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Case report

Serotypes and antibiotic susceptibility patterns of *Salmonella* spp. isolates from spur-thighed tortoise, *Testudo graeca* illegally introduced in Italy

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Abstract. The prevalence of *Salmonella* carriage and distribution of serotypes in spur-thighed tortoises, *Testudo graeca* Linnaeus, 1758 illegally introduced in Italy was studied to assess the risk of disease exposure for humans once these specimens were traded as pets. Antibiotic susceptibility patterns were also analyzed to estimate the emergence of antibiotic-resistant *Salmonella* strains. One hundred forty-six cloacal swabs of spur-thighed tortoises were tested by standard bacteriological methods. Antimicrobial susceptibility tests on *Salmonella* strains isolated were also performed. Ninety-one *Salmonella* spp. strains were isolated in 74 of 146 turtles examined and a total of 20 different serotypes were found. Out of the 91 isolates, 67 were grouped in the *Salmonella enterica* subspecies I. *Salmonella* isolates were susceptible to most of the antibiotics tested. Resistance was most commonly observed against tetracycline (57.1%) followed by ampicillin (33.0%), streptomycin (13.2%) and amoxicillin-clavulanic acid (11.0%). Our findings confirm that wild-caught spur-thighed tortoises can carry different serotypes of *Salmonella*. Accordingly, strict preventive sanitation measures should be adopted when handling reptiles.

Key Words: *Salmonella*, serotypes, disk diffusion antimicrobial tests, *Testudo*, illegal import.

Riassunto. Obiettivo: Gli Autori hanno studiato la prevalenza e la distribuzione dei sierotipi di *Salmonella* isolati in esemplari di *Testudo graeca* Linnaeus, 1758 illegalmente introdotti in Italia per valutare il rischio di esposizione umana all'infezione conseguente alla loro commercializzazione come animali da compagnia. Sono stati effettuati anche tests di suscettibilità agli antimicrobici per valutare l'eventuale emergenza di ceppi di *Salmonella* antibioticoresistenti. Materiali e metodi: 146 tamponi cloacali di *Testudo graeca* sono stati sottoposti a metodiche batteriologiche standard e la suscettibilità ad antimicrobici dei ceppi di *Salmonella* isolati è stata saggiata mediante metodica di diffusione su agar. Risultati: Sono stati isolati 91 ceppi di *Salmonella* spp. in 74/146 Testudo esaminate e sono stati identificati 20 differenti sierotipi. Dei 91 ceppi 67 appartenevano alla sottospecie I. Le salmonelle isolate erano sensibili alla maggior parte delle molecole testate. Sono state registrate diverse resistenze nei confronti di tetraciclina (57.1%) seguita da ampicillina (33.0%), streptomicina (13.2%) e amoxicillina-acido clavulanico (11.0%). Conclusioni: I nostri risultati confermano che le Testudo graeca catturate dall'ambiente selvatico possono veicolare diversi sierotipi di *Salmonella*, di conseguenza quando tali animali vengono manipolati bisogna adottare rigorose misure igienico-sanitarie.

Parole chiave: *Salmonella*, sierotipi, antibiogramma, *Testudo*, importazione illegale.

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Introduction

It was estimated that in 2009 between 5.9 and 9.8 million live reptiles were imported into the EU (RSPCA 2011), reflecting a similar previously trend in the USA (Franke & Telecky 2001). These animals are known to shed *Salmonella* frequently (Bauwens *et al* 2006; Hidalgo-Vila *et al* 2007; Pedersen *et al* 2009). Reptiles have been increasing in popularity as pets, resulting in an increase in the number of reptile-associated salmonellosis in USA (CDC 2008) and European countries (Corrente *et al* 2006; Bertrand *et al* 2008; Eurosurveillance 2008). *Salmonella* can be transmitted from reptiles to humans both by direct contact or indirect contact with surfaces contaminated with reptile feces. Even if less frequent than foodborne related salmonellosis, outbreaks of reptile-associated salmonellosis in humans have been widely reported, in particular affecting infants, children and immunocompromised subjects (Nowinski & Albert 2000; Nagano *et al* 2006; Hames *et al* 2008; Böhme *et al* 2009; Harris *et al* 2009; Van Meervenne *et al* 2009), but also healthy persons (Harris *et al* 2010). The reptiles imported by the EU Member States were reported to derive from various sources, as captive-bred or taken from the wild (Auliya 2003).

Many species of Chelonia, among which *Testudo graeca* Linnaeus, 1758 so-called commonly spur-thighed tortoise, are now frequently kept as companion animals (CAWC 2003). *Testudo graeca* is an endangered species with a broad distribution range. It can be found in northern Africa, the Middle East, Europe and Asia (van der Kuyl *et al* 2005). It is nowadays regularly offered as captive bred, although specimens from the

Testudinidae family were most frequently illegally imported (Auliya 2003). *Testudo graeca* has a high international and national protection status. It is listed as threatened under Appendix II of the Convention on International Trade in Endangered Species (CITES) (EC 1997; EC 2010) and is subjected to strict trade regulation and require by law a CITES certificate in order to be sold (CITES 2009). It is also included in Appendix II of the Bern Convention (1979) and is classified as vulnerable in the IUCN Red List of Threatened Species (IUCN 2002). In the European Union the species is included in Appendix II and IV of the Habitat Directive (EEC 1992). Moreover European Union import regulation requires certificates stating that imported animals were free of *Salmonella* (de Jong *et al* 2005). Despite this, tortoises are subject to extensive smuggling from North African countries to Sicily, Italy. There are no real data on the number of tortoises raised as pets in Italy, even if a CITES registration scheme is compulsory in Italy since 1995. Clinical cases of *Salmonella* infection in spur-thighed tortoises are infrequently documented (González Candela *et al* 2005); however several studies on *Salmonella* incidence in free living tortoises have been shown that these specimens may be important reservoirs of this zoonotic microorganism (Hidalgo-Vila *et al* 2007, 2008ab). So together with species conservation issues, the illegal introduction of tortoises raises public health concerns because they can be reservoirs of zoonotic pathogens including *Salmonella* (Percipalle *et al* 2011).

The aim of this study was to analyse the prevalence of *Salmonella* carriage, distribution of serotypes, and antibiotic susceptibility patterns in *Testudo graeca* illegally introduced in Italy from Tunisia in April 2008, and to evaluate the risk of disease exposure for humans once these specimens were traded as pets.

Material and Methods

One hundred forty-six spur-thighed tortoises (*Testudo graeca*) were the subjects of this study. These tortoises were seized by port police officers as they were being illegally imported in Italy. The sampled individuals were from a total of 1400 spur-thighed tortoises seized in a single shipment. They were transferred to Sicily (Italy) by a ferry from Tunisia, hidden in the trunk of a car. It was the smell of a number of dead tortoises coming from the car that has revealed a suspect content to the police officers. It was confirmed by CITES Service of the Italian State Forestry Corps that tortoises were taken from the wild to supply the illegal trade of endangered species, but remain unknown the history of these specimens until their seizure. During their travel they were packed into the vehicle trunk closely together, even piled on top of each other, without access to food and water. Mortality was high yet at their arrival. The survivors, quickly transferred at two rescue centers, were dehydrated, malnourished, someone even injured and they were also highly infested by ticks. The sampling was performed two hours after their arrival at the shelters. A total of 75 and 71 samples were respectively collected in tortoises sheltered in the two wildlife rescue centres of Messina and Palermo (Sicily, Italy). All procedures were conducted according to the guidelines for the accommodation and the care of animal used for experimental and other scientific purposes (Legislative Decree 116/92 and Directive 2007/526/EC).

Bacteriological analysis was performed on cloacal swabs inserted into the coprodeum of each animal tested. They were stored at 4°C in Stuart's transport medium (Meus, Piove di Sacco, Italy) until they were processed at the Department of Veterinary Public Health's Microbiology Laboratory of Messina University.

Non selective-pre-enrichment was performed in 10 mL of Buffered Peptone Water (BPW) (Biolife Italiana, Milan, Italy) incubated at 37°C for 16-20 h. Selective enrichment was then carried out by inoculating 1 mL of BPW in 10 mL of Selenite Broth (Oxoid, Basingstoke, UK). Following 24-48 h of selective enrichment at 37°C broths were subcultured on Brilliant green agar, *Salmonella*-Shigella agar (Oxoid) and Hektoen enteric agar (Liofilchem, Teramo, Italy), solid selective media. After 24-48 h of culture at 37°C, up to five presumptive *Salmonella* colonies from positive plates, were subcultured in Brain Heart Infusion agar (Oxoid) and submitted to biochemical identification by the API 20 E system (Biomerieux, Marcy l'Etoile, France). The following screening tests were also performed on all isolated strains: Gram-staining, motility, urease, catalase and oxidase reactions. *Salmonella* subspecies were recognized on the basis of biochemical characteristics (LeMinor *et al* 1982) *Salmonella* spp. isolates were serotyped using commercial polyvalent anti-sera (Difco Laboratories, Detroit, USA) for somatic (O) and flagellar (H) antigens followed by type-specific monovalent anti-sera (Staten Serum Institut, Copenhagen, Denmark). The complete antigenic formula was determined according to the Kaufmann-White scheme (Grimont & Weill 2007). All *Salmonella* isolates were tested for antimicrobial susceptibility against a panel of 13 different antibiotics using the Kirby-Bauer disk diffusion technique (Bauer *et al* 1966). The following antibiotics (disc concentration in μg) were tested: ampicillin (10), cefotaxime (30), chloramphenicol (30), gentamicin (10), cotrimoxazole (25), trimethoprim (5), tetracycline (30), amoxicillin-clavulanic acid (30), ciprofloxacin (5), ceftazidime (30), sulphonamide (300), nalidixic acid (30), streptomycin (25). Isolates were classified as susceptible, intermediate or resistant according to the interpretative criteria provided by the M02-A10 (CLSI 2009) and M100-S21 (CLSI 2011) documents of the Clinical and Laboratory Standards Institute.

Results

Salmonella was found in 74 (50.7%) of the 146 samples collected from the tortoises. A total of 20 different serotypes were identified (see Table 1).

Seventeen samples yielded two different serotypes. All 91 isolates belonged to *Salmonella enterica*. In total 67 isolates could be classified as subsp. enterica (group I), whereas 18 isolates and 2 isolates belonged to subsp. salamae (group II) and diarizonae (group IIIb) respectively. The serotype of four *Salmonella* isolates could not be identified. The most frequently identified serotypes within the subsp. enterica were Kottbus (n=18) and Sheffield (n=13). Other less frequently isolated serotypes were Potsdam (n=8) and Halle (n=6). Serotypes belonging to subspecies usually found in cold-blooded animals (II to IV) were detected in 20 isolates. Among which, Canastel (n=14) was the most frequently isolated serotype. No isolates were attributed to subspecies IIIa, IV and VI or the subspecies bongori (group V). Eighty-four (92.3%) *Salmonella* isolates were resistant or intermediate to at least one of the antimicrobials tested. The results of the antimicrobial susceptibility test are reported in Table 2.

Table 1. Subspecies and Serotypes of *Salmonella enterica* isolates (n=91) from *Testudo graeca*

Subspecies	Serotype	Number of isolates
Subsp. enterica or I	Kottbus	18
	Sheffield	13
	Potsdam	8
	Halle	6
	Ferruch	4
	Langford	4
	Abony	3
	Solna	2
	Westafrica	2
	4,12:b:-	2
	Heron	1
	Richmond	1
	Salford	1
	38:-:-	1
	48:-:1,5	1
Subsp. salamae or II	Canastel	14
	4,12:b:-	2
	Uphill	1
	9,12:z29:-	1
Subsp. diarizonae or IIIb	50:r.-	2
Unable to serotyped		4
Total		91

Table 2. Susceptibility (percent of resistant, intermediate and susceptible) of *Salmonella* isolates (n=91) from *Testudo graeca* to a panel of antimicrobial drugs

Antimicrobial agents	Sensitive (%)	Intermediate (%)	Resistant (%)
Ampicillin	65.9	1.1	33
Amoxicillin/clavulanic acid	82.4	6.6	11
Ceftazidime	100	0	0
Cefotaxime	100	0	0
Chloramphenicol	98.9	1.1	0
Gentamicin	98.9	1.1	0
Streptomycin	14.3	72.5	13.2
Nalidixic acid	100	0	0
Ciprofloxacin	100	0	0
Sulphonamide	97.8	2.2	0
Trimethoprim	100	0	0
Sulfamethoxazole/ trimethoprim	100	0	0
Tetracycline	34.1	8.8	57.1

Resistance was most commonly observed against tetracycline (57.1%) followed by ampicillin (33.0%), streptomycin (13.2%) and amoxicillin-clavulanic acid (11.0%). A total of 27 *Salmonella* strains (29.7%) showed resistance to two or more antibacterial drugs. Of these 24.2%, 2.2% and 3.3% were resistant to two, three and four antimicrobial agents respectively. In particular, 17 were resistant to ampicillin and tetracycline, three to ampicillin and streptomycin, three to amoxicillin-clavulanic acid, ampicillin, streptomycin and tetracycline, two to ampicillin, streptomycin and tetracycline and two to streptomycine and tetracycline. Each of only two isolates was intermediate respectively to chloramphenicol and gentamicin, and two ones were both intermediate to sulphonamide. All *Salmonella* isolates were susceptible to a large proportion of the antimicrobial drugs tested. Seven *Salmonella* isolates (7.7%) were susceptible to all of the antibiotics tested; of these five belonged to the Canastel serotype and two to the Potsdam and Kottbus serotypes respectively. All isolates proved to be susceptible to nalidixic acid, ciprofloxacin, third generation cephalosporins, cotrimoxazole and trimethoprim.

Discussion

In this study we found a recovery rates of *Salmonella* of 50.7% in 146 free-living spur-thighed tortoises coming from Tunisia. It is unclear whether these tortoises acquired *Salmonella* in nature or following to their capture by ingestion of contaminated meat, or by contact with contaminated faeces of other tortoises during their trade to Italy. Previously researches report several levels of infection in both captive and free-living tortoises. *Salmonella* was detected in 61% of cloacal samples collected in captive *Testudo hermanni* and *Testudo graeca* tortoises in France (Strohl *et al* 2004). Among the *Testudo graeca* sampled specimens of this study, *Salmonella* was detected in eight healthy household pets (n=6 isolates, 75%) and two tortoises from a rescue centre (n=2 isolates, 100%) (Strohl *et al* 2004). In a study carried out in two distinct populations of free living *Testudo graeca* tortoises in Morocco, *Salmonella* was isolated from 100% of samples collected in one population and 89.4 % in the other (Hidalgo-Vila *et al* 2008b). In Italy only a few studies have been carried out in tortoises which were sheltered in wildlife rescue centres (Pasmans *et al* 2000; Percipalle *et al* 2011). Pasmans *et al* (2000) found a *Salmonella* prevalence rate of 79% in tortoises sheltered in a rescue centre in Italy. In a recent study of Percipalle *et al* (2011) was determined the prevalence of *Salmonella* infection in a group of *Testudo graeca* seized during two smuggling attempts from North African countries. The *Salmonella* prevalence rates were of 36.8%.

Our findings confirm that wild-caught Spur-thighed tortoises can carry different serotype of *Salmonella*. It is known that stress and poor breeding conditions in captivity favor the excretion of *Salmonella* by chelonians, without any clinical sign being present in most cases (Duponte *et al* 1978). So the excretion may be likely higher when these animals are obtained from illicit sources and trade routes, due to the poor adherence to hygienic practices during transport and commercialization.

In our case the shipment of tortoises was likely intended to supply the international trade of wild species, by which protected species illegally circulate in association with false permits and certificates. The zoonotic risk for household tortoise owners may be highlighted by the fact that the major serotypes isolated from the sampled tortoises (n=67; 73.6%) belong to the *Salmonella enterica* subspecies *enterica*. We also found 20 isolates (22%) of serotypes belonging to *Salmonella enterica* subspecies salamae (group II) and diarizonae (group IIIb), which are commonly

isolated in reptiles, but are considered of low pathogenicity to humans. The serotypes Kottbus, Sheffield (both group I) and Canastel (group II) were the most frequently isolated. Serotypes commonly associated with human salmonellosis such as Enteritidis and Typhimurium were not found. The serotypes Abony, Halle, Heron, Kottbus, Potsdam, Richmond, Salford, Sheffield, Solna and Canastel have been previously isolated in both captive and free-living tortoises in Europe (Alin 1956; Pasmans *et al* 2000; Briones *et al* 2004; Strohl *et al* 2004; Hidalgo-Vila *et al* 2007) and in North Africa (Hidalgo-Vila *et al* 2008b; Percipalle *et al* 2011). The presence of serotypes, such as Kottbus, that have been reported to be responsible for both sporadic and epidemic human cases of enteritis, is a further issue of concern (Palmera-Suárez *et al* 2007). The serotype Ferruch was already isolated in pet chelonians (Kodjo *et al* 1997).

Resistance to antibiotics was infrequent, except for some antibacterial drugs such as ampicillin, streptomycin and tetracycline. In particular, more than half of the *Salmonella* strains were resistant to this last antibiotic. Tetracyclines are still clinically important drugs, commonly used to control a wide range of human and animal diseases (Chopra & Roberts 2001). Antibiotic resistant nontyphoidal *Salmonella* infections have been associated with invasive diseases (blood stream infections) and hospitalizations (Varma *et al* 2005; Crump *et al* 2011), so the presence of *Salmonella* resistant strains among our isolates, may represent a relevant date. Sale and distribution of reptiles and turtles are officially strictly regulated, but these animals are easily obtainable from various sources, including pet shops, street vendors, and Internet Web sites. Additionally, only a small proportion of owners - one fifth of 60 infected individuals according to an epidemiological investigation conducted by the Centers for Disease Control and Prevention (CDC) – seems to be aware of the link between *Salmonella* infection and contact with reptiles, suggesting that measures to educate the public about this risk are insufficient or ineffective (CDC 2008). Despite continuing efforts to regulate the global trade of exotic animals, illegal importation of wildlife species has increased worldwide. Our results confirm that wild-caught Spur-thighed tortoises can carry different serotypes of pathogenic *Salmonella*, demanding the necessity of regulation of pet tortoises trade in Europe. The scale of the illegal trade in CITES-listed reptile species cannot be easily quantified (Auliya 2003). Fortunately these tortoises were intercepted before they can be brought into a person's home where humans may be at greater risk of disease exposure. Cases of *Salmonella* infections attributed to direct o indirect contact with reptiles have been described in a number of European countries (Bertrand *et al* 2008). Accordingly, strict preventive sanitation measures should be adopted when handling reptiles from all sources (CDC 2007), and tortoises should be always regarded as a potential source of pathogenic *Salmonella* strains for humans.

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