination of antimicrobial resistant gene determinants (Lewis 2002). A study performed with wild-caught reptiles from remote areas of Java and Krakatau found antibiotic resistance to chloramphenicol among *Citrobacter* spp. (Graves et al. 1988). The mechanism for how they acquired resistance is unknown; however, what is of most concern is the transportation of these reptiles, for the pet trade, across international lines and their potential to transfer these novel resistance genes to local bacteria populations.

In addition to the importation of reptiles harboring non-pathogenic bacteria they are also known to shed *Salmonella* spp., which is a pathogen of humans. *Salmonella* spp. can cause reptile-associated salmonellosis in people, particularly in children under 5 years old. This continues to be a growing concern for public health agencies, considering the CDC estimates 1.4 million cases annually and reptile-associated salmonellosis accounts for 5%(74,000) of those cases per year (CDC 2002). The majority of reptile-associated salmonellosis cases are infections resulting from contact with common pets (Mermin et al. 1997).

We hypothesized that the pet trade, particularly because of the conditions under which wildlife is captured and handled, has the potential to influence the composition of culturable *Salmonella* spp. and lactose fermenting *Enterobacteriaceae* and the selection of antimicrobial

resistance in tokay geckos. The first objective of this study was to describe the culturable lactose fermenting *Enterobacteriaceae* bacteria (from here forward, the enteric commensal flora) from the feces of tokay geckos. Our second objective was to describe the effects that overcrowding had on the diversity and composition of enteric commensal flora and on the shedding of lactose positive *Salmonella* spp. We hypothesized that stressful conditions have the potential to alter the normal flora composition resulting in a decreased diversity of enteric bacterial isolates. As gecko density was artificially increased we expected to see less diversity in their enteric genera. Our third objective was to describe the antimicrobial resistance patterns of lactose fermenting *Enterobacteriaceae* isolates from fecal samples, and how antimicrobial resistance patterns changed after mimicking the conditions under which reptiles are imported for the pet trade. We hypothesized that the prevalence of antimicrobial resistant lactose fermenting *Enterobacteriaceae* isolates cultured from individually housed tokay geckos (*Gekko gecko*) would increase as animal density and capture/transport/holding-related stress and crowding occurred. Our fourth objective was to describe the antibiotic resistance profiles of *Salmonella* spp. cultured from individually housed geckos immediately after importation and then again after geckos had been housed at different densities. We also hypothesized that *Salmonella* spp. cultured from these geckos would express antibiotic resistance, and the prevalence of resistance would increase as we experimentally increased the density of geckos.

LITERATURE REVIEW

The Family Enterobacteriaceae

The family *Enterobacteriaceae*, is a diverse group of both lactose and non-lactose fermenters that are ubiquitous in the environment, plants, and animals. They are characterized as Gram-negative, rod shaped, facultative anaerobic bacteria. While many members of this family

are commensal intestinal organisms, several are also considered important pathogens and are thought to cause half of all nosocomial infections in the United States (Segen 2002) The taxonomy of this family has been under dispute since its formation in early 1937. The first member of this family, *Serratia marcescens*, was described much earlier, in 1823 (Janda 2006). The debate about which genera belong to this family continued until the 1980's when molecular techniques, like DNA sequencing, had advanced enough to be considered a reliable method for classification purposes. Through the 1980's- 2000's the family has continued to grow with the discovery of a few new genera. Currently there are greater than 40 genera and over 200 species known to belong to the family *Enterobacteriaceae* (Janda 2006). There are many genera in this family and they are found in a diverse set of environments including humans, other animals, plants, and the environment where they can be pathogenic or commensal (Janda 2006). Several members of this family are important human pathogens like *Yersinia*, *Salmonella*, and *Shigella.* Other members of this family are mainly opportunistic pathogens like *Escherichia, Enterobacter, Klebsiella*, *Citrobacter*, and *Serratia*. The genera in this family are implicated in opportunistic infections in hospitals and usually harbor antimicrobial resistance mechanisms (Kariuki et al. 2001; Kim et al. 2003).

Commensal Enterobacteriaceae

Different areas of the body have different compositions of normal flora, which provide many benefits to their host. Normal flora can be made up of a variety of bacteria, fungi, and sometimes single celled eukaryotes depending on the host and the region of the body (Todar 2008). Generally, the presence of normal flora is considered to diminish the ability of pathogens to colonize due to competition and to prevent dissemination of pathogens (Wells et al. 1987). A high diversity of normal flora in the gastrointestinal tract is thought to help stabilize the

community but this can be disrupted by antibiotics, which allow a limited number of genera to persist and over colonize (Katouli et al. 1994). Normal flora also assists immune function and nutrient processing (Hooper et al. 2001; Kitano et al. 2006). Several genera of *Enterobacteriaceae* are considered non-pathogenic commensal bacteria that make up part of the normal flora of the gastrointestinal tract of animals (Janda 2006).

The family *Enterobacteriaceae* is a diverse group of bacteria comprised of significant human pathogens and non-pathogenic species that are part of the normal flora of humans including *Escherichia, Klebsiella, Enterobacter, Citrobacter,* and *Serratia* (Guentzel 1996; Todar 2008). The non-pathogenic genera are lactose fermenters while the pathogenic genera are generally non-lactose fermenters (Janda 2006). While these organisms are normally non-pathogenic, they have the potential to become opportunistic infections when they are introduced into areas of the body where they are not normally found, when they infect immunocompromised individuals, or when the normal flora composition is disrupted (Guentzel 1996). A study conducted in Japan described antimicrobial resistance in Gram-negative normal flora from captive mammals, reptiles, and birds (Ahmed et al. 2007). From the 232 isolates collected in this study 21.1% were resistant to more than one drug. This study demonstrates that zoo animals harbor commensal bacteria with antimicrobial resistant genes. The majority of research has explored the prevalence of antimicrobial resistance in pet and captive reptiles but very few studies have explored this topic in free-living reptiles. One study suggests that the prevalence of antibiotic resistance in *Enterobacteriaceae* from captive reptiles can range from 30-40% (Gopee et al. 2000). Graves et al. described the fecal flora and antimicrobial resistance patterns in wild-caught reptiles from remote areas of Java and Krakatau (Graves et al. 1988). They found antimicrobial resistance in enteric isolates from snakes, geckos, and monitors. *Citrobacter* spp.

was the most common isolate from reptiles and they observed that *Citrobacter freundii* was consistently resistant to chloramphenicol. However, the sample size of that study was relatively small (n=19) (Graves et al. 1988). The emergence of antibiotic resistance in normal flora of wildlife poses a global public health threat.

Global Antimicrobial Resistance

While antimicrobial resistance emergence and prevalence is often monitored on the regional level, its potential to disseminate transforms this problem into a global issue (O'Brien 1997). We live in a global world where consumer commodities and people can travel anywhere making it possible for the international spread of antimicrobial resistance (Levy et al. 2005). However, developing nations are disproportionately affected by antimicrobial resistance because the infectious disease burden is high, preventive medicine efforts are poor, diagnostic capabilities are hampered and poverty restricts the amount of newer more expensive antimicrobial agents available for treatment (Okeke et al. 2005). There are other factors that contribute to the prevalence of antimicrobial resistance in a nation, they include: 1) inappropriate antimicrobial use, 2) complex political agendas, 3) social and behavioral influences, and 4) biomedical infrastructure and personnel (Nweneka et al. 2009). The prevalence of multidrug resistance is rising worldwide in the family *Enterobacteriaceae* (Nordmann 2006). Recently the emergence of extended spectrum beta-lactamases and resistance to quinolones has appeared in Southeast Asia at relative high rates in clinical isolates (Okeke et al. 2005; Hawser et al. 2009). Unfortunately, a recent study suggests that even non-pathogenic commensal *E.coli* are developing resistance in the Indonesia population (Lestari et al. 2008).

Indonesia Antimicrobial Resistance

The prevalence of antimicrobial resistant bacteria in Southeast Asia and Indonesia is a particular focus of this research project because it represents the tokay gecko's native range. These geckos may have been exposed to antimicrobial resistant bacteria of domestic animals or humans in the peri-domestic setting in which they are often found. In Thailand and Indonesia there is insufficient data on the types and amounts of antibiotics being used, probably due to the lack of control over distribution of these drugs (Sarmah et al. 2006). Since there is a lack of data on antibiotic-resistant bacteria found in animals the discussion will be limited to antibiotic resistance from human isolates. There have been several reports on antibiotic use and the prevalence of resistance from human patients visiting hospitals. In Indonesia, antibiotics, with or without a prescription, are readily available from primary health centers, government or private hospitals, private doctor or midwife practices, public pharmacies, drug stores and roadside stalls (Simanjuntak et al. 2004). One study in Indonesia investigated the use of antibiotics in patients visiting healthcare facilities. This same study determined that in most cases the antibiotics prescribed were either unnecessary or ineffective (Hadi et al. 2008). In Jakarta, doctors prescribed antibiotics to 94% of young children, despite their belief that the infections were of viral origin (Gani L 1991). Hadi et al. also discussed prescribing ineffective antibiotics for acute bacterial diarrheal cases, such as enterotoxigenic *Escherichia coli* (ETEC) in children and adults in Indonesia. The resistance rates for ETEC are 67% for the heat-labile toxin (LT) and 83% heat-stable (ST) for ampicillin, 48% LT and 70% ST for trimethoprim-sulfamethoxazole, and 95% LT and 85% ST for tetracycline (Hadi et al. 2008). This is important because Hadi et al. determined that aminopenicillins and tetracyclines accounted for 80% of the prescribed antibiotics. Aminopencillins used were older generations and low-cost antibiotics. The use of amphenicols

was relatively high in Indonesia, Chloramphenicol and thiamphenicol represented 6% of courses taken by adults and 12% by children (Hadi et al. 2008), compared to their limited used in most developed nations due to resistance. This study also determined that an alarming 17% of antibiotic users in their study were self-medicating.

Additional studies have investigated the prevalence of antibiotic resistance isolates cultured from acute diarrheal patients. In one study by Tjaniadi et al. they used a disk-diffusion method to determine the antimicrobial susceptibility of 630 strains of *Salmonella* spp. to eight different antibiotics including: ampicillin, trimethoprim-sulfamethoxazole, chloramphenicol, tetracycline, cephalothin, ceftriaxone, norfloxacin, and ciprofloxacin. Their results indicated that both *S. typhi* and *S. paratyphi* A were susceptible to all the antibiotics tested (Tjaniadi et al. 2003). These results contrast the findings of a previous study done in human diarrheal patients in Indonesia, which investigated *Salmonella typhi* strains and revealed resistance to trimethoprim-sulfamethoxazole, chloramphenicol, streptomycin, and tetracycline (Sanborn et al. 1975). In Tjaniadi et al. they detected antibiotic resistance in *Salmonella* group B, C, D, and E and *S. enteritidis* to most of the antibiotics tested. *Salmonella* group B was resistant to both fluoroquinolones tested, while all the other isolates were susceptible.

These studies illustrate the prevalence of antimicrobial resistance of mainly pathogenic isolates from patients that go to health care facilities. Lestari et al. screened individuals at two major urban centers for commensal strains of *Escherichia coli* and *Staphylococcus aureus*. Antimicrobial susceptibility was determined using a disk diffusion method as described by the Clinical and Laboratory Standards Institute. *E. coli* was tested for resistance against gentamicin, chloramphenicol, trimethoprim-sulfamethoxazole, ampicillin, cefotaxime, and ciprofloxacin. Isolates where collected from four different groups: group 1 consist of people being admitted to a

hospital, group 2 were those being discharged, group 3 was made up of individual ambulatory patients, and group 4 consisted of relatives of patients in group 1 (Lestari et al. 2008). A total of 3,284 isolates were tested. The results show a surprisingly high amount of resistance among the isolates. Resistance was lowest in the group made up of relatives of those in the hospital (group 4) and highest in those being discharged from the hospital (group 2). Upon admission to the hospital, *E.coli* resistance rates were low for the following antibiotics gentamicin, cefotaxime, and ciprofloxacin, but was significantly higher at the time of the patients discharged (Lestari et al. 2008). In addition, there were relatively high rates of resistance among *E. coli* cultured from patients visiting primary health centers to chloramphenicol, trimethoprim-sulfamethoxazole, and ampicillin compared with *E. coli* from the relatives group (Lestari et al. 2008). Interestingly, the study by Lestari et al. was the first to demonstrate relatively high rates of resistance in commensal *E.coli* strains to ciprofloxacin (6% in group 1 and 22% in group 2). This is relatively high compared to 1990's when 100% of clinical isolates of *E. coli* from Taiwan were susceptible to fluoroquinolones (ciprofloxacin) and rapid resistance emerged thereafter (Sheng et al. 2002). The isolates studied in Lestari et al. are from a similar geographic region to Taiwan and they were commensal isolates which expressed resistance, which is less expected than pathogenic isolates expressing resistance. Overall, Lestari et al. demonstrated that resistance rates among commensal *E.coli* strains are significantly higher in the group of patients being discharged from the hospital then previously described. Understanding and monitoring the prevalence of antimicrobial resistance is necessary to prevent the spread of antibiotic resistance organisms.

Enterobacteriaceae and Potential Antibiotic Resistance Mechanisms

Antibiotic resistance in commensal and pathogenic organisms can be intrinsic based on the biology and genetics of an organism, or acquired via horizontal transfer from other bacteria

(Houndt et al. 2000). The location of the enteric bacteria in the gastrointestinal tract predisposes them to encountering bacteria harboring antimicrobial resistance genes or to antibiotics directly that are consumed (Kariuki et al. 2001). Under these circumstances, when bacteria are not intrinsically resistant, they adapt either by mutation or horizontal transfer (Kariuki et al. 2001). One study in 2000 compared the antibiotic resistance patterns of pre-antibiotic era strains to the contemporary populations of *Escherichia coli* and *Salmonella enterica* (Houndt et al. 2000). Strains were collected from a variety of hosts including healthy humans, humans with infections, and a variety of domestic and wild mammals, reptiles and birds. The study concluded there were higher background levels of resistance to commercially applied antibiotics in commensal *E. coli* strains commonly associated with humans and domesticated mammals compared to the *Salmonella* spp. strains. This was expected because the *Salmonella* spp. strains were mainly associated with non-mammalian populations, like birds and reptiles, which are less likely to receive commercially applied antibiotics (Houndt et al. 2000). Their findings supports the idea that increased background levels of resistance in contemporary isolates of *Escherichia coli* and *Salmonella enterica* compared to pre-antibiotic era isolates is associated with the widespread application of antibiotics.

A similar study explored the antimicrobial resistance in *Enterobacteriaceae* from human fecal flora to determine if a specific bacterial species was responsible for high levels of resistance. Three populations were utilized: the first were isolates collected from healthy individuals with no exposure to antibiotics in past three months (HP), the second were from patients admitted to the hospital for a duration of five days (UP), and the third group were long term patients (LTP) (Osterblad et al. 2000). The minimum inhibitory concentration (MIC) was determined for ampicillin, trimethoprim, sulfamethoxazole, chloramphenicol, cephalothin,

cefotaxime, gentamicin, tetracycline, and nalidixic acid. In that study, *E. coli* isolates possessed the most resistance and 18% of the no exposure individuals had resistance to two or more classes of antibiotics (Osterblad et al. 2000). It is interesting that the other species of *Enterobacteriaceae* were generally susceptible to the antibiotics tested and multidrug resistance was absent. However, clinical isolates, those collected from diseased patients, of *Klebsiella* spp. and *Enterobacter* spp. were significantly more resistant than other non-pathogenic strains. The authors of this study conclude that antimicrobial use increased the prevalence of resistance but conditions that facilitated transfer of resistance genes were essential for maintaining high levels of resistance (Osterblad et al. 2000). This is significant because it suggests that the conditions which promote the exchange of transferable genetic elements are important for controlling the threat of multidrug resistance in clinical environments.

There are several mechanisms that are responsible for antimicrobial resistance among *Enterobacteriaceae.* Depending on the species and the type of antibiotic, bacteria may be intrinsically resistant. If an organism is not inherently resistant to an antibiotic, they can become resistant through mutation or horizontal transfer. A mutation occurs spontaneously and is independent of antimicrobial agents. When a mutation occurs that produces antibiotic resistance while the bacterium is growing in the presence of that antibiotic then selection will favor that gene because it is beneficial (Kariuki et al. 2001). In addition to mutation, genes encoding for resistance can be transferred vertically to progeny and horizontally to other species and genera of bacteria via extrachromosomal genetic elements which include plasmids, transposons, integrons, and bacteriophages (Kariuki et al. 2001). A study published in 2003 sought to determine the extent to which multidrug resistance is intergron-related in *Enterobacteriaceae* (Leverstein-van Hall et al. 2003). They also investigated what resistance patterns were indicative

of the presence of integrons. They found that only resistance to sulfamethoxazole, cotrimoxazole, gentamicin, tobramycin, ampicillin, piperacillin, and cefuroxime predicted the presence of integrons. They concluded that intergron-carrying elements are essential for the development of multi-drug resistance in *Enterobacteriaceae* (Leverstein-van Hall et al. 2003). Integrons are made up of genes integrated into the chromosomal DNA from plasmids or transposons and can be excised to become mobile genetic elements. While extrachromosomal genetic elements can be responsible for antimicrobial resistance, so can genes located on the chromosome.

Regions of bacterial chromosomes can be responsible for resistance to a single class of antimicrobial agents or to several. A recent study looked at a chromosomal locus from *Escherichia coli*, called *mar* (multiple antibiotic resistance), that regulates the susceptibility to multiple antibiotics. It had been previously demonstrated that the deletion of this locus from E. coli increases its susceptibility to eight antibiotics (Cohen et al. 1993). Cohen et al. investigated the prevalence of this locus in other species of *Enterobacteriaceae* and detected multiple resistant mutants of *Enterobacter agglomerans* and *Salmonella* spp. that showed increased expression of *mar*-specific RNA. Some of the *E. coli mar* mutants demonstrated increased resistance to tetracycline, chloramphenicol, norfloxacin, and ampicillin.

Similarly, the extended spectrum afforded by beta-lactamases possessed by many gram-negative bacteria is chromosomally mediated. However, in the 1960's the first plasmid mediated beta-lactamase, TEM-1, was described and within a few years was found worldwide in many different families such as *Enterobacteriaceae, Pseudomonas aeruginosa, Haemophilus influenza*, and *Neisseria gonorrhoeae* (Bradford 2001). As fast as new beta-lactam drugs were developed, so did the emergence of new beta-lactamases that were resistant to that drug. More

recent development of a class of oxyimino-cephalosporins has given rise to a new class of extended spectrum beta-lactamases (ESBLs).Worldwide there have been over 150 different ESBLs in many different genera of *Enterobacteriaceae* (Bradford 2001). As the ESBLs have spread globally, some regions have been more impacted than others. In particular, the Asia-Pacific region has seen significant increases in the prevalence of ESBLs. ESBL frequencies in the Asia-Pacific region in 2007 was 40% compared with 30%, 17%, 10%, and 8% for Latin America, the Middle East and Africa, the European Union, and North America, respectively (Hawser et al. 2009). The Asia-Pacific region has seen a rapid increase in prevalence of ESBLs from just 15% in 2003 to 40% in 2007 (Hawser et al. 2009). A recent study investigating ESBLs in clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae* from Indonesia found that CTX-M-15 was the most widespread ESBL in 94.5 % of *E. coli* strains and 55.6 % of *K. pneumoniae* strains (Severin et al. 2010). CTX-M is a relatively new family of plasmid-mediated ESBLs, that preferentially hydrolyze cefotaxime, (Bradford 2001). The study by Severin et al. (2010) demonstrates that ESBLs are a serious concern in the clinical environment and have the potential to disseminate widely because they are plasmid mediated.

Expanded spectrum beta-lactamase is not the only class of antimicrobial agents against which resistance is on the rise. The prevalence of resistance against quinolones in *Enterobacteriaceae* is also increasing worldwide (Garau et al. 1999; Hooper 2001; Nordmann 2006). The antimicrobial class referred to as quinolones are bactericidal. They enter bacteria through porins where they target and bind to DNA gyrase and related DNA topoisomerases (Drlica et al. 1997). The binding and forming of a complex effectively inhibits supercoiling, chromosome decatenation, and induces DNA lesions which triggers an SOS response, a DNA repair system, and eventually leads to cell death (Lewis K. 2002). The majority of resistance to

quinolones is a result of mutations in the chromosome. However, a multiresistance plasmid that encodes for resistance to quinolones was discovered from a clinical isolate of *Klebsiella pneumoniae*. The isolate contained a broad host range plasmid that in *E. coli* transconjugants increased resistance to nalidixic acid from 4 to 32μg/ml, and to ciprofloxacin from 0.008 to 0.25μg/ml. (Martinez-Martinez et al. 1998). Further analysis of this plasmid provided the molecular characterization of gene *gnr* responsible for the transferrable resistance to quinolones. The gene product, QnrA, is a 218-amino-acid protein that protects DNA gyrase from the inhibitory activity of quinolones (Tran et al. 2002). QnrA is just one of several Qnr determinants recently described. There is high prevalence of the Qnr proteins (QnrA-like, QnrB and QnrS) in Asian isolates that are often associated with clavulanic acid inhibited expanded-spectrum beta-lactamases and plasmid-mediated cephalosporinases (Nordmann 2006). There is a rising concern for the potential association between quinolone resistance and extended-spectrum beta-lactamases (Paterson et al. 2000). This is especially alarming because it is unclear whether there is a specific link between the two emerging mechanisms of resistance in *Enterobacteriaceae* (Nordmann 2006).

A follow up study to the survey done by Hadi et al. (2008), of approximately 4,000 patients in two Indonesia cities, investigated the epidemiology and virulence characteristics of fluoroquinolone resistant *E. coli* (Kuntaman et al. 2005). The prevalence of resistance to ciprofloxacin was 8% but increased to 23% at the time of discharge from the hospital. They were unable to confirm the presence of a transferable plasmid-mediated quinolone resistance mechanism. The authors suggest that a combination of limited clonal spread and the emergence of resistant strains resulted in the high prevalence of fluoroquinolone resistant *E. coli* in the hospital environment (Kuntaman et al. 2005). Another study done in Indonesia investigated the

antimicrobial susceptibility patterns and the presence of gyrA mutations in *Salmonella typhi* isolated in 2006 (9 strains) and 2008 (8 strains) (Yanagi et al. 2009). All nine of the strains from 2006 were sensitive to all the antibiotics and had no mutation *gyrA* gene. However, the eight of the isolates from 2008 were all resistant to nalidixic acid and ampicillin. There was also a gyrA mutation present in these isolates. There were three of the eight isolates that had multidrug resistance to chloramphenicol, trimethoprim–sulfamethoxazole, and ciprofloxacin (Yanagi et al. 2009). This was the first report of fluoroquinolone resistance in clinical strains of *S. typhi* with *gyrA* mutation in Indonesia. The development of fluoroquinolone resistance poses a serious public health threat to Indonesia, because there are exceptionally high rates of enteric fever, with greater than 1,000 incident cases per 100,000 population annually, and fluoroquinolones are the preferred treatment choice (Hume et al. 2009). A study done in Japan investigated clinical strains of *Salmonella* spp. isolated from patients with traveler's diarrhea for the presence of the *gnr* gene (Taguchi et al. 2009). Twenty-eight of 302 strains *Salmonella* spp. showed decreased susceptibility to nalidixic acid and ciprofloxacin over a six year period. Twenty five of the twenty eight strains contained the *gnr* gene. Three of these strains were isolated from patients who had visited Indonesia. This supports Kuntaman et al. assertion that future research in Indonesia should investigate the prevalence of fluoroquinolone resistance that is mediated via plasmids.

The Pet Trade

The pet trade in the United States is an extensive industry that imported nearly 1.5 billion live animals between 2000 and 2006 (Smith et al. 2009). Approximately 92% of imports were designated for commercial purposes, the majority of which were for the pet trade. Smith et al concluded that little mandatory pathogen screening is implemented and 80% of shipments

contained wild caught animals. Of these shipments, 69% of live animal imported into the US originated in Southeast Asia, which is a hotspot for emerging infectious diseases (Smith et al. 2009). The poor regulations on wildlife trade combined with the conditions in which live animals are shipped and the geographic regions from which they are shipped should be of great concern because of the risks it can pose to public health. Smith et al. analyzed all Law Enforcement Management Information System (LEMIS) shipment records gathered by the U.S. Fish and Wildlife Service (USFWS) for live wildlife imports and exports for the period 2000 to 2006. They concluded that the current regulations are insufficient to assess the biological diversity of wildlife entering the United States and determine the threat wildlife pose as possible invasive species or as hosts of potential harmful pathogens (Smith et al. 2009). Smith et al. (2009) also recommended: 1) that stricter measures be taken to enforce federal regulations regarding taxonomic status, 2) that a third party pathogen screening system for known and unknown pathogens be implemented, 3) development of risk analyses to identify risk factors associated with the potential of an imported species becoming an invasive species or for potentially carrying a pathogen, and 4) increased efforts for public education concerning the threat that the pet trade industry poses to the health of the environment, animals, and the public. There have been several scholarly articles published on the threats the pet trade poses to both public health and ecosystem health from introducing invasive species or pathogens (Daszak et al. 2000; Pavlin et al. 2009). However, there is also the possibility that the pet trade promotes and facilitates the global spread of unique non-pathogenic commensal organisms and pathogenic enteric bacteria, all of which may harbor antimicrobial resistance. The goal of this research was to explore this potential threat by mimicking stressful pet trade conditions and describe the composition of culturable lactose fermenting *Enterobacteriaceae* and the antimicrobial susceptibility patterns.

There is evidence from domestic animal production facilities that crowding and stress can potentially promote the colonization and shedding of harmful bacteria, like *Salmonella* spp., and the exchange of antimicrobial resistance genes in non-pathogenic bacteria (Molitoris et al. 1987; Moro et al. 1998; Sorum et al. 2001). The type of crowding and stress that occurs at animal production facilities is similar to the stress associated with overcrowding and handling in the wildlife trade (Bowman 1998). Reptiles imported under stressful conditions may shed pathogens, like *Salmonella* spp. at a higher prevalence. An increase in shedding could promote the exchange of bacteria among reptiles. In addition to promoting shedding of pathogens, wild caught reptiles may also harbor antibiotic-resistant bacteria. Particularly in the case of the tokay gecko, they may have acquired antibiotic-resistant bacteria through exposure to domestic animals or humans in their native range because they are typically found in close proximity. The majority of reptiles being imported originate from Southeast Asia, which is also an area of the world where there is a high prevalence of antibiotic resistance in the human population (Okeke et al. 2005). The importation of reptiles poses a threat to public health because of the increase in popularity of reptiles as pets (Shepherd 2008)

Tokay Gecko's (Gekko gecko) Life History:

The tokay gecko belongs to the family *Gekkonidae*. The tokay gecko can grow to a length of 35cm, making it the second largest gecko in the world. Their native range includes India and Southeast Asia. Tokay geckos have been introduced to Belize, Florida, Texas, Hawaii and some Caribbean islands where they are considered an invasive species (2009). They are nocturnal, arboreal, insectivores and their life span in the wild is approximately 7 to 10 years. Their usual habitat consists of trees and cliffs in tropical rain forest. However, in their native range they are often found dwelling in human habitations where shelter and food is readily available (Corl

1999). Tokay geckos are solitary creatures and only interact with the opposite sex during the breeding season. Males establish territories and will aggressively defend them against other tokay geckos or other species (Corl 1999).

Tokay geckos are a common pet, often perceived as attractive display animals because of their bright coloration (Cavendish 2001). The majority of captive tokay geckos sold today in the USA are still wild caught (2008). There is a huge export industry of wild tokay geckos from Southeast Asia for the pet trade but also for use in traditional Chinese medicine (Bauer 2009). Records from 1998 to 2002 reveal that the United States alone imported several million wild caught amphibians and reptiles annually for commercial use. This number likely underestimates the true amount of wildlife in the pet trade since shipments are rarely recorded to the species level of the animal being shipped (Schlaepfer et al. 2005). While a small fraction of the geckos imported into the United States are used in traditional Chinese medicine, China imports substantially higher quantities of geckos for this use (Bauer 2009). A study done in China investigating the wildlife trade, consumption and the conservation awareness of people in southwest China (Zhang et al. 2008) found that the majority of trade occurs in markets located in cities that border along Vietnam. In China, tokay geckos have been protected since 1988, are currently considered endangered and are listed in the Chinese Red Data Book (Zhao 1998). The number of wildlife being imported into China for use in traditional Chinese medicine is staggering. In a survey of wildlife at live animal markets in China 20,000-30,000 tokay geckos were sold annually in Pingxiang City. In another market along the border, Dongxing City, there was approximately 2,400–6,000 tokay geckos sold annually. In a five year period, Pingxiang City would have imported approximately 100,000 to 150,000 geckos and that is only one city in

China. In addition, millions are being exported internationally for the pet trade, it is easy to understand how this abundant species is being extirpated from parts of its native range (Bauer 2009).

Reptiles and Salmonella spp.

There are two species of *Salmonella* spp. which is divided into 6 subspecies, then 60 serogroups, and greater than 2,300 serotypes. Of these, 40% have been cultured from reptiles (Mermin et al. 2004). It is thought that *Salmonella* spp. is a part of the normal gastrointestinal flora of both captive and free-ranging reptiles (Jacobson 2007). The majority of cases where reptiles are colonized with *Salmonella* spp. do not result in overt disease or clinical signs (Onderka et al. 1985; Jacobson 2007). Healthy reptiles have the potential to serve as reservoirs because they can intermittently shed *Salmonella* spp.

There are many reports on captive reptiles. Some studies found the prevalence of *Salmonella* spp. as low as 11.3% (Pasmans et al. 2002) compared to 90% in another study (Hatt 2009). There are only a few studies on free-ranging reptiles, two of which report zero to 12% prevalence of *Salmonella* spp. shedding in aquatic turtles (Johnson-Delaney 1996; Richards et al. 2004), but as high as 100% in terrestrial turtles (Hidalgo-Vila et al. 2007). There is a wide range in the prevalence of detected *Salmonella* spp. which could reflect different techniques for isolation, species, diets, captive conditions, etc. Additionally, the stress associated with the overcrowding conditions of the pet trade might amplify the shedding *Salmonella* spp. in reptiles.

CONCLUSIONS

The stressful conditions that tokay geckos experience during shipment in the pet trade have the potential to influence the composition of commensal flora. This disruption can decrease the diversity of normal flora and promote pathogen colonization or the overgrowth of resident

flora (Hentges 1983; Katouli et al. 1994). The stress due to overcrowding may promote the shedding of pathogenic bacteria, a phenomenon that has been shown with the shedding of *Salmonella* spp. in livestock (De Passillé et al. 2005; Ball et al. 2011). Reptiles are known reservoirs of *Salmonella* spp. and pose a risk to their owners.

The pet trade has the potential to import wild-caught geckos harboring antibiotic resistance. This is a serious public health concern because geckos are being imported from a known hotspot of emerging infectious disease and a high prevalence of antibiotic resistance in the family *Enterobacteriaceae*. The stressful conditions which the reptiles experience may promote the shedding of commensal and pathogenic bacteria, which could ultimately increase the rate of exchange of bacteria between geckos. The exchange of antibiotic-resistant bacteria could increase the prevalence of antibiotic resistance.

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CHAPTER 2

UNDERSTANDING THE EFFECTS OF IMPORTATION ON COMMENSAL ENTERIC BACTERIAL DIVERSITY TOKAY GECKO (*GEKKO GECKO*)

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ABSTRACT

We propose the pet trade, particularly the conditions under which wildlife are captured and transported, has the potential to influence the community composition and the prevalence of pathogens of the gastrointestinal flora. Through experimental manipulations using the tokay gecko (*Gekko gecko*) as a model for the pet trade, we described how the conditions in which reptiles are imported for the pet trade may impact the composition of their enteric flora.

One hundred and eight six wild tokay geckos were imported from their native range and housed individually after collection and during import. A total of one hundred and eighty nine lactose positive *Enterobacteriaceae* isolates were cultured from these animals. The top three most frequently cultured genera were *Citrobacter* spp., *Klebsiella* spp., and *Enterobacter* spp. Our results suggest the trend that the diversity of a small subset of culturable lactose positive *Enterobacteriaceae* decreased when animals were housed in groups rather than individually. Maintaining diverse microbial communities in the gut is important for preventing pathogen colonization. In fact, the prevalence of *S. arizonae* was ≤1% in geckos individually housed compared to 11.5% from animals living in varying densities. The changes in culturable lactose fermenting *Enterobacteriaceae* shed by reptiles are important to monitor because they could provide insight into how stressful overcrowding conditions similar to those currently used in the pet trade influence enteric commensal flora composition.

INTRODUCTION

The commercial pet trade is a massive worldwide industry and is often a source of concern in the conservation, ecology and public health fields (Chomel et al. 2007). Besides being implicated in the introduction of invasive species, the pet trade is also a route for the dissemination and introduction of pathogens to naïve animal and human populations. The commercial pet trade has been implicated in public health disasters including the introduction of zoonotic pathogens like the 2003 Monkeypox outbreak (Stephenson 2003) Severe acute respiratory syndrome (SARS) was linked to the trade of wild civets and bats (Bell et al. 2004; Lau et al. 2005) and the trade in wild bird species has contributed to the movement of avian influenza and Newcastle's disease (Karesh et al. 2007)

The commercial pet trade is just small component of the larger global wildlife trade industry. Quantifying the total number of animals in the global industry is extremely difficult due to its scale both at the regional and international level, inconsistent monitoring efforts, and illegal trafficking of animals. A rough estimate of the global wildlife trade from the World Wildlife Fund suggests that approximately 350 million live fish, 4 million live birds, 600,000 reptiles, and 40,000 primates are traded annually (Karesh et al. 2005). The United States is one of the world's largest importers of live animals (USFWS 2003) and a review of the US Fish and Wildlife Service's Law Enforcement Management Information System (LEMIS) revealed that between 2000 and 2006 the US imported over 1.4 billion live animals (Smith et al. 2009). Reptiles were the third most abundant class of animals being imported into the US, fish and corals were the first and second respectively.

The movement of wild animals in the pet trade is a significant problem because it has the potential to introduce pathogens and this is exacerbated by the fact that the majority of wild

animals imported into the US originate from Southeast Asia (Smith et al. 2009), which is considered a hot spot for emerging infectious diseases (Jones et al. 2008). Reptiles are known reservoirs of *Salmonella* spp. (Jacobson 2007). Reptiles as pets represent a public health concern because they are responsible for 74,000 cases of salmonellosis annually (CDC 2003). There are few studies that detail the prevalence of *Salmonella* spp. in wild reptiles (Hoff et al. 1977; Richards et al. 2004) and they suggest that *Salmonella* spp. is shed at a low prevalence. In contrast, the prevalence of *Salmonella* spp. in captive reptiles has been reported at a relatively high prevalence (Gopee et al. 2000; Nakadai et al. 2005; Hatt 2009), presumably from stress. There could be other reasons why the composition of normal is altered, like diet and environmental conditions (O'Hara et al. 2006). Another explanation could be that stressful conditions under which animals are shipped alter commensal enteric flora composition. Research on the effects of crowding and associated stress promoting the colonization and shedding of pathogenic bacteria, like *Salmonella* spp., is limited to domestic animal production facilities and captive wildlife species (Richards et al. 2004; Hatt 2009). Normal flora enhances the host's defense against potential pathogens by occupying space and competing for essential nutrients (Lu et al. 2001; Sekirov et al. 2009). In domestic chickens it was demonstrated that a more diverse normal flora created a stable microbial community (Santos et al. 2008). There have been no extensive studies that have described the gastrointestinal normal flora of a wide range of reptiles, but rather a few reports for snakes, turtles, and lizards from different geographical regions (Mathewson 1979; Cooper et al. 1985; Jacobson 2007). Understanding the microbial diversity of commensal enteric flora in reptiles commonly imported into the US for the pet trade is important because it may influence the potential for pathogen colonization, such as *Salmonella* spp.

Wild caught tokay Geckos (*Gekko gecko*), a common reptile used in the pet trade, were imported from Java, Indonesia. The species occurs throughout the Indo-Australian Archipelago but are imported to the U.S. almost entirely from Indonesia and Malaysia (Smith et al. 2009). The first objective of this study was to describe the culturable lactose fermenting *Enterobacteriaceae* bacteria (from here forward, the enteric commensal flora) from the feces of tokay geckos. We expected to find *Salmonella enterica arizonae*, *Citrobacter* spp., *Enterobacter* spp., *Klebsiell*a spp. and *E. coli* based on previous studies describing common enteric flora of reptiles (Mathewson 1979; Cooper et al. 1985; Graves et al. 1988; Mahajan et al. 2003; Mader 2006). Our second objective was to describe the effects that overcrowding had on the diversity and composition of enteric commensal flora and on the shedding of lactose positive *Salmonella* spp. We hypothesized that stressful conditions have the potential to alter the normal flora composition resulting in a decreased diversity of enteric bacterial isolates. As gecko density was artificially increased we expected to see less diversity in their enteric genera.

METHODS

Animal Collection, Shipping, and Housing

Wild caught adult tokay Geckos were imported from an international wildlife distributor based in Indonesia. Two batches of geckos were captured by the distributor from two locations on the island of Java. Once captured, the geckos were housed individually and shipped to the University of Georgia's College of Veterinary Medicine. All animal handling and care was approved by the University of Georgia's Institutional Animal Use Care Committee. Animals were housed in temporary mouse containers then transferred to enclosures measuring 24"W x 36"D x 72" H. They were fed a standard diet of crickets and mealworms. The temperature was 26.6º±4ºC and the humidity was maintained at 50% - 70% and the lighting was on for a 12 hour

period. They were monitored twice a day. The first batch arrived in March 2009. They were housed individually and fecal samples were collected. The geckos remained individually housed for 10 days to allow feces to be collected from each animal. In this time period 6 geckos died and extras were used in their place. Individuals were pre-assigned to different groups with varying densities and added to them once feces was collected. The groups were designated: Group A which represented a "low" density (low-A) and contained 5 animals, Group B was a "medium" density (med-B) and contained 15 animals, and Group C was a "high" density (high-C) and contained 30 animals. After the first batch of individuals had been combined into groups 9 animals died within the first week. A second batch of individually-housed geckos was imported in June. Once all fecal samples were collected from this group, 15 geckos were assigned to each of the pre-existing groups (low-A, med-B, and high-C) and a fourth group D was created. Group D contained 15 animals (low-D). After the second batch of geckos were added to the previous groups (low-A, med-B, and high-C) and the new low-D group, another 13 animals died in the three month period before they were euthanized. In September, fecal samples were collected from all remaining geckos and then humanely euthanized according with our AUP (Figure 2.1).

Isolation and Identification

Fecal samples were collected from individually housed geckos and from animals living in varying densities. To ensure animals were not accounted for more than once, geckos were removed from the group and placed in containers until they defecated. Fecal sample were processed within 24 hours of collection. Sterile water was added to the cryovial containing the feces. Then a 10µl loop was used to streak out the diluted fecal solution on MacConkey media. The plates were incubated at 37°C for 24 hours. Lactose positive growth on enteric media resulted from 71% of the samples obtained. One sample of each morphologically distinct group

of colonies was collected to be restreaked separately. If the colonies were later identified as the same genus and species, only one of the samples was used. Pure isolates were suspended in freezer media consisting of 1% peptone and 15% glycerol and frozen at -80°C until further analysis. Standard methods were used to determine the identity of isolated organism including plating on a selective and differential media, MacConkey. Standard biochemical tests including oxidase, citrate, motality/indole/ornithine, triple sugar iron, phenylalanine, rhamnose, malonate, sorbitol, methyl red/Voges Proskauer, and lysine decarboxylase were performed as recommended in the Manual of Clinical Microbiology (Murray 2003). If there were conflicting biochemical test results, rapid Analytical Profile Index (API20E) strips (Biomerieux, Durham, NC) were used to determine the identification of the isolate.

Data Analysis

Statistical analyses were performed using SAS V 9.2 (SAS Institute Inc. Cary, NC). An analysis of covariance (ANCOVA) was used to compare diversity (total number of genera) between combined and individually housed geckos. Housing density (low, medium, and high), number of animals, and number of isolates were included as covariates. A chi-square analysis was used to compare the prevalence of each species between individual and combined groups for each housing density level. All hypothesis tests were 2-sided and the significance level was set at $\alpha = 0.05$.

RESULTS

There were a total of 186 geckos used in this study. From these geckos, 189 lactose positive *Enterobacteriaceae* isolates were cultured. The top four most frequently cultured genera were *Citrobacter* spp. (114), *Klebsiella* spp. (29), and *Enterobacter* spp. (15) and. *Kluyvera* spp. (15). Seven isolates of both *Salmonella enterica* subspecies *arizonae* and *Echerichia coli* were

cultured. *Serratia* spp. (4) and *Pantoea* spp. (2) made up the least common genera cultured. The data from the individuals on prevalence of isolates that were lactose negative was not recorded. However, from the geckos after they were housed at varying densities 33% (25/76) of the samples were lactose negative and 17% produced no growth.

A total of 110 geckos were housed individually after capture and during import until their arrival at the University of Georgia. Lactose positive fecal samples were isolated from 88 of the 110 individually housed geckos. The second batch of 60 individually housed geckos produced more lactose positive isolates, 83, compared to the first batch of 50 individually housed geckos, from which we cultured 54 isolates (Table 2.1). *Klebsiella* spp. was the only genus cultured from all the individual pre-assigned groups. *Enterobacter* spp. and *Citrobacter* spp. were the only genera to be cultured from all of the individual pre-assigned groups except one. The number of genera cultured from the first batch of individuals ranged from 3 in low-A group and 5 in both med-B and high-C. The number of genera cultured from the second batch of individuals ranged from 4 in low-D and high-C to 6 genera cultured from low-A and med-B.

After the density experiment, 76 animals survived. Of those, forty-four geckos produced 52 lactose positive samples (Table 2.2). Groups made up of individually housed animals produced four to seven genera compared to the combined groups which varied from two to four genera cultured (Figure 2.2). In all cases except low-D, there was a higher diversity in the number of genera cultured in the individually housed animals when compared with animals living in combined groups (Figure 2.2). This difference was not statistically significant. *Citrobacter* spp. was the most frequent (or common) isolate from all combined treatment groups and was cultured from every pre-assigned individual group except the Batch 1 low-A. The overall prevalence of *Citrobacter* spp. increased in all four of the combined groups versus the

individually housed animals. The only statistically significant difference was the increase in *Citrobacter* spp. from 36.4% in the individual low-A group to 81.8% in the combined low-A group (p-value = 0.0138; Figure 2.3, 2.4, 2.5, 2.6).

Salmonella enterica subspecies *arizonae* was cultured from three of the four combined density groups and one individual group low-A. The prevalence of *S. arizonae* from the combined high-C group was higher, 18.75%, compared to 0% from individual high-C group (p-value = 0.0005; Figure 2.3, 2.4, 2.5, 2.6). *Klebsiella* spp. and *Enterobacter* spp. were cultured from all of the individual groups and combined groups high-C and low-D, and combined med-B group respectively. The prevalence of *Enterobacter* spp. was higher, 31.25%, in individual low-D group compared to the combined low-D group 0% (p-value =0.0326). *Kluyvera* spp. was present in three of the individual groups (low-A, med-B and high-C) and combined med-B. There were only two isolates of *Pantoea* spp., one from individual med-B group and one from combined low-D group. *Serratia* spp. and *Echerichia coli* were only cultured from the individual groups.

DISCUSSION

Our study describes a subset of the enteric flora of tokay geckos under specific conditions. The enteric flora we described was isolated once geckos arrived at the University of Georgia and may be affected by factors such as transport-associated stress. Thus our description of enteric flora should be interpreted with caution. Regardless, the commensal enteric flora (the isolates that were cultured from geckos prior to experimental manipulation) was compromised of genera previously cultured from reptiles (Mader 2006). There are very few reports that describe the commensal enteric flora of geckos (Graves et al. 1988), because the majority of studies focus on the prevalence of *Salmonella* spp. (Hoff et al. 1977; Murphy et al. 1993; Callaway et al.

2010). The majority of reports describing non-*Salmonella* spp. enteric bacteria include a variety of reptile species (Mathewson 1979; Cooper et al. 1985). However, these reports have to be interpreted with caution, because some were descriptions from clinically diseased reptiles (Jacobson 2007; Johnson et al. 2008). The three most common genera generally isolated from all of the individual geckos were *Citrobacter* spp. (54.7%), *Klebsiella* spp. (19.7%), and *Enterobacter* spp. (9.5%). Given that shipping conditions for batch 1 and batch 2 were different, we compared the most frequently isolated genera and found little difference (Table 2.3). The prevalence of lactose negative isolates, 33%, we recovered is difficult to compare to other studies because we did not identify the genera. We may have cultured *Salmonella* spp. which has been reported at high prevalences from captive reptiles (Corrente et al. 2004; Chen et al. 2010) or a different genera like *Proteus* spp. or *Edwardsiella* spp. which have been reported at a low occurrence (Mathewson 1979; Thaller et al. 2010).

Citrobacter spp. is commonly associated with the gastrointestinal flora of reptiles (Janda 2006). However, it has also been isolated from opportunistic infections in reptiles (Jacobson 2007) illustrating its potential as a pathogen. *Citrobacter* spp. was cultured from all individual pre-assigned groups except batch 1 low-A. The prevalence of *Citrobacter* spp. cultured was similar among both batches of individuals (59.2% and 51.8%), which is consistent with the study by Graves et al. which demonstrated that *Citrobacter* spp. was the predominant Our study is consistent with previous reports that claim that *Klebsiella* ssp. is a common component of the resident flora of many reptiles, as it was isolated from every pre-assigned group from each batch of individuals and was the second most frequently isolated bacteria among individual geckos (Mader 2006). *Enterobacter* spp. was the third most common genera cultured from all the individuals. *Enterobacter* spp. has been isolated from both healthy and diseased reptiles

(Mathewson 1979; Ebani et al. 2005), although a specific report that included geckos did not report this bacteria in them (Graves et al. 1988). *Salmonella arizonae* was isolated from <1% of individual geckos. The presence of *S. arizonae* was expected since reptiles are considered reservoirs for *Salmonella* spp. and is commonly associated with wild reptiles at a low prevalence (Richards et al. 2004; Jacobson 2007). It was not surprising to culture *E. coli* and *Serratia* spp. because they have been previously reported in healthy and sick reptiles (Mader 2006). Less has been published about *Kluyvera* spp. but it has also been reported in reptiles (Bastos et al. 2008). All of the studies reporting the isolation of *Pantoea* spp. came from clinically diseased reptiles were multiple bacterial cultures were recovered (Jacobson 2007; Johnson et al. 2008). The information regarding *Pantoea* spp. could be misleading because this genus was not established until 1989 (Janda 2006). *Enterobacter agglomerans* was recently renamed to the genus *Pantoea* spp. Regardless, *Enterobacter agglomerans* has also been reported in both sick and healthy reptiles (Santoro et al. 2006; Jacobson 2007). It is difficult to compare the prevalence of the genera we isolated to other studies because of the lack of information published on tokay gecko or similar species. The prevalence of bacteria is highly variable among species because the normal flora composition depends on a lot factors such as: age, gender, diet, and reproductive status (Gordon et al. 2003; Brown et al. 2007; Martin et al. 2010).

There were two major findings that are related to the influence of density on diversity and composition. Firstly, there was an increase in the overall prevalence of *Citrobacter* spp. from 54.7% cultured from individuals to 75% in combined groups (Table 2.3). Even though the difference was not statistically significant, the prevalence of *Citrobacter* spp. increased in every combined group compared to individual pre-assigned groups (Figure 2.3, 2.4, 2.5, 2.6). The difference in the increase in prevalence of *Citrobacter* spp. cultured from the individual low-A

compared to the combined low-A was significant. Additionally, we documented an increase of *S. arizonae* prevalence from 0% to 19% in the individual high-C group. A possible explanation why only a few groups demonstrated statistical significance is related to sample size. The observed increase in prevalence of these two genera could be a result of a physiological change in the host due to stress, an inherent trait of the particular bacteria that allows it to out compete other resident and transient flora for resources, or a combination of these two mechanisms (Ehrlich et al. 2008; Lutgendorff et al. 2008; Sekirov et al. 2010).

In our study, we expected that stress due to overcrowding may promote the shedding of pathogenic bacteria, a phenomenon that has been shown with the shedding of *Salmonella* spp. in livestock (De Passillé et al. 2005; Ball et al. 2011). We thought increasing density would be stressful, because tokay geckos are solitary animals, very territorial, and naturally aggressive toward other geckos. Therefore, we expected to see a higher number of isolates of *Salmonella* spp. shed from animals in the higher density groups both due to an increase in stress associated with overcrowding and more frequent contact with shed *Salmonella* spp. isolates. However, we only saw an increase in *Salmonella* spp. isolation between the individual high C and the combined C group, although, we did observe an increase in the overall prevalence *S. arizonae* isolated. The overall prevalence represents the total number of *S. arizonae* across all the isolates cultured for either individuals (≤1%) or combined (11.5%). This is different from detecting a difference in prevalence between groups because only the isolates cultured from a specific group, like low-A, med-B, or high-C, are compared to each other. An explanation for the increase in the overall prevalence, but not an increase in prevalence between different density treatments maybe that stress from simply co-habitating was enough to induce a change in the normal flora and promote *S. arizonae* shedding. Therefore, this would make any additional stress from the

increase in density irrelevant because the geckos were already sufficiently stressed. It is important to understand how stress related to overcrowding effects pathogen shedding because of the zoonotic potential these pets represent to their owners. If stress does result in a higher prevalence of pathogen shedding, like in livestock species, then it is prudent to reduce stress in order to diminish the potential public health risk.

Interestingly, when comparing the overall prevalence of genera from the individuals to the combined groups, although there was an increase in *Citrobacter* spp. and *Salmonella arizonae* there was also a decrease in *Klebsiella* spp., *Enterobacter* spp., and *Kluyvera* spp. prevalence and a complete loss of 2 genera (*Serratia* and *E. coli*). Thus, our initial prediction that as the density of geckos was artificially increased the diversity of culturable lactose fermenting enteric flora would decrease, was not validated. However, we did observe a consistent decrease in diversity between individuals and combined groups across all groups except low-D (Figure 2.2). To illustrate this point the third most frequently isolated genus from the individual groups, *Enterobacter* spp., was not recovered from 3 out of 4 combined groups. However, the differences in the diversity of genera between individual and combined groups were not statistically significant. The lack of statistical significance could be a result of the inability of analyses to detect differences when the range of numbers of genera is too narrow, (eg, from 7 in the individual to 2 in the combined groups). There was also no change in the number of genera cultured from the individual low-D and the combined low-D groups, which could have skewed the overall number of genera.

There is evidence that suggests a high diversity in normal flora promotes the stability of microbiota in the intestine (Hentges 1983) and when disturbed by stress, use of antibiotics, or host physiological changes, transient, as well as resident bacteria may colonize and overgrow,

resulting in a decreased diversity (Katouli et al. 1994). There have been several studies that investigate specifically the ability of stress to disrupt the stability of the intestinal microbial ecosystem (Holdeman et al. 1976; Lizko 1987; Bailey et al. 1999; Knowles et al. 2008). Our research explores the influence of stress on the diversity of composition using the tokay gecko and a subset of its gastrointestinal flora as a model. In the human gastrointestinal tract, the family *Enterobacteriaceae* represents a minority (≤1%) of the composition of the commensal flora (Finegold 1969; Drasar 2003). To our knowledge, there is little known about the flora(Costello et al. 2010). Therefore, it is difficult to definitively state, without further investigation, whether the observed decrease in diversity among a subset of *Enterobacteriaceae* represents a similar change in the rest of the gastrointestinal microbiota of tokay geckos. Furthermore, in this experiment, we did not control for sex, age, reproductive status, and seasonal or temporal effects. Although, a definitive link between stress and the decrease in diversity observed was not established it is still an important trend because of the public health implications associated with the family *Enterobacteriaceae*.

The impact of stress on pathogen shedding is possibly related to the decrease in diversity. The disruption of normal flora via stress results in a decreased diversity which may promote pathogen colonization. For example, a study in mice demonstrated that food deprivation and harassment destabilized their enteric microbial communities, diminishing their enteric microbial diversity and increasing their susceptibility to colonization by *Citrobacter rodentium,* an enteric pathogen (Bailey et al. 2010). Therefore, maintaining a diverse healthy gastrointestinal microbial community is important because helps prevent pathogen colonization (Lu et al. 2001; Dillon et al. 2005; Dowd et al. 2008; Chinnadurai et al. 2009). One specific example, is a study that demonstrated manipulating the diets of turkeys promoted the gastrointestinal microbial diversity

and discouraged *Salmonella* spp. colonization (Santos Jr 2006). There are three generally accepted mechanisms by which the presence of commensal bacteria prevent the colonization of pathogens: competitive exclusion where normal flora outcompete transient bacteria for nutrients and receptors, the production of bacteriocins, and the activation of the host immune response (Alverdy et al. 2005; Stecher et al. 2010). Most of the research on the physiological interactions between microbial communities and their hosts is limited to humans, mouse models, domestic livestock and poultry. Germ free animals have allowed researchers to explore the effects normal flora have on stimulating an immune response and preventing pathogen colonization (Guarner et al. 2003; Dillon et al. 2005). It is apparent from previous research that healthy individuals with stable diverse gastrointestinal microbial communities are important for preventing the colonization and overgrowth of transient or resident flora. From a public health perspective it is wise to promote conditions that do not decrease gastrointestinal microbial diversity in pet reptiles that are known reservoirs of human pathogens because of the potential to increase the prevalence of pathogen colonization and ultimately shedding.

Interestingly, we saw no decrease in the diversity of *Enterobacteriaceae* cultured between the individual low-D and combined low-D groups. This group was created to detect whether the time the geckos spent in the groups affected the composition of normal flora. The lack of change in diversity could be due to the shorter period of time these animals spent in the combined low-D group, or because it was compromised solely of animals from the second batch. Therefore, these geckos never had an opportunity to acquire any of the enteric bacteria from the geckos in the first batch. Since the combined low-D group spent the least amount of time together, and the diversity remained the same compared to the low-A, med-B, and high-C it would appear that as geckos spend more time together, the diversity of commensal enteric flora

is a function of the time geckos are housed in groups. However, a limitation of this study was the lack of time points to demonstrate that diversity truly decreased and it was not just normal fluctuation of the microbial community.

Future studies should focus on teasing apart the complex interactions between the host and the microbial communities and how gender, reproductive status, diet, stress, and environment influence the composition. It would be interesting to investigate whether the observed decrease in diversity in our subset of *Enterobacteriaceae* isolates is also observed in other bacteria normally present in the enteric flora of geckos. Future work would also benefit from collecting isolates at more than two time points to distinguish whether or whether observed differences are just seasonal fluctuations in composition. Our study purposely limited the number of sampling times to reduce the stress associated with capture and handling to collect fecal samples. This created more time for the geckos to interact undisturbed and allowed for shedding and exchange of commensal organisms without the stress of handling. It would also be beneficial to track the changes in normal flora at the individual level. This was our initial goal; however, we lost the ability to track individuals because the skin markers we utilized were prematurely shed. Thus we were forced to analyze our data at the group level. Subcutaneous tags are a more effective method for tracking individual reptiles (Galliard et al. 2011). Finally, acquiring samples prior to transport and immediately after importation would ultimately allow the best representation of true shipping conditions.

CONCLUSIONS

The changes in culturable lactose fermenting *Enterobacteriaceae* shed by reptiles are important to monitor because they could provide insight into how stressful overcrowding conditions similar to those currently used in the pet trade influence enteric commensal flora

composition. Our results describing the commensal enteric flora similar to previous studies of reptiles, but provides a unique description of commensal enteric flora of specifically tokay geckos. We focused on the *Enterobacteriaceae* because several members of this family are primary pathogens and some are important opportunistic pathogens.

Promoting humane conditions for the importation of pet reptiles is important for welfare concerns, but may also help to maintain intact diverse microbial communities that protect individual animals from pathogen colonization and prevent pathogen dissemination. Normal flora has many benefits to health and our research suggests that stress alters a small subset of the normal flora, decreasing diversity of commensal enteric bacteria and promoting the shedding of *S. arizonae*. The increased prevalence of *S. arizonae* is an important finding since reptile-associated salmonellosis a public health concern (CDC 2003). The popularity of reptiles as pets is increasing, which makes reptile-associated salmonellosis a public health concern (Sanyal et al. 1997), especially when these pets are placed in the homes of young children or immunocompromised individuals.

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Table 2.1. The Composition of Normal Flora from Individually Housed Tokay Geckos

	Bacteria	Batch 1(Indv)		Bacteria	Batch 2(Indv)	
A (Low 1)	*S. arizonae*	1	25%	*S. arizonae*	0	
	Kluyvera spp.	1	25%	*Kluyvera spp.*	4	22.2%
	Klebsiella spp.	2	50%	*Klebsiella spp.*	2	11.1%
	Citrobacter spp.	0		*Citrobacter spp.*	8	44.4%
	Echerichia coli	0		*Echerichia coli*	1	5.5%
	Enterobacter spp.	0		*Enterobacter spp.*	2	11.1%
	Pantoea spp.	0		*Pantoea spp.*	0	
	Serratia spp.	0		*Serratia spp.*	1	5.5%
	# animals = 5	n= 4		# animals = 15	n= 18	
D (Low 2)	Bacteria	Batch 1(Indv)		Bacteria	Batch 2(Indv)	
	N/A			*S. arizonae*	0	
				Kluyvera spp.	0	
				Klebsiella spp.	1	6.25%
				Citrobacter spp.	9	56.25%
				Echerichia coli	1	6.25%
				Enterobacter spp.	5	31.25%
				Pantoea spp.	0	
				Serratia spp.	0	
				# animals = 15	n= 16	
B (Med)	Bacteria	Batch 1(Indv)		Bacteria	Batch 2(Indv)	
	S. arizonae	0		*S. arizonae*	0	
	Kluyvera spp.	2	15.4%	*Kluyvera spp.*	1	4.2%
	Klebsiella spp.	2	15.4%	*Klebsiella spp.*	4	16.7%
	Citrobacter spp.	6	46.2%	*Citrobacter spp.*	13	54.2%
	Echerichia coli	0		*Echerichia coli*	3	12.5%
	Enterobacter spp.	2	15.4%	*Enterobacter spp.*	1	4.2%
	Pantoea spp.	1	7.7%	*Pantoea spp.*	0	
	Serratia spp.	0		*Serratia spp.*	2	8.3%
	# animals = 15	n= 13		# animals = 15	n = 24	
C (High)	Bacteria	Batch 1(Indv)		Bacteria	Batch 2(Indv)	
	S. arizonae	0		*S. arizonae*	0	
	Kluyvera spp.	1	2.7%	*Kluyvera spp.*	0	
	Klebsiella spp.	7	19%	*Klebsiella spp.*	9	36%
	Citrobacter spp.	26	70%	*Citrobacter spp.*	13	52%
	Echerichia coli	0		*Echerichia coli*	2	8%
	Enterobacter spp.	2	5.4%	*Enterobacter spp.*	1	4%
	Pantoea spp.	0		*Pantoea spp.*	0	
	Serratia spp.	1	2.7%	*Serratia spp.*	0	
	# animals = 30	n = 37		# animals = 15	n=25	

Table 2.2. Total Number of Isolates from Individuals (Batch 1 and 2) and Combined Groups

	Bacteria	Individuals		Bacteria	Combined - Low 1	
A (Low 1)	*S. arizonae*	1	4.5%	*S. arizonae*	2	18.2%
	Kluyvera spp.	5	22.7%	*Kluyvera spp.*	0	
	Klebsiella spp.	4	18.2%	*Klebsiella spp.*	0	
	Citrobacter spp.	8	36.4%	*Citrobacter spp.*	9	81.8%
	Echerichia coli	1	4.5%	*Echerichia coli*	0	
	Enterobacter spp.	2	9.1%	*Enterobacter spp.*	0	
	Pantoea spp.	0		*Pantoea spp.*	0	
	Serratia spp.	1	4.5%	*Serratia spp.*	0	
	# animals = 20	n= 22		# animals = 15	n= 11	
D (Low 2)	Bacteria	Individuals		Bacteria	Combined - Low 2	
	S. arizonae	0		*S. arizonae*	1	8.33%
	Kluyvera spp.	0		*Kluyvera spp.*	0	
	Klebsiella spp.	1	6.25%	*Klebsiella spp.*	1	8.33%
	Citrobacter spp.	9	56.25%	*Citrobacter spp.*	9	75%
	Echerichia coli	1	6.25%	*Echerichia coli*	0	
	Enterobacter spp.	5	31.25%	*Enterobacter spp.*	0	
	Pantoea spp.	0		*Pantoea spp.*	1	8.33%
	Serratia spp.	0		*Serratia spp.*	0	
	# animals = 15	n= 16		# animals = 15	n= 12	
B (Med)	Bacteria	Individuals		Bacteria	Combined - Med	
	S. arizonae	0		*S. arizonae*	0	
	Kluyvera spp.	3	8.1%	*Kluyvera spp.*	2	15.4%
	Klebsiella spp.	6	16.2%	*Klebsiella spp.*	0	
	Citrobacter spp.	19	51.3%	*Citrobacter spp.*	9	69.2%
	Echerichia coli	3	8.1%	*Echerichia coli*	0	
	Enterobacter spp.	3	8.1%	*Enterobacter spp.*	2	15.4%
	Pantoea spp.	1	2.7%	*Pantoea spp.*	0	
	Serratia spp.	2	5.4%	*Serratia spp.*	0	
	# animals = 30	n = 37		# animals = 17	n = 13	
C (High)	Bacteria	Individuals		Bacteria	Combined - High	
	S. arizonae	0		*S. arizonae*	3	18.75%
	Kluyvera spp.	1	1.6%	*Kluyvera spp.*	0	
	Klebsiella spp.	16	25.8%	*Klebsiella spp.*	1	6.25%
	Citrobacter spp.	40	64.5%	*Citrobacter spp.*	12	75%
	Echerichia coli	2	3.2%	*Echerichia coli*	0	
	Enterobacter spp.	2	3.2%	*Enterobacter spp.*	0	
	Pantoea spp.	0		*Pantoea spp.*	0	
	Serratia spp.	1	1.6%	*Serratia spp.*	0	
	# animals = 45	n=62		# animals = 29	n= 16	

Table 2.3. Overall Prevalence for Individuals and Combined

	Batch 1	batch 2	total (batch 1 + 2)	Combined
Citrobacter spp.	59.2 % (32/54)	51.8% (43/83)	54.7% (75/137)	75% (39/52)
Klebsiella spp.	20.4% (11/54)	19.3% (16/83)	19.7% (27/137)	3.8% (2/52)
Enterobacter sp	7.4% (4/54)	10.8% (9/83)	9.5% (13/167)	3.8% (2/52)
Kluyvera spp.	7.4% (4/54)	6.0% (5/83)	6.5% (9/137)	3.8% (2/52)
Echerichia coli	0	8.4% (7/83)	5.1% (7/137)	0
Serratia spp.	1.8% (1/54)	3.6% (3/83)	2.9% (4/137)	0
Pantoea spp.	1.8% (1/54)	0	0.7% (1/137)	1.9% (1/52)
S. arizonae	1.8% (1/54)	0	0.7% (1/137)	11.5% (6/52)
Total #	54	83	137	52

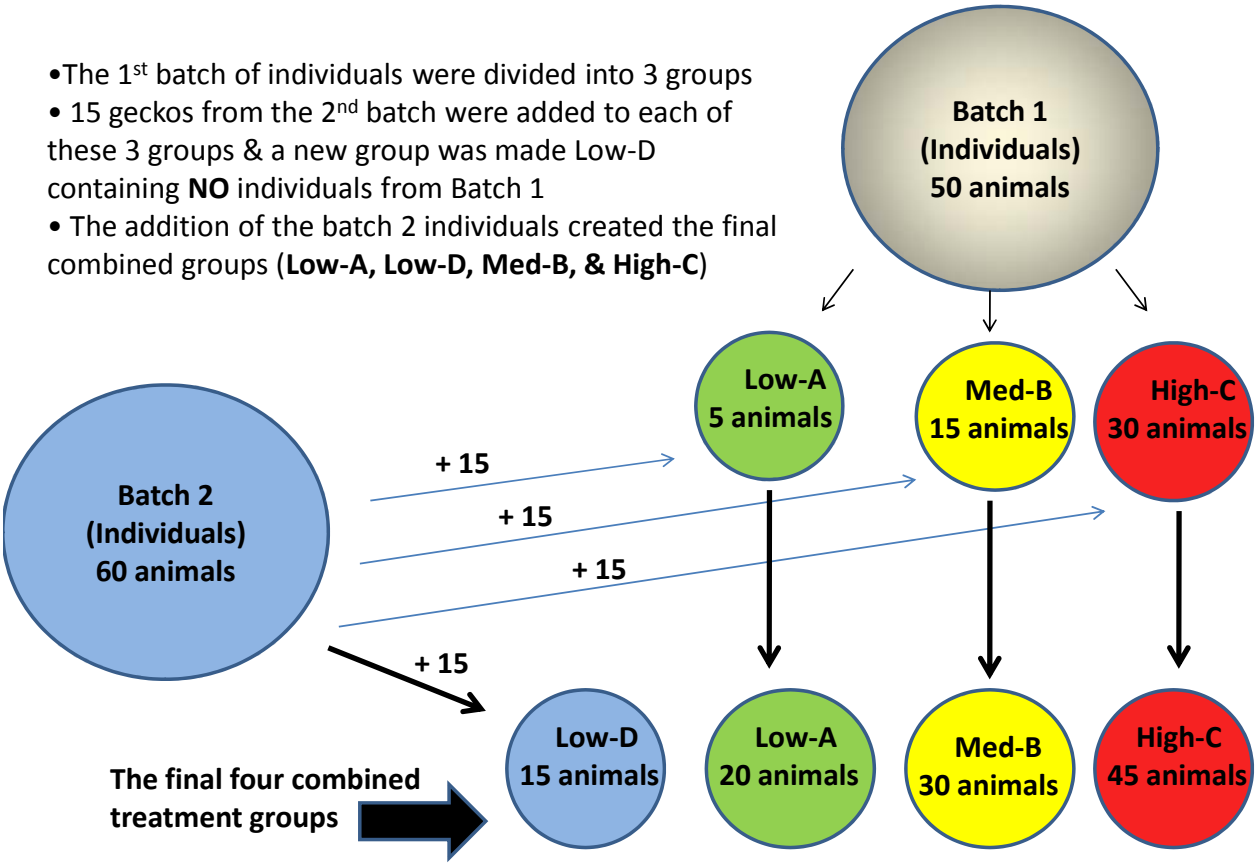


Figure 2.1. Diagram of How Geckos Were Assigned to Different Groups

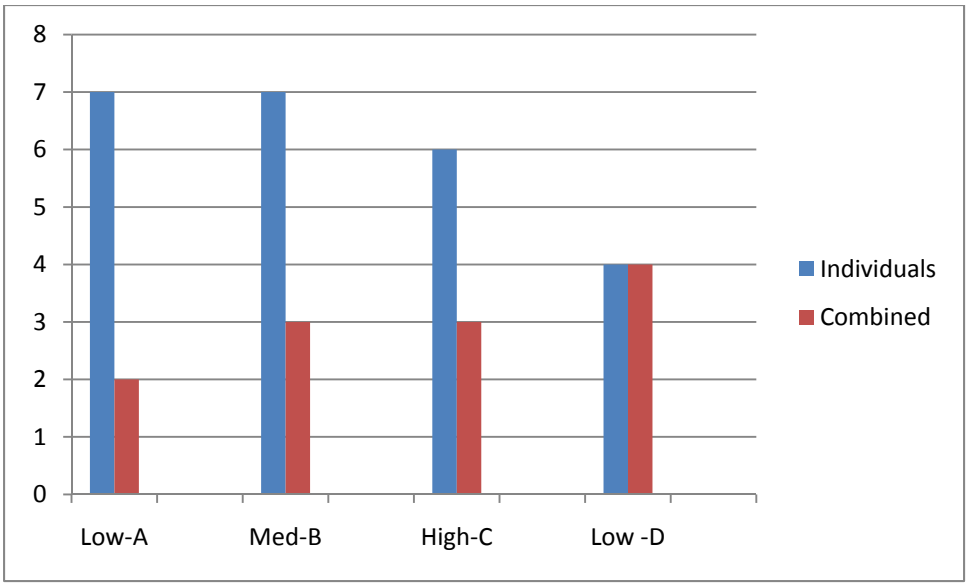


Figure 2.2. Diversity of Genera between Individual and Combined Groups

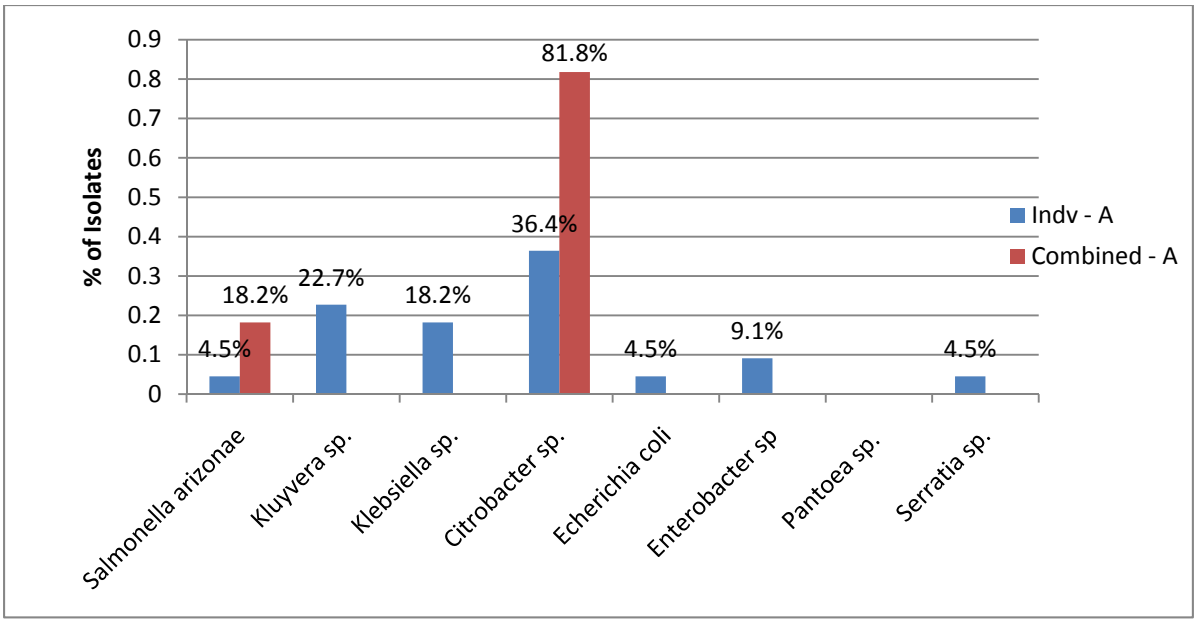


Figure 2.3. Prevalence of Isolates in Group A

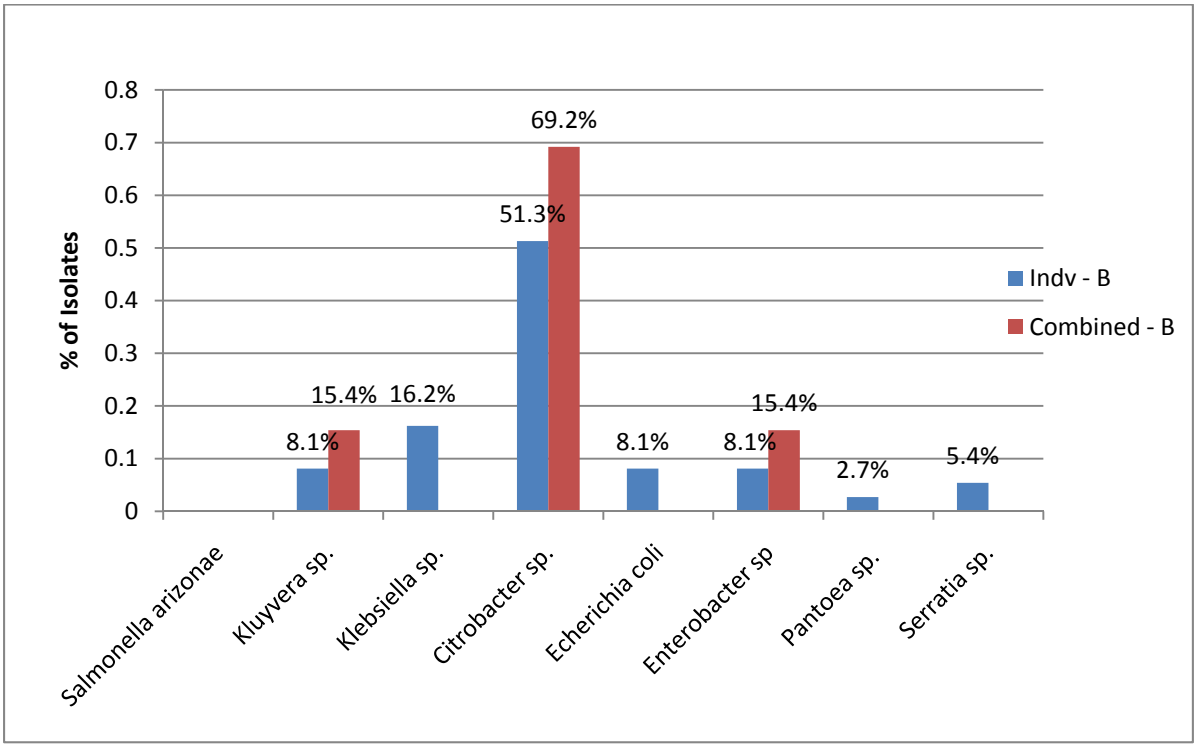
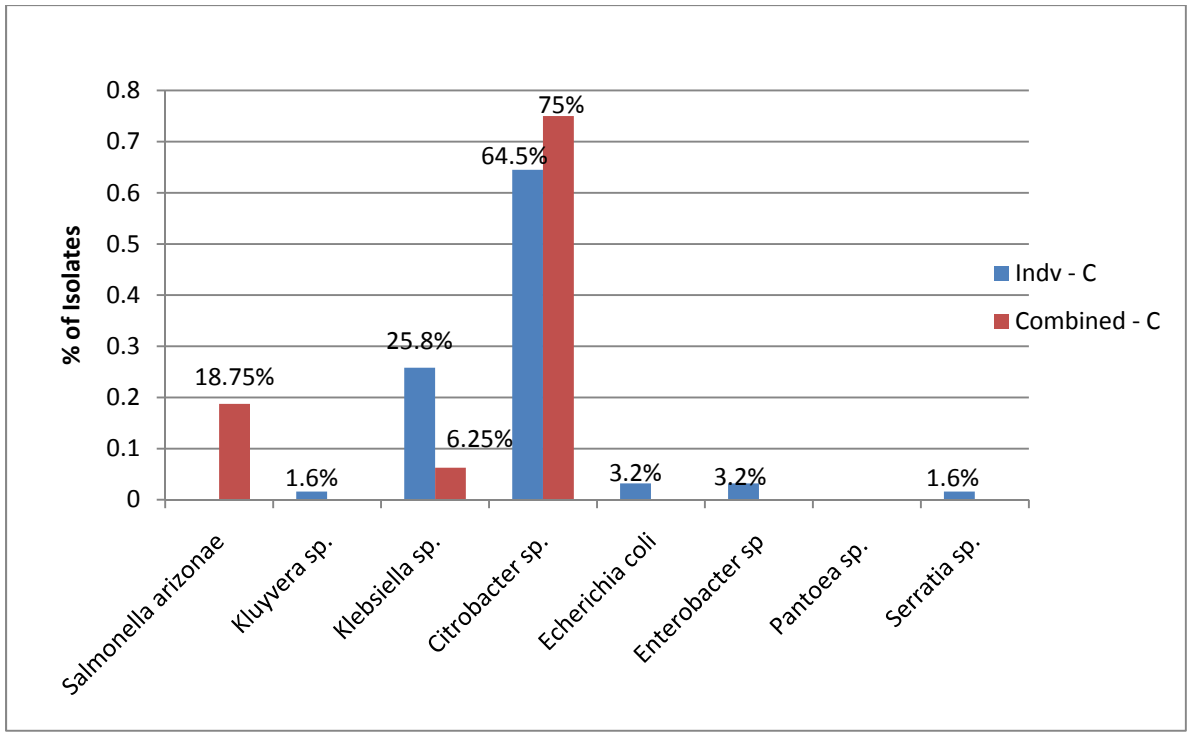
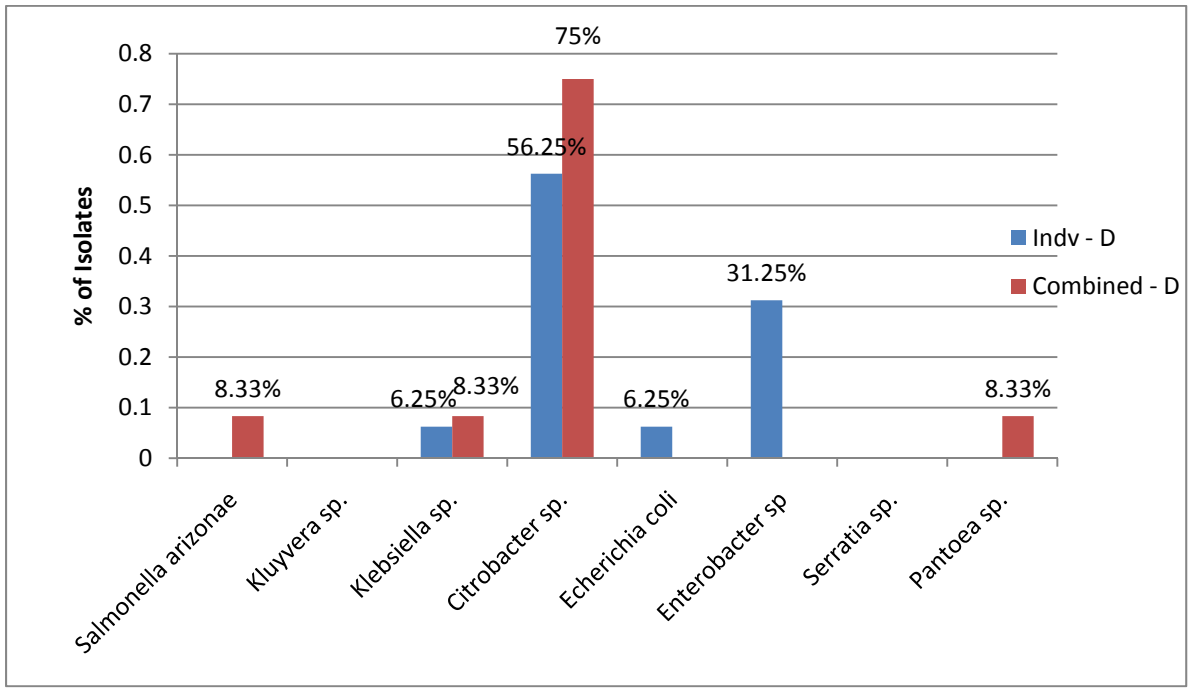


Figure 2.4. Prevalence of Isolates in Group B



2.5. Prevalence of Isolates in Group C



2.6. Prevalence of Isolates in Group D

CHAPTER 3

UNDERSTANDING THE EFFECTS OF IMPORTATION HAS ON ANTIMICROBIAL RESISTANCE IN COMMENSAL ENTERIC BACTERIA FROM THE TOKAY GECKO (*GEKKO GECKO*)

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To be submitted to *Ecohealth.*

ABSTRACT

Wildlife traded for the pet market has the potential to contribute to dissemination of multidrug-resistant bacteria. We propose the pet trade, particularly the conditions under which wildlife are captured and transported has the potential to influence the rate of antimicrobial resistance development. Through experimental manipulations, using the Tokay gecko (*Gekko gecko*) as a model for the pet trade, we to described 1) the culturable lactose fermenting *Enterobacteriaceae* 2) the antimicrobial resistance of *Enterobacteriaceae* 3) how the conditions which reptiles are imported, for the pet trade, impact diversity of composition and antimicrobial resistance in *Enterobacteriaceae*. The shift in normal flora shed by reptiles are important to monitor because it provides insight into how stressful overcrowding situations, similar to pet trade conditions, influence commensal lactose fermenting *Enterobacteriaceae* composition and promote antibiotic resistance. Both individually housed geckos and those living in varying densities harbored isolates resistant to multiple antibiotics. Several isolates possessed intermediate resistance to chloramphenicol. Extended spectrum beta-lactases (ESBLs) are increasing in prevalence in the gecko's native range; however none were detected among the isolates.

INTRODUCTION

Given the global increase in antimicrobial resistance and the fact that the growth in pharmacological drug development is currently stagnant, understanding the selection pressures that contribute to the development of antimicrobial resistance in bacterial populations is essential (Moellering 1995). The cumulative effects of a global society and economy, the lack of novel classes of drugs being produced, and an increase in antimicrobial resistance has the potential to create a serious public health threat (Levy et al. 2005). Antimicrobial resistance is often approached via the study of pathogens in clinical situations and less is known about the mechanisms contributing to the development of antimicrobial resistance involving commensal bacteria, domestic animal consumption of antimicrobials, and environmental contamination (Martínez 2008). However, the importance of normal flora as a reservoir of antibiotic resistance genes for pathogenic bacteria has gained more recognition in recent years (van den Bogaard et al. 2000). Several reviews of the epidemiology of antibiotic resistance assert that current research should pursue the investigation of low level antibiotic resistance because it is the foundation for the development of high level, clinically relevant resistance (Baquero 2001; Normark et al. 2002).

Wildlife can acquire antibiotic-resistant bacteria and potentially act as reservoirs of antimicrobial resistance genes (Pallecchi et al. 2008; Blanco et al. 2009; Kozak et al. 2009). Specifically, the pet trade has been implicated in the introduction of several pathogens; therefore it is plausible that commensal bacteria harboring antimicrobial resistance genes could also be disseminated via the pet trade (Okeke et al. 2001; Stephenson 2003; Bell et al. 2004; Karesh et al. 2007). Research has demonstrated that transport conditions such as overcrowding, thermal extremes, diet, and poor ventilation are stressful to livestock (Hartung 2003; Schumacher 2006;

Weston et al. 2009) and possibly more stressful to wild animals because, unlike domestic animals, they are not acclimated to novel stimuli (Grandin 1997). Stressful conditions experienced by animals often translate into physiological changes within the host and these changes have been linked to immunosuppression, increased mutations and exchange of antimicrobial resistance among the pathogenic and commensal bacteria, and increased shedding of pathogens (Molitoris et al. 1987; Jacobson 1993; Sorum et al. 2001; Blázquez 2003; Silbergeld et al. 2008).

The unfavorable conditions and the sheer volume of animals being exported for the pet trade create an ideal environment for the movement of commensal bacteria harboring antimicrobial resistance genes (McEwen et al. 2002; Aarestrup 2006; Brown 2006). A review completed by the U.S. Fish and Wildlife Service's Law Enforcement Management Information System (LEMIS) revealed that between 2000 and 2006 the US imported over 1.4 billion live animals of which 80% were from wild populations (USFWS 2000-2006). Of the 80% of wild animals imported, 69% of these animals originated from Southeast Asia, a known hotspot of emerging infectious disease. Southeast Asia has also experienced an increased prevalence of extended spectrum beta-lactamases and resistance to quinolones in clinical isolates from the family *Enterobacteriaceae* (Okeke et al. 2005; Hawser et al. 2009). In fact, the prevalence of multidrug resistance is rising worldwide in the family *Enterobacteriaceae* (Nordmann 2006). One example is the emergence of multidrug resistant strains of *Salmonella enterica* subspecies *enterica* serotype Typhi, in South Asia (Okeke et al. 2005). Several members of the family *Enterobacteriaceae* are considered important pathogens while others are commonly associated with commensal gastrointestinal flora. Lactose fermenting *Enterobacteriaceae* are usually associated with commensal bacteria and include several genera: *Escherichia* spp., *Citrobacter*

spp., *Klebsiella* spp., *Serratia* spp., and *Enterobacter* spp. (Janda et al. 2006). However, even commensal bacteria can be important opportunistic pathogens and are estimated to cause half of all nosocomial infections in the United States (Segen 2002).

The potential for dissemination of antibiotic-resistant bacteria via the transport of livestock and produce has been examined in several studies (Okeke et al. 2001). However, until now, wildlife transported across international borders has not been examined as hosts of antimicrobial resistance. The current knowledge on antimicrobial resistance in wild animals comes from captive mammals and birds (Souza et al. 1999). For example, a study investigating the ability of zoonotic pathogens harbored by zoo animals demonstrated the potential for the dispersion of multidrug-resistant bacteria harboring resistant genetic determinants to humans (Ahmed et al. 2007). The prevalence of antibiotic resistance of *Enterobacteriaceae* from reptiles has only been examined in pets and captive populations (Gopee et al. 2000). We proposed that the pet trade, particularly the conditions under which wildlife is captured, handled, and transported, would influence the development of antimicrobial resistance. We hypothesized that the prevalence of antimicrobial resistant lactose fermenting *Enterobacteriaceae* isolates cultured from individually housed tokay geckos (*Gekko gecko*) would increase as animal density and capture/transport/holding-related stress and crowding occurred. Through experimental manipulations, using the tokay gecko as a model for the pet trade, we investigated 1) the antimicrobial resistance patterns of lactose fermenting *Enterobacteriaceae* isolates from fecal samples, and 2) how antimicrobial resistance patterns changed after mimicking the conditions under which reptiles are imported for the pet trade.

METHODS

Animal Collection, Shipping, and Housing

Wild caught tokay Geckos were imported from an international wildlife distributor based in Indonesia. Two batches of geckos were captured by the distributor from two locations on the island of Java. Once captured, the geckos were individually housed and shipped to the University of Georgia's College of Veterinary Medicine. All animal handling and care was approved by the University of Georgia's Institutional Animal Use Care Committee. Animals were housed in temporary mouse containers then transferred to enclosures measuring 24"W x 36"D x 72" H. They were fed a standard diet of crickets and mealworms. The temperature was controlled at 26.6º±4ºC and the humidity maintained at 50% - 70% and the lighting was on for a 12 hour period each day. They were monitored twice daily. The first batch arrived in March 2009. Fecal samples were collected during a 10 day period while they were housed individually. After feces were collected from all animals, individuals were randomly assigned to different groups with varying densities: Group A (low-A) represented a "low" density and contained 5 animals, Group B (med-B) was a "medium" density and contained 15 animals, and Group C (high-C) was a "high" density and contained 30 animals (Figure 3.1). A second batch of individually-housed geckos was imported in June. Once all fecal samples were collected from this batch, groups of 15 geckos were assigned to each of the pre-existing groups (low-A, med-B, and high-C) and a fourth group D (low-D) was created. Low-D contained 15 animals and was compromised solely of geckos from the second batch (low density). After the geckos from batch 1 and 2 had been housed together for three months, fecal samples were collected from all remaining geckos.

Isolation and Identification

Fecal samples were collected from individually housed geckos and from animals living in varying densities. At the time of fecal collection, to ensure animals were not sampled more than once, geckos were removed from the group and placed in containers until they defecated. Fecal samples were processed within 24 hours of collection. Fecal samples were placed in cryovials that contained 0.25 ml of sterile water. A sterile 10µl loop was used to streak the diluted fecal solution on MacConkey media. The plates were incubated at 37°C for 24 hours. Growth of lactose positive bacteria on enteric media resulted from 71% of the samples obtained. One sample of each morphologically distinct group of colonies was collected to be restreaked separately. If the colonies were later identified as the same genus and species, only one of the samples was used. We recognize that it is possible that they could be the same species but different strains with potentially different antibiotic resistance patterns, but for the purpose of this study we were interested in reporting the antibiotic resistance of different genera present in the fecal matter of tokay geckos. Once colonies were selected they were re-plated for isolation and again for purity. Pure isolates were suspended in freezer media consisting (1% peptone and 15% glycerol) and frozen at -80°C until further analysis. Standard methods were used to determine the identity of isolated organisms including plating on a selective and differential media, MacConkey. Standard biochemical tests, including oxidase, citrate, motality/indole/ornithine, triple sugar iron, phenylalanine, rhamnose, malonate, sorbitol, methyl red/Voges Proskauer, and lysine decarboxylase, were performed as recommended in the Manual of Clinical Microbiology (Murray 2003). If there were conflicting biochemical test results, rapid Analytical Profile Index (API20E) strips (Biomerieux, Durham, NC) were used to determine the identification of the isolate.

Minimum Inhibitory Concentrations

Minimum Inhibitory Concentrations (MIC) were determined by using broth microdilution methods described by the Clinical Laboratory Standards Institute (CLSI) guidelines (CSLI 2008) and validated dry-form panels (CMV1AGNF) produced by TREK Diagnostics (Cleveland, OH). Aseptic technique was to transfer colonies into sterile water to create a 0.5 Mcfarland standard. From the 0.5 Mcfarland solution 10µl were added to 10ml of cation-adjusted Mueller-Hinton broth and vortexed. Then 50µl of the broth was pipetted into each well of the MIC plate. Multiple classes of antimicrobials were tested. We selected a commercially-available standard susceptibility plate containing antibiotics that reportedly inhibit the growth of the Gram negative enteric bacteria that we expected to culture in reptiles (Mathewson 1979; Mader 2006). The antibiotics and the concentrations included on the plate were amikacin (0.5- 32µg/ml), ampicillin (1-32µg/ml), amoxicillin/ clavulanic acid (1/0.5-32/16µg/ml), cefoxitin (0.5-32µg/ml), ceftriaxone (0.25-64µg/ml), ceftiofur (0.12-8µg/ml), chloramphenicol (2-32µg/ml), ciprofloxacin (0.015-4µg/ml), gentamicin (0.25-16µg/ml), kanamycin (8-64µg/ml), nalidixic Acid (0.5-32µg/ml), sulfisoxazole (16-256µg/ml), streptomycin (32-64µg/ml), trimethoprim/sulfamethoxazole (0.12/2.38-4/76µg/ml), and tetracycline (4-32 µg/ml). The plates were incubated at 37°C±2° for 18 hours. Interpretations of susceptibility for all the antimicrobials tested were compared to CLSI criteria (CLSI 2010) for *Enterobacteriaceae*. *Escherichia coli* ATCC 25922 was routinely included as a quality control. The reference for *Enterobacteriaceae* isolates used were from known human data. There are no standards produced by CLSI for MIC values for *Enterobacteriaceae* isolates from reptiles.

Data Analysis

Statistical analyses were performed using SAS V 9.2 (SAS Institute Inc. Cary, NC). The MIC values were assigned numerical values of 0 if susceptible, 0.5 if intermediate resistance, and 1 for resistant. Logistic Regression was used to look at the prevalence of resistance under different scenarios or conditions like: combined groups low-A, low-D, med-B, and high-C versus each matching individual groups low-A, low-D, med-B, and high-C for the 15 different antibiotics. We also looked at the different genera for each combined group versus the individual groups for the antibiotics and finally we compared each genus within just the combined groups to each combined treatment group for the different antibiotics. Due to the low number of observations not all genera or antibiotics were able to be compared for every group. All hypothesis tests were 2-sided and the significance level was set at $\alpha = 0.1$.

RESULTS

A total of 189 lactose positive *Enterobacteriaceae* isolates were recovered. There were 137 isolates cultured from the 110 individuals comprising batch 1 and 2 (Table 3.1). The three most common genera isolated from all of the individual geckos were *Citrobacter* spp. (54.7%), *Klebsiella* spp. (19.7%), and *Enterobacter* spp. (9.5%). The diversity of genera in the individuals ranged from 4 in the pre-assigned low-D group to a high of 7 in the pre-assigned medium-B group. The analysis of antibiotic resistance of *S. arizonae* will not be discussed in this chapter.

All isolates of *Citrobacter* spp. cultured from individuals were susceptible to two 3rd generation cephalosporins: ceftriaxone and ceftiofur. However, 81% (60/74) of the isolates of *Citrobacter* spp. cultured from the individuals were resistant against the 2nd generation cephalosporin, cefoxitin (Table 3.2). The overall prevalence of resistance against amoxicillin with clavulanic acid among *Citrobacter* spp. isolates was 45.9%. The prevalence of intermediate

resistance against chloramphenicol among *Citrobacter* spp. isolates was 33.8%. One isolate of *Citrobacter* sp. cultured from the individuals was resistant against chloramphenicol (Table 3.2).

One isolate of *Citrobacter* sp. was resistant against kanamycin and one isolate expressed intermediate resistance. One isolate of *Citrobacter* sp. was resistant against streptomycin. One isolate of *Citrobacter* sp. was resistant against nalidixic acid. One isolate of *Citrobacter* sp. was resistant against trimethoprim/sulfamethoxazole. One isolate of *Citrobacter* sp. was resistant against tetracycline and two isolates of *Citrobacter* spp. expressed intermediate resistance.

There were a total of 27 isolates of *Klebsiella* spp. cultured from the individually housed geckos. One isolate of *Klebsiella* sp. from the individuals was resistant against cefoxitin and one isolate expressed intermediate resistance. There was one isolate of *Klebsiella* sp. resistant against amoxicillin with clavulanic acid from the individuals (Table 3.2). One isolate of *Klebsiella* sp. from the individuals expressed intermediate resistance to chloramphenicol. Four isolates of *Klebsiella* sp. cultured from the individuals were resistant against tetracycline and one isolate was resistant against streptomycin.

There were a total of 13 isolates of *Enterobacter* spp. cultured from the individuals (Table 3.2). One isolate of *Enterobacter* sp. cultured from the individual low-D group showed intermediate resistance against ceftiofur and another isolate from the same group was the only isolate to have a MIC equal to 1μg/ml to ceftriaxone. The prevalence of resistance against cefoxitin was 84.6% of isolates cultured from the individually housed geckos (Table 3.2). All the isolates of *Enterobacter* spp. cultured from the individually housed geckos, except one, were resistant against amoxicillin with clavulanic acid. Two of the 13 isolates (15.3%) of *Enterobacter* spp. cultured from the individually housed geckos group were resistant to chloramphenicol and 3/13 isolates (23.1%) expressed intermediate resistance. The overall

prevalence of resistance against ampicillin among *Enterobacter* spp. isolates cultured from individuals was 46.1% (Table 3.2).

There were nine isolates of *Kluyvera* spp. cultured from the individuals, seven isolates of *E.coli*, four of *Serratia* spp., and one isolates of *Pantoea* sp (Table 3.3). One isolate of *E.coli* was resistant against kanamycin and ampicillin while the other was resistant to cefoxitin. All isolates of *Kluyvera* spp., *Serratia* spp., *E.coli,* and *Pantoea* spp. were susceptible to the third generation cephalosporins. All nine isolates of *Kluyvera* spp. cultured from the individually housed geckos were susceptible to cefoxitin. The one isolate of *Pantoea* spp. cultured from the individuals was resistant to cefoxitin (Table 3.3). All four isolates of *Serratia* spp. cultured from the individual geckos produced intermediate resistance against cefoxitin (Table 3.3) and expressed resistance against amoxicillin with clavulanic acid. The one isolate of *Pantoea* spp. from the individuals expressed an intermediate resistance against amoxicillin with clavulanic acid. Overall 55.5% of *Kluyvera* spp. cultured from individuals expressed intermediate resistant and one isolate was resistant against ampicillin. The prevalence of resistance against ampicillin among *Serratia* spp. cultured from individually housed geckos was 25% with one isolate expressing intermediate resistance. Both *Pantoea* spp. isolates were susceptible to ampicillin. Two of *Kluyvera* spp. isolates had intermediate resistance against chloramphenicol. Two isolates of *Serratia* spp. expressed intermediate resistance to chloramphenicol. Three isolates of *Serratia* spp. were resistant against tetracycline.

There were 52 isolates cultured from the remaining 76 geckos housed in varying densities (Table 3.1). *Citrobacter* spp. (75%) and *Salmonella arizonae* (11.5%) were the two most common genera cultured from all the combined groups. In general, the diversity of genera cultured decreased in the combined treatments groups compared to isolates from individuals. In

the combined treatment groups, the diversity ranged from 1 genus recovered in low-A group to a high of 3 genera recovered from the low-D group. This does not include the lactose positive *Salmonella* genus recovered. Overall, 39 isolates of *Citrobacter* spp. were cultured from the combined treatment groups, 2 *Klebsiella* sp., 2 *Enterobacter* spp., 2 *Kluyvera* sp., and 1 *Pantoea*. The data from low-D group should be interpreted with caution because it was compromised of only geckos from the second batch while all the other combined groups were a mix of geckos from both batch 1 and 2.

All isolates of *Citrobacter* spp. cultured from the combined groups were susceptible to three 3rd generation cephalosporins: ceftriaxone and ceftiofur. However, 84.6% (33/39) of the isolates cultured from the combined groups were resistant against the 2nd generation cephalosporin, cefoxitin. Prevalence of resistance among *Citrobacter* spp. to amoxicillin with clavulanic acid among individuals in all four pre-assigned treatment groups was higher when compared to the combined treatment groups (Table 3.4). The overall prevalence of resistance against amoxicillin with clavulanic acid among *Citrobacter* spp. isolates decreased from 45.9% for the individual geckos to 35.8% for the combined groups. The overall prevalence of intermediate resistance against ampicillin for isolates cultured from individually housed geckos and the combined groups was 13.5% and 10.25% respectively (Table 3.4). Overall the prevalence of intermediate resistance to chloramphenicol decreased from 33.8% among isolates cultured from individuals to 17.9% of the isolates cultured from the combined groups. Overall 17.5% of the total isolates of *Citrobacter* spp. cultured from individually housed geckos were resistant against sulfisoxazole and 12.8% of the isolates cultured from the combined groups were resistant.

There were only two isolates of *Klebsiella* sp. cultured from the combined treatment groups. One of those isolates was cultured from the combined high-C group. This isolate was resistant to cefoxitin and expressed intermediate resistance to amoxicillin with clavulanic acid (Table 3.5). The isolates of *Klebsiella* spp. cultured from the combined high-C group also demonstrated intermediate resistance to chloramphenicol. The overall prevalence of resistance against sulfisoxazole among *Klebsiella* spp. isolates cultured from individuals was 51.8% and 50% from isolates cultured from the combined groups.

There were two isolates of *Enterobacter* spp. cultured from the combined treatment groups and both were resistant against cefoxitin (Table 3.6). These two isolates of *Enterobacter* spp. cultured from the combined treatment group were both resistant against amoxicillin with clavulanic acid. The resistance to ampicillin decreased from 46.1% among individuals to 1 isolated of 2 cultured from the combined group which expressed intermediate resistance (Table 3.6). The two isolates of *Enterobacter* spp. cultured from the combined medium-B group demonstrated intermediate resistance to chloramphenicol. The same two isolates of *Enterobacter* spp. cultured from the combined medium-B group were resistant against gentamicin and one isolate expressed intermediate resistance against kanamycin. These two isolates and one isolate from the individual groups were also resistant against streptomycin. The two isolates of *Enterobacter* spp. from the combined med-B group were resistant against nalidixic acid and one expressed intermediate resistance against ciprofloxacin. The same two isolates of *Enterobacter* spp. from the combined med-B group were resistant against trimethoprim /sulfamethoxazole. The prevalence of resistance among *Enterobacter* spp. isolates cultured from individual geckos against sulfisoxazole was 76.9% and 100% for the two isolates of *Enterobacter* spp. cultured from the combined medium-B group

There were only two isolates of *Kluyvera* spp. and one isolate of *Pantoea* sp. cultured from the combined groups. Both isolates of *Kluyvera* spp. were susceptible to cefoxitin (Table 3.7). The one isolate of *Pantoea* sp. cultured from the combined groups displayed intermediate resistance to cefoxitin. Both isolates of *Kluyvera* spp. and one isolates of *Pantoea* sp. from combined groups were susceptible to amoxicillin with clavulanic acid. One isolate of *Kluyvera* spp. cultured from the combined medium-B group expressed intermediate resistance against ampicillin and the other was susceptible. *Pantoea* sp. was susceptible to ampicillin (Table 3.7).

DISCUSSION

Our data supports the idea that some of the commensal bacteria from a common pet, the tokay gecko, imported into the United States are resistant to antibiotics. Interpreting the importance of the resistance among the commensal isolates is dependent on several factors such as the importance of low-level resistance, the potential mechanism for resistance, and the clinical relevance of the MIC values. Our initial prediction was that as gecko density increased in the treatment groups, the stress associated with housing solitary animals in crowded conditions would influence the amount of commensal flora shed. The close contact of these animals would facilitate the exchange of bacteria among geckos and potentially result in an increased prevalence of resistant bacteria.

The overall prevalence of resistance against cefoxitin (81.1%) among *Citrobacter* spp. isolates from individuals was expected. This is consistent with a study that described the enteric flora from a snake, *Bothrops jararaca*, found in Brazil which reported 62.5% of the *Citrobacter* spp. isolates recovered were resistant to cefoxitin (Bastos et al. 2008). Another study using clinical isolates of *Citrobacter freundii* from humans reported an 85% prevalence of resistance against cefoxitin (Gootz et al. 1984). We also found a high prevalence of resistance among

Enterobacter spp. (84.6%). This was also expected and similar to the Bastos et al. study that reported that 100% of the isolates of *Enterobacter* spp. were resistant to cefoxitin. The resistance to cefoxitin among *Citrobacter* and *Enterobacter* species were expected because these genera commonly express β-lactamase activity, which can confer this type of resistance (Lewis 2002; Janda et al. 2006). While cefoxitin would not be used to treat a *Citrobacter* spp. or *Enterobacter* spp. infection, there is still clinical relevance in reporting this data. Isolates that are resistant to cefoxitin may also be resistant to expanded spectrum cephalosporins that may be used to treat these types of infections (Livermore 1987).

It is important to understand the potential mechanisms that confer resistance in order to appropriately interpret our results. A possible explanation for the observed resistance is the presence of a commonly reported inducible chromosomal AmpC β-lactamase found in *Citrobacter freundii*, *Enterobacter* spp. and *Serratia* spp. (Bradford 2001). AmpC β-lactamases usually confer resistance against penicillins, cephalosporins, and classic β-lactam/ β-lactamases inhibitor combinations, like clavulanic acid (Lewis K. 2002). Bacteria that express an inducible AmpC will separate into derepressed mutants. Derepressed mutants constitutively overexpress AmpC and have the potential to be resistant to broad-spectrum cephalosporins including cefotaxime, ceftazidime, and ceftriaxone, making them a serious threat to public health (Livermore 1987; Jacoby 2009). Clinical isolates that hyperproduce AmpC β-lactamases may appear susceptible, on initial tests, to third generation cephalosporins but upon treatment, selection favors derepressed mutants and patients experience clinical failure of antibiotic. Isolates of *Citrobacter freundii* and *Enterobacter* spp. that express resistance to cefoxitin and amoxicillin with clavulanic acid but susceptibility to third generation cephalosporins are indicative of an inducible AmpC β-lactamases (Livermore 1987).

The resistance patterns of our isolates of *Citrobacter* spp. and *Enterobacter* spp. are consistent with this typical AmpC β-lactamases pattern (Table 3.8). Eighty one percent of the *Citrobacter* spp. isolates were resistant to cefoxitin and 45.9% were resistant to amoxicillin with clavulanic acid and another 27% expressed intermediate resistance to amoxicillin with clavulanic acid. Eighty four percent of the *Enterobacter* spp. isolates were resistant to cefoxitin and 92.3% were resistant to amoxicillin with clavulanic acid. The prevalence of resistance against cefoxitin and either resistance or intermediate resistance to amoxicillin with clavulanic acid that we observed is characteristic of an AmpC β-lactamases, but without further investigation we cannot definitively determine its presence. There are other mechanisms that could be responsible for the observed cefoxitin resistance among *Citrobacter* spp. and *Enterobacter* spp. however, they seem less plausible.

Another interesting finding among the individual isolates was the observed resistance against cefoxitin in two isolates of *Klebsiella* sp. and one of *E. coli.* Of the two *Klebsiella* sp. resistant to cefoxitin, one was resistant and one expressed intermediate resistance against amoxicillin with clavulanic acid. The *E. coli* isolate that was resistant was also the only *E. coli* isolate to have the highest MIC value that is still considered susceptible. *Klebsiella* spp. is not known to have a chromosomal AmpC β-lactamase, but plasmid-mediated AmpC β-lactamase have been reported in *Klebsiella* sp. A few recent studies have reported plasmid-mediated AmpC β-lactamase from human isolates of *Klebsiella* sp. at varying rates, 3.3% (Moland et al. 2006) and 8.5% (Alvarez et al. 2004). The possibility that an AmpC plasmid is present in reptiles should be investigated further to determine the mechanism responsible, especially because of the clinical relevance associated with this observed resistance pattern. If an AmpC plasmid is responsible, then just like the movement of people has allowed for the global dispersion of

AmpC plasmid-mediated β-lactamases (Philippon et al. 2002), imported reptiles could also potentially disseminate resistance determinants.

The majority of isolates were susceptible to the other antibiotics such as, amikacin, ceftriaxone, ceftiofur, ciprofloxacin, gentamicin, kanamycin, nalidixic acid, streptomycin, trimethoprim/sulfamethoxazole, and tetracycline. However, there were a few isolates that expressed resistance against some of these clinically relevant antibiotics. One isolate of *Citrobacter* sp. was resistant against kanamycin and one isolate expressed intermediate resistance. Kanamycin is an aminoglycoside which is a common class of drugs used to treat *Citrobacter* spp. infections (Kasper et al. 2005). One isolate of *Citrobacter* sp. was resistant against nalidixic acid, which is a first generation quinolone. This is a concern because there has been an increase in quinolone resistance in regions of Southeast Asia, from where these geckos originated, (Okeke et al. 2005). One isolate of *Citrobacter* sp. was resistant against trimethoprim/sulfamethoxazole, which is a common drug used to treat urinary tract infections. (Jancel et al. 2002). Forty to fifty percent of *Citrobacter* spp. infections in humans are urinary tract infections (Kasper et al. 2005). One isolate of *Enterobacter* sp. expressed intermediate resistance against ceftiofur and another isolate from the same group was the only isolate to have a MIC equal to 1μg/ml to ceftriaxone, which is the highest value still considered susceptible. These are both third generation cephalosporins and resistance against these should be monitored due to the increase in extended spectrum β-lactamases in the native range of these geckos (Hawser et al. 2009).

Our initial hypothesis that the prevalence of antibiotic-resistant bacteria would increase as gecko density increased was not supported. For the interpretation of our results in terms of density effects on the prevalence of antibiotic-resistant bacteria, the antibiotic resistance patterns

from the isolates from low-D group will be omitted from the group comparisons. Low-D group was compromised of individuals from just batch 2 compared to the other three groups (low-A, med-B, and high-C) which had individuals from both batches. The low-D group was designed to detect any temporal discrepancies. However, since the low-D group did not have the opportunity to mix with individuals from batch 1 and exchange bacteria, it is not reasonable to compare them to the other three groups.

Interestingly, when the data for the individual low-D and combined low-D groups were removed, a trend in the distribution of resistant, intermediate, and susceptible isolates of *Citrobacter* spp. against cefoxitin from the combined treatment groups was visible (Figure 3.4). The distribution of susceptible isolates varied depending on density (Figure 3.4). The treatment groups reflect a range of susceptible, intermediate, and resistant isolates. The low density group is composed of resistant and susceptible isolates while in the medium density group there are resistant, intermediate, and susceptible isolates, and in the high density group there are only resistant and intermediate isolates. There appears to be a shift in the antibiotic resistance pattern such that as density increases, less isolates are susceptible to cefoxitin.

The resistance against cefoxitin among *Citrobacter* spp. isolates increased as the diversity of culturable genera from the combined groups decreased compared to the individual pre-assigned groups (Figure 3.6). The combined groups low-A, med-B, and high-C differed from their respective individual pre-assigned groups by at least 4 or 5 less genera detected (Table 3.1). This is most likely explained by the composition of the combined groups. The diversity of genera decreased among the combined groups (Table 3.1) and *Citrobacter* spp. was the predominant isolate cultured. Therefore, the resistance patterns are heavily influence by this single genus. *Citrobacter* spp. is commonly resistant to cefoxitin, which explains why the resistance against

cefoxitin increased in the combined groups (Figure 3.6). *Citrobacter* spp. was the pre-dominant lactose fermenting *Enterobacteriaceae* genera cultured which is consistent with a study that reported a high prevalence of *Citrobacter* spp. from reptiles from Indonesia (Graves et al. 1988). The increase in resistance against cefoxitin between the individual and combined low-D groups is not noticeable (Figure 3.5). This might be due to the minimal change in diversity of genera cultured between individual low-D and combined low-D. Combined low-D has one less genera then individual low-D.

Commensal bacteria can harbor resistance against antibiotics which can potentially act as a reservoir for other commensal and pathogenic organisms (Blake et al. 2003). Due to the close contact humans have with pets there is a possible route of dissemination of these antibiotic-resistant bacteria to humans. The degree of resistance expressed by an organism is important in determining the best clinical treatment. However, the prevalence of low-level resistance in a population of microbes is also important because they have the potential to contribute to the selection of antibiotic-resistant strains (Andersson et al. 1999; Baquero 2001). An *in vitro* study using *E.coli* harboring β-lactamases challenged by low-level concentrations of cefotaxime selected for low-level resistant variants (Baquero et al. 1996). We examined the potential for decreased susceptibility by dividing the susceptibles into two groups based on the MIC value. Isolates were considered to have *decreased susceptibility* if the MIC value was equal to the highest MIC value still considered susceptible and anything below that value was truly susceptible. For example, the prevalence of *Citrobacter* spp. isolates with decreased susceptibility to chloramphenicol, where the MIC was equal to 8μg/ml, increased for combined group low-A, med-B, and high-C compared to the individual groups. This suggests there was an increase in the amount of isolates expressing low-level resistance. There was no change in low-

level resistance to chloramphenicol between the individual and combined low-D groups. Low-level resistance is typically similar to clinical breakpoints of susceptibility. However, they are based on the epidemiological distribution of antimicrobial susceptibility within the population (Baquero 2001; Schwarz et al. 2010), which is unknown for *Enterobacteriaceae* from tokay geckos. Monitoring low-level resistance to chloramphenicol is important because this drug is used to treat a wide variety of bacterial infections (Brock 1961).

Two isolates of *Enterobacter* spp. (two different species from the same animal) produced similar resistance patterns, expressing resistance against trimethoprim/sulfamethoxazole, nalidixic acid, and gentamicin. These isolates both displayed intermediate resistance to chloramphenicol, and one isolate was resistant to kanamycin and ceftiofur. Of the fifteen total isolates of *Enterobacter* spp., 2/15 were resistant and 5/15 had intermediate resistance to chloramphenicol. Three of the fifteen isolates were resistant to tetracycline and another three were resistant to streptomycin. Even when only a few isolates of *Enterobacter* spp. were recovered, they appeared to be resistant to a more diverse array of antimicrobial classes. It is difficult to say what mechanisms are at work here, but given the breadth of classes of drugs covered, it is likely plasmid-mediated. *Enterobacter* spp. was the third most frequent isolate cultured prior to increasing housing density. The common occurrence of *Enterobacter* spp. and the diversity of antimicrobial resistance of these species should be a concern because of the possibility of exchanging these antibiotic-resistant bacteria with humans (Kasper et al. 2005).

Our data illustrates that imported tokay geckos harbor bacteria resistant to antibiotics. Tokay geckos are insectivorous lizards native to Southeast Asia. The tokay geckos in this study were captured in peri-domestic settings, such as human dwellings or barns. Tokay geckos are commonly found in these habitats in their native range (Corl 1999). It is possible that these

geckos came into direct contact with surfaces contaminated with enteric bacteria from either humans or livestock or indirectly through their prey's contact with human or domestic animal commensal flora. To our knowledge, only one other report exists on the antimicrobial susceptibility of enteric bacteria of reptiles from this geographic region (Graves et al. 1988). That study found a high resistance to chloramphenicol (43%); however, they did not describe the methodology used to test antimicrobial resistance, and thus we cannot compare our results (Schwarz et al. 2010). The high prevalence of chloramphenicol resistance in developing nations, such as Indonesia might explain why we obtained isolates with decreased susceptibility to chloramphenicol, and Graves et al. found a high prevalence of resistance (Okeke et al. 2005).

In this report, we expressed our MICs as clinical breakpoints because we wanted to be able to analyze our results in terms of clinical relevance. We postulated that antibiotic-resistant commensal bacteria from tokay geckos could potentially be acquired by humans in close contact with reptiles. The acquired reptile bacteria could then act as potential reservoir of resistance for commensal flora or pathogens in humans. If we wanted to describe the epidemiological distribution of antimicrobial susceptibility of *Enterobacteriaceae*, we would have referred to MIC values in terms of epidemiological cut-off values (Schwarz et al. 2010). Since we evaluated our data in terms of clinical importance, it is also essential to recognize that susceptibility testing done *in vitro* does not necessarily reflect the *in vivo* phenotype. *In vitro* susceptibility testing can be misleading because the inoculum amount standardized by CLSI does may not necessarily reflect the reality of an *in vivo* infection (Gould et al. 1997). Also it is important to note that a clinical breakpoint is based on the concentration of antibiotic that must be obtained at the site of infection, but it is not always possible to reach the minimum inhibitory concentration. This should be considered when interpreting our results because if an opportunistic infection occurred

in a human as a result from one these microbes, it would be important to know whether the appropriate minimum inhibitory concentration could be reached at the site of infection.

There were several important trends observed in the data. However, due to the high mortality of geckos from batch 1, the sample sizes within our treatment groups decreased and made it difficult to detect statistical significance. In addition, as density increased, the diversity of genera decreased. This made it difficult to compare the same genera between individuals and combined. Comparisons between the combined treatment groups were hindered because the same isolates were not cultured in every group making it difficult to determine the effect, if any, that density had on the antimicrobial resistance patterns of specific genera.

A future study to further determine how the capture, handling, and shipping conditions of the pet trade influences the dispersion of antibiotic resistance determinants should consider using genetic techniques to identify specific resistance mechanisms. Use of specific screening and confirmatory tests for general identification of ESBLs and AmpC β-lactamases would be useful. Another consideration would be to include more time points to get a better idea of whether the composition and antimicrobial susceptibility patterns are constant. Obtaining samples animals that have been maintained for an extended period of time would be interesting to compare whether the presence of resistance diminishes or is maintained after import. Additionally, collecting fecal samples immediately after capture and comparing those resistance patterns to treatment groups would be interesting and provide insight into the effects of stress. Lastly large samples would be beneficial so that between group comparisons for different genera could be done.

CONCLUSIONS

Commensal enteric bacteria from tokay geckos imported for the pet trade display resistance against some common antibiotics. A trend observed from an increase in housing density meant to mimic shipping conditions was the decrease in diversity of a small subset of *Enterobacteriaceae*. The identity of a small subset of culturable lactose positive *Enterobacteriaceae* and the frequency they were cultured influenced the prevalence of antibiotic resistance detected. The combined treatment groups were dominated by *Citrobacter* spp. isolates which commonly possess β-lactamases, which would account for the high prevalence of cefoxitin resistance. Further investigation should be done to determine if the observed resistance against cefoxitin was a result of an AmpC β-lactamase because they are becoming increasingly more common in clinical isolates and have significant implications for clinical therapy. The antibiotic susceptibility patterns that demonstrate resistance against more than one class of antibiotic could be associated with a plasmid. The presence of plasmids could potentially promote the exchange of antibiotic resistance determinants among commensal bacteria and pathogens. The data supports that tokay geckos imported into the United States, as pets, harbor commensal bacteria with antibiotic resistance. Further work is needed to understand whether the close contact between pet owners and reptiles harboring antibiotic-resistant bacteria will facilitate the exchange of these organisms to humans and the potential to transfer antibiotic resistance to human commensal flora and pathogens.

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Table 3.1. Number and Genera of Isolates Cultured from Different Treatment Groups

	Bacteria	Individuals	Combined
A (Low 1)	*Salmonella arizonae*	1	2
	Kluyvera spp.	5	0
	Klebsiella spp.	4	0
	Citrobacter spp.	8	9
	Echerichia coli	1	0
	Enterobacter spp.	2	0
	Pantoea spp.	0	0
	Serratia spp.	1	0
D (Low 2)	*Salmonella arizonae*	0	1
	Kluyvera spp.	0	0
	Klebsiella spp.	1	1
	Citrobacter spp.	9	9
	Echerichia coli	1	0
	Enterobacter spp.	5	0
	Pantoea spp.	0	1
	Serratia spp.	0	0
B (Med)	*Salmonella arizonae*	0	0
	Kluyvera spp.	3	2
	Klebsiella spp.	6	0
	Citrobacter spp.	19	9
	Echerichia coli	3	0
	Enterobacter spp.	3	2
	Pantoea spp.	1	0
	Serratia spp.	2	0
C (High)	*Salmonella arizonae*	0	3
	Kluyvera spp.	1	0
	Klebsiella spp.	16	1
	Citrobacter spp.	39	12
	Echerichia coli	2	0
	Enterobacter spp.	3	0
	Pantoea spp.	0	0
	Serratia spp.	1	0
	Total # of Isolates	137	52

Table 3.2. Table of Antimicrobial Susceptibility for a few Antibiotics to *Citrobacter* spp., *Klebsiella* spp., and *Enterobacter* spp. from Individuals

R = resistance, ≥32 is the MIC breakpoint, I = intermediate resistance, = 16 is the MIC breakpoint, S = susceptible, = 8 is the highest MIC value still considered susceptible, < 8 is the MIC breakpoint considered susceptible

	***Citrobacter* spp. Cefoxitin**		***Citrobacter* spp. Chloramphenicol**		***Citrobacter* spp. Amoxicillin/ Clavulanic Acid**		***Citrobacter* spp. Ampicillin**	
MIC breakpoints	total # of isolates	%	total # of isolates	%	total # of isolates	%	total # of isolates	%
R ≥ 32	60/74	81.1	1/74	1.3	34/74	45.9	5/74	6.7
I = 16	8/74	10.8	25/74	33.8	20/74	27	10/74	13.5
S = 8	4/74	5.4	40/74	54	13/74	17.6	3/74	4
S < 8	2/74	2.7	8/74	10.8	7/74	9.4	56/74	75.7
	***Klebsiella* spp. Cefoxitin**		***Klebsiella* spp. Chloramphenicol**		***Klebsiella* spp. Amoxicillin/ Clavulanic Acid**		***Klebsiella* spp. Ampicillin**	
MIC breakpoints	total # of isolates	%	total # of isolates	%	total # of isolates	%	total # of isolates	%
R ≥ 32	1/27	3.7	0/27	0	1/27	3.7	19/27	70.4
I = 16	0/27	0	1/27	3.7	0/27	0	4/27	14.8
S = 8	2/27	7.4	7/27	25.9	1/27	3.7	4/27	14.8
S < 8	24/27	88.9	19/27	70.4	25/27	92.5	0/27	0
	***Enterobacter* spp. Cefoxitin**		***Enterobacter* spp. Chloramphenicol**		***Enterobacter* spp. Amoxicillin/ Clavulanic Acid**		***Enterobacter* spp. Ampicillin**	
MIC breakpoints	total # of isolates	%	total # of isolates	%	total # of isolates	%	total # of isolates	%
R ≥ 32	11/13	84.6	2/13	15.3	12/13	92.3	6/13	46.1
I = 16	1/13	7.7	3/13	23.1	0/13	0	3/13	23.1
S = 8	0/13	0	3/13	23.1	0/13	0	2/13	15.3
S < 8	1/13	7.7	5/13	38.5	1/13	7.7	2/13	15.3

Table 3.3. Antimicrobial Susceptibility for *E.coli, Kluyvera spp., Serratia spp.,* and *Pantoea spp.* Number of Isolates

R = resistant I.R. = intermediate resistance, FOX = cefoxitin, AUG = amoxicillin with clavulanic acid, KAN = Kanamycin, AMP = ampicillin, CHL = chloramphenicol, TET = tetracycline

	Total # of isolates	R FOX	I.R. FOX	R AUG	I.R. AUG	R Kan	R AMP	I.R. AMP	I.R. CHL	R TET
E. coli	7	1				1	1			
Kluyvera spp.	9						1	5	2	
Serratia spp.	4		4	4			1	1	2	3
Pantoea spp.	1	1			1					

Table 3.4. The Prevalence of Resistance, Intermediate Resistance, and Decreased Susceptibility and Susceptible of *Citrobacte*r spp. to four types of antimicrobials

Treatment Groups		*Citrobacter* spp. Cefoxitin		*Citrobacter* spp. Chloramphenicol		*Citrobacter* spp. Amoxicillin/Clavulanic Acid		*Citrobacter* spp. Ampicillin	
		Individuals	Combined	Individuals	Combined	Individuals	Combined	Individuals	Combined
		%	%	%	%	%	%	%	%
Low A	R ≥ 32	62.5(5/8)	77.8(7/9)	0	0	25(2/8)	22.2(2/9)	12.5(1/8)	0
	I = 16	25(2/8)	0	37.5(3/8)	0	0	0	12.5(1/8)	11.1(1/9)
	S = 8	0	0	62.5(5/8)	77.8(7/9)	75(6/8)	55.5(5/9)	0	22.2(2/9)
	S > 8	12.5(1/8)	22.2(2/9)	0	22.2(2/9)	0	22.2(2/9)	75(6/8)	66.6(6/9)
Low D	R ≥ 32	100(9/9)	100(9/9)	0	0	77.7(7/9)	66.6(6/9)	0	11.1(1/9)
	I = 16	0	0	22.2(2/9)	33.3(3/9)	22.2(2/9)	11.1(1/9)	11.1(1/9)	0
	S = 8	0	0	66.7(6/9)	66.7(6/9)	0	22.2(2/9)	11.1(1/9)	0
	S > 8	0	0	11.1(1/9)	0	0	0	77.7(7/9)	88.8(8/9)
Med B	R ≥ 32	83.3(15/18)	77.8(7/9)	0	0	55.5(10/18)	44.4(4/9)	0	0
	I = 16	16.7(3/18)	11.1(1/9)	33.3(6/18)	22.2(2/9)	22.2(4/18)	11.1(1/9)	22.2(4/18)	22.2(2/9)
	S = 8	0	0	61.1(11/18)	66.7(6/9)	11.1(2/18)	33.3(3/9)	5.6(1/18)	11.1(1/9)
	S > 8	0	11.1(1/9)	5.6(1/18)	11.1(1/9)	11.1(2/18)	11.1(1/9)	72.2(13/18)	66.6(6/9)
High C	R ≥ 32	79.5(31/39)	83.3(10/12)	2.5(1/39)	0	38.4(15/39)	16.6(2/12)	10.2(4/39)	0
	I = 16	7.7(3/39)	8.3(1/12)	35.9(14/39)	16.7(2/12)	35.9(14/39)	50(6/12)	10.2(4/39)	8.3(1/12)
	S = 8	10.3(4/39)	0	46.1(18/39)	58.3(7/12)	12.8(5/39)	25(3/12)	2.6(1/39)	16.6(2/12)
	S > 8	10.3(4/39)	8.3(1/12)	15.4(6/39)	25(3/12)	12.8(5/39)	8.3(1/12)	76.9(30/39)	75(9/12)

Table 3.5. The Prevalence of Resistance, Intermediate Resistance, and Decreased Susceptibility and Susceptible of *Klebsiella* spp. to four types of antimicrobials

Treatment Groups		*Klebsiella* spp. Cefoxitin		*Klebsiella* spp. Chloramphenicol		*Klebsiella* spp. Amoxicillin/Clavulanic Acid		*Klebsiella* spp. Ampicillin	
		Individuals	Combined	Individuals	Combined	Individuals	Combined	Individuals	Combined
		%	%	%	%	%	%	%	%
Low A	R ≥ 32	0	0	0	0	0	0	100(4/4)	0
	I = 16	0	0	0	0	0	0	0	0
	S = 8	0	0	50(2/4)	0	25(1/4)	0	0	0
	S > 8	100(4/4)	0	50(2/4)	0	75(3/4)	0	0	0
Low D	R ≥ 32	0	0	0	0	0	0	0	100(1/1)
	I = 16	0	0	0	0	0	0	100(1/1)	0
	S = 8	0	0	100(1/1)	0	0	0	0	0
	S > 8	100(1/1)	100(1/1)	0	100(1/1)	100(1/1)	100(1/1)	0	0
Med B	R ≥ 32	0	0	0	0	0	0	83.3(5/6)	0
	I = 16	0	0	0	0	0	0	0	0
	S = 8	0	0	33.3(2/6)	0	0	0	16.6(1/5)	0
	S > 8	100(6/6)	0	66.6(4/6)	0	100(6/6)	0	0	0
High C	R ≥ 32	6.25(1/16)	100(1/1)	0	0	6.25(1/16)	0	62.5(10/16)	0
	I = 16	0	0	6.25(1/16)	100(1/1)	0	100(1/1)	18.75(3/16)	0
	S = 8	12.5(2/16)	0	12.5(2/16)	0	0	0	18.75(3/16)	0
	S > 8	81.25(13/16)	0	81.25(13/16)	0	93.75(15/16)	0	0	100(1/1)

Table 3.6. The Prevalence of Resistance, Intermediate Resistance, and Decreased Susceptibility and Susceptible of *Enterobacter* spp. to four types of antimicrobials

Treatment Groups		*Enterobacter* spp. Cefoxitin		*Enterobacter* spp. Chloramphenicol		*Enterobacter* spp. Amoxicillin/Clavulanic Acid		*Enterobacter* spp. Ampicillin	
		Individuals	Combined	Individuals	Combined	Individuals	Combined	Individuals	Combined
		%	%	%	%	%	%	%	%
Low A	R ≥ 32	100(2/2)	0	0	0	100(2/2)	0	0	0
	I = 16	0	0	50(1/2)	0	0	0	50(1/2)	0
	S = 8	0	0	50(1/2)	0	0	0	50(1/2)	0
	S > 8	0	0	0	0	0	0	0	0
Low D	R ≥ 32	100(5/5)	0	40(2/5)	0	100(5/5)	0	60(3/5)	0
	I = 16	0	0	20(1/5)	0	0	0	0	0
	S = 8	0	0	20(1/5)	0	0	0	20(1/5)	0
	S > 8	0	0	20(1/5)	0	0	0	20(1/5)	0
Med B	R ≥ 32	66.6(2/3)	100(2/2)	0	0	66.6(2/3)	100(2/2)	66.6(2/3)	0
	I = 16	0	0	33.3(1/3)	100(2/2)	0	0	0	50(1/2)
	S = 8	0	0	33.3(1/3)	0	0	0	0	50(1/2)
	S > 8	33.3(1/3)	0	33.3(1/3)	0	33.3(1/3)	0	33.3(1/3)	0
High C	R ≥ 32	66.6(2/3)	0	0	0	100(3/3)	0	33.3(1/3)	0
	I = 16	33.3(1/3)	0	0	0	0	0	66.6(2/3)	0
	S = 8	0	0	0	0	0	0	0	0
	S > 8	0	0	100(3/3)	0	0	0	0	0

Table 3.7. Number of Isolates that expressed resistance or intermediate resistance for *E.coli, Kluyvera* spp., *Serratia* spp., & *Pantoea* spp.

		# of isolates	R FOX	I.R FOX	R AUG	IR AUG	R Kan	R AMP	IR AMP	IR CHL	R TET
	Individual	7	1				1	1			
E. coli	Combined	0									
	Individual	9						1	5	2	
Kluyvera spp.	Combined	2							1		
	Individual	4		4	4			1	1	2	3
Serratia spp.	Combined	0									
	Individual	1	1			1					
Pantoea spp.	Combined	1		1							

Table 3.8. The Prevalence of Isolates Resistance or Resistance and Intermediate Resistance against Cefoxitin and Amoxicillin with Clavulanic Acid for Combined versus Individual

Citrobacter spp.					
Treatment Group	(# of total isolates)	% R to Cefoxitin	% R to Amoxicillin w/ Clavulanic Acid	% R to Cefoxitin & Amox w/ Clavulanic Acid	% R Cefoxitin & R & I.R. to Amox w/ Clavulanic Acid
Low-A	Indv (8)	62.5	25	25	25
	Comb (9)	77.7	22.2	22.2	22.2
Low-D	Indv (9)	100	77.7	77.7	100
	Comb (9)	100	66.6	66.6	77.7
Med- B	Indv (18)	83.3	55.5	50	77.7
	Comb (9)	77.7	44.4	44.4	55.5
High-C	Indv (39)	79.5	38.4	38.4	74.3
	Comb (12)	83.3	16.2	16.2	66.6

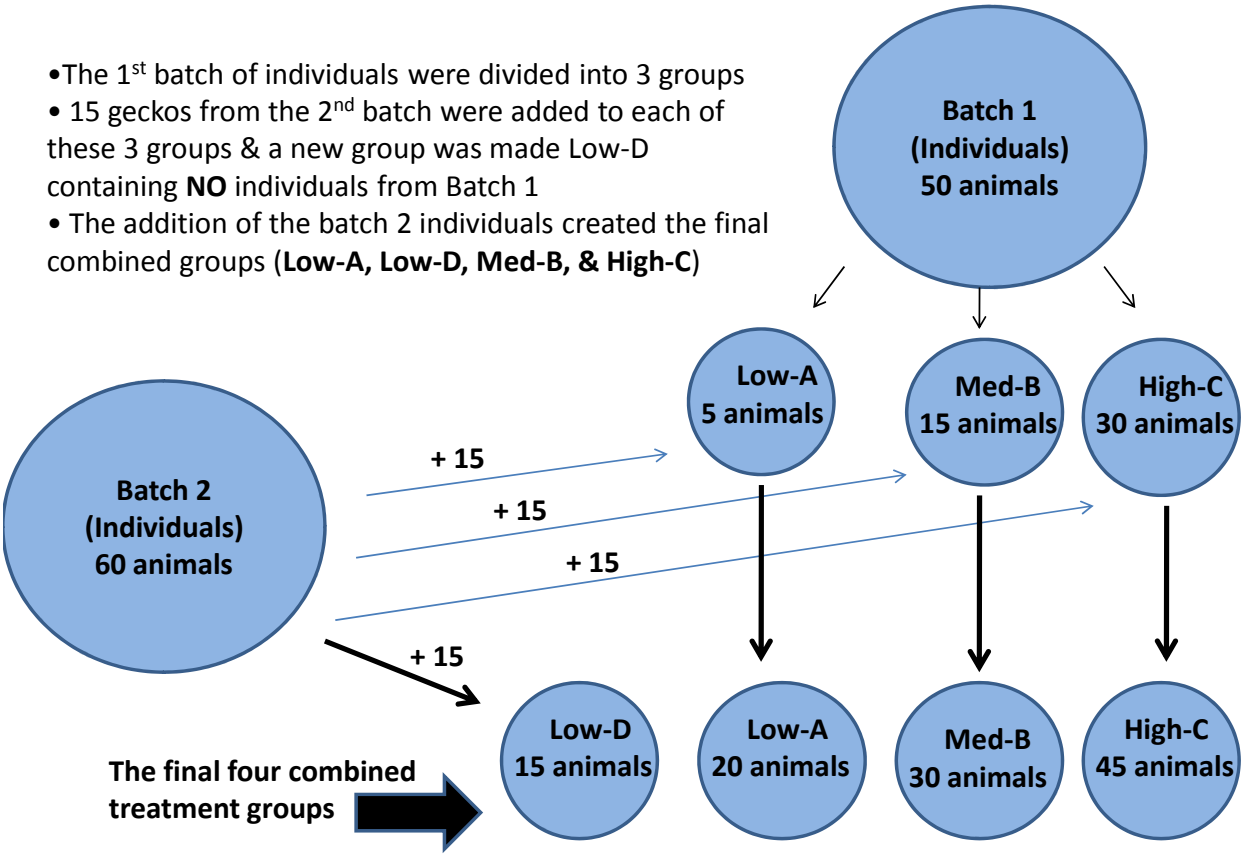

Figure 3.1. Diagram of How Geckos Were Assigned to Different Groups

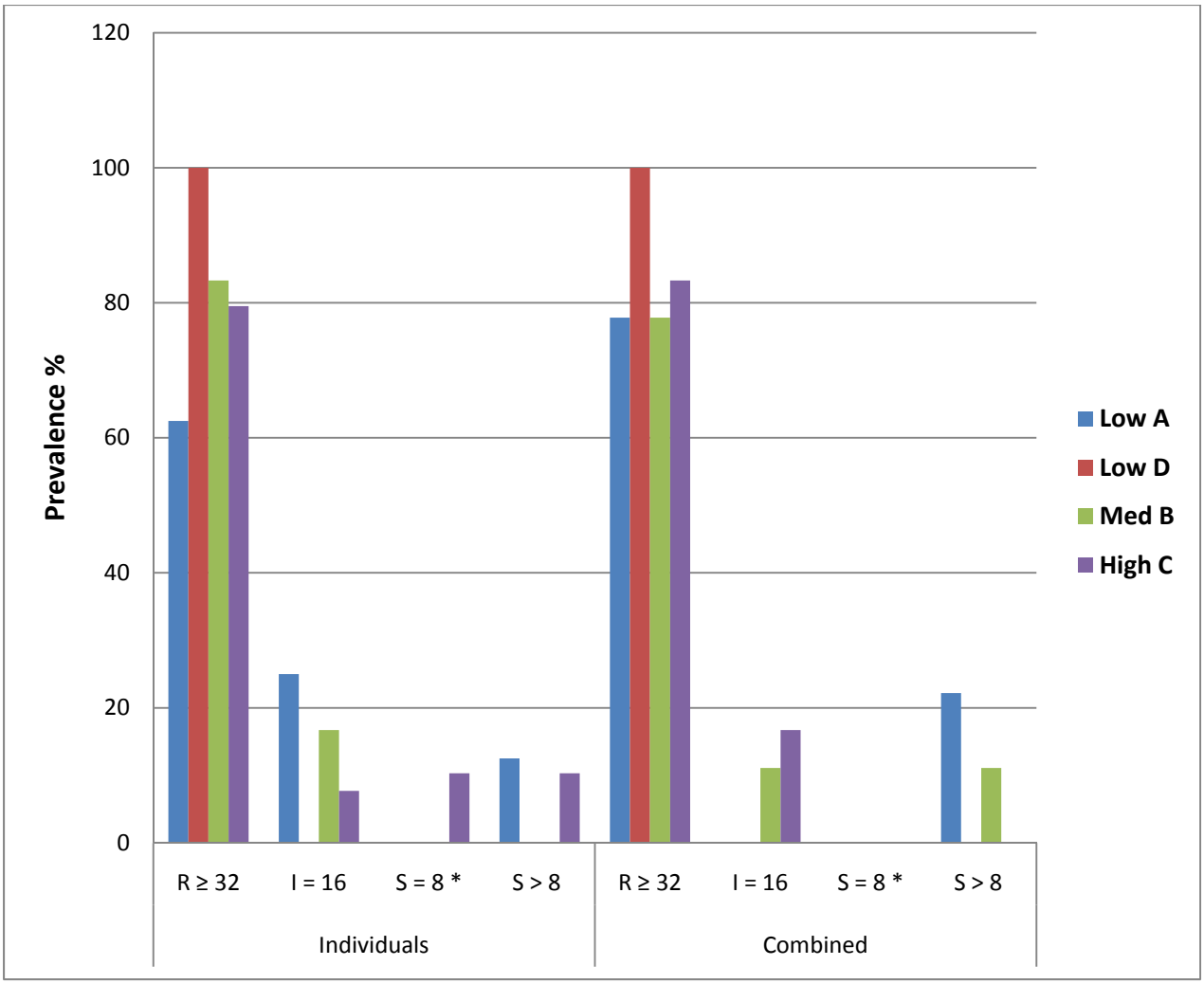

Figure 3.2. The Prevalence of Susceptibility of *Citrobacter* spp. Against Cefoxitin Among Groups

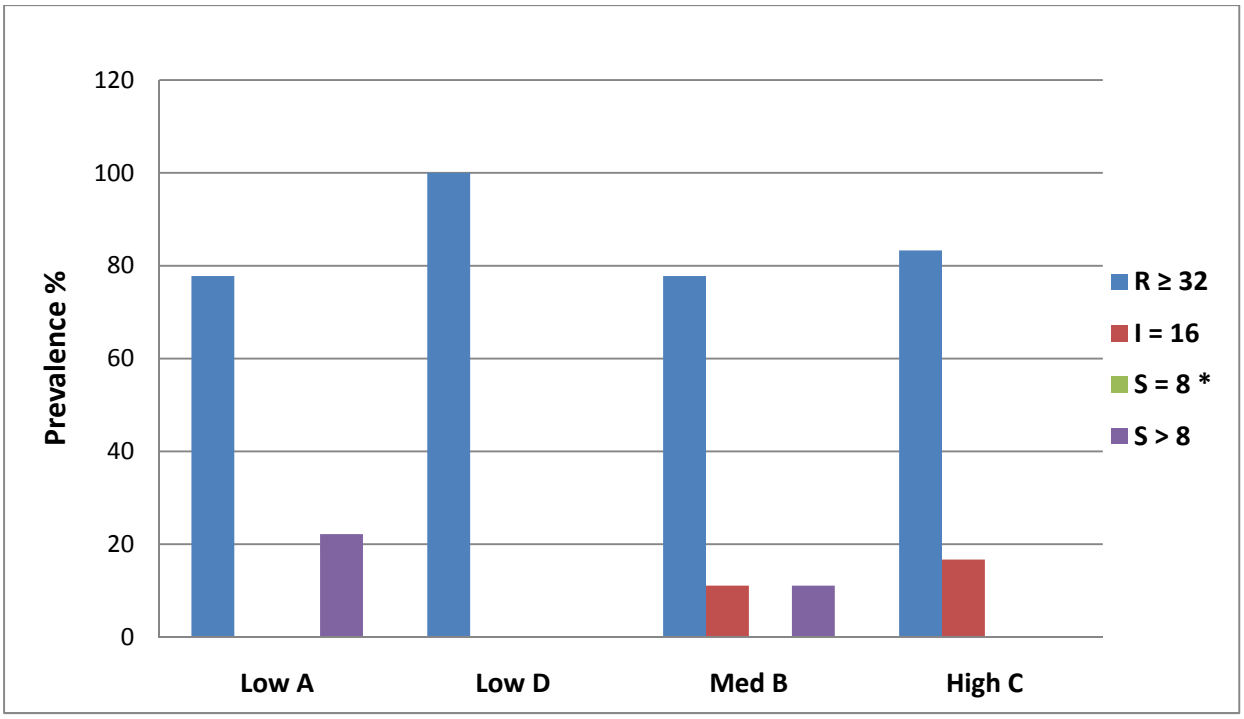

Figure 3.3. The Prevalence of Susceptibility of *Citrobacter* spp. Against Cefoxitin Among Combined Groups

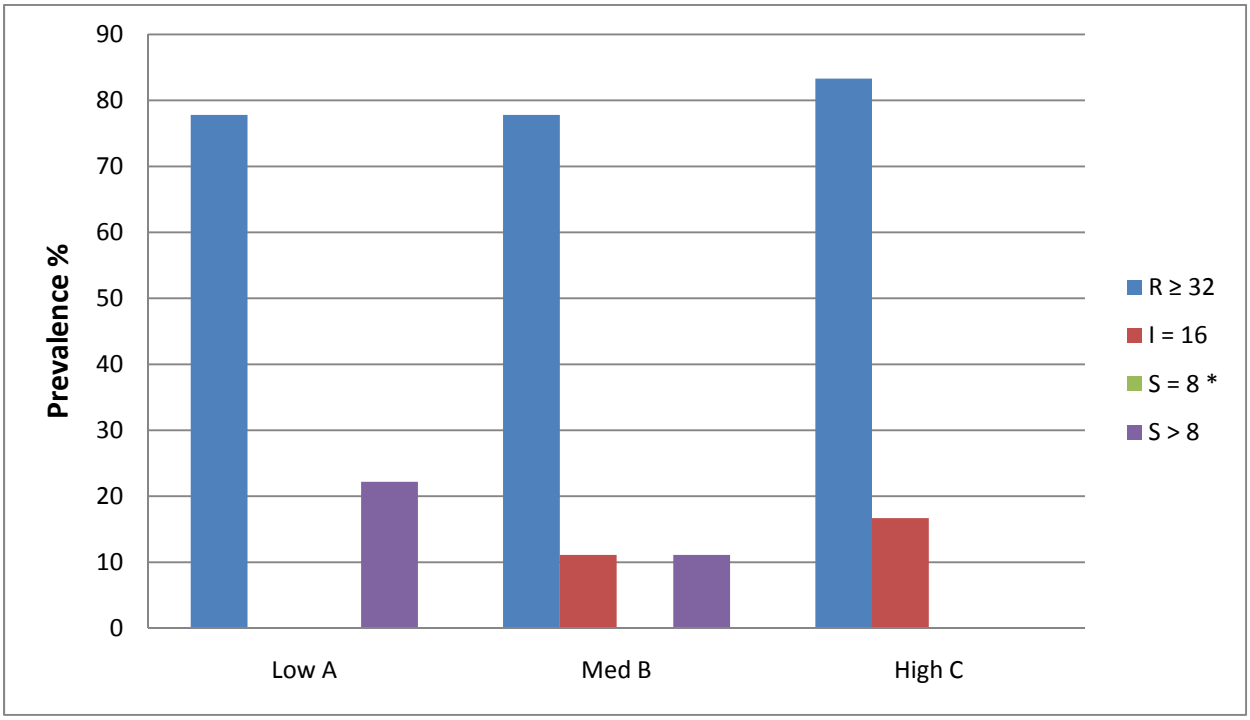

Figure 3.4. The Prevalence of Susceptibility of *Citrobacter* sp Against Cefoxitin Excluding Treatment Low-D

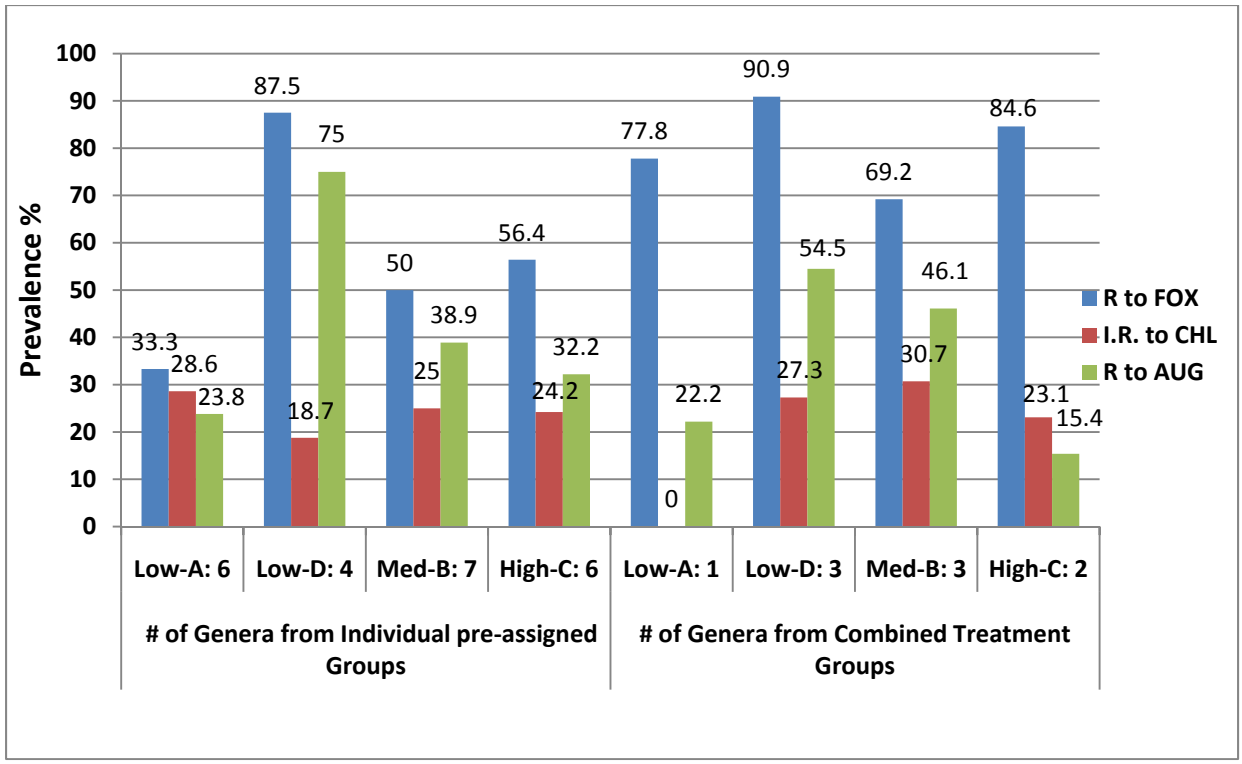

Figure 3.5. Diversity of Genera Compared to the Prevalence of Resistance

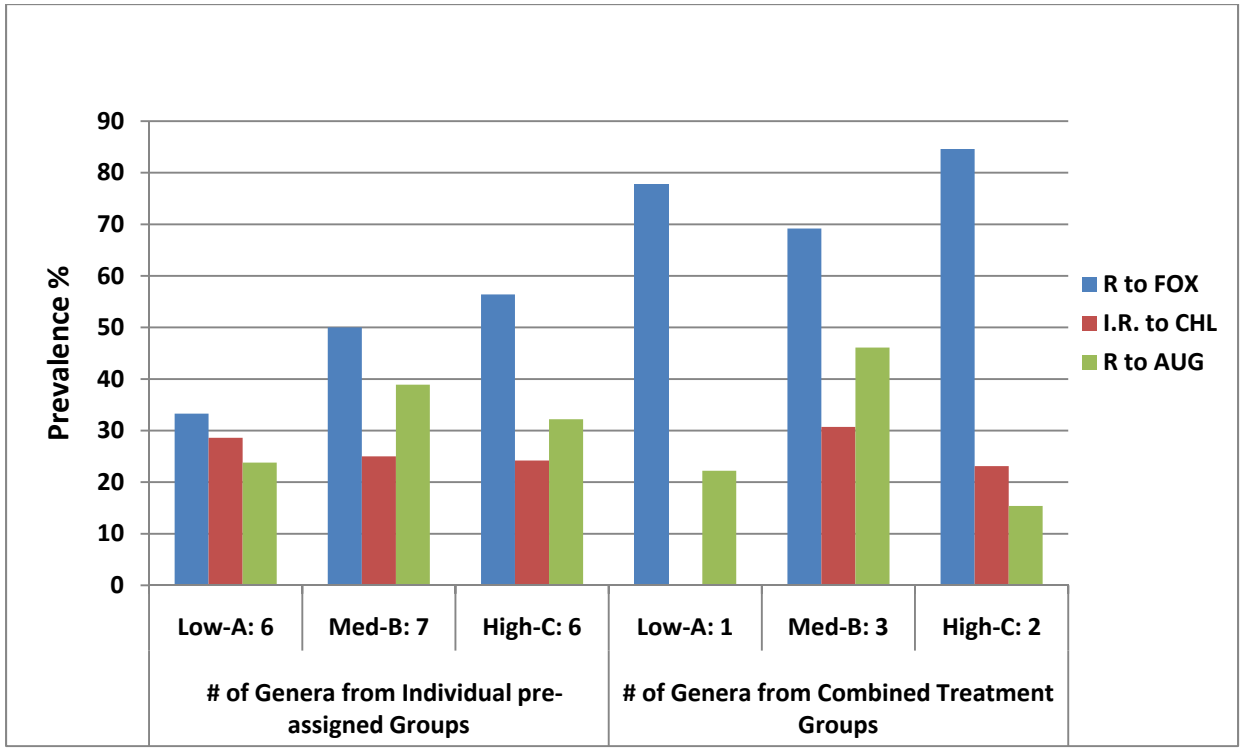

Figure 3.6. Diversity of Genera Compared to Prevalence of Resistance Excluding Low-D

CHAPTER 4

ANTIBIOTIC RESISTANCE OF *SALMONELLA* SPP. CULTURED FROM TOKAY GECKOS

INTRODUCTION

Worldwide infections with *Salmonella* spp. have serious implications for public health. In the United States alone there are approximately 1.4 million cases of salmonellosis annually (Mead et al. 1999). The increase in prevalence of antibiotic resistance and the potential for exchange of antibiotic-resistant bacteria have dire consequences for the treatment of these infections. There are two species, 6 subspecies, and 60 serogroups which are comprised of over 2,400 serotypes of *Salmonella* spp. but only 20 are commonly associated with salmonellosis in humans in the United States. These 20 serotypes are all *Salmonella* species *enterica*. Of the 20 serotypes commonly associated with human salmonellosis, 45% of cases are associated with Typhimurium, Enteritidis, Newport, and Heidelberg. Sporadically, serotype Javiana replaces Heidelberg as the fourth most common serotype (CDC 2006).

Reptile-associated salmonellosis accounts for roughly 5% of all human cases in the United States, 74,000 cases annually (CDC 2003). In 1975 a commercial ban on the sale of turtles of less than 4 inches was established, to reduce the number of reptile-associated salmonellosis cases. This ban decreased the number of cases by an estimated 100,000 cases per year (Cohen et al. 1980). However, as the popularity of reptiles as pets has increased in recent years, (Shepherd 2008) so has the incidence of reptile-associated salmonellosis (Cieslak et al. 1994; Mitchell et al. 2001). *Salmonella* spp. is generally considered to be a part of the normal flora of the gastrointestinal tract (Jackson JR et al. 1971; MacNeill et al. 1986; Jacobson 2007) and less frequently as a cause of clinical salmonellosis in reptiles (Onderka et al. 1985). Forty

percent of all *Salmonella* spp. serotypes have been predominately cultured from reptiles and are rare in other animals (Mermin et al. 2004). A review of several studies reported on the types of *Salmonella* spp. serotypes present in both captive and wild reptiles from all different geographic regions (Jacobson 2007). In general, subspecies III and IV are cultured from reptiles (Sanyal et al. 1997; Briones et al. 2004; Pasmans et al. 2005; Bauwens et al. 2006; Pedersen et al. 2009). The variety of serotypes recovered is difficult to define because it is dependent on many variables like species, captive versus wild, geographic location, and other factors.

The United States alone reportedly imports several million wild caught reptiles annually for commercial use (Brown 2004; Schlaepfer et al. 2005). A review completed by the US Fish and Wildlife Service's Law Enforcement Management Information System (LEMIS) suggested that 69% of the live animals imported into the United States between 2000 and 2006 originated from Southeast Asia, an area of the world experiencing increased prevalence of antimicrobial resistance (Okeke et al. 2005; Hawser et al. 2009). The movement of wildlife provides mechanisms for the dispersion of pathogens (Karesh et al. 2007). It also has the potential to distribute reptiles with an enormous diversity of *Salmonella* spp. serotypes, some which may be resistant to antibiotics. Most of the information we have about the serotypes recovered from reptiles is from captive populations and less is known about those from wild populations (Jacobson 2007). The research described in this report provides unique insight into the composition of culturable *Salmonella* spp. serotypes and antimicrobial susceptibility profiles of isolates from a wild population of tokay geckos destined for use in the pet trade. Tokay geckos utilized in this study were collected from their native range, Indonesia, where they are often found living among houses or agricultural buildings. This creates a unique circumstance where the geckos are potentially exposed to the commensal flora of humans and domestic livestock. We

also hypothesized that *Salmonella* spp. cultured from these geckos would express antibiotic resistance, and the prevalence of resistance would increase as we experimentally increased the density of geckos. We expected the prevalence of *Salmonella* spp. to increase due to the stress of overcrowding, which has been shown with the shedding of *Salmonella* spp. in livestock (De Passillé et al. 2005; Ball et al. 2011). The geckos were expected to be more stressed when housed with other geckos at varying densities because tokay geckos are typically solitary animals and aggressive. Our objective was to describe the antibiotic resistance profiles of *Salmonella* spp. cultured from individually housed geckos immediately after importation and then again after geckos had been housed at different densities.

METHODS

Animal Collection, Shipping, and Housing

Wild caught tokay Geckos were imported from an international wildlife distributor based in Indonesia. Two batches of geckos were captured by the distributor from two locations on the island of Java. Once captured, the geckos were housed individually and shipped to the University of Georgia's College of Veterinary Medicine. All animal handling and care was approved by the University of Georgia's Institutional Animal Use Care Committee. Animals were housed in temporary mouse containers then transferred to enclosures measuring 24"W x 36"D x 72" H. They were fed a standard diet of crickets and mealworms. The temperature was 26.6º±4ºC, the humidity was maintained at 50% - 70% and the lighting was on for a 12 hour period. They were monitored twice a day. The first batch arrived in March 2009. They were housed individually and fecal samples were collected. The geckos remained individually housed for 10 days to allow feces to be collected from each animal. After feces were collected, individuals were randomly assigned to different groups with varying densities: Group A represented a "low" density and

contained 5 animals, Group B was a "medium" density and contained 15 animals, and Group C was a "high" density and contained 30 animals. A second batch of individually-housed geckos was imported in June. Once all fecal samples were collected from this group, 15 geckos were assigned to each of the pre-existing groups (A, B, and C) and a fourth group D was created. Group D contained 15 animals (low density). In September, fecal samples were collected from all remaining geckos and they were then humanely euthanized in accordance with our AUP.

Isolation and Identification

For *Salmonella* spp. isolation, fecal samples were enriched by inoculation into tetrathionate brilliant green broth (TTB) (Difco; Detroit, MI) and dulcitol selenite media and incubated at 41.5°C for 18 hours. Enrichment broths were streaked onto *Salmonella* spp. selective media (XLT4 and BGN bi-plates) (Difco) followed by 37°C incubation overnight. Four to five H_2S positive colonies were used to inoculate Triple Sugar Iron (TSI) slants (Difco), which were incubated overnight at 37°C. A delayed-secondary enrichment was done for those samples that cultured negative following primary enrichment with TTB. *Salmonella* spp. isolates were forwarded to the National Veterinary Service Laboratory at Ames, Iowa for definitive serotyping. As part of a concurrent study on the diversity of *Enterobacteriaceae* of tokay geckos, a loop of the fecal sample was streaked on MacConkey plates. On rare occasions, a lactose positive *Salmonella* spp. was isolated that wasn't detected by the above method. These isolates were identified as *Salmonella arizonae* by rapid Analytical Profile Index (API20E) strips (Biomerieux, Durham, NC).

Antibiotic Susceptibility

Minimum Inhibitory Concentrations (MIC) were determined by using broth microdilution methods described by the Clinical Laboratory Standards Institute (CLSI)

guidelines (CSLI 2008) and validated dry-form panels (CMV1AGNF) produced by TREK Diagnostics, Cleveland, OH. Aseptic technique was used to transfer colonies into sterile water to create a 0.5 Mcfarland standard. 10µl of this solution was added to 10ml of cation adjusted Mueller-Hinton broth and vortexed. 50µl of the inoculated Mueller Hinton broth was pipetted into each well of the MIC plate. Multiple classes of antimicrobials were tested. We selected a standard susceptibility plate that contained antibiotics that reportedly inhibit the Gram negative enteric bacteria we expected to culture, which included *Salmonella* spp. and other lactose positive *Enterobacteriaceae* as part of another study. The antibiotics and the concentrations included on the plate were amikacin (0.5- 32µg/ml), ampicillin (1-32µg/ml), amoxicillin/ clavulanic acid (1/0.5-32/16µg/ml), cefoxitin (0.5-32µg/ml), ceftriaxone (0.25-64µg/ml), ceftiofur (0.12-8µg/ml), chloramphenicol (2-32µg/ml), ciprofloxacin (0.015-4µg/ml), gentamicin (0.25-16µg/ml), kanamycin (8-64µg/ml), nalidixic Acid (0.5-32µg/ml), sulfisoxazole (16-256µg/ml), streptomycin (32-64µg/ml), trimethoprim/sulfamethoxazole (0.12/2.38-4/76µg/ml), and tetracycline (4-32 µg/ml). The plates were incubated at 37°C±2° for 18 hours. Interpretations of susceptibility for all antimicrobials tested were compared to CLSI criteria (CLSI 2010) for *Enterobacteriaceae*. *Escherichia coli* ATCC 25922 was routinely included as a quality control.

RESULTS

There were a total of 88 isolates from 186 animals. The 110 individually housed geckos produced 42 isolates of *Salmonella* spp. while 76 geckos from the combined housing groups produced 46 isolates. Four animals shed more than one serotype of *Salmonella* spp. There were 14 different serotypes cultured from individuals and 8 serotypes cultured from the combined groups, for a total of 17 different serotypes (Table 4.1). Five serotypes were cultured from both the individuals and the combined geckos. Nine serotypes recovered from the individuals were

not cultured from the combined geckos. Likewise, three serotypes cultured from the combined groups were not cultured from the individuals (Table 4.1). *Salmonella enterica* serotype Fresno (29%) and *Salmonella enterica* serotype Houten (21%) were the two most common serotypes and *Salmonella enterica* serotypes Weltevreden (9%) and Adelaide (9.5%) were tied for third most common serotypes cultured from the individual animals. *Salmonella enterica* serotype Houten (46%), *Salmonella enterica* serotype Apapa (17%), and *Salmonella enterica* subspecies *arizonae* (13%) were the first, second and the third most common serotypes cultured from the combined groups, representing 76% of the isolates cultured from the combined groups.

Of the 88 isolates of *Salmonella* spp., 7 expressed resistance to one or more antibiotics (Table 4.2). Five of the isolates were cultured from individuals and two were from the combined groups, both were *S. arizonae*. All four isolates of *Salmonella enterica* serotype Adelaide cultured from the individuals were resistant to nalidixic acid. One isolate of *Salmonella enterica* serotype Fresno was resistant to amoxicillin with clavulanic acid, and streptomycin and expressed intermediate resistance to chloramphenicol, ceftiofur, and kanamycin. Additionally, this isolate had reduced susceptibility to both amikacin and ampicillin. One isolate of *S. arizonae* expressed resistance to cefoxitin and ampicillin and intermediate resistance to amoxicillin with clavulanic acid. In addition to the susceptibility patterns, 43 other isolates were resistant to sulfisoxazole and a total of 53 isolates had decreased susceptibility, an MIC of 8, to chloramphenicol (Table 4.2).

DISCUSSION

Our results are consistent with the findings of previous studies that demonstrate there is a large amount of diversity in the serotypes of *Salmonella* spp. recovered from reptiles (Briones et al. 2004; Pedersen et al. 2009). We cultured *Salmonella* spp. from 84/186 animals (45.6%). Four

animals produce two serotypes for a total of 88 isolates. This prevalence of *Salmonella* spp. recovered in our study can be broken down into the prevalence of *Salmonella* spp. shed upon arrival while geckos were housed individually (38.2%, 42/110) and after being in captivity while housed with other geckos (60.5%, 46/76). The prevalence of *Salmonella* spp. shed from wild reptiles varies widely (Briones et al. 2004; Richards et al. 2004). This is probably due to the variability associated with the species sampled, geographic range, and other factors. Two examples of the prevalence of *Salmonella* spp. recovered from geckos and lizards are: 30% from 90 intestinal samples of tokay geckos and *Hemidactylus* sp. collected from houses in Nigeria and 40.9% in fecal samples from wild lizards in Spain (Oboegbulem et al. 1985; Briones et al. 2004). The prevalence of *Salmonella* spp. shed upon arrival while geckos were housed individually (38.2%) is similar to previously reported rates. However, our results are different from the prevalence reported (23%) from 114 intestinal samples of tokay geckos caught from homes in Singapore (Murphy et al. 1993). The prevalence of *Salmonella* spp. after the geckos were housed in varying densities (60.5%) is similar to high rates of shedding previously reported from captive reptiles, such as 50% in lizards from a zoo in Italy, 62.8% in pet lizards in Taiwan, and 66.1% in pet lizards from Japan (Corrente et al. 2004; Nakadai et al. 2005; Chen et al. 2010). One explanation for reptiles having higher prevalence of *Salmonella* spp. shedding is the stress associated with captivity (Hoelzer et al. 2011).

The prevalence of shedding appears to increase after geckos have been housed together in captivity (Hoelzer et al. 2011). This should be of concern to their owners or handlers since reptiles often shed *Salmonella* spp. without showing clinical signs (Jacobson 2007), particularly since reptiles are increasing in popularity as pets (Shepherd 2008). Additionally, exotic pet reptiles are commonly imported from a wide variety of geographic locations and may be

important carriers of *Salmonella* spp. serotypes associated with their native range (Murphy et al. 1993; Karesh et al. 2005; Schlaepfer et al. 2005).

We documented a change in the diversity of serotypes cultured from animals when they were housed individually compared to combined housing. Interestingly, two isolates of *Salmonella enterica* serotype Newport, the third most common serotype associated with salmonellosis in humans in United States (CDC 2006), were isolated from the individual group and two isolates of serotype Newport were cultured from animals in the combined treatment groups. *Salmonella enterica* serotype Newport has been commonly isolated from poultry sources, other food production animals, and humans (Baudart et al. 2000). Similarly, another serotype recovered from individual geckos, *Salmonella enterica* serotype Adelaide, has been cultured from poultry (CDC 2006). Perhaps, the detection of these two serotypes among individuals is due to the exposure to domestic animals or humans in peri-domestic settings where tokay geckos are often found in their native range. Another interesting serotype that was only cultured from the individual geckos was *Salmonella enterica* serotype Weltevreden, commonly reported from geckos in Asia, Australia, and Nigeria (Oboegbulem et al. 1985; Murphy et al. 1993; Callaway et al. 2010) and specifically it is the serotype most commonly reported in Southeast Asia. In addition to being reported in reptiles, it is commonly reported from well water, animals products, and vegetables (Thong et al. 2002). In fact, from 1993 to 2002 serotype Weltevreden was the most common cause of human salmonellosis in Thailand (Bangtrakulnonth et al. 2004). The World Health Organization (WHO) reports that serotype Weltevreden is largely restricted to Southeast Asia (Galanis et al. 2006). In the United States, serotype Weltevreden is usually associated with imported foods from Asia (Zhao et al. 2006; Ponce et al. 2008). Tokay geckos imported from the pet trade appear to be a new way that this serotype is imported into the

USA. Of the four isolates of Weltevreden we obtained, none were resistant to any of the antibiotics we tested. However, one study published an overall resistance of 9.5% for Weltevreden against one or more antibiotics such as ampicillin (1.8%), chloramphenicol (1.6%), nalidixic acid (1.6%) and trimethoprim (1.4%) (Aarestrup et al. 2003).

A common serotype associated with reptiles, Houten, was isolated with increasing prevalence from the individuals compared to combined groups. The serotypes that were cultured from the combined groups are more commonly associated with reptiles, like *Salmonella enterica* serotype Apapa, *Salmonella enterica* subspecies *houtenae* serotype Houten and *Salmonella enterica* subspecies *arizonae* (Sanyal et al. 1997; Willis et al. 2002; Cooke et al. 2009), which were also the three most prevalent serotypes in the combined groups. The prevalence of *S. arizonae* increased from 2.4% in the individuals to 13% cultured from the combined animals. Whether this was due to added stress associated with density or simply the increased contact time with other animals which facilitated the exchange of commensal bacteria is unknown, but the importance is the increase in prevalence of a well known causative agent of reptile-associated salmonellosis in humans (Mahajan et al. 2003). In addition to the change from human or non-human serotypes to more commonly reptile associated serotypes between the individually housed animals and the combined, there was also a decrease in diversity of serotypes cultured. Overall, 14 different serotypes were cultured from the individual geckos compared to only 8 serotypes cultured from the combined groups.

The lack of detection of resistance among the *Salmonella* spp. isolates is reassuring, especially since there has been an increase in the tokay geckos native range of resistance against quinolones in *Salmonella enterica* serotype Typhi and Extended Spectrum β-lactamases (ESBLs) in *Klebsiella pneumonia* and *E. coli* (Okeke et al. 2005; Hawser et al. 2009). However, there

were still a few isolates that expressed unique resistance patterns of concern. Four isolates of *Salmonella* serotype Adelaide were resistant against nalidixic acid out of 42 isolates cultured from the individuals upon arrival (9.5%). This is of particular concern because nalidixic acid is a first generation quinolone. Later generations of fluoroquinolones, like ciprofloxacin, are commonly used to treat invasive *Salmonella* spp. infections (Piddock et al. 1998). In some instances, resistance against nalidixic acid in *Salmonella* spp. has been shown to precede resistance against fluoroquinolones (Turnidge 1995). Additionally, some isolates resistant to nalidixic acid have shown decreased susceptibility to fluoroquinolones, like ciprofloxacin (Herikstad et al. 1997; Stevenson et al. 2007). One study investigated 9 clinical strains of serotype Typhi from Indonesia collected in 2006 and 8 strains from 2008. They found that all 9 strains from 2006 were susceptible to all antibiotics tested, including nalidixic acid, while all 8 strains from 2008 were resistant to nalidixic acid and ampicillin (Yanagi et al. 2009). Another study that collected strains of serotype Typhi from multiple countries across Asia reported an increase in nalidixic acid resistance from 5% to 51% from 2002 to 2004 (Chau et al. 2007). The prevalence of resistance against nalidixic acid from non-Typhi *Salmonella* spp. isolates was reported as zero from pet lizards from Taiwan and 13.1% from captive reptiles in Italy (Corrente et al. 2004; Chen et al. 2010). The prevalence of nalidixic acid resistance we detected (9.5%) is a public health concern because infection with such strains could lead to clinical failure due to a decrease in susceptibility to ciprofloxacin, a drug commonly used to treat *Salmonella* spp. infections. In immunocompromised individuals, like the elderly and children, non-Typhi *Salmonella* spp. infections can be devastating if not treated (Salyers et al. 1994; Janda et al. 2006). The tokay geckos in this study were destined for the pet trade and are often purchased for households with children.

Another interesting finding was an isolate of *S. arizonae* which expressed resistance to cefoxitin and ampicillin and intermediate resistance to amoxicillin and clavulanic acid. Cefoxitin is a second generation cephalosporin, which is not used to treat *Salmonella* spp. infections. However, like fluoroquinolones, expanded-spectrum cephalosporins are the other drug of choice for treatment. While we did not observe any resistance to the third generation cephalosporin we tested, ceftriaxone, the observed resistance to cefoxitin could potentially indicate a mechanism that would allow for the selection of isolates resistant to the third generation cephalosporins *in vivo* but may not be observed *in vitro* (Livermore 1987). Any isolates of *Salmonella* spp. that could potentially be resistant to a third generation cephalosporin warrant attention due to the clinical relevance and increase in resistance to expanded-spectrum cephalosporins in recent years (Winokur et al. 2000; Su et al. 2005). Further investigation is required to determine the specific mechanism behind this observed resistance.

Lastly one isolate, serotype Fresno, expressed resistance or intermediate resistance to several classes of drugs including aminoglycosides, β-lactam/β-lactamases inhibitor combination, chloramphenicol, and had decreased susceptibility to a third generation cephalosporin used in veterinary medicine, ceftiofur, and trimethoprim/sulfamethoxazole. The resistance to a variety of antimicrobials is a common finding for plasmid-mediated resistance. Without genetic typing it is difficult to determine what type of plasmid is responsible for the resistance pattern observed with this isolate. Chloramphenicol, ampicillin, and trimethoprim/ sulfamethoxazole were common drugs used to treat salmonellosis infections prior to the emergence multidrug in the late 1980's (Kasper et al. 2005; Yanagi et al. 2009). The decreased susceptibility to a veterinary third generation cephalosporin is interesting, potentially resulting

from previous exposure to domestic animals receiving antibiotic treatment in the native range of these tokay geckos.

There were some interesting trends in the changes of serotypes recovered from the individuals upon arrival compared to combined groups of geckos. Further research that includes more time points is required to determine if this trend we observed was a fluctuation or truly a stable pattern. Future studies into how the importation of reptiles for the pet trade under stressful conditions influences the shedding of *Salmonella* spp. could include determining the mechanisms that confer resistance in these isolates via molecular techniques. Collecting samples from the geckos immediately after capture and prior to importation would provide a unique insight into the types of serotypes shed prior to the stress of shipping. Another follow-up study could also investigate differences in the prevalence of shedding of *Salmonella* spp. between species, since, for example, turtles and snakes shed different serotypes and at different prevalences (Jacobson 2007).

CONCLUSIONS

A major finding was the change in prevalence and serotypes present between the individual geckos that had just arrived from their native range compared to geckos that had been housed together for several months under stressful conditions similar to the transport conditions of the pet trade. We observed an increase in prevalence from 38.2% in individuals to 60.5% from combined geckos. Further work is needed to confirm this trend, but our data supports that transport and captivity stress may increase the prevalence of *Salmonella* spp. shed (Hoelzer et al. 2011). The serotypes Newport, Weltevreden, and Adeleida were all cultured from the individual geckos. Of these three serotypes, only Newport was cultured again in the combined groups. It is important to note that serotype Newport, the third most common serotype associated with

salmonellosis in humans in the United States, was detectable in both individuals and combined groups (CDC 2006). Perhaps the presence of these serotypes is indicative of the contact between geckos and domestic animals or humans in their native range, especially since Weltevreden is one of the most common serotypes found in Southeast Asia (Thong et al. 2002). To, our knowledge this is the first report that has detected the common Southeast Asian serotype Weltevreden in geckos.

It is important to keep monitoring the serotypes and antimicrobial susceptibility patterns of *Salmonella* spp. imported with reptiles because they commonly shed these pathogenic organisms without showing clinical signs (Jacobson 2007). This study was important because it provided insight about the serotype composition and the resistance profiles of *Salmonella* spp. from animals originating from Southeast Asia. There is a concern because Southeast Asia is known to have an increased prevalence of resistance to quinolone and ESBLs. Tokay geckos usually live in close proximity to humans and domestic animals, which might increase the possibility of exchange of antibiotic-resistant bacteria with humans and domestic animals. The resistance noted in some isolates is of concern because it was against antimicrobials commonly used to treat *Salmonella* spp. infections in both children and adults. If antibiotic-resistant bacteria are exchanged with humans, they could serve as reservoirs of resistance to human commensal flora or pathogens. Finally, our data suggests that, due to the health risks posed, commensal bacteria harbored by reptiles imported from Southeast Asia should be screened for antimicrobial resistance.

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Table 4.1. Number of Serotypes Present Among Different Groups

Serotype	Individual				Total	Serotype	Combined				Total
	Low - A	Low-D	Med-B	High-C			Low - A	Low-D	Med-B	High-C	
Adelaide			2	2	4	Adelaide					
Apapa	1			1	2	Apapa		6		2	8
Bangkok				1	1	Bangkok					
Eastbourne		1			1	Eastbourne					
Fresno	4	1	2	5	12	Fresno	4				4
Houten		4		5	9	Houten	2	2	9	8	21
Lexington			1		1	Lexington					
Lohbruegge			1		1	Lohbruegge					
Newport				2	2	Newport				2	2
Orientalis				1	1	Orientalis					
Oslo	1			1	2	Oslo					
IV Rough_O:z4,z23						IV Rough_O:z4,z23	1	1		1	3
Rough O:m,t:-						Rough O:m,t:-		1			1
Rubislaw				1	1	Rubislaw					
S. arizonae	1				1	S. arizonae	2	1		3	6
Weltevreden			3	1	4	Weltevreden					
Waycross						Waycross				1	1
Total	7	6	9	20	42 (47.7% of 88)	**Total**	9	11	9	17	46 (52.3% of 88)
# Isolates/ total Indv (42)	16.6%	14.3%	21.4%	47.6%		# Isolates/ total Comb (46)	19.5%	23.9%	19.5%	36.9%	

Table 4.2. The Resistance Profiles of Serotypes of *Salmonella* spp.
FOX(Cefoxitin), AMI(Amikacin), CHL(Chloramphenicol), TET(Tetracycline), AXO (Ceftriaxone), AUG(Amoxicillin/ Clavulanic Acid), CIP(Ciprofloxacin), GEN(Gentamicin), NAL(Nalidixic Acid), TIO(Ceftiofur), FIS(Sulfisoxazole), SXT(Trimethoprim/ Sulfamethoxazole), KAN(Kanamycin), AMP(Ampicillin), STR(Streptomycin)

	Group ID	Serotype or Subspecies	FOX	AMI	CHL	TET	AXO	AUG	CIP	GEN	NAL	TIO	FIS	SXT	KAN	AMP	STR
Individual	B11	Adelaide	4	1	8^{*S}	< 4	<0.25	< 1	0.25	<0.25	**> 32^{R}**	1	**>256^{R}**	<0.12	< 8	< 1	< 32
	B 13	Adelaide	4	1	8^{*S}	< 4	<0.25	< 1	0.25	<0.25	**> 32^{R}**	1	**>256^{R}**	<0.12	< 8	< 1	< 32
	C 3	Adelaide	4	1	8^{*S}	< 4	<0.25	< 1	0.25	<0.25	**> 32^{R}**	1	**>256^{R}**	<0.12	< 8	< 1	< 32
	C 4	Adelaide	4	1	8^{*S}	< 4	<0.25	< 1	0.25	<0.25	**> 32^{R}**	1	**>256^{R}**	<0.12	< 8	< 1	< 32
	C 26	Fresno	2	**16^{*S}**	**16^{I}**	< 4	0.5	**32^{R}**	0.25	2	8	**4^{I}**	128	1	**32^{I}**	8^{*S}	**>64^{R}**
Combined	A 15	S. arizonae	4	2	4	< 4	<0.25	<1	0.03	<0.25	2	1	**>256^{R}**	<0.12	< 8	< 1	**64^{R}**
	D 9	S. arizonae	**>32^{R}**	1	8^{*S}	< 4	<0.25	**16^{I}**	0.12	0.5	2	1	**>256^{R}**	<0.12	< 8	**32^{R}**	<32

CHAPTER 5

CONCLUSIONS

The commercial pet trade is a massive industry and the United States is a major importer of wildlife for commercial use. The majority of these animals originate from wild populations in Southeast Asia. The movement of wild animals in the pet trade is a significant problem because it has the potential to introduce pathogens. It is also plausible that the pet trade can introduce commensal bacteria harboring antimicrobial resistance genes that could also be disseminated via the pet trade. We hypothesized that the pet trade, particularly the conditions under which wildlife are captured and transported, has the potential to influence the composition of commensal flora and the prevalence of antibiotic-resistant bacteria.

To achieve our goal of investigating the potential effects of stressful shipping conditions associated with the pet trade on commensal flora, we described changes in composition and antimicrobial susceptibility. Our results indicate that the commensal enteric flora of tokay geckos is similar to previous studies of reptiles, but provides a unique description of commensal enteric flora of specifically this species. Normal flora has many benefits to health and our research suggests that stress alters a small subset of the normal flora, decreasing diversity of commensal enteric bacteria and promoting the shedding of *S. arizonae*. The increased prevalence of *S. arizonae* is an important finding since reptile-associated salmonellosis a public health concern (CDC 2003). Additionally, the popularity of reptiles as pets is increasing, which increases the risk of reptile-associated salmonellosis. This a public health concern, especially when these pets are placed in the homes of young children or immunocompromised individuals.

The identity of a small subset of culturable lactose positive *Enterobacteriaceae* and the frequency with which they were cultured influenced the prevalence of antibiotic resistance detected. The combined treatment groups of geckos were dominated by *Citrobacter* spp. isolates which commonly possess β-lactamases, which could account for the high prevalence of cefoxitin resistance. Further investigation should be done to determine if the observed resistance against cefoxitin was a result of an *ampC* β-lactamase because they are becoming increasingly more common in clinical isolates and have significant implications for clinical therapy. Several antibiotic susceptibility patterns of lactose fermenting *Enterobacteriaceae* and non-lactose fermenting *Salmonella* spp. demonstrate resistance against more than one class of antibiotics, which could be indicative of a plasmid-mediated resistance. Future work should investigate the mechanisms behind the resistance patterns observed.

A major finding was the change in prevalence and serotypes *Salmonella* spp. present between the individual geckos that had just arrived from their native range compared to geckos that had been housed together for several months under stressful conditions similar to the transport conditions of the pet trade. We observed an increase in prevalence in *Salmonella* spp. shedding from 38.2% in individuals to 60.5% from combined geckos. Further work is needed to confirm this trend, but our data supports that transport and captivity stress may increase the prevalence of *Salmonella* spp. shed. The serotypes Newport, Weltevreden, and Adeleida were all cultured from the individual geckos. Perhaps the presence of these serotypes is indicative of the contact between geckos and domestic animals or humans in their native range, especially since Weltevreden is one of the most common serotypes found in Southeast Asia (Thong et al. 2002). To, our knowledge this is the first report that has detected the common Southeast Asian serotype

Weltevreden in geckos. It is important to keep monitoring the serotypes and antimicrobial susceptibility patterns of *Salmonella* spp. imported with reptiles because they commonly shed these pathogenic organisms without showing clinical signs (Jacobson 2007). This study was important because it provided insight about the serotype composition and the resistance profiles of *Salmonella* spp. from animals originating from Southeast Asia. There is a concern because isolates of *Salmonella* sp. from Southeast Asia are known to have an increased prevalence of resistance to quinolone and ESBLs. The resistance noted in some isolates is of concern because it was against antimicrobials commonly used to treat *Salmonella* spp. infections in both children and adults. Finally, our data suggests that, due to the health risks posed, commensal bacteria harbored by reptiles imported from Southeast Asia should be screened for antimicrobial resistance.

This study has created a solid foundation for future work involving the dissemination of antimicrobial resistant bacteria via the importation of wildlife. It is clear that some commensal flora harbored by reptiles imported into the United States for use in the pet trade express resistance to antimicrobials. The data supports the need for enhanced surveillance and more research into the genetic mechanism responsible for the resistance patterns we observed. Due to the close contact that owners have with these pets, it is important to understand how stressful conditions can promote the shedding of commensal flora and pathogens which increases the risk of exposure to humans.