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Evaluating the effect of two commercial antimicrobial products on Salmonella spp. in the aquatic habitat of the red-eared slider turtle (Trachemys scripta elegans)

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EVALUATING THE EFFECT OF TWO COMMERCIAL ANTIMICROBIAL PRODUCTS ON *SALMONELLA* SPP. IN THE AQUATIC HABITAT OF THE RED-EARED SLIDER TURTLE (*TRACHEMYS SCRIPTA ELEGANS*)

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ABSTRACT

Turtle-associated salmonellosis was recognized as a public health concern in the 1960's, particularly due to an increase in the incidence of disease among children. In response to the public health threat, the United States Food and Drug Administration (FDA) implemented regulations in 1975 restricting the sale of turtle eggs and turtles with a carapace length less than 10.2 cm. Since that time, attempts to eliminate *Salmonella* from turtles using antibiotics have been unsuccessful and lead to antibiotic resistant strains of *Salmonella* on turtle farms. Recent work has focused on identifying non-antibiotic products to suppress or eliminate *Salmonella* and reverse the FDA regulations. Baquacil® and Sanosil® are commercial non-antibiotic, antimicrobial products. Eighty-four red-eared slider turtle hatchlings (*Trachemys scripta elegans*) were used to evaluate the efficacy of these products as a method to suppress *Salmonella* in the turtles' habitats. The turtles were maintained individually in plastic containers that contained chlorinated tap water, chlorinated tap water and 10, 50, or 100 ppm Sanosil®, or dechlorinated tap water and 5, 10, or 50 ppm Baquacil®. Water samples from each container were collected twice weekly for two months, and the *Salmonella* status determined by standard microbiological culture, including delayed secondary enrichment (DSE). Water samples from containers with 50 ppm Baquacil® were less likely to be positive for *Salmonella* than those from the control group ($p < 0.0001$). Water samples from containers with 10 ppm Sanosil® were more likely to be positive for *Salmonella* spp. than those from the control group ($p < 0.0001$). The use of DSE significantly increased the recovery of *Salmonella* spp. from the water samples ($p < 0.0001$). The intestinal tracts of the turtles were cultured for *Salmonella* spp. at the conclusion of the study. There was no significant difference in the *Salmonella* status of the intestinal cultures from any of the turtles ($p = 0.08$). No gross or histopathologic lesions in the

turtles were found to be associated with any of the Baquacil® or Sanosil® concentrations. A concentration of 50 ppm Baquacil® may be used to decrease the prevalence of *Salmonella* in the aquatic habitat of red-eared slider turtles.

CHAPTER ONE

INTRODUCTION

Reptiles represent an important segment of the pet market in the United States of America (US). According to a 2005 - 2006 National Pet Owners Survey, more than 69 million households own a pet, and approximately 4.4% of these households have reptiles (APPMA 2006). The same survey reported that there is an average of 3.7 reptiles per household, or a total of 11 million pet reptiles are in the US. The number of reptiles being imported into the US each year is also on the rise, with over one million animals being imported annually (Chomel et al. 2007). This close association between reptiles and humans has been associated with an increased risk of zoonotic disease transmission, particularly salmonellosis.

Salmonella was first identified in snakes in 1943 (Hinshaw and McNeil 1944), and was found soon thereafter in chelonians and lizards (McNeil and Hinshaw 1946). The first reported case of turtle-associated salmonellosis (TAS) occurred in 1953 (Boycott et al. 1953), while the first reported case of TAS in a child was reported in 1963 (Hersey and Mason 1963). During the 1960's and early1970's, the incidence of TAS grew, particularly in children. It was estimated that by 1971, 15 million pet turtles were being sold annually, 4.2% of US households owned a pet turtle, and that these animals accounted for 14% (280,000) of human salmonellosis cases annually (Lamm et al. 1972).

The Washington State Health Department investigated a series of 21 index cases of TAS in the Seattle area in 1965, all but one of which involved a child (Baker et al. 1972). This helped in part to prompt a ban on the sale of pet turtles by the Washington State Board of Health in 1968. In 1972, the US Food and Drug Administration (FDA) imposed regulations that required *Salmonella*-free certification of all interstate turtle shipments. Subsequently, a study conducted

by the Centers for Disease Control found that 38% of the certified turtles harbored *Salmonella* (CDC 1974). In 1975, the FDA implemented more stringent regulations that restricted the intra- and interstate sale of turtle eggs and live turtles less than 10.2 cm (4 in) in carapace length. These regulations effectively halted the sale and ownership of turtles in the US. After the enforcement of the regulations, there was a marked reduction (approximately 77%) in the number of cases of TAS in children by 1976, which equated to approximately 200 cases (Cohen et al. 1980).

The 1975 FDA regulations limiting pet turtle sales did contain exceptions for marine turtles and turtles sold for educational and scientific purposes. Herpetoculturists have circumvented the ban by selling chelonians under the guise of the latter two purposes, and chelonians with a carapace length of less than 10.2 cm are readily available for purchase on the internet and at hundreds of reptile expositions and swap meets in the US. Due to the public health concern for TAS, and the continued availability of pet turtles, a need to develop intervention methods that minimize the likelihood of disseminating *Salmonella* spp. between chelonians and their environment exists.

Various procedures for reducing or eliminating *Salmonella* spp. from turtles or turtle eggs have been investigated since the 1975 FDA regulations. Siebeling et al. (1975) found that hatchling red-eared slider turtles (*Trachemys scripta elegans*) (RES) treated with terramycin or tylosin in their tank water for up to 14 days had reduced shedding of *Salmonella*. However, the antibiotic treatment did not affect enteric colonization. Gentamicin, an aminoglycoside, was evaluated as a potential treatment using techniques described in poultry production (Siebeling et al. 1984). Exposing RES eggs to gentamicin via temperature- or pressure-differential methods was found to decrease the prevalence in the eggs to < 2% (Siebeling et al. 1984). In 1985, this

treatment method was adopted by the Louisiana Department of Agriculture and became a mandatory requirement.

The use of antibiotics to treat hatchling turtles and turtle eggs has led to multiple findings of resistance in strains of *Salmonella*. D'Aoust et al. (1990) tested 28 lots of RES eggs that were exported from Louisiana to Canada and found that six lots (21%), representing three of four exporters, yielded *Salmonella* from the eggs or the packing moss in which they were shipped. Gentamicin resistance was found in 81% (30/37) of the *Salmonella* strains recovered. In a study of two turtle farms in southern Louisiana, hatchlings from one farm yielded *Salmonella* strains resistant to erythromycin, gentamicin, tetracycline, and sulfonamides. *Salmonella* isolates from pond water on both farms showed similar resistance patterns (Shane et al. 1990). An examination of the records of 115 batches of hatchlings from 28 turtle farms delivered to the Louisiana Department of Agriculture and Forestry in 1988 revealed an additional four *Salmonella* isolates that were resistant to the same antibiotics (Shane et al. 1990).

Due to the emergence of antibiotic-resistant *Salmonella* strains from turtles and eggs, more recent work has focused on the use of non-antibiotic antimicrobials for treatment. Mitchell et al. (2005) showed that the application of the sanitizing agent polyhexamethylene biguanide (PHMB) in the water of RES at concentrations of 25 and 50 ppm significantly reduced the prevalence of *Salmonella* in the aquatic medium, but did not eliminate it from the turtles. The compound was found to be safe for the turtles at both the 25 and 50 ppm concentrations. In a RES egg treatment study, a combination of sodium hypochlorite and PHMB was used as a bath or pressure-differential dip. Both methods were found to significantly reduce *Salmonella* spp. from the egg or the resulting hatchlings compared to controls (bath odds ratio = 0.2, dip odds

ratio = 0.01) (Mitchell et al. 2007). It was also found that PHMB used in a pressure-differential dip was more effective than when it was used in a bath.

The application of any one method to suppress or eliminate *Salmonella* from captive chelonians is not likely to be successful. Mitchell et al. (2005) have suggested that to minimize the zoonotic risk associated with these animals, that a program focused on minimizing the amount of *Salmonella* spp. at each life stage of the turtles in captivity would be needed. These different life stages include the adult breeding animals, eggs, and hatchlings. Minimizing *Salmonella* spp. in the environment of these animals would also be considered important. Because the hatchlings serve as the primary source of infection for humans, this would appear as the most appropriate point to start a control program. The purpose of this study was to evaluate Salmonella spp. control methods for captive hatchling turtles.

Two non-antibiotic, antimicrobial products have been identified for this purpose. Baquacil® (Arch Chemicals, Inc., Norwalk, Connecticut 06856, USA) contains the PHMB derivative poly-iminoimidocarbonylimino-hexamethylene hydrochloride. It is a chlorine alternative for use as a pool sanitizer and algistat. Sanosil® (Sanosil Ltd., Hombrechtikon, Switzerland) contains a combination of 7.5% hydrogen peroxide and 0.0075% silver. It is a disinfectant that is labeled for use in industrial, agricultural, medical, and recreational fields, among others.

The primary objective of this study was to evaluate the efficacy of Baquacil® and Sanosil® against *Salmonella* spp. in the aquatic habitat of RES. A secondary objective was to determine the safety of Baquacil® and Sanosil® for RES. A final objective was to determine if delayed secondary enrichment increases the likelihood of isolating *Salmonella* spp. from the aquatic habitat of RES.

CHAPTER TWO

LITERATURE REVIEW

2.1 *Salmonella*

Salmonella are Gram-negative, typically motile, facultative anaerobes that belong to the family Enterobacteriaceae. This bacterium has a cosmopolitan distribution (Acha and Szyfres 2001). *Salmonella* are tolerant of dehydration and salinity, which enable them to persist in the environment (Acha and Szyfres 2001). Most of the described *Salmonella* should be considered pathogenic (Smith 1991) with a zoonotic potential (Acha and Szyfres 2001). The majority of *Salmonella* utilize citrate as their primary carbon source and do not ferment lactose, although *S. arizonae* uses malonate and does ferment lactose (LeMinor 1984). Hydrogen sulfide is produced by most *Salmonella*, except for certain strains of *S*. Paratyphi and *S*. Cholerasuis (LeMinor 1984).

Members of the family Enterobacteriaceae share DNA homology, and the *Salmonella* are closely related to *Escherichia coli*. The genetic map of *S*. Typhimurium (Sanderson and Hartman 1978) is extremely similar to that of *E. coli* K12 (Bachmann and Low 1980). Conjugation can occur between *Salmonella* and *E. coli* and between *Salmonella* serotypes, frequently transferring plasmids carrying genes for antibiotic resistance and pathogenicity factors (Guthrie 1992).

The nomenclature for *Salmonella* has gone through a number of historical changes. Currently, there are two recognized species of *Salmonella*: *S. enterica* and *S. bongori*. *Salmonella enterica* is further divided into six subspecies: *enterica* (subspecies I), *salamae* (subspecies II), *arizonae* (subspecies III), *diarizonae* (subspecies IIIb), *houtenae* (subspecies IV), and *indica* (subspecies V) (Popoff and LeMinor 1997). Serotypes of subspecies I are designated

by the name of the geographical location where they were first identified, while the rest of the subspecies are identified by their antigenic formula (Grimont et al. 2000). Of the 2,435 described serotypes of *Salmonella*, the majority (1,435) are found within the subspecies I classification (Popoff and LeMinor 1997).

The serotypes of *Salmonella* are based on their O (heat-stabile somatic), Vi (heat-labile capsular), and H (flagellar) antigens (Grimont et al. 2000). The antigenic formulae are expressed according to the Kauffmann-White scheme: O antigen(s), Vi antigens (when present): H antigen(s) (phase 1): H antigen(s) (phase 2; when present) (McWhorter-Murlin and Hickman-Brenner 1994). The O antigens represent a specific component of the bacterial lipopolysaccharide, and are used to group organisms into common groups (Grimont et al. 2000). The structure of the O antigen can be modified by converting bacteriophages (Grimont et al. 2000), which affect its specificity by changing the polysaccharide portion of the lipid-polysaccharide-polypeptide complex.

Protocols to characterize *Salmonella* organisms beyond the serotype level have been developed. These include phage typing, bateriocin typing, biotyping, plasmid profiling, and ribotyping, among others (Grimont et al. 2000). Techniques to identify *Salmonella* organisms are routinely employed during epidemiological investigations.

2.2 *Salmonella* Pathogenesis

Certain *Salmonella* serotypes are considered to be "host-adapted" and are limited to a narrow range of species (e.g., *S*. Dublin in cattle), while others are "non-host adapted" and can affect many different species (e.g., *S*. Typhimurium) (Hollinger 2000). Host-adapted serotypes are more commonly associated with carrier states (Smith 1991). *Salmonella* can cause a spectrum of disease expression and outcomes, from subclinical infection to fatality (Hollinger

2000). Salmonellosis in humans usually affects the gastrointestinal system and is self-limiting after a 2- to 4-day course (Acha and Szyfres 2001). A 6- to 72-hour incubation period leads to the sudden onset of fever, myalgias, cephalalgia, and malaise, followed by abdominal pain, nausea, vomiting, and diarrhea (Acha and Szyres 2001). The organism is shed for a variable amount of time after recovery occurs in both man and animals (Hollinger 2000).

Salmonella possess several virulence factors that allow them to invade and survive within a host. The motile serotypes of *Salmonella* use flagella for motility, allowing them to come into contact with enterocytes (Finlay and Falkow 1989). Jones et al. (1992) demonstrated that *S.* Typhimurium with flagella that organize at one pole of the organism are more invasive than those with unorganized flagella due to mutation. The unorganized flagella projected from the bacteria and were unable to orient their movement in the proper manner.

The lipopolysaccharide (LPS) of *Salmonella* is a central factor in the virulence of the organism. It consists of three regions: lipid A, a hydrophobic portion that resides in the outer membrane; the core oligosaccharide; the O side-chain, the hydrophilic portion that is extended in to the extracellular environment (Rycroft 2000). Lipid A is the component that gives LPS its endotoxic properties. The O side-chain, or O antigen, is involved in evading the host defenses via interaction with the humoral immune system and phagocytic cells (Rycroft 2000). *Salmonella* with normal O antigen are considered "smooth" in comparison to "rough" mutants that lack O antigen and are less virulent (Rycroft 2000).

By far the most important mechanisms in the virulence and pathogenicity of *Salmonella* are the type three secretion systems (TTSS). The TTSS are complex structures that allow protein secretion and delivery (Francis et al. 2002). *Salmonella* have two major TTSS, each coded by genes in a *Salmonella* pathogenicity island (SPI). One of the TTSS is involved in adherence and

invasion of the organism into enterocytes, and is encoded by SPI-1. The other TTSS is involved in intracellular survival and systemic spread of the organism by modulating the host immune response, and it is encoded by SPI-2 (Schlumberger and Hardt 2006). Both of the SPI, and thus the TTSS, are controlled by two-component regulators, such as PhoP/PhoQ and OmpR/EnvZ (Francis et al. 2002).

2.3 Methods for Isolation of *Salmonella*

2.3.1 Microbiological Methods

Microbiological culture is the standard methods for detecting *Salmonella* in tissue, excretory, and environmental samples. The general method includes pre-enrichment, selective enrichment, and selective isolation media. The use of delayed secondary enrichment (DSE) may be used to increase the likelihood of isolating *Salmonella*. Finally, screening media and confirmatory tests may be used to help identify isolated colonies of *Salmonella*.

Pre-enrichment media are non-selective, and are used to resuscitate injured bacterial cells prior to inoculation in selective enrichment media (Waltman 2000). *Salmonella* can be stressed by the amount of available water, pH, and temperature, among other conditions, in fecal and environmental samples (Waltman 2000). Lactose broth (LB), buffered peptone water, M9, and universal pre-enrichment are common pre-enrichment media. The use of LB may not be indicated for the recovery of *Salmonella*, since the fermentation of lactose may increase the acidity to inhibitory or lethal levels (Waltman 2000). Siebeling et al. (1975) found evidence to support this when turtle samples were pre-enriched in LB before inoculation in tetrathionate broth, and the recovery of *Salmonella* was reduced.

Selective enrichment media that are commonly used include tetrathionate broth (TB), selenite broth (SB), and Rappaport-Vassiliadis broth (RV), each of which has had different

formulations developed. Tetrathionate broth may have brilliant green added to it, SB may have cystine or brilliant green added to it, and RV may be modified into a semi-solid media (Waltman 2000).

Many comparative studies have been performed to assess which selective enrichment media is best for isolation of *Salmonella*. (Waltman 2000). The selection of an enrichment media should be based on the subspecies or serotype of *Salmonella* that is to be isolated. For example, a small inoculum of a certain serotype may be inhibited in TB (van Schothorst et al. 1977). Smith (1952) reported that SB is toxic to *S*. Cholerasuis, and Greenfield and Bigland (1970) found that its use was not efficacious for growth of *S*. Pullorum and *S*. Gallinarum. Although RV is adequate for isolating many *Salmonella* taxa, Vassiliadis (1968) found that only 3 of 11 (27.3%) of strains of *S*. e*nterica* subspecies III *arizonae* could be isolated from it. Modified semi-solid RV is not indicated for the isolation of non-motile *Salmonella*, such as *S*. Pullorum and *S*. Gallinarum (Waltman 2000). Mitchell (2006) also reports difficulty with isolating *Salmonella* from green iguana (*Iguana iguana*) samples using RV.

There are many plating media that can be used for the selective isolation of *Salmonella*. The selectivity of the different media varies. Plating media with low selectivity, such as MacConkey and eosin methylene blue, are not commonly used due to the difficulty in using colony morphology in identification (Mitchell 2006). More selective media, such as xylose-lysine-desoxycholate (XLD) and *Salmonella-Shigella* agar, have a major disadvantage in that they have high-false positive rates, primarily due to *Proteus* spp. (Waltman 2000). The most selective media include bismuth sulfite and xylose-lysine-tergitol 4 (XLT-4) agars (Mitchell 2006). All of the medium and highly selective plating media contain hydrogen sulfide (H_2S) indicators. The modification of XLD to XLT-4 agar involved substituting tergitol 4, which is

inhibitory to *Proteus*, *Pseudomonas*, and *Providencia* spp., and the addition of peptone to enhance the H_2S reaction (Waltman 2000). Mitchell (2006) prefers the use of XLT-4 agar for isolation of *Salmonella* from reptiles.

The use of DSE may increase the likelihood of isolating *Salmonella* from samples when organisms are found in low numbers or are inhibited by their environmental conditions. After the initial incubation and culture attempt, an enriched sample is held at room temperature for a period of 5 -7 days (Waltman 2000). An aliquot is then placed in fresh enrichment media, incubated, and another attempt at culture is made. In a study investigating the use of DSE for the isolation of *Salmonella* from poultry and their environment, it was found that DSE significantly improved the rate of recovery (Waltman et al. 1991). A total of 4,377 samples were collected and subjected to a standard microbiological culture procedure employing a 5-day DSE. Four-hundred and sixty-four (11%) of the samples yielded *Salmonella*. The initial culture recovered 269 (58%) of these, 43 (9%) of which were only positive at that time. The use of DSE recovered 421 (91%) of the positive cultures, 195 (42%) of which were only positive at that time. The overall recovery of *Salmonella* was increased by approximately 64% with the use of DSE. Rybolt et al. (2005) evaluated the use of DSE compared to different enrichment media without DSE for the recovery of *Salmonella* from poultry environmental samples. The use of DSE recovered *Salmonella* from 94.5% of the positive samples and provided the highest agreement when used with two different plating media.

Suspect *Salmonella* colonies can be evaluated with screening media such as lysine iron (LIA), triple sugar iron (TSI), and urea agar. *Salmonella* decarboxylate lysine and produce H_2S in LIA. The organism ferments glucose and produces gas and H_2S in TSI. *Salmonella* are negative for urease. Confirmation of *Salmonella* can be done with a slide agglutination test with

antisera for O antigen groups or with biochemical tests, such as the API 20E system (bioMerieux, Inc., Durham, North Carolina 27712) (Mitchell 2006).

Salmonella grow at temperatures between 8 - 45^{0}C (46 - 113^{0}F), and cannot survive temperatures greater than 70^{0}C (158^{0}F) (Acha and Szyfres 2001). The optimal growth temperature of *Salmonella* is approximately 37^{0}C (98.6^{0}F), and samples that have low levels of background flora, such as organs and tissues, are generally incubated at this temperature (Waltman 2000). However, samples that have high levels of bacteria, such as intestinal and environmental samples, can be incubated at temperatures of 40 - 43^{0}C (104 - 109^{0}F) to select against bacteria that compete with *Salmonella* (Waltman 2000).

The sensitivity and specificity of microbiological culture has not been determined, but it is considered to be the standard for the detection of *Salmonella* (Mitchell 2001). Several factors can affect the reliability of culture, including temporal variation in shedding, sample collection method, sample quantity, and method of culture (Smith 1991; Hird et al. 1984; Owens et al. 1983).

2.3.2 Isolation from Reptiles

The techniques used to culture *Salmonella* from reptiles are based on those that have been developed from domestic animals, with minor differences. For example, selenite enrichment is commonly used for culture from reptiles, while the poultry industry prefers the use of tetrathionate broth (Mitchell 2006). Also, since *Salmonella* serotypes from reptiles commonly belong to subspecies III and IIIb, they may use different biochemical pathways than other subspecies. Thus, they may need different enrichment and isolation media (Mitchell 2006). Research to determine optimal techniques for the microbiological culture of *Salmonella* from

reptile samples is needed. Comparative studies of selective enrichment and isolation media are limited (Harvey and Price 1983; Kodjo et al. 1997).

Fecal material from 20 different reptile species at the Bristol Zoological Gardens was used to evaluate techniques for *Salmonella* isolation (Harvey and Price 1975). One gram from each sample was placed into both selenite F and Muller-Kauffmann tetrathionate broth. The samples were then incubated aerobically at 43^0C (109^0F) for 24 hours. Each of the enrichment broths were then plated on brilliant green MacConkey, desoxycholate citrate, and de Loureiro's three stock solution modification of bismuth sulfite agar. Plates were incubated at 37^0C (98.6^0F) for 24 hours. Sixteen *Salmonella* serotypes were identified by biochemical tests of suspected colonies. *Salmonella* were recovered from 81% of the samples enriched with selenite F, compared to 63% with Muller-Kauffmann tetrathionate. When the results from the two media are combined, the number of isolates is increased by 38%. These results suggest that there are differences in the recovery of S*almonella* based on the enrichment media used. Also, the use of multiple enrichment media may increase the recovery of *Salmonella* from reptiles, since they are able to harbor multiple serotypes

In a study of fecal samples from 32 chelonians, Kodjo et al. (1997) evaluated two enrichment and 3 isolation media for recovery of *Salmonella*. Samples were inoculated into SB or TB and incubated. An aliquot from each enrichment was then cultured on Rambach, *Salmonella-Shigella*, and XLT-4 agar. All incubation was conducted at 37^0C (98.6^0F) for 24 hours, and suspect colonies were evaluated with biochemical tests. Thirteen of the 32 samples were positive for *Salmonella*, with all of the isolates recovered using SB and only 11 when using TB. After SB enrichment, all 13 of the isolates grew on Rambach and *Salmonella-Shigella* agar, but only 12 grew on XLT-4.

Wells et al. (1974) conducted a study to evaluate three types of samples for isolating *Salmonella* from turtles: habitat water, individual visceral organs, and whole carcasses. Fifty individually housed turtles were fasted for one week. A water sample was collected from each, and the turtles were euthanized and samples collected from their bile, liver, spleen, yolk, kidney, small intestine, and colon. Another 50 turtles were individually homogenized with a blender. All samples were inoculated in TB with brilliant green and incubated. An aliquot was then plated on brilliant green agar and incubated. All incubation was conducted at 37^0C (98.6^0F) for 24 hours. *Salmonella* was recovered from 98% and 96% of the whole carcass and water samples, respectively. Only 72% of the organ samples yielded *Salmonella*. *Salmonella* was isolated from 34% of the small intestine samples and 50% of the colon samples, compared to ≤ 12% for each of the other organ samples. These results suggest that the type of sample collected may affect the recovery rate of *Salmonella* from reptiles. Also, multiple samples may be needed to increase the probability of recovering *Salmonella*.

The results of these two studies, along with the known intermittent shedding pattern of *Salmonella* from reptiles (Burnham et al. 1998; Mitchell 2001), indicate that there is a high chance of false-negative results when attempting to isolate *Salmonella* from reptiles. To determine the *Salmonella* status of a reptile, Mitchell (2006) recommended a minimum of five samples collected over a 30-day period, or longer if the reptile has been treated with antibiotics.

2.3.3 Other Diagnostic Modalities

Although the use of microbiological culture is the standard technique detecting *Salmonella* in biological and environmental samples, its use is fraught with certain shortcomings. The sensitivity and specificity have not been determined. A multitude of factors can affect the outcome, including temporal variation in shedding, type of sample, sample collection method,

sample quantity, and method of culture (Smith 1991; Hird et al. 1984; Owens et al. 1983). Microbiological culture also requires a relatively long period of time to complete, with isolation and identification requiring up to 96 hours (Cohen et al. 1996). Certain organisms may not be recovered with the use of culture. Mitchell (2001) estimated that the sensitivity of culture of the detection of *Salmonella* in the green iguana (*Iguana iguana*) to be 70%, which could lead to false-negative results. The delay or lack of therapy and control procedure implementation due to such deficiencies could significantly affect the outcome of clinical cases and epidemiologic investigations. The study of more rapid and reliable diagnostic techniques has resulted in the development of enzyme-linked immunosorbent (ELISA) and polymerase chain reaction (PCR) assays.

Enzyme-linked immunosorbent assays are rapid and have high sensitivity and specificity relative to microbiological culture. These traits, together with their ease of use and the relatively small amount of equipment involved, make them popular for large-scale use (Barrow 2000). The food industry employs widespread use of ELISA for the detection of *Salmonella* (Ibrahim 1986; Todd et al. 1987; Flowers et al. 1989; van Poucke 1990). The ELISA procedure utilizes immunoglobulins conjugated with an enzyme to detect specific microbial antigens. Depending on the type of ELISA, immunoglobulins without conjugation may also be included. The detection of *Salmonella* in animal and environmental samples using ELISA has been studied (Pelton et al. 1994; Desmidt et al. 1994; Tan et al. 1997).

In a study of equine, avian, and reptile fecal samples, an antigen-capture ELISA was compared to microbiological culture for the detection of *Salmonella* (Pelton et al. 1994). Culture was used to identify 35 negative and 35 positive samples, which were then evaluated with the ELISA. One (3%) of the negative samples and 11 (31%) of the positive samples were found to

be positive by the ELISA. This indicated that the ELISA had a sensitivity of 69% and a specificity of 97%. The positive predictive value was 96%, indicating a low rate of false-positive results. However, the negative predictive value was 75%, suggesting a moderate rate of false-negative results.

Desmidt et al. (1994) compared the *Salmonella*-Tek ELISA system (bioMerieux, Inc., Durham, North Carolina 27712) with microbiological culture for the detection of *Salmonella* in cloacal swabs and litter samples from experimentally infected chicks. Forty-two litter and 73 cloacal samples were collected. The ELISA and culture were positive for 22 (30%) and 25 (33%) of the cloacal samples, respectively. Of the 42 litter samples, 39 (88%) and 22 (48%) were positive with ELISA and culture, respectively. There was no difference between the two methods when used to detect *Salmonella* from cloacal swabs. The ELISA was found to be more sensitive when used with litter samples. This was attributed to antigen from *Salmonella* killed by the bactericidal effects of poultry litter.

In a study using chicken fecal and organ samples, a competitive ELISA was compared to a motility enrichment culture using modified semisolid Rappaport-Vassiliadis medium for the detection of *Salmonella* (Tan et al. 1997). The ELISA was specific for the lipopolysaccharide of *Salmonella*. A total of 3,928 samples were collected. The ELISA was positive for 1,085 (28%) and the culture was positive for 1,067 (27%) of the samples. The sensitivity and specificity of the ELISA were determined to be 93% and 97%, respectively.

The introduction of new techniques for the detection of *Salmonella*, such as PCR, can involve a gap between what is available and what is suitable for practical application (van der Zee and Huis in't Veld 2000). However, PCR is increasingly being used for disease diagnosis and investigation (Fredericks and Relman 1999), and pre-fabricated test kits have been

developed (van der Zee and Huis in't Veld 2000). The process of PCR involves amplification of a part of the *Salmonella* genetic material through the use of specific, complementary oligonucleotide primers (van der Zee and Huis in't Veld 2000). Target DNA is denatured, primers are annealed to it, and a polymerase extends the primers. When repeated multiple times, this process results in logarithmic increased in copies of the target DNA. The PCR assay has a high specificity due to the use of the oligonucleotide primers. It also has a high sensitivity due to the increase in the amount of target material. There are multiple methods used to confirm the presence of target DNA, including gel electrophoresis, ELISA, fluorescence immunoassay (van der Zee and Huis in't Veld 2000). There are also multiple versions of PCR available.

Despite the advantages of speed, sensitivity, and specificity, PCR does have disadvantages. The most common of these is contamination. Improper processing of samples can lead to false-negative or false-positive results. Contaminant DNA may give a false-positive result. A false-negative result may be due to biological inhibitors, such as blood, blood culture media, urine, vitreous humor, and sputum (Fredericks and Relman 1999). False-negative results may also occur with inadequate DNA extraction or loss of DNA during processing, both of which decrease the quantity of DNA for use in the assay (Fredericks and Relman 1999).

Cohen et al. (1993) developed a PCR assay specific for a 496-base pair segment of the histidine transport operon of the genus *Salmonella*. This assay was then tested to determine the minimum number of organisms it could detect in a sample (Cohen et al. 1994b). Culture-negative samples of horse feces were inoculated with various levels of *Salmonella* organisms. The PCR assay could detect *Salmonella* at levels of 10^3 CFU/g or greater, whereas culture techniques could detect levels as low as 10^2 CFU/g.

Cohen et al. (1996) compared this same PCR assay to microbiological culture using naturally infected horse feces and environmental samples from a veterinary teaching hospital. One-hundred and fifty-two samples were collected from outpatient horses. Twenty-six (17%) of these were positive by PCR, but none were positive by culture. One-hundred and ten ill horses were sampled for a total of 282 fecal samples. Eleven (10%) of these were positive by culture, compared to 71 (65%) by PCR. The PCR detected all of the culture-positive samples. Three-hundred and thirteen environmental samples were tested. Eight (3%) were positive by PCR, but none by culture. In each set of samples, the prevalence of *Salmonella* detected by PCR was significantly higher than that detected by microbiological culture.

This same PCR assay was again compared to microbiologic culture using environmental samples, but poultry houses were substituted for horse barns (Cohen et al. 1994a). Fifty drag swabs from multiple poultry houses were collected. The PCR assay detected *Salmonella* in 47 (94%) of the samples, while culture yielded *Salmonella* in 29 (58%). Enrichment of the samples significantly increased the sensitivity of the PCR assay, as only one sample was positive before the use of enrichment broth.

Charlton et al. (2005) compared a *S.* Enteritidis-specific PCR assay with delayed secondary enrichment (DSE) culture using drag swabs from a poultry ranch. A total of 942 samples were collected. The PCR assay detected significantly more positive samples (43, 4.6%) than the DSE culture (24, 2.5%).

The use of enrichment to increase the sensitivity of PCR is common. However, enrichment increase the amount of time needed to get a result from the test. Wolffs et al. (2006) reported the development of a two-step filtration method to directly detect and quantitate *Salmonella* from biological samples. Experimentally inoculated chicken carcass rinse and spent

irrigation water were passed through two filters, one with > 40 µm pores and one with 0.22 µm pores. The second filter was vortexed in saline to release attached cells and PCR was applied. Detection level was as low as 220 CFU/100 ml, and quantitation level was as low as 750 CFU/100 ml. The process was determined to take only three hours.

Techniques to detect *Salmonella* will continue to increase in speed, sensitivity, and specificity. Future methods that are in development include immunosensors, immunomagnetic separation, and genetic microarray assays.

2.4 *Salmonella* in Reptiles

2.4.1 Reptile Salmonellosis

The first confirmed report of the isolation of *Salmonella* from a reptile occurred in 1943, and involved an apparently healthy gopher snake (*Pituophis catenifer deserticola*) killed on a turkey farm (Hinshaw and McNeil 1944). The snake harbored three serotypes: *Salmonella* Maleagridis, *S*. Panama, and an unidentified *S.* Paracolon type. Several other snakes from the farm were subsequently found to harbor the same *S.* Paracolon type, and the snakes were believed to be the source of a *Salmonella* outbreak in the turkey flock, although this could not be verified. Caldwell and Ryerson (1939) had reported the isolation of a bacterium from three dead wild-caught Gila monsters (*Heloderma suspectum*) that was similar in biochemical characteristics to *Salmonella*, but this was unconfirmed. The first report of true *Salmonella* isolated from a lizard involved a dead Gila monster from the San Diego Zoo (McNeil and Hinshaw 1946). *Salmonella* was first isolated from a chelonian by McNeil and Hinshaw (1946) at about the same time. Two Galapagos tortoises (*Geochelone giganteas*) were found dead at the San Diego Zoo, and *Salmonella* Newport was isolated from the liver, spleen, lungs, and intestine of the first, and the abdominal fluid, intestine, and bladder of the second.

Salmonellosis in reptiles is usually subclinical, but in cases where pathology is present, possible signs include enteritis, septicemia, necrotic foci in the viscera, diarrhea, anorexia, and listlessness (Marcus 1971). Cambre and McGuill (2000) list other disease conditions in which *Salmonella* has been implicated, including spinal osteomyelitis, infectious stomatitis, pneumonia, hepatitis, splenitis, nephritis, gastritis, enteritis, coelomitis, epicarditis, and myocarditis. Regurgitation may also be a clinical sign. Oophoritis and endocarditis can also be caused in reptiles by infection with *Salmonella* (Onderka and Finlayson 1985).

Keymer (1978) sampled 144 tortoises of 17 different species for *Salmonella* at necropsy at the Zoological Society of London. *Salmonella* was isolated from four (2.8%) of the animals, in three of which disease was attributed to salmonellosis. A retrospective study of the disease patterns in the reptile and amphibian collection at the Detroit Zoo from 1973 to 1983 determined that the greatest number of deaths (36.6%) was due to microbial agents. *Salmonella* was included in the list of primary agents; however, no further classification was reported (Kaneene et al. 1985). In a retrospective study of records at the Emperor Valley Zoo in Trinidad from 1993 to 1996, thirteen (76%) of the reptile fatalities were attributed to *Salmonella*. In a six month longitudinal study at the same institution, the morbidity and mortality of reptiles from salmonellosis were both 1% (Gopee et al. 2000).

Onderka and Finlayson (1985) sampled 150 pet reptiles for *Salmonella* at necropsy. *Salmonella* was isolated from forty-six (51%) of the snakes, 22 (48%) of the lizards, and one (7%) of the chelonians. Salmonellosis was implicated as the cause of death for fifteen (17%) of the snakes, and five (11%) of the lizards. In a study of fifteen privately owned snakes of various species with proliferative osteoarthropathy, six (40%) yielded identical blood and bone cultures of *Salmonella* (Isaza et al. 2000). Huchzermeyer (1991) reported an outbreak of salmonellosis in

farmed hatchling Nile crocodiles (*Crocodylus niloticus*) in South Africa. Septicemia caused by several *Salmonella* serotypes produced clinical signs of lethargy, weakness, and acute death.

Challenge studies have been performed to determine the susceptibility of reptiles to *Salmonella* infection. In a group of 28 snakes, divided equally between brown snakes (*Sonnoria dekayi*) and garter snakes (*Thamnophis sirtalis*), infections were established by either the oral, intracardiac, or intracoelomic route. Inocula consisted of either *Salmonella* Typhimurium or *S. arizonae*, with equal numbers of each snake species infected with each serotype. The orally infected snakes suffered no disease or pathologic lesions, and experimental serotypes were isolated form the intestinal tract. Two (16.8%) of the snakes infected via the intracardiac route died of pericarditis and myocarditis. None of the snakes infected via the intracoelomic route suffered disease, although a mild coelomitis was present. For the latter two groups of snakes, experimental serotypes were isolated from all of the visceral organs of all the subjects (Chiodini 1982). Caldwell and Ryerson (1939) infected five Gila monsters and five horned lizards (*Phrynosoma solare*) with *Salmonella* intracoelomically. Four (80%) of the Gila monsters died and *Salmonella* was isolated from them. None of the Gila monsters had gross pathologic lesions, and the last was unaffected. All of the horned lizards died and yielded *Salmonella* when sampled at necropsy. Several of the horned lizards were found to have splenomegaly.

2.4.2 Salmonella as a Component of the Indigenous Gastrointestinal Flora

Salmonella are routinely isolated from reptiles that are apparently healthy. Hinshaw and McNeil (1945) surveyed 55 snakes from the San Diego Zoo and wild-caught in California via necropsy. Eleven (26.8%) of the 41 wild snakes and one (7.1%) of the 14 captive snakes yielded *Salmonella*. Three different serotypes were identified, but only one from any individual snake. In a survey of samples from wild lizards and snakes in Ghana, 37.5% of lizards and 29.6% of

snakes were positive for *Salmonella*, with many different serotypes identified (Zwart 1962). Kourany and Telford (1981) examined samples from 447 wild lizards in Panama, representing seven families and 34 species. *Salmonella* was isolated from 131 (29.4%) of the lizards, and 36 different serotypes were identified. In a survey of the oral flora of 50 captive garter snakes (*Thamnophis* spp.), three *Salmonella* isolates were recovered (Goldstein et al. 1981). Geue and Loschner (2002) examined captive and wild reptile fecal samples from Germany and Austria, and isolated *Salmonella enterica* from 86 of 159 (54.1%). Cloacal swabs of 67 wild Nile crocodiles in Zimbabwe yielded 18 (26.9%) that were positive for *Salmonella*, including eight serotypes (Madsen et al 1998).

Manolis et al. (1991) undertook a study on two crocodile farms in Australia, both of which raised Johnston's crocodiles (*Crocodylus johnstoni*) and estuarine crocodiles (*Crocodylus porosus*). Fecal or cloacal swabs or both were taken from animals just before slaughter. The proportion of animals that were positive for *Salmonella* was 25.4% (75/295) on one farm and 85.7% (18/41) on the other. A total of 23 *Salmonella* serotypes were identified. In a survey of Nile crocodiles on a farm in Zimbabwe, *Salmonella* isolation from jejunal samples taken at slaughter was 16% (8/50). All isolates were *S. arizonae*, except one Group F (Obwolo and Zwart 1993). In two cross-sectional studies on an iguana farm in El Salvador, Mitchell (2001) found the prevalence of *Salmonella* in iguanas of all ages to be 40.7% (337/827) and 56.1% (812/1447), respectively. *Salmonella* was also recovered from an extensive number of soil and water samples from the iguanas' environments.

Refai and Rohde (1969) isolated *Salmonella* from 14 of 25 (56%) reptiles sampled at the Gizeh Zoological Gardens. The fecal samples contained 11 total serotypes. Three hundred and seventeen reptiles were surveyed for *Salmonella* at the US National Zoological Park by Cambre

et al. (1980). Samples consisted of cloacal swabs, and 117 (37%) were positive overall. Sixty-nine of 125 (55%) of snakes, 46 of 129 (36%) of lizards, and 2 of 63 (3%) of chelonians were positive. A similar survey at the Emperor Valley Zoo in Trinidad found 17 of 35 (48.6%) of snakes, 2 of 2 (100%) of lizards, and one of 20 (5%) chelonians to be positive for *Salmonella* from either fecal samples or cloacal swabs (Adesiyun et al. 1998). Kennedy (1973) collected fecal samples and cloacal swabs from 14 different species of reptile, including snakes and crocodilians, from a museum display in Canada. *Salmonella* was isolated from seven (50%) of the animals.

One hundred and twenty-four turtles from zoological institutions across the US were examined for *Salmonella* by Jackson and Jackson (1971). Cultures from cloacal swabs were positive in 12.1% (15/124) of the cases, including seven serotypes. A similar study conducted by Otis and Behler (1973) at the New York Zoological Park revealed that 29.1% (37/127) of the turtles examined by cloacal swab were positive for *Salmonella*, all of which were identified as *S*. Durham. In a study of 27 red-eared slider turtles (*Trachemys scripta elegans*) obtained from pet stores, cultures of cloacal swabs and water samples yielded *Salmonella* in 20% (5/27) (McCoy and Seidler 1973). Strohl et al. (2004) found that among captive chelonians in France, 44% (23/52) of tortoises and none of five turtles yielded S*almonella* from fecal samples. Prevalence in sick or injured animals was 40% and 45% in healthy animals. Of the tortoises at a rescue center in Italy, cultures of 106 of 135 cloacal swabs were positive for *Salmonella*, including 16 serotypes (Pasmans et al. 2000). A survey of fecal samples from tortoises (*Testudo* spp.) imported into the United Kingdom from Turkey, Yugoslavia, and Russia demonstrated that *Salmonella* could be isolated from 38% (62/163) (Savage and Baker 1980). The samples were collected from the boxes in which the tortoises were shipped. Pasmans et al. (2002) collected 28

cloacal swabs and 34 aquarium soil samples from captive freshwater turtles of various species. Overall, 11.3% (7/62) of the samples were positive for *Salmonella*, including three (37.5%) cloacal swabs and four (11.8%) soil samples. A survey of wild diamondback terrapins (*Malaclemys terrapin centrata*) in Duval County, Florida revealed that 5.1% (2/39) harbored *Salmonella* (Harwood et al. 1999).

Interestingly, two recent studies have found a 0% prevalence of *Salmonella* in wild reptiles from the U.S. Richards et al. (2004) obtained cloacal swabs from 75 animals presented to the Wildlife Center of Virginia, representing eight species of turtles and snakes. Saelinger et al. (2006) tested 97 cloacal swabs, one fecal sample, and 16 mucosal samples from 94 turtles. Forty-six were presented to the North Carolina State University College of Veterinary Medicine Turtle Rescue Team and 48 were captured from ponds near the institution. The variation in results between the various studies described herein is interesting. There are a number of different reasons for this, the most obvious of which is that each examines a different population of animals. Other factors that can affect the recovery of *Salmonella* from samples, and thus the prevalence, are type of sample, sampling technique, transport and storage of sample, culture methods, culture media, identification modality, and the intermittent pattern of *Salmonella* shedding in reptiles. It should be noted that all of the studies relied on culture methods for recovery of *Salmonella*, and almost all of the studies examined only one sample from any individual animal. Thus, the chances of false-negative results were increased.

2.5 Reptile-Associated Salmonellosis in Humans

2.5.1 Turtle-Associated Salmonellosis

The first reported case of turtle-associated salmonellosis (TAS) occurred in 1953 (Boycott et al. 1953). The first reported case of TAS in a child was reported in 1963 (Hersey and

Mason 1963). A 7-month-old infant was found to harbor *Salmonella* Hartford after the onset of diarrhea, vomiting, and fever. During an investigation into the source of the organism, *S.* Hartford was isolated from the family's pet turtle, and also from 25 turtles obtained from the same source. During the 1960's and early1970's, the incidence of TAS grew, particularly in children.

The Washington State Health Department (WSHD) investigated a series of 21 index cases of TAS in the Seattle area in 1965, all but one of which involved a child 13-years-old or younger (Baker et al. 1972). The association of salmonellosis and pet turtle ownership prompted the WSHD to impose a state-wide ban in January, 1968 on the sale of turtles that were not certified *Salmonella*-free. Because it is almost impossible to prove that a turtle is *Salmonella*-free, the ban effectively ended pet turtle sales in the state (CDC 1971). The Washington ban was effective in decreasing the rates of TAS, especially in children. The number of cases and outbreaks of salmonellosis decreased in the Seattle area and the State of Washington as a whole. In children less than ten years of age in the state, salmonellosis attack rates decreased in 1968 - 1969 compared to 1966 - 1967. In the rest of the United States (US) during this period, the salmonellosis attack rate increased for children of the same age group (Lamm et al. 1972).

During the 1960's and early 1970's, attempts were made to estimate the magnitude of the problem of TAS. Retrospective studies were conducted to determine the frequency of turtle ownership or exposure in cases of salmonellosis confirmed by laboratory methods. In Minnesota, an uncontrolled study found turtles associated with 25% of salmonellosis cases (Williams and Heldson 1965). In New Jersey, a controlled study found that 22.6% of salmonellosis cases were associated with turtles, compared to 5.7% of the control group (Altman et al. 1972). Lamm et al. (1972) conducted surveys in several areas of the US and found that

turtles were associated with 15.6% of salmonellosis cases in Utah; 10.9% in Atlanta, Georgia; 18% in Santa Clara County, California; and 11.6% in Seattle, Washington. The report also included a retrospective case-control study in Connecticut, which found that 24% of salmonellosis cases were associated with turtles, compared to 2% of control cases. Lamm et al. (1972) then calculated an estimate of the number of TAS cases in the US in 1971. The data from Williams and Heldson (1965) and Altman et al. (1972) was combined with their own to get an average of 18.2% of salmonellosis cases associated with exposure to turtles. An estimate of two million cases of salmonellosis in the US was obtained (Aserkoff et al. 1970). An estimate of 4.2% for the number of households in the US that contained a pet turtle was made based on an estimate of 15 million pet turtles sold and 60 million households present in the US in 1971. A calculation based on all of these numbers determined that 14%, or approximately 280,000, of the cases of salmonellosis were associated with turtles in 1971.

In response to the public health risk posed by pet turtle ownership, the US Food and Drug Administration (FDA) imposed regulations in 1972 that required any interstate shipment of turtles to be certified as *Salmonella*-free. Similar to what had been the case in Washington State, verification of a *Salmonella*-free status for turtles proved nearly impossible. The Centers for Disease Control (CDC) conducted a study that found that 38% of the turtles certified as *Salmonella*-free were actually contaminated with the organism (CDC 1974). Since the regulatory measures of 1972 were not effective in decreasing the dissemination of turtles carrying *Salmonella*, the FDA imposed more stringent regulations in 1975. These measures included a ban on all intra- and interstate shipments of turtle eggs and live turtles with a carapace length less than 10.2 cm (4 in). The choice of 10.2 cm was made based on the assumption that turtles with a carapace of that length or shorter were attractive for young children to put into their

mouths. The 1975 FDA ban did lead to a 77% decrease in the number of cases of salmonellosis due to serotypes associated with turtles (*Salmonella* Java, *S*. Urbana, *S*. Litchfield) in children less than ten years of age (Cohen et al. 1980). Interestingly, this reduction was not found in states in which turtle production is prominent, such as Louisiana and Mississippi. In these states, the number of case of TAS was found to be relatively constant.

The exportation of turtles from the US was not restricted by the 1975 FDA regulations. *Salmonella* has been isolated from shipments of turtles to various countries, including the United Kingdom (Borland 1975), Japan (Fujita et al. 1981), Israel (Chassis et al. 1986), Guam and Yugoslavia (Tauxe et al. 1985), and France (Sanchez et al. 1988). An outbreak of *Salmonella* Pomona in 1984 in Puerto Rico was traced to an imported shipment of turtles, and it was determined that approximately 15% of all the cases of child salmonellosis could be attributed to that shipment (Rigau-Perez 1984). Tauxe et al. (1985) conducted a case-control study in Puerto Rico that found 17% of infant salmonellosis cases and none of the matched controls were associated with exposure to a pet turtle. Also, lots of red-eared slider turtles collected from pet retailers were cultured, and S*almonella* was isolated from all of them. *Salmonella* Pomona was the most common serotype identified, appearing in 89% of the lots. The ethical question of exporting a product that is known to cause disease—in this case, turtles—has been raised (Gangarosa 1985).

The 1975 FDA regulations made exceptions for marine turtle and turtles sold for educational and scientific purposes. Violators of the law are provided a written demand to destroy the animals under FDA supervision within 10 days. Violators are also subject to a fine of not more than $1,000 and/or imprisonment of not more than one year for each violation. Herpetoculturists have circumvented the ban by selling chelonians under the guise of educational

purposes. Chelonians with a carapace length of less than 10.2 cm are readily available for purchase on the internet and at hundreds of annual reptile expositions and swap meets in the U.S. The sale of small pet turtles by retailers has led to recent cases of TAS in Wisconsin and Wyoming (CDC 2005). Some of these retailers were not aware of the FDA regulations, while others claimed that their turtle sales fell under the educational purposes exemption. However, the exemption for educational purposes applies to sales to institutions and organizations only.

2.5.2 Reptile-Associated Salmonellosis from Other Reptile Species

Despite the 1975 FDA ban on small pet turtles, the number of cases of reptile-associated salmonellosis (RAS) has increased. The CDC estimated that in 1996, 3 - 5% of the cases of salmonellosis in the US were RAS (Cambre and McGuill 2000). By 2006, that number had increased to approximately 7% (Chomel et al. 2007). This increase has coincided with an increase in the number of reptiles kept as pets. In a 2005 - 2006 National Pet Owners Survey, over 11 million reptiles were kept as pets in the US (APPMA 2006), an increase from the 7.3 million in 1994 (Mitchell 2001). By 1995, more than 2.5 million reptiles were imported into the US each year (Redrobe 2002), the majority of which were iguanas. Importation of green iguanas increased 431% from 143,000 to 760,000 animals during the period of 1989 to 1993 (Ackman et al. 1995). Increased ownership of reptiles other than chelonians may account for the fact that, despite the 1975 FDA ban on turtle sales, it has been estimated that RAS may account for 75,000 cases in the US annually (Bradley et al. 2001). It is clear that the risk of RAS reaches far beyond the realm of aquatic chelonian ownership.

While the risk of salmonellosis from turtles is relatively well known, many people are not aware that other reptile species also pose a risk, including veterinarians (Meehan 1996). Mermin et al. (1997) found that in a survey, fewer than half the families with salmonellosis and known

iguana exposure suspected that the iguanas might have been the source of the illness. In the last fifteen years, the CDC has gotten numerous reports of RAS from around the US. During 1994 - 1995, 13 states reported cases of RAS from species that included the green iguana, the corn snake (*Elaphe guttata guttata*), the savannah monitor (*Varanus exanthematicus*), and an unidentified species of aquatic turtle (CDC 1995). Sixteen states reported RAS cases during 1996 - 1998, and the majority of cases were again caused by iguanas and snakes (CDC 1999). The CDC received reports of RAS from 12 states during the period of 1998 - 2002, with iguanas, turtles, an unidentified boa species, and a bearded dragon (*Pogona vitticeps*) implicated as the sources of the infections (CDC 2003).

Other cases of RAS have proven to be more unusual, but no less hazardous. Willis et al. (2002) report a case of RAS in a 3-month-old infant who was infected via a pet chameleon that had been given to him by his grandmother. Two recipients of a platelet transfusion contracted cases of salmonellosis, one of them lethal (O'Rourke 2002). The donor of the blood products had acquired asymptomatic salmonellosis from his pet boa constrictor. Kraus et al. (1991) reported 11 cases of severe infection with *S. arizonae* contracted from ingestion of desiccated, uncooked rattlesnake flesh as a "natural" folk remedy for arthritic conditions. Many of the patients had concurrent immunosuppression from their condition or the medical treatment they were receiving, which may have promoted the infections, and one patient died.

2.5.3 Epidemiology of Reptile-Associated Salmonellosis

Only a handful of epidemiologic studies have been conducted in recent years to investigate RAS. The epidemiology of RAS was defined and described by Cieslak et al. (1994). *Salmonella* serotypes that were associated with reptiles were defined as those in which reptilian sources formed the majority of non-human isolates reported to the CDC during the period of

1981 - 1990. Isolates of reptile-associated serotypes that were reported to the CDC between 1970 and 1992 were analyzed. Incidence rates were calculated by age, sex, state, and year. Rates of reptile-associated serotypes isolated by state were compared to reptile ownership as reported in a 1991 American Veterinary Medical Association survey. The annual incidence of reptile-associated serotypes increased from $2.4/10^7$ persons per year in 1970 to $8.4/10^7$ persons per year in 1992. Based on US population estimates, this constitutes 150 new cases per year. The number of cases of RAS in males ($4.1/10^7$/year) was greater than in females ($3.5/10^7$/year). The 17 states that had the highest rate of reptile ownership also had the highest rate of reptile-associated serotype isolation. Infants less than one year of age had a higher incidence of infection ($66.1/10^7$/year) than older individuals. Infants also accounted for a greater number of reptile-associated serotypes (27%) than other *Salmonella* serotypes (19%).

In response to three cases of RAS in New York State in 1993 involving rare serotypes (*S.* Matadi and *S.* Poona) attributed to iguana exposure, Ackman et al. (1995) conducted a matched case-control study for that year. Cases were selected due to isolation of *Salmonella* serotypes commonly associated with reptiles or those that had been associated with reptile exposure in case reports. These serotypes included 35 in *Salmonella* subspecies I, all in subspecies II - IV, and 10 other serotypes from reported cases of RAS. Cases were matched by age (< 5 years of age within 2 years; 5 - 21 years of age within 3 years; > 21 years of age within 10 years) and date of diagnosis (within 30 days) to one or two controls. Control cases consisted of cases of shigellosis reported to the New York State Health Department during the same year. Data on symptoms, hospitalization, underlying illnesses, pet ownership, reptile exposure, and dietary history were obtained through telephone surveys with all available cases and controls. *Salmonella* was isolated from 1,362 people, 42 (3%) of which had reptile-associated serotypes. Telephone

interviews were conducted in 24 (57%) of these cases and 28 controls. Thirteen (54%) of the case patients were less than one year of age, compared to only one (4%) control. Reptile ownership was reported in 12 (50%) of the cases, compared to 2 (7%) of controls (odds ratio: 6.6; 95% confidence interval: 1.4 - 31.0). There was only one significant association with ownership of pets: 10 of the cases, and no controls, owned iguanas.

Mermin et al. (1997) investigated the clinical aspects and risk factors associated with *Salmonella* Marina infection in humans in 1994. Attempts were made to contact all patients with *S*. Marina isolates reported to the National *Salmonella* Surveillance System. Data on demographics, clinical course, diet, travel history, and reptile contact during the week before illness onset were collected by phone interview. *Salmonella* Marina was isolated from a person in 40 different households in 16 states. Interviews were conducted for 32 (80%) of those cases. Twenty-six (81%) of the 32 cases were in infants less than one year of age. Twenty-four (75%) of the cases were in males. In 28 (88%) of the cases there was a history of exposure to an iguana, including ownership in 22 households, visitation to a household with an iguana in three cases, and visitation by a relative who owned an iguana in three cases. In only 4 (14%) of the 28 cases was there a history of contact with an iguana. Only 12 (43%) of the 28 patients with exposure to an iguana suspected that the animal was the source of the infection.

Despite the relatively large number of reptiles on both private and public display, there are few outbreaks of RAS. Freidman et al. (1997) report on an outbreak of *Salmonella* Enteritidis associated with a Komodo dragon (*Varanus komodoensis*) exhibit at the Denver Zoological Gardens. In January 1996, several children in Jefferson County, Colorado were found to have *S*. Enteritidis in their stool. The only common link between them was that they had all attended a 9-day Komodo dragon exhibit at the zoo. A matched case-control study was

conducted to investigate the extent of the outbreak. Confirmed cases were defined as persons with gastrointestinal illness and *S*. Enteritidis isolated from their stool that had visited the dragon exhibit during the 9-day period. Suspect cases were defined as persons who had attended the exhibit during the specified time period and experienced illness within eight days of their visit. Secondary cases were defined as persons meeting the criteria of a confirmed or suspect case, but who became ill subsequent to a confirmed or suspect case in the household. Controls were selected from a list of zoo patrons who had attended a promotional event during the exhibit. Cases and controls were matched by age group (nine total) and day of the exhibit. Telephone interviews were conducted to gather information on demographics, medical history, clinical illness, exposures, and behaviors at the zoo. Culture samples were collected from the four Komodo dragons, their food source (frozen rats), zoo personnel that worked with them, and the exhibit environment.

Sixty-five persons with illness were identified, 39 cases confirmed by culture and 26 suspect cases. Forty-eight patients, 33 confirmed and 15 suspected cases, were the first to become ill in their households. The median age of the patients was 7 years (range: 3 months - 48 years), with 53 (82%) under 13 years of age and 34 (55%) male. The median time to onset of illness was 3.5 days. For the case-control study, 26 cases were matched to 49 controls. Three behaviors were found to be significant. Touching the barrier around the dragon exhibit was associated with significant risk (odds ratio: 4.0; 95% confidence interval: 1.2 - 13.9). Hand washing after visiting the exhibit (OR: 0.14; 95% CI: 0.03 - 0.68) and hand washing before the next meal (OR: 0.10; 95% CI: 0.02 - 0.47) were found to significantly decrease the risk of infection. Culture results were positive for *S*. Enteritidis from one of the dragons and five of 29 samples collected from the exhibit barrier. Zoo patrons were allowed to put their hands on the

barriers, which were also accessible to the dragons. Phage typing confirmed that all of the isolates from the cases, barriers, and dragon were the same.

In response to the continued problem of RAS, the Association of Reptilian and Amphibian Veterinarians (ARAV), in cooperation with the CDC, produced a client education handout (ARAV 1998). The handout explains the zoonotic risk of *Salmonella* associated with reptile exposure. It also proposes several guidelines for decreasing that risk, emphasizing proper hygiene standards and limiting reptile exposure for those groups of people at the greatest risk (e.g., children < 5 year of age and the immunocompromised).

2.6 Methods to Eradicate or Suppress *Salmonella*

2.6.1 Antimicrobials

After the 1972 and 1975 FDA regulations on turtles and turtle eggs were implemented, investigations began to find procedures for reducing or eliminating *Salmonella* in these animals. Kaufmann et al. (1972) attempted investigated the use of copper to decontaminate turtle ponds. Starting just before breeding season, a turtle pond was maintained at 2 -5 ppm copper for six months. Though the prevalence of *Salmonella* in the water was reduced, the copper had no effect on *Salmonella* in the surrounding egg nest soil, adult animals, or hatchlings. Siebeling et al. (1975) found that hatchling red-eared slider turtles (*Trachemys scripta elegans*) (RES) treated with terramycin or tylosin in their tank water for up to 14 days had reduced shedding of *Salmonella*. However, the antibiotic treatment did not affect enteric colonization. Gentamicin, an aminoglycoside, was evaluated as a potential treatment using techniques described in poultry production (Siebeling et al. 1984). Exposing RES eggs to gentamicin via temperature- or pressure-differential methods was found to decrease the prevalence in the resulting hatchlings to < 2%, compared to > 40% of the control hatchlings (Siebeling et al. 1984). In 1985, this

treatment method was adopted by the Louisiana Department of Agriculture, and became a mandatory requirement.

The use of antibiotics in turtles and their eggs has led to findings of resistance in strains of *Salmonella*, an added public health concern. D'Aoust et al. (1990) tested 28 lots of RES eggs that were exported from Louisiana to Canada and found that six lots (21%), representing three of four exporters, yielded *Salmonella* from the eggs or the packing moss in which they were shipped. Gentamicin resistance was found in 81% (30/37) of the *Salmonella* strains recovered. In a study of two turtle farms in southern Louisiana, hatchlings from one farm yielded *Salmonella* strains resistant to erythromycin, gentamicin, tetracycline, and sulfonamides. *Salmonella* isolates from pond water on both farms showed similar resistance patterns (Shane et al. 1990). An examination of the records of 115 batches of hatchlings from 28 turtle farms delivered to the Louisiana Department of Agriculture and Forestry in 1988 revealed an additional four *Salmonella* isolates that were resistant to the same antibiotics (Shane et al. 1990). During the period 1999 - 2002, *Salmonella* and other potential pathogenic bacteria isolated from Louisiana turtle farms were demonstrated to have high levels of resistance to gentamicin and other aminoglycosides, such as kanamycin and tobramycin (Diaz et al. 2006). Isolates of *Salmonella* were found to have resistance to gentamicin at levels of > 2,000 µg/ml.

Due to the emergence of antibiotic-resistant *Salmonella* strains from turtles and eggs, there has been a focus on the use of other non-antibiotic antimicrobials for treatment. Recent work has followed in the footsteps of the poultry industry. Cox et al. (1994) evaluated different microbicidal compounds for their effectiveness at eliminating S. Typhimurium from experimentally inoculated chicken eggs. Of the chemicals tested by spray application, including quaternary ammonium, peroxygen compounds, hydrogen peroxide (H_2O_2), ethylene oxide,

phenols, and sodium and potassium hydroxide, only the 0.035% polyhexamethylene biguanide (PHMB) solution was 100% effective. In another study, eggs were immersed in one of three different antimicrobial solutions at 1 minute, 5 minutes, 4 hours, or 24 hours after inoculation with *Salmonella* (Cox et al. 1998). Of the three solutions, 0.05% PHMB was the most effective (93%) at reducing the prevalence of *Salmonella* (0.78% phenol: 80% reduction; 1% H_2O_2: 85% reduction). The effectiveness of all three solutions decreased with time, until there was no significant reduction in prevalence by 24 hours. Cox et al. (1999) applied 0.035% PHMB or 1.4% H_2O_2 to eggs experimentally inoculated with *S*. Typhimurium or *S*. Heidelberg. Half of the eggs were exposed to the treatments with a vacuum cycle followed by an atmospheric pressure dip; the other half received only the atmospheric pressure dip. Both of the solutions reduced the prevalence of *Salmonella* with the pressure dip alone (PHMB: 82% elimination; H_2O_2: 68% elimination). With the vacuum, the prevalence was further reduced (PHMB: 88% elimination; H_2O_2: 90% elimination).

The antimicrobial agent PHMB has been safely used in humans (Rosin et al. 2001) and animals (Panda et al. 2003). One derivative of PHMB is poly-iminoimidocarbonylimino-hexamethylene hydrochloride (Baquacil®, Arch Chemicals, Inc., Norwalk, Connecticut 06856), which is used as a swimming pool sanitizer and algistat. Mitchell et al. (2005) showed that the application of this product in the water of RES at concentrations of 25 and 50 ppm significantly reduced the prevalence of *Salmonella* in the aquatic medium, but did not eliminate it from the turtles. The compound was found to be safe for the turtles at both the 25 and 50 ppm concentrations, based on gross and histologic examination. In a RES egg treatment study, eggs from eight aquatic chelonian farms in Louisiana were collected from nests and exposed to different treatments of antimicrobial products (Mitchell et al. 2007). Eggs were exposed to a

combination of sodium hypochlorite (NaOCl) (1,000 ppm) and PHMB (50 ppm) as a bath, pressure-differential dip, or NaOCl and gentamicin (1,200 μg/ml) as a pressure-differential dip. Eggs were then incubated in sanitized containers and the hatchlings were cultured for *Salmonella*. All the treatment methods were found to significantly reduce the prevalence of *Salmonella* compared to a control group. The PHMB as a pressure-differential was significantly more effective than the bath. The final odds ratios (OR) for the likelihood that *Salmonella* would be cultured from the treated hatchlings compared to controls were: PHMB pressure-differential dip 0.01, PHMB bath 0.2, gentamicin pressure-differential dip 0.1.

2.6.2 Competitive Exclusion

Competitive exclusion (CE) is a phenomenon in which certain constituents of the intestinal microbial flora inhibit the multiplication of and colonization by other bacteria, particularly pathogens. The concept of CE has been acknowledged for some time, dating back at least to Metchnikoff (1908), who suppositioned that beneficial organisms could displace pathogens, thus improving intestinal health and prolonging life. However, the study of the mechanisms and application of CE to reduce food-borne illness did not begin in earnest until more recently.

In many countries, *Salmonella* are the principal cause of human food-borne infections (Stavric and D'Aoust 1993), and the importance of poultry as a source of this illness has increased in parallel with the global consumption of poultry meat (Todd 1980). Nurmi and Rantala (1973) were the first to demonstrate that 1 - 2 day old chicks given a crop gavage of gut contents from healthy adult chickens afforded significant inhibition of *Salmonella* colonization after challenge infection compared to untreated controls. This was quickly followed by a larger study in which different CE treatments were used (Rantala and Nurmi 1973). Newly-hatched

chicks were given a crop gavage of fresh crop and intestinal contents from a healthy adult cock; cultured, *Salmonella*-free intestinal flora from a healthy adult cock; or a mixture of cattle rumen contents and horse feces. An untreated control group was also included, and all the chicks were challenged with an inoculum of *Salmonella* Infantis. *Salmonella* was not isolated from the ceca of any of the animals treated with the fresh or cultured cock gut contents, whereas *Salmonella* was readily cultured from the ceca of the chicks in the other two groups. It was suggested that CE was most effective during the first few days post-hatch, establishing a more stable bacterial population when chicks are most susceptible to *Salmonella* colonization.

Competitive exclusion products can be divided between those that are undefined and those that are defined. Defined CE cultures utilize bacteria that are classified as "generally regarded as safe" by the FDA. There are 42 organisms in this category, the majority of which are *Lactobacillus* spp. (Corrier and Nesbit 1999).

Undefined CE cultures are prepared from the intestinal bacterial flora of healthy, preferably *Salmonella*-free, adult animals. It is interesting to note that CE cultures from specific pathogen-free (SPF) birds are generally less protective than those from birds that are not raised as SPF (Stavric and D'Aoust 1993). While the majority of CE cultures are developed anaerobically, it has been shown that both strictly aerobic and strictly anaerobic cultures afford significant protection from *Salmonella* colonization in chicks (Revolledo et al. 2003). However, a mixed culture showed more protection than either. Nisbet et al. (1993) developed a continuous-flow steady-state culture system derived from the cecal microflora of chickens. It has been shown to be protective against *Salmonella* Typhimurium colonization in chicks (Nisbet et al. 1994), and led to the development of a patented characterized continuous-flow culture (CCF) containing 15 facultative and 14 obligate anaerobes representing 10 genera (Nisbet et al.

1995). Corrier et al. (1995b) demonstrated that this CCF was effective in chicks against experimental challenge with *Salmonella* Typhimurium. It has also been demonstrated to be effective in protecting chicks from challenge by *Salmonella* from environmental sources (Corrier et al. 1995a).

While undefined CE cultures do have the potential to transmit microbial pathogens, nearly 20 years of experimentation with them has shown no instances of this concern (Stavric and D'Aoust 1993). Viruses and protozoans are unlikely to multiply in bacteriological culture media, and cultural conditions that are used do not favor the growth of potential pathogenic bacteria, such as *Listeria monocytogenes* and *Campylobacter jejuni* (Stavric and D'Aoust 1993). Blankenship et al. (1993) demonstrated that an undefined CE culture can be free of specific pathogens.

The mechanisms by which CE cultures exclude or inhibit the growth of enteropathogens are not fully understood. Four possible mechanisms were suggested by Rolfe (1991): 1) creation of a microecology that is hostile to other bacterial species, 2) synthesis of antibacterial compounds, 3) elimination of available bacterial receptor sites, and 4) depletion of essential nutrients. Microbes that synthesize volatile fatty acids (VFA) can reduce the intestinal pH to levels which inhibit the growth of enteric pathogens. Meynell (1963) demonstrated that an acidic intestinal pH can reduce the survival of *Salmonella* and other species in the family Enterobacteriaceae. It has been shown in mice that the increase in intestinal VFA coincides with a decrease in the level of coliform bacteria (Lee and Gemmell 1972). However, the results of studies that examine levels of VFA associated with CE administration are mixed. Nisbet et al. (1994) applied a continuous-flow culture to chicks on the day of hatch, and found significant correlations between total VFA and propionic acid increases on day three and decreases in

Salmonella on day ten in the cecum. In a similar study, Corrier et al. (1995b) reported results that were much the same. It was determined that the acetate and proprionate produced by a CE culture of *Lactobacillus crispatus* and *Clostriudium lactatifermentans* inhibited *Salmonella* Enteriditis in an *in vitro* mimic of chicken cecal conditions (van der Wielen et al. 2002). Hudault et al. (1985) administered either dilutions or cultures of chicken cecal contents to groups of chicks. Despite a level of cecal VFA three-fold greater in one group than the other, both groups had significant suppression of cecal levels of *Salmonella* Typhimurium.

The production of antimicrobial metabolites by bacteria used in CE cultures has been investigated to some degree. Reuterin production by *Lactobacillus reuteri* has received the most attention from scientists. Reuterin is a broad-spectrum antimicrobial compound produced in association with the anaerobic metabolism of glycerol (Edens et al. 1997). Concentrations as low as 10 to 30 µg/ml can kill *Salmonella* spp., *Escherichia coli*, and *Campylobacter* spp. within 30 to 90 minutes, while 15 to 30 µg/ml can inhibit the growth of Gram-negative and most Gram-positive bacteria, yeast, fungi, and protozoa (Edens et al. 1997). However, concentrations of 60 to 150 µg/ml are needed to kill lactic acid bacteria, including *L. reuteri* (Chung et al. 1989), possibly due to ecosystem implications in the intestinal tract (Edens. et al. 1997). Microcins are peptides produced by the Enterobacteriaceae that kill by inhibition of DNA gyrase or protein synthesis (Wooley et al. 2001). In an *in vitro* study using *Salmonella* recovered from reptiles, microcin-producing *E. coli* inhibited all but one of 57 isolates (Wooley et al. 2001). Forty-four (77.2%) were strongly inhibited, and 13 (22.8%) were weakly inhibited.

The competition for receptor sites on enterocytes and essential nutrients can help to suppress enteropathogens in the intestinal tract. Common bacteria have cell wall components that mediate adherence to each other and the intestinal epithelium (Edens et al. 1997).

Application of CE cultures can increase the numbers of these bacteria, thus increasing the effectiveness of this block on receptor sites. Increased numbers of common bacteria also causes greater depletion of nutrients from enteropathogens, helping to limit their ability to establish and multiply in the intestinal tract. However, this is probably not the most effective control mechanism of CE (Edens et al. 1997), particularly since many organisms are able to utilize alternative metabolic pathways (Mitchell 2001).

The application of CE has been manipulated in different ways in attempts to increase efficacy and efficiency. Efforts have been made to determine the number of bacteria species needed in defined CE cultures to maintain efficacy. Stavric et al. (1985) inoculated groups of chicks with mixtures of 50, 40, 25, or 10 bacterial isolates, and then challenged them with S*almonella* Typhimurium. Only the mixture of 50 isolates afforded protection comparable to undefined cultures of fecal or cecal material, although the other mixtures provided some level of protection. A study using a mixture of 48 bacterial isolates in chicks resulted in similar levels of protection (Impey et al. 1982). Stavric and D'Aoust (1993) reference several studies in which single bacterial isolates or several strains of a single genus have been used and conclude that protection of chicks against *Salmonella* challenge with these techniques is inconsistent. It has been noted that cultures of a limited number of organisms (i.e., < 14) do not afford adequate protection from *Salmonella* colonization Stavric et al. (1992). It is likely that the proper number and combination of bacterial isolates needed to maximize the efficiency of defined CE will be difficult to ascertain.

Competitive exclusion combined with fructooligosaccharide administration has been attempted. Results are generally inconclusive (Stavric and D'Aoust 1993). Indigenous intestinal bacterial flora can metabolize lactose as a primary carbon source, in contrast to most strains of

Salmonella. Therefore, the addition of dietary lactose would theoretically increase the ability of indigenous flora to compete with *Salmonella* (Mitchell 2001). Also, the production of acid by the metabolism of lactose would reduce the pH of the intestinal tract, further affecting the survival of *Salmonella*. Oyofo et al. (1989) demonstrated that the addition of lactose and mannose to the drinking water of chicks at 2.5% (w/v) significantly reduced the intestinal colonization of *Salmonella* Typhimurium after challenge. Similar administrations of dextrose, maltose, and sucrose were not effective. Waldroup et al. (1992) administered lactose to chickens at concentrations of 0, 2.5, 5, and 7.5% in the diet. Not only did birds that received lactose have an increased prevalence of *S*. Typhimurium after challenge compared to controls, they also had significant reductions in body weight. Better results are reported when the use of dietary lactose is combined with administration of CE cultures. Corrier et al. (1991) demonstrated that the use of a cecal-derived CE culture combined with dietary lactose administration significantly reduced intestinal colonization, tissue invasion, and organ colonization in chicks challenged with *S*. Enteritidis, compared to either CE culture or dietary lactose used alone. These findings were supported by a subsequent study (Corrier and Nesbit 1999?), and similar results were obtained in a study in which chicks were challenged with *S*. Typhimurium (Hinton et al. 1990).

The use of CE cultures combined with antibiotic administration has been examined. In a study conducted over two years and involving chickens, turkeys, and ducks, Mead (1991) administered various antibiotics in feed for 14 days, followed by one dose of CE culture via drinking water. Chickens received chlortetracycline (200 ppm) or furazolidine (400 ppm), turkeys received neomycin (200 ppm) and chlortetracycline (200 ppm), and ducks received neomycin (200 ppm). In 20 of 22 trials, protection from *Salmonella* was achieved, and flocks remained negative for *Salmonella* for three months following treatment. Seo et al. (2000)

investigated the use of enrofloxacin and CE culture in chicks. Chicks were infected with *S*. Enteritidis and then given 10 mg/kg of enrofloxacin for 10 days in the drinking water. In one trial, the chicks were then given doses of CE culture at 8 and 10 weeks of age. In a second trial, the chicks were given doses of CE culture at 10 and 12 weeks of age. A significant reduction in the number of infected chickens and the amount of cecal *Salmonella* colonization was found in the second trial, compared to control birds treated with enrofloxacin only. No significant difference was found in the first trial.

One report of the use of CE culture combined with vaccination has been published (Methner et al. 2001). Specific pathogen free chicks were administered a live *S*. Typhimurium vaccine, a CE culture, or both at 1 and 2 days of age. On day 3, they were challenged with an inoculum of *S*. Typhimurium, and the amount of *Salmonella* present in the ceca was examined on day 43. There was no significant difference found between the combined treatment and either of the treatments alone.

The technique by which CE cultures are applied can influence efficacy. Cox et al. (1990) demonstrated that chicks administered CE culture either orally or intracloacally were equally protected from a challenge with *S*. Typhimurium. A comparison of application methods, including crop gavage, upper body spray, and vent lip administration was performed by Corrier et al. (1994). Chicks in each group had significant decreases in *S*. Enteritidis colonization compared to a control group, and all of the treatment groups had comparable results. It has been shown that a two-step method of CE culture administration, involving spraying chicks and inoculating drinking water, is effective at reducing *Salmonella* prevalence in both animals and their environment (Blankenship et al. 1993). Efforts to perform *in ovo* CE administration have met with variable success. Administration of an undefined CE culture into the air cell of 18 day

old chicken eggs produced newly hatched chicks that had significant resistance to challenge with *S*. Typhimurium compared to controls (Cox et al. 1990). Edens et al. (1997) describe numerous studies in which *in ovo* administration of *L. reuteri* has been effective at reducing *Salmonella* in chicks. In a study involving 18 day old eggs, a cecal-derived CE culture, a diluted culture, or *L. salivarius* was administered into the air sac (Filho et al. 2006). The resulting chicks were challenged with *S*. Enteritidis at 2 days of age, and at 5 days of age, the livers and ceca were examined for *Salmonella.* None of the treatments afforded significant protection against *Salmonella* colonization in those organs.

The application of CE cultures may be a viable method for reducing or preventing S*almonella* infection in reptiles. The initial step toward such a product is the characterization of the intestinal bacterial flora of reptiles. This has been undertaken in the green iguana (*Iguana iguana*) (Salb et al. 2007) and the Tokay gecko (*Gecko gecko*) (Tan et al. 1978). Current efforts are underway to identify the intestinal microflora associated with the red-eared slider turtle (*Trachemys scripta elegans*) (M. Mitchell, Louisiana State University, personal communication). Further studies to evaluate the efficacy of this control method in reptiles are required.

2.6.3 Vaccination

The use of vaccines is intended to stimulate the immune system of a host by exposing it to a non-virulent antigen(s) from a pathogen. Stimulation of the immune system may involve cell-mediate immunity (CMI), humoral immunity (HI), or both, depending on the antigen(s) used. Satisfactory vaccines should be avirulent in animals and humans, highly immunogenic, genotypically stable, unaffected by diet or host, and simple to manufacture and administer (Curtiss et al. 1993).

Salmonella is considered a ubiquitous colonizer of farm animals, and vaccination may not be a feasible approach for its control (Curtiss et al. 1993). However, investigation into the use of vaccination to control or eliminate *Salmonella* in domestic livestock and poultry is ongoing. Vaccines can be made from whole or parts of pathogens, and there are various manifestations of each of these types.

Whole pathogenic organisms can be used as live or inactivated vaccines. Inactivated vaccines mainly stimulate the HI system, can be rapidly eliminated from the host, only express the antigens present at the time of *in vitro* harvesting, and cannot participate in colonization of the gastrointestinal system (van Immerseel et al. 2005). These attributes make them less desirable as vaccine candidates. However, they do have less potential for virulence in the host. Gast et al. (1993) evaluated the protective ability of an acetone-killed *Salmonella* Enteritidis vaccine in chickens. Parenteral administration was carried out twice, four weeks apart, followed by challenge with a virulent strain of *S*. Enteritidis. Vaccinated chickens had significantly higher specific antibody levels and significantly lower fecal shedding of *Salmonella* than unvaccinated control birds. *Salmonella* was not eliminated, however, as more than 50% of the vaccinated chickens continued to shed the challenge strain. Timms et al. (1990) evaluated a formalin-killed *S*. Enteritidis vaccine given to chickens at three weeks or three and six weeks of age, followed by challenge with a virulent strain of the bacteria. The vaccine was administered parenterally, as were the challenges. Moderate antibody response was seen after one vaccination, and a strong response was elicited after two. All of the vaccinated birds were afforded significant protection from organ colonization. Again, despite vaccination, many of the birds continued to shed the challenge strain.

Live vaccines are preferred to inactivated vaccines because they stimulate both the CMI and HI systems, express all antigens *in vivo*, participate in genus-specific colonization-inhibition, and stimulate primed polymorphonuclear cells in the intestine (van Immerseel et al. 2005). Live vaccines can be made from natural avirulent strains, by attenuation, or by creating deletion mutants. Roof and Doitchinoff (1995) vaccinated young pigs with a single intranasal dose of a naturally-avirulent *Salmonella* Cholerasuis strain, followed by challenge with a virulent strain at 2, 4, or 20 weeks. Significant protection and reduction in organ colonization was afforded up to 20 weeks after initial vaccination. In a second study, a different avirulent strain of *S*. Cholerasuis was administered to pigs conjunctivally at different doses (Kramer et al. 1987). Similar results were seen after challenge with a virulent strain.

Live vaccines created from deletion mutants have been shown to be effective. The deletions are intended to reduce the growth and virulence of *Salmonella* without affecting key virulence determinants (van Immerseel et al. 2005). Curtiss and Kelly (1987) found that deletion of genes for adenylate cyclase and cyclic AMP receptor protein created *S*. Typhimurium mutants that afforded good protection to challenge in mice. It was later found that this mutant strain afforded significant protection to chickens from both homologous and heterologous *Salmonella* challenge (Hassan and Curtiss 1994). Dueger et al. (2003) vaccinated newly hatched chicks with *S*. Typhimurium containing a deletion in a gene for DNA adenine methylase. This mutant strain protected vaccinated chicks against homologous and heterologous *Salmonella* challenge. Organ colonization was significantly less than controls. Methner et al. (2004) found that *S*. Typhimurium and *S*. Enteritidis with a deletion mutation of the *phoP* gene were able to participate in significant colonization-inhibition in chicken intestines. Both were able to afford

significant protection from and decreased liver colonization by homologous and heterologous *Salmonella* challenge.

The use of subunit vaccines has also been investigated. Subunit vaccines include one or more specific epitopes from a pathogen. Bouzoubaa et al. (1987) investigated the use of a S*almonella* Gallinarum outer membrane protein (OMP) extract as a vaccine. Chickens were administered two injections of the extract, three weeks apart, at concentrations of 50, 100, 200, or 400 µg/100 g body weight. Three weeks after the second dose, the birds were challenged with virulent *S*. Gallinarum orally. Birds were euthanized 30 days post-challenge and internal organs were examined for colonization. All the concentrations were found to be significantly protective. Organ colonization appeared to be positively associated with OMP concentration. Khan et al. (2003) evaluated the use of two *S*. Enteritidis OMP that are typically involved in *Salmonella* attachment to host cells. Chickens were given two 10-µg, parenteral injections of either a 75.6 kDa or an 82.3 kDa OMP, two weeks apart. Birds were challenged with an oral dose of virulent *S*. Enteritidis one week after the second injection. After 24 hours, all the inoculated birds exhibited mild to moderate diarrhea, but no mortalities. Significant reduction in prevalence of the challenge strain was found in the ceca and small intestine, compared to uninoculated controls.

Compared to mammalian species, little is known about the immune responses to virulent and attenuated *Salmonella* strains in poultry (van Immerseel et al. 2005). Even less is known about the immune responses of reptile species in general. Vaccination as a method to control *Salmonella* in reptiles may be possible. However, in a study to evaluate the effect of a commercial, attenuated *Salmonella* Typhimurium vaccine (Megan®Vac1, AVANT Immunotherapeutics, Inc., Needham, Massachusetts 02494) on colonization in green iguanas

(*Iguana iguana*), no significant effect was found (Mitchell 2001). Iguanas were cleared of *Salmonella* by antibiotic administration before the vaccine trial began. Iguanas were given two injections, two weeks apart. Animals received saline twice or vaccine twice. One week after the second injection, half the iguanas in each group received a challenge dose of *S*. Typhimurium orally. Cloacal swabs were collected once every seven days and necropsy tissue samples of internal organs were collected after three weeks. All samples were examined for the challenge strain with microbiologic culture and polymerase chain reaction. There was no difference in the amount of *Salmonella* shedding or organ colonization between the vaccinated animals and controls.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Hypotheses

The primary objective of this study was to evaluate the effect of Baquacil® and Sanosil® on *Salmonella* in the aquatic habitat of red-eared slider turtles (RES). The secondary objective was to determine the safety of Baquacil® and Sanosil® for RES. There were four hypotheses evaluated in this study:

1. Water samples from turtle habitats treated with Baquacil® or Sanosil® would be less likely to be *Salmonella*-positive than samples from the control group (H_0: $\mu_T = \mu_C$; H_1: $\mu_T < \mu_C$).

2. There would be no difference in the prevalence of *Salmonella* in gastrointestinal samples from the turtles in the Baquacil® or Sanosil® groups and the control group (H_0: $\mu_T = \mu_C$; H_1: $\mu_T \neq \mu_C$).

3. There would be no difference in the frequency of *Salmonella* isolation between the initial and the delayed secondary enrichment (DSE) cultures (H_0: $\mu_I = \mu_{DSE}$; H_1: $\mu_I \neq \mu_{DSE}$).

4. There would be no difference in the pathological findings between the Baquacil® or Sanosil® groups and the control group (H_0: $\mu_T = \mu_C$; H_1: $\mu_T \neq \mu_C$).

3.2 Study Design and Sample Collection

This study was approved by the Louisiana State University Institutional Animal Care and Use Committee. Eighty-four hatchling RES were obtained from an aquatic chelonian farm in Ponchatoula, Louisiana. The turtles were collected from a group of animals that were not treated as eggs for *Salmonella* using the techniques outlined in Mitchell et al. (2007). The turtles were housed in individual plastic containers and provided a mortar brick for basking and

approximately two liters of chlorinated tap water. The turtles were examined for any obvious physical abnormalities or behaviors. Each turtle was weighed to the nearest 0.1 gram.

Before the treatments were applied, all the turtles were kept in chlorinated tap water for a two-month acclimation period and confirmed to be shedding *Salmonella* using standard microbiological techniques. A total of 3 ml of water was collected from around the base of the brick in each enclosure using a new, sterile 3-ml syringe. The samples were added to 7 ml of selenite enrichment broth and incubated at 37 ^{O}C (98.6 ^{O}F) for 48 h under aerobic conditions. After incubation, the enriched selenite cultures were mixed on a Vortex agitator for five seconds. A heat-sterilized bacterial loop was used to transfer an aliquot of enriched broth to the surface of a petri dish containing xylose-lysine-tergitol agar (Hardy Diagnostics, Santa Maria, California 93455). Streaked plates were incubated at 37 ^{O}C (98.6 ^{O}F) for 48 h under aerobic conditions. Presumptive *Salmonella* colonies were identified based on colony characteristics, including irregular shape, clear growth, hydrogen sulfide (H_2S) production, and lack of lactose fermentation. Selected colonies were evaluated on indicator media including urea agar, lysine iron agar (LIA), and triple sugar iron agar (TSI). A heat-sterilized bacterial loop was used to streak a portion of a suspect colony onto slants of urea, LIA, and TSI agar. A heat-sterilized inoculation needle was used to stab a portion of a suspect colony twice into the LIA and once into the TSI. The preparations were incubated aerobically at 37 ^{O}C (98.6 ^{O}F) for 24 h. The presence of *Salmonella* was denoted by a negative urea test, positive LIA with H_2S production, and alkaline over acid with H_2S production in the TSI.

A random number generator was used to assign the animals to seven different groups: Group 1) 10 ppm Baquacil®, Group 2) 5 ppm Baquacil®, Group 3) 50 ppm Sanosil®, Group 4) 50 ppm Baquacil®, Group 5) 10 ppm Sanosil®, Group 6) chlorinated tap water, Group 7) 100

ppm Sanosil®. Each treatment group was comprised of 12 turtles. The water from the enclosures was changed on the same day each week, sanitized plastic containers were exchanged for the old ones, and fresh treatment solutions were made weekly. The solutions of Baquacil® were made by dechlorinating (Aqua-Plus, Rolf C. Hagen Corp., Mansfield, Massachusetts 02048) and adjusting the pH of the tap water to 7.5 (Proper pH® 7.5, Aquarium Pharmaceuticals, Inc., Chalfont, Pennsylvania 18914). No special treatments were applied to the Sanosil® solutions. The ambient air temperature of the room the animals were housed in was maintained between 24 - 27 ^{O}C (76 - 80 ^{O}F), and the water temperature was maintained between 21 - 23 ^{O}C (70 - 73 ^{O}F). The turtles were provided a 12-hour photoperiod. A *Salmonella*-free commercial turtle pellet food (Fluker's Aquatic Turtle Diet, Fluker Farms, Port Allen, Louisiana 70767) was offered to the animals daily.

After the initial treatment water change, water samples were collected twice a week for eight weeks. Sample collection was always performed on days three and six for a given week. Every sample was evaluated for *Salmonella* using the techniques described previously. A DSE was performed on each negative sample to increase the likelihood of identifying low concentrations of *Salmonella*. For the procedure, the original selenite samples were placed at room temperature (24 ^{O}C, 75 ^{O}F) for 96 h. A 3-ml aliquot of the original selenite sample was added to 7 ml of selenite enrichment broth and incubated aerobically at 37 ^{O}C (98.6 ^{O}F) for 48 h. The samples were processed using the techniques described previously.

At the conclusion of the eight-week trial, all of the turtles were weighed and anesthetized with ketamine hydrochloride (100 mg/kg/turtle, intramuscular) (Ketaset®, Fort Dodge Animal Health, Overland Park, Kansas 66225). The turtles were then humanely euthanized using an overdose of pentobarbital sodium and phenytoin sodium (0.1 ml/turtle, intracoelomic)

(Beuthanasia®-D Special, Schering-Plough Animal Health Corp., Union, New Jersey 07083). The firmness of the turtles' shells was measured using an ordinal scale: 0 = firm shell, 1 = mildly distensible shell, 2 = easily distensible shell. To determine the shell score, the bridge of the shell was gripped between the thumb and index finger of one hand and squeezed. All of the turtles were scored by one evaluator.

A random number generator was used to select nine turtles from each treatment group for evaluation of the gastrointestinal tract for presence of *Salmonella*. Sterile techniques were used to remove the gastrointestinal tract (Mitchell et al. 2005). Scissors were used to cut the bridge of the shell and facilitate removal of the plastron. The gastrointestinal tract was excised, placed into 7 ml of enriched selenite broth, and a sterile cotton-tipped applicator used to macerate the tissues. The samples was further macerated and mixed using a Vortex agitator for five seconds. The samples were incubated aerobically at 37 ^{O}C (98.6 ^{O}F) for 48 h and processed using the techniques described. A 96-hour DSE was performed on all the samples that failed to yield *Salmonella* from the initial culture.

The three remaining turtle carcasses from each treatment group were submitted for histopathologic evaluation. Scissors were used to cut the bridge of the shell and remove the plastron. The carcass was then placed into 100 ml of 10% neutral buffered formalin and fixed for seven days. A complete histologic review of all the tissues was performed. The tissues were processed routinely, sectioned (4 μm thicknesses), stained with hematoxylin and eosin, and examined microscopically. The amount of ossification at the midline and the bridge of the carapace of each turtle was measured with an ordinal scale, from 0 = no ossification to 4 = nearly complete ossification.

3.3 Sample Size Determination

The sample size required for this study was calculated under the following assumptions and criteria: that the proportion of *Salmonella* in the water column of the turtles in the control group would be > 0.9, the prevalence of *Salmonella* in the water column of the turtles in the treatment groups would be < 0.1, that the $\alpha = 0.5$, and that the power = 0.8.

3.4 Statistical Analyses

The 95% binomial confidence intervals (CI) were calculated for each of the proportion estimates and odds ratios. The first hypothesis tested in this study was that the water samples from turtle habitats treated with Baquacil® or Sanosil® would be less likely to be *Salmonella*-positive than samples from the control group (H_0: $\mu_T = \mu_C$; H_1: $\mu_T < \mu_C$). A Cochran's Q test was used to determine if there was a significant difference in the *Salmonella* status of the water samples over time. A multinomial logistic regression model was developed to predict the *Salmonella* status of a sample using the variables treatment group (Group), day of sample collection (Day), and week of study (Week). The second hypothesis was that there would be no difference in the prevalence of *Salmonella* in gastrointestinal samples from the turtles in the Baquacil® or Sanosil® groups and the control group (H_0: $\mu_T = \mu_C$; H_1: $\mu_T \neq \mu_C$). A chi-square test was used to determine if there was a significant difference between treatment groups for prevalence of *Salmonella*. The third hypothesis was that there would be no difference in the frequency of *Salmonella* isolation between the initial and the delayed secondary enrichment cultures (H_0: $\mu_I = \mu_{DSE}$; H_1: $\mu_I \neq \mu_{DSE}$). All of the paired initial and DSE cultures throughout the study were tabulated. A McNemar's change test was used to determine if there was an overall significant difference in the frequency of *Salmonella* isolation between the initial and DSE cultures. Each treatment group was then evaluated with a McNemar's change test using a

binomial distribution. The final hypothesis tested was that there would be no difference in the pathological findings between the Baquacil® or Sanosil® groups and the control group (H_0: $\mu_T = \mu_C$; H_1: $\mu_T \neq \mu_C$). A Kruskal-Wallis one-way analysis of variance (ANOVA) was used to determine if there was a significant difference in shell score, carapace midline ossification, or bridge ossification between treatment groups. The difference in pre- and post-treatment weights was calculated for each turtle. A Shapiro-Wilk test was used to determine the normality of the weight difference data. A Kruskal-Wallis ANOVA was used to determine if there was a significant difference in weight change between treatment groups. To determine differences in the prevalence of histopathologic abnormalities between treatment groups, the 95% CI were compared visually. Values of $p < 0.05$ were considered statistically significant. Statistical analysis was performed using SPSS 11.0 (SPSS, Inc., Chicago, Illinois, 60606).

CHAPTER FOUR

RESULTS

All of the turtles remained clinically normal throughout the study period. There was a significant within-groups difference over time (Cochran's Q = 103.3, $p < 0.0001$). The multinomial logistic regression model was found to be significantly different from a null model at predicting the outcome (Table 1), and demonstrated adequate goodness-of-fit (Pearson chi-square = 108.76, $p = 0.19$). No significant interactions among the variables were found. Each of the variables was found to have a significant effect in the model (Table 1). Groups 4 (50 ppm Baquacil®) and 5 (10 ppm Sanosil®) were significantly different from the control group (Table 2 and 3); day 6 was significantly different from day 3 (Tables 2 and 4); weeks 5 - 8 were significantly different from the first week (Tables 2 and 5). There was no significant difference in the prevalence of *Salmonella* in the gastrointestinal samples between any of the treatment groups ($\chi^2 = 11.3$, $p = 0.08$) (Table 6). There was an overall significant difference in the frequency of *Salmonella* isolation between the initial and DSE cultures ($\chi^2 = 49.0$, $p < 0.0001$). There was disagreement between the two groups in 51 of the 775 times (6.6%) that DSE was employed. Interestingly, all the treatment groups but Group 7 (100 ppm Sanosil®) also showed a significant difference in the frequency of *Salmonella* isolation between the initial and DSE cultures (Table 7). There was no significant difference in the shell score ($\chi^2 = 3.29$, $p = 0.77$), carapace midline ossification ($\chi^2 = 10.23$, $p = 0.12$), or bridge ossification ($\chi^2 = 2.56$, $p = 0.86$) between any of the treatment groups. The pre- and post-treatment differences in weight for the turtles were not normally distributed ($p < 0.0001$). There was no significant difference in the weight differences between any of the treatment groups ($\chi^2 = 6.1$, $p = 0.41$). All but one of the

turtles gained weight over the course of the study. The median weight gain was 2.2 g (min/max: -1.1/10.7; 10 - 90% quantiles: 0.8 - 5.1).

All of the turtles had mild granulocytic accumulations in the liver and kidney consistent with extramedullary hematopoiesis. Two turtles had noteworthy histopathologic findings. One turtle from Group 7 (100 ppm Sanosil®) had mild granulomatous omphalophlebitis with fibrinous and granulomatous coelomitis associated with a ruptured yolk sac. One turtle in Group 4 (50 ppm Baquacil®) had severe heterophilic cuffing of the hepatic and renal vasculature with perivascular heterophils observed in the vasculature of the carapace. The heterophilic cuffing was often severe enough to displace the surrounding tissue, but no significant tissue damage was observed to be associated with the inflammatory cells. Differential diagnoses for this condition included: septicemia, aberrant/extreme extramedullary hematopoiesis, myeloproliferative disease.

Table 1. The -2 Log Likelihood (-2LL) of the multinomial logistic regression model.

Variable	-2LL	χ^2	p
Model	406.86	263.58	< 0.0001
Group	591.26	184.4	< 0.0001
Day	427.72	20.86	< 0.0001
Week	477.81	70.95	< 0.0001

Table 2. Statistics for variables in the multinomial logistic regression model: p-value, odds ratio (OR), and 95% confidence interval (CI) of the odds ratio.

Variable	p	OR	CI
Group 1	0.13	1.38	0.91 - 2.11
Group 2	0.29	0.79	0.53 - 1.21
Group 3	0.52	1.15	0.75 - 1.74
Group 4	< 0.0001	3.85	2.4 - 6.17
Group 5	< 0.0001	0.17	0.1 - 0.27
Group 6[a]	NA	NA	NA
Group 7	0.52	1.15	0.75 - 1.74
Day 3[a]	NA	NA	NA
Day 6	< 0.0001	0.58	0.45 - 0.73
Week 1[a]	NA	NA	NA
Week 2	0.72	0.92	0.58 - 1.46
Week 3	0.64	1.12	0.7 - 1.77
Week 4	0.48	0.85	0.53 - 1.34
Week 5	0.04	1.6	1.01 - 2.55
Week 6	0.002	2.08	1.3 - 3.32
Week 7	< 0.0001	3.54	2.17 - 5.78
Week 8	< 0.0001	2.91	1.8 - 4.7

[a] Used as a control in the regression model.
NA = not applicable.

Table 3. Prevalence and 95% confidence intervals (CI) of *Salmonella* by Group.

Group	Prevalence	CI
1	100%	NA
2	100%	NA
3	91.7%	90.4% - 92.9%
4	66.7%	63.0% - 70.3%
5	100%	NA
6	100%	NA
7	100%	NA

NA = not applicable.

Table 4. Prevalence and 95% confidence intervals (CI) of *Salmonella* in the water samples by Day.

Day	Prevalence	CI
3	40.5%	36.8% - 44.2%
6	51.6%	47.9% - 55.4%

Table 5. Prevalence and 95% confidence intervals (CI) of *Salmonella* in the water samples by Week.

Week	Prevalence	CI
1	54.2%	46.6% - 61.7%
2	57.1%	49.7% - 64.6%
3	51.8%	44.2% - 59.3%
4	58.9%	51.5% - 66.4%
5	45.2%	37.7% - 52.8%
6	39.3%	31.9% - 46.7%
7	29.2%	22.3% - 36.0%
8	32.7%	25.6% - 39.8%

Table 6. Prevalence and 95% confidence intervals (CI) of *Salmonella* in the gastrointestinal samples by Group.

Group	Prevalence	CI
1	66.7%	35.9% - 97.5%
2	100%	NA
3	88.9%	68.4% - 100.0%
4	88.9%	68.4% - 100.0%
5	100%	NA
6	100%	NA
7	100%	NA

NA = not applicable.

Table 7. Results by treatment group for McNemar's change test to evaluate intial versus delayed secondary enrichment (DSE) cultures: p-value, percent disagreement (number of disagreements/total instance of DSE).

Group	p	% disagreement
1	0.002	7.9% (10/127)
2	0.004	8.8% (9/102)
3	0.031	5.2% (6/116)
4	0.031	3.7% (6/161)
5	0.008	18.6% (8/43)
6	0.008	7.1% (8/112)
7	0.13	3.5% (4/114)

CHAPTER FIVE

DISCUSSION

Since the implementation of the 1975 FDA regulation restricting the sale of turtles < 10.2 cm in the USA, the aquatic chelonian industry has been focused on identifying non-antibiotic methods to eliminate/suppress *Salmonella* spp. in turtle hatchlings and their environment. Because it is financially and logistically difficult to treat the adult breeding turtles on a farm, the logical point of intervention is with the hatchling turtles. This also makes sense because the young turtles are the animals that will be distributed among the public and serve as the primary source of infection for humans.

Since it has been shown that the primary route of *Salmonella* infection in RES is via a horizontal route through egg contamination during deposition (Izadjoo et al. 1987), there has been a focus on eliminating *Salmonella* from RES eggs and the hatching environment. Current methods for treating eggs include a combination of washing with water or sodium hypochlorite solution and temperature- or pressure-differential treatment using gentamicin (Mitchell et al. 2005) or Baquacil®. The eggs are then incubated in sanitized plastic containers without substrate until hatching. These methods have led to an apparent low prevalence of *Salmonella* among RES eggs. However, the use of antibiotics for this purpose has led to reports of *Salmonella* isolates that are resistant (D'Aoust et al. 1990; Shane et al. 1990), further increasing the public health risk of these animals.

Another concern for the aquatic chelonian industry is that once the hatchlings are shipped from the farms and disseminated into the pet trade, there are no methods in place to control re-colonization by or shedding in animals that are positive for *Salmonella*. This concern, along

with the problems of TAS and antibiotic-resistant *Salmonella*, has lead to research into non-antibiotic antimicrobials that are effective against *Salmonella* in turtle eggs and hatchlings.

Polyhexamethylene biguanide (PHMB) is an antimicrobial that has been found to be safe for use as a mouthrinse in humans (Rosin et al. 2001) and as a treatment for keratitis in animals (Panda et al. 2003). Cox et al. (1994, 1998, 1999) evaluated the effect of different antimicrobial compounds against *Salmonella* organisms on experimentally inoculated chicken eggs. The compounds tested included quaternary ammonium, peroxygen compounds, hydrogen peroxide, ethylene oxide, phenols, and sodium and potassium hydroxide, and PHMB. The PHMB was the only compound that had a consistently high efficacy against *Salmonella*. In a recent study of RES egg treatment methods, it was found that 50 ppm Baquacil® combined with sodium hypochlorite used as a bath or pressure-differential treatment significantly reduced the prevalence of *Salmonella* on egg surfaces and the resulting hatchlings (Mitchell et al. 2007). It has also been shown that Baquacil® significantly reduces the prevalence of *Salmonella* spp. in the aquatic habitat of RES when added to the water of captive hatchlings at concentrations of 25 and 50 ppm (Mitchell et al. 2005). Anecdotally, aquatic chelonian farmers report that 5 - 10 ppm concentrations of Baquacil® are effective as RES egg treatments based on polymerase chain reaction testing of treatment solutions.

The findings of this study suggest that Baquacil® can significantly reduce the prevalence of *Salmonella* in the habitat of RES, but only at a concentration of 50 ppm. These results are consistent with those obtained by Mitchell et al. (2005). The results are also consistent with the fact that PHMB is variable in its antimicrobial effect, with bacteriostatic action at low concentrations and bactericidal action at higher concentrations (Mitchell et al. 2005). The fact

that the present study did not find significance when Baquacil® was used at 5 ppm or 10 ppm suggests that aquatic chelonian farmers may need to modify their current treatment protocols.

The results of this study suggest that Sanosil® at concentrations of 10, 50 and 100 ppm cannot significantly decrease the prevalence of *Salmonella* in the aquatic habitat of RES. The fact that the 10 ppm Sanosil® treatment (Group 5) was found to be significantly less protective against *Salmonella* than the control (Group 6) is likely a cause of variation in *Salmonella* excretion by RES and the number of organisms collected in a given sample. Hydrogen peroxide or silver would not promote the growth of bacteria, despite their apparent lack of antimicrobial efficacy in this study. The antimicrobial effects of hydrogen peroxide (Juven and Pierson 1996; Aarestrup and Hasman 2004) and silver particles (Feng et al. 2000; Morones et al. 2005) are well known. However, hydrogen peroxide is rapidly degraded when exposed to light and the environment, and its antimicrobial effect may therefore be quite transient and insubstantial.

Silver particles exert their antibacterial effect principally through interaction with cell membranes. This interaction can disrupt the permeability of membranes (Sondi and Salopek-Sondi 2004), leading to cell lysis (Gogoi et al. 2006). It can also interfere with cell respiration by disrupting the proton motive force involved in oxidative phosphorylation (Dibrov et al. 2002). It has also been shown that the antibacterial effect of silver particles is dependent on size (Morones et al. 2005; Panacek et al. 2006), with small particles being more effective due to their large surface area to volume ratio. This allows more surface area for interaction with cell membranes. The silver particles in Sanosil® may have been too large to have a significant effect on the *Salmonella* in the RES water. It is also possible that they settled to the bottom of the water column, limiting their interaction with *Salmonella* organisms in suspension. Another possibility is that the silver particles were inactivated by binding to organic material from RES

excretions or the daily food ration. Silver ions are known to bind to proteins and nucleic acids (Brett 2006).

The effect of time was significant in this study, as expressed by the variables Day and Week. Samples collected on Day 6 were significantly more likely to be positive for *Salmonella* than those collected on Day 3. This is likely due to the degradation of the treatment chemicals between water changes, and therefore the decrease in their antimicrobial efficacy. During this same period, the RES would have continued to shed *Salmonella*, increasing the number of organisms in the water column. Based on these findings, the authors recommend changing the water of a RES habitat frequently (every 24-72 hours) to reduce the likelihood of exposure to *Salmonella* spp.

Water samples collected during Weeks 5 - 8 were significantly more likely to be free of *Salmonella* compared to Week 1. Because there was no significant interaction between the variables Group and Week in the regression model, it is unlikely that this effect was due to the Baquacil® or Sanosil® treatments. It is possible that the amount of *Salmonella* spp. shed by the RES decreased during the course of the study. These animals were hatched in sanitized incubators and did not feed for the first few weeks of life while absorbing their yolk sacs, which limited their exposure to microorganisms that could colonize their intestinal tract. Once enrolled in this study, they were exposed to water, bricks, and food rations. These could have acted as sources of colonizing microflora that then had a competitive exclusion effect on the *Salmonella* spp.

In this study, Baquacil® was not 100% effective at eliminating *Salmonella* spp. from the RES water. Overall, only five of the 84 turtles had no *Salmonella* spp. cultured from any of the samples taken during the study, although four of these were in Group 4 (50 ppm Baquacil®).

Only two of the 84 turtles were positive for *Salmonella* spp. on every sample, and these were in Group 5 (10 ppm Sanosil®). The rest of the turtles had variable shedding over the course of the study. This is not surprising, since it has been shown that reptiles can shed *Salmonella* spp. intermittently (Burnham et al. 1998; Mitchell 2001). This also suggests that the risk of TAS is not constant. An additional consideration is the amount of physiologic stress with which an animal deals. Inappropriate husbandry and other causes of stress can increase the rate of *Salmonella* shedding, as has been demonstrated with the ability of dehydration to activate latent *Salmonella* infections in RES (DuPonte et al. 1978). This may partially help to explain the public health concern of TAS identified in the 1960's and early 1970's. At that time, the captive management of aquatic chelonians was inadequately understood and executed, leading to an average captive life span of just two months for RES hatchlings (Lamm et al. 1972).

The purpose of this study was to determine the prevalence of *Salmonella* in the habitats of RES, and in order to increase the likelihood of isolating the organism, an enrichment broth and delayed secondary enrichment (DSE) were used. Attempts to isolate *Salmonella* without enrichment may result in sample misclassification (false-negative), since the number of organisms present is likely to be small. The overall significant difference found between the initial and DSE cultures in this study also suggests that the number of *Salmonella* organisms in the samples was small. Mitchell et al. (2005) reported that samples of water treated with Baquacil® resulted in a reduction in the numbers of *Salmonella* to $<10^3$. Thus, enrichment of samples may inflate the true risk associated with *Salmonella* in the RES habitat, since approximately 10^3 - 10^6 organisms are needed to infect a human. However, factors such as age, previous exposure, and immune status can affect a human's susceptibility to TAS.

The majority of gastrointestinal samples (58/63, 92%) were positive for *Salmonella*, and there was no significant difference in the prevalence of positive samples between the treatment groups. These findings are similar to those found by Mitchell et al. (2005), and suggest that neither Baquacil® nor Sanosil® has an effect on the colonization of *Salmonella* in the intestinal tract of RES. The fact that approximately 8% of the samples failed to yield *Salmonella* could be a result of the use of microbiological methods to detect the organisms or self-clearance by the RES. It has been estimated that the sensitivity of culture without DSE as a method to detect *Salmonella* spp. in organ samples from green iguanas (*Iguana iguana*) is approximately 70% (Mitchell 2001). Therefore, the possibility exists that up to 30% of samples could have been misclassified as false negatives. However, the real estimate is likely much smaller due to the use of DSE to increase the chance of isolating *Salmonella*. It is also possible that the RES cleared themselves of *Salmonella*, though this is not a likely occurrence. In mammals, including humans, *Salmonella* infections are generally self-limiting (Acha and Szyfres 2001), but infections in reptiles are considered to be persistent. *Salmonella* spp. are considered to be part of the indigenous gastrointestinal flora of reptiles, making them carriers of the organism. It is also unlikely that the treatment chemicals had an effect on *Salmonella* colonization of the RES. Though the animals imbibed the treated water, the chemicals would likely have been degraded by the acidic environment of the stomach. Reports of gastric pH in feeding chelonian species have ranged from 2.0 to 6.0 (Skoczylas 1978).

Though there was no significant difference in the prevalence of *Salmonella*-positive gastrointestinal samples between the treatment groups, the p-value of the chi-square test ($p = 0.08$) approached the significance level of $p < 0.05$ for this study. Because of this, a power analysis was performed, and the power of the chi-square test was found to be 0.6. This value is

lower than the accepted robust power level of 0.8 or greater, suggesting the potential of a type II error. This is not surprising, considering the small sample size of nine RES from each treatment group. However, when examining the data, only Group 1 (10 ppm Baquacil®) appears different from the other groups. The authors have no biological explanation for this finding.

Only two of the RES submitted for necropsy and histopathologic examination had significant lesions identified. The lesions in both of these animals were internal processes and were unlikely to have been induced by the treatments used in this study. Both Baquacil® and Sanosil® exert their effects in the environment, and lesions associated with either product would be predicted to occur in the integumentary system or external organs (e.g., conjunctiva and cornea of the eyes). This was not seen in any of the animals examined. The differences seen in the shell and ossification scores were not significant and were most likely due to natural variation.

CHAPTER SIX

CONCLUSIONS

The findings of this study confirm what has been previously shown by Mitchell et al. (2005), that the polyhexamethylene biguanide (PHMB) product Baquacil® can effectively decrease the prevalence of *Salmonella* in the water column of hatchling red-eared slider turtles (*Trachemys scripta elegans*) (RES). Unlike the previous study, it was found that only a concentration of 50 ppm in the RES habitat was significant. This is an indication that the efficacy of lower doses of PHMB against *Salmonella* is not adequate and use of such should be avoided. While a concentration of 50 ppm Baquacil® reduced the prevalence of *Salmonella*, there was no expectation that this concentration would eliminate the organism from the RES habitat. Indeed, the treatment had no effect on the colonization of *Salmonella* in the gastrointestinal tract of the hatchlings, so they would continue to be a source of the organisms. The Baquacil® also appeared to lose its efficacy over the time between water changes. However, there was a general trend over the course of the study in which the prevalence of *Salmonella* decreased. Investigations into whether this trend would continue with prolonged use of PHMB treatment should be conducted. It also remains to be seen if Baquacil® use at greater concentrations may be more effective at reducing the prevalence of *Salmonella* in the habitat of RES. Higher concentrations of PHMB would also have to be investigated for safety. In this study, no significant lesions were caused by either the Baquacil® or the Sanosil® products.

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