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This is the peer-reviewed, manuscript version of an article published in *Veterinary Record*. The final version is available online via <http://dx.doi.org/10.1136/vr.104457>.

The full details of the published version of the article are as follows:

TITLE: Zoonotic potential of *Salmonella enterica* carried by pet tortoises

AUTHORS: Bruce, HL., Barrow, PA., Rycroft, AN.

JOURNAL TITLE: *Veterinary Record*

PUBLISHER: BMJ Publishing Group

PUBLICATION DATE: 7 December 2017 (online)

DOI: 10.1136/vr.104457

Zoonotic potential of *Salmonella enterica* carried by pet
tortoises.

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Abstract.

The prevalence of *Salmonella* in chelonians is not known in the UK and it is not clear whether such *Salmonella* strains would be pathogenic for humans. Some strains, such as members of the Arizonae sub group, may be unable to cause anything more than very mild disease. To determine the carriage of *Salmonella* in pet tortoises, cloacal swabs were taken for culture. *Salmonella enterica* Group D was isolated from 5 of the 89 samples. All 5 were from the same household of 7 tortoises. *Salmonella* isolates were shown by PCR to carry the *invA* and *spiC* genes associated with Pathogenicity Islands 1 & 2. Each isolate carried both genes indicating they had the genetic basis for disease and enterocyte invasion in humans. The study indicates a low rate of asymptomatic carriage among the general population of pet tortoises. However, it does suggest that those *Salmonella* strains colonising the tortoise can carry SPI-1 and SPI-2 conferring the potential to cause disease in humans and other animals.

1. Introduction.

Approximately 1.3 million reptiles and amphibians were kept as pets in the United Kingdom in 2014 according to the PFMA Pet Population report in 2014 (Pfma.org.uk, 2014). Since reptiles are increasing in popularity as pets, so reptile-associated *Salmonella enterica* may constitute a public health concern. However, the prevalence of *Salmonella* in chelonians in the UK is unknown. *S. enterica* has been described as a component of the normal chelonian gastrointestinal flora (Hinshaw and McNeil, 1947) and both captive and wild chelonians have been considered carriers of *S. enterica* in their intestinal tract without it causing clinical disease (Chiodini and Sundberg, 1981). They have been recorded as carrying *Salmonella* intermittently for up to nine years (Boycott, 1962) but without the development of a detectable immune response in the turtle (Pasmans and others, 2002).

In New Zealand, 11.4% of 378 reptiles tested positive for *Salmonella*, but lizards contributed more positive samples than chelonians (Kikillus and others, 2011). In

Croatia, Lukac and others (2015) found that 13% of captive reptiles were positive, but only 3.8% of the chelonians were positive and in Italy, Ebani and others (2005) found that 29.9% of 305 reptiles carried *Salmonella*: 14 of these were chelonian and 19.8% of these were positive. A study in Bulgaria showed that 10.5% of 691 wild tortoises were excreting *Salmonella enterica* subspecies Arizona (Dimow, 1965).

The current advice given to owners is to assume that a tortoise is a carrier of *Salmonella* until proven otherwise (Barrow and others, 2013). In humans, salmonellosis presents as gastroenteritis, commonly causing diarrhoea, stomach cramps and sometimes vomiting and fever. The disease can be relatively mild to potentially life threatening. Those at higher risk of infection include children under 5 years old and those that are immune-compromised (nhs.gov.uk, 2017). There are estimated to be 93.8 million cases of gastroenteritis due to *Salmonella* globally each year (Majowicz and others, 2010). In July 2015 alone, there were 906 reported cases of *Salmonella* in the United Kingdom, the majority being foodborne (PHE, 2015). Worldwide, an estimated 3 to 5% of salmonellosis cases in humans are associated to exotic pet exposure (Woodward and others., 1997).

Human infections with *Salmonella bongori* and *Salmonella enterica* subspecies *salamae*, *arizonae*, *diarizonae*, *houtenae*, and *indica* are infrequent and are considered more likely to be from contact with reptiles (Woodward and others., 1997; Aleksic and others., 1996). Public Health England advises that all *Salmonella* serovars should be considered potentially pathogenic (PHE, 2015). However, not all strains of every subspecies are known to cause enteric disease in humans. The bacteria must be able to invade the host enterocyte to enter the intestinal tract mucosa and avoid destruction by innate and adaptive host defences. Enabling these properties in *S. enterica* are the *Salmonella* Pathogenicity Islands (SPIs). These are sequences of genes in the bacterial chromosome that encode Type Three Secretion Systems necessary for bacterial virulence (Marcus and others, 2000; Waterman and Holden, 2003; Hensel, 2004). While there are currently 17 identified SPIs that contribute to pathogenicity (Srivastava and others, 2010), carriage of SPI-1 and SPI-2 are necessary for enterocyte invasion and survival in macrophages respectively.

The objective of this study was to estimate, using enrichment culture of cloacal samples, the prevalence of *S. enterica* in pet tortoises and to determine, by detection of key Pathogenicity Island-associated genes, whether they would be likely to offer a zoonotic risk to human beings.

2. Materials and Methods

3.1 Sample Collection

Direct cloacal swabs were collected from 89 pet tortoises at a veterinary practice in West Sussex with owners' written consent. Ethical approval for sampling was obtained from the Royal Veterinary College Ethics and Welfare Committee. The samples were taken using Ames Transport Medium swabs and stored at 3°C for no longer than 3 weeks.

3.2 Culture and identification

Each swab was placed into 10 ml of sterile buffered peptone water (BPW) and incubated at 37°C for 24 hours as pre-enrichment. One ml of the BPW culture was transferred into 10ml of Rappaport-Vassiliadis (RV) enrichment broth (Oxoid, Basingstoke, UK) and incubated for 24 hours at 37°C. Samples were then plated onto Xylose Lysine Deoxycholate (XLD) agar (Oxoid) and incubated for 24 hours at 37°C. Colonies showing H₂S production, or without sucrose or lactose fermentation, were sub-cultured to Nutrient agar and Urease medium (Oxoid) and incubated for 18 hours at 37°C. Urease-negative colonies were identified using the Analytical Profile Index (API 20E) profile (bioMérieux) according to the manufacturer's instructions.

Serotyping was carried out using standard slide agglutination with polyvalent O and H antiserum (Murex Biotech, Dartford).

3.3 Polymerase Chain Reaction

Oligonucleotide primers specific for the *S. enterica*-specific gene *himA* were used to confirm the identity of presumptive *Salmonella* (Bej and others 1994). To demonstrate the presence of SPI-1 and SPI-2 in isolates of *S. enterica*, primers for

recognition of the *invA* gene (Rahn and others 1992) or the *spiC* gene (Dione and others 2011) were chosen (Table 1). All three sets of primers were verified using the NCBI Standard Nucleotide Basic Local Alignment Search Tool (BLAST).

Template DNA was prepared from presumptive *S. enterica* strains by re-suspending 5-7 colonies in 100 µl of Tris EDTA buffer (10 mM Tris and 1 mM EDTA, pH 8.0). These were held over boiling water for five minutes and then centrifuged at 11,000 xg to remove debris. PCR was carried out separately for each gene using 14.4 µl nuclease-free water, 2 µl PCR buffer, 0.5 µl dNTP mix, 1.0 µl of a 10 µM solution of each oligonucleotide primer and 0.1 µl HotStarTaq DNA Polymerase (all from New England Biolabs). One µl of template DNA was added to each tube to make a final volume of 20 µl. A positive control of DNA prepared from a strain of *S. enterica* previously identified as serovar Typhimurium was included in each test.

All PCR reaction mixtures were cycled at 95°C for 15 minutes followed by 30 cycles of denaturing at 94°C for 1 minute, annealing for 1 minute at 67°C for *invA* and 52°C for *spiC* and *himA*. Primer extension was at 72°C for 70 seconds. A final extension step of 10 minutes at 72°C was used for all PCRs.

The PCR products (10 µl) were separated using agarose gel electrophoresis on 1.5% agarose (Ultrapure; ThermoFisher Scientific) in 40mM Tris acetate buffer (pH 8.0) with 1mM EDTA. Gels were stained with 15 µL of 10 mg/ml GelRed (Biotium). Size estimation was using 100 bp DNA ladder (Promega)

3.4 Prevalence Determination

Prevalence and confidence limit were calculated using the method of Putt and others (1988).

3. Results

4.1 Culture and Identification of *Salmonella*

Cloacal swabs from 89 tortoises were cultured for *Salmonella*. All 89 samples grew bacterial colonies on XLD medium of which 32 included colonies showing either H₂S production or inability to ferment sucrose and lactose. Of these, 25 were both urease and oxidase negative.

The API20E biochemical profile (6104102) suggested 3 of these isolates to have a 35% likelihood of being *S. enterica* serovar Gallinarum, and a further 3 (4104112) to have a 66% likelihood of being *S. enterica* serovar Pullorum. However, none of these isolates agglutinated with polyvalent O or H antiserum. Furthermore, none gave positive amplification of the *Salmonella*-specific *himA* gene or for the two pathogenicity island-associated genes. Based on API profile they were probably strains of *Escherichia coli* or *Hafnia alvei* and were not investigated further.

Another 5 isolates showed a profile (6704552) with a 90% likelihood of being *Salmonella*. These latter five isolates agglutinated with polyvalent O antiserum and were further serotyped revealing them to be serogroup D. These five also agglutinated with H polyvalent phase 1 + 2 (a-z₂₉) antiserum but no specific phase 1 or phase 2 H agglutination was detected. PCR analysis confirmed the presence of the *himA* gene was present in all of these five isolates and the positive control (data not shown). The *invA* gene of SPI-1 was present in all five isolates and the positive control (Figure 1A) and the *spiC* gene of SPI-2 was also present in all five isolates and control (Figure 1B).

4. Discussion

Tortoises are common pets in the UK and they are often kept by children. It is known that chelonians can be carriers of *Salmonella* but the importance of this in transmission of disease to humans is not clear. In this study *S. enterica* was detected with a prevalence of 5.6% (5 of 89 tortoises). That estimate could be lower than the true figure because storage of cloacal swabs for up to 3 weeks might reduce the sensitivity of culture.

The 5 tortoises shown to carry *S. enterica* were not distributed among the 19 households sampled but were all from the same household. The household prevalence of *Salmonella* (one out of 19; 5.3%) is low when compared to another captive chelonian study in central Italy, where the prevalence was found to be 19.8% (Ebani and others, 2005). Nevertheless, that sample size was only 14 animals. The result in this study was closer to the 3.8% prevalence of *Salmonella* in 79 captive chelonians in Croatia (Lukac and others, 2015).

All 5 *S. enterica* isolates belonged to Group D, but the H antigen type could not be determined and hence the serovar could not be recognised. The isolates were further confirmed as *S. enterica* by identification of the *himA* gene. Each isolate also possessed both the *invA* gene, a component of SPI-1 conferring the ability to invade epithelial cells of a host, and the *spiC* gene, a component of SPI-2 associated with intra-macrophage survival (Hansen-Wester and Hensel, 2001). Carriage of the SPI-1 and SPI-2, encoding the key virulence mechanisms of *S. enterica*, suggests that these strains, isolated from tortoises, do have the potential to cause disease in humans.

The five strains isolated were indistinguishable in biochemical profile or serotyping. All the tortoises that tested positive for *S. enterica* were relatively young at four years of age. Since they were from the same household and had been together all their lives, it is likely the *S. enterica* carried was transmitted between them. The tortoises originated from a different household at which they were hatched and kept. However, none of the 35 tortoises at their original household were found to carry *S. enterica*, suggesting exposure through their eggs to be unlikely. These tortoises had always been kept inside, so the primary source of infection was likely to be from contact with humans or from a contaminated food source. It has been reported that the longer reptiles stay in captivity, the higher the probability that they would be infected with *Salmonella* (Pfleger and others, 2003). However, these tortoises were the youngest of those sampled in this study and therefore had been in captivity for the shortest time.

In conclusion, tortoises have been shown to harbour *S. enterica* strains. These carried the genes demonstrating the presence of those pathogenicity islands (SPI-1 and -2) known to be required for virulence in the human host. This indicates the need for

general hygiene measures when handling and cleaning these animals and such advice from the veterinary surgeon may be warranted.

Acknowledgements

The authors acknowledge the kind help of staff at Seers Croft Veterinary Surgery, Horsham, West Sussex, and support from the BBSRC, Grant BB/G020744/1.

Figure 1. Agarose gel electrophoresis of DNA from the PCR amplification of *S. enterica* isolated from tortoises. (a) Amplification using primers invA 139. F and invA 141. R. (b) Amplification with the primers spiC F and spiC R. The first lane shows the positive control, followed by sample numbers 88, 87, 85, 84 and 79 in that order. The first negative control was sample number 64, and the second negative control has nuclease-free water in place of DNA.

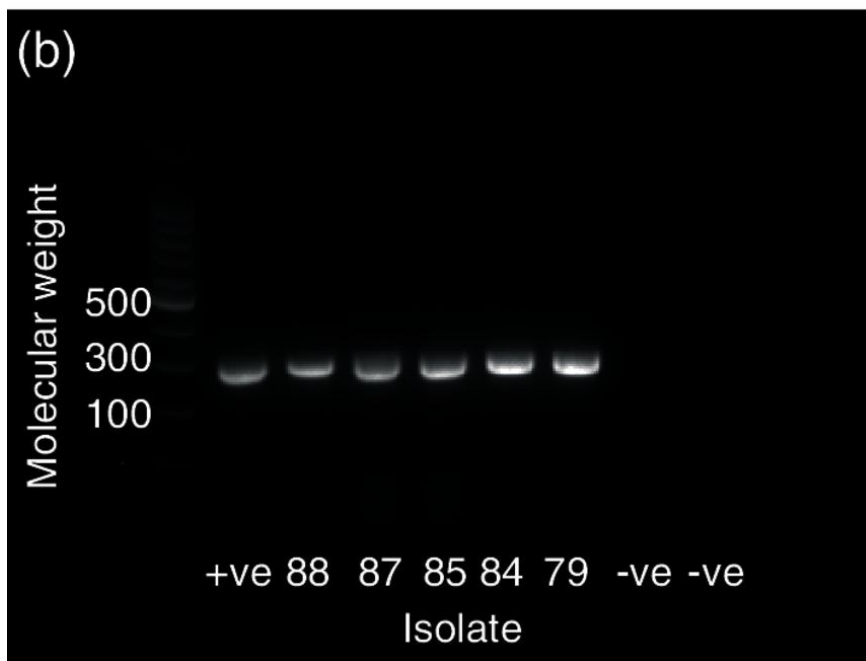
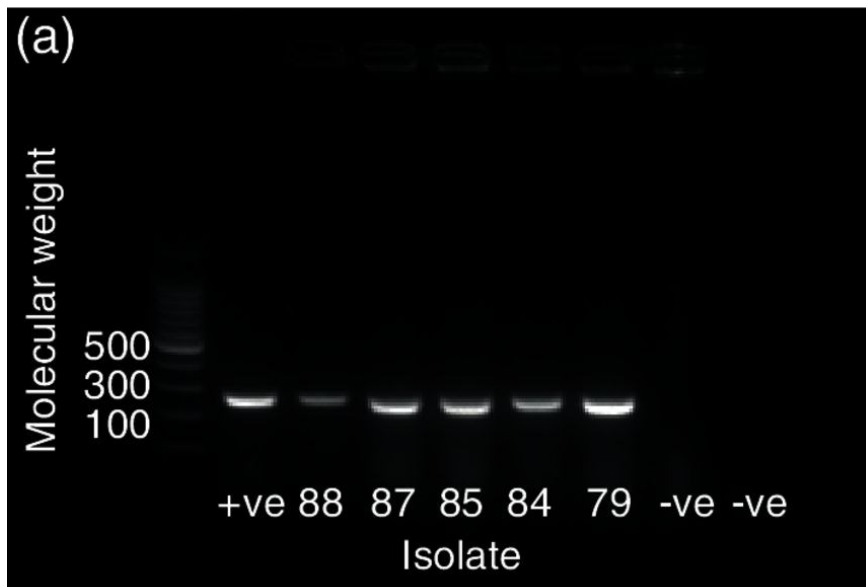


Table 1. Oligonucleotide primers used to detect SPI-associated genes and the *S. enterica* specific gene *himA*.

Gene	Primers	Reference
<i>invA 139 F</i>	5'-GTG AAA TTA TCG CCA CGT TCG GGC AA	Rahn and others, 1992
<i>invA 141 R</i>	5'-TCA TCG CAC CGT CAA AGG AAC C	
<i>spiC F</i>	5'-CCT GGA TAA TGA CTA TTG AT	Dione and others, 2011
<i>spiC R</i>	5'-AGT TTA TGG TGA TTG CGT AT	
<i>himA F</i>	5'-CGT GCT CTG GAA AAC GGT GAG	Bej and others, 1994
<i>himA R</i>	5'-CGT GCT GTA ATA GGA ATA TCT TCA	

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