Microbiological and parasitological investigation on chelonians reared in Italian facilities

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Summary
The rapid rise in the number of pet chelonians and their illegal trade can modify the ecology, involving exotic pets, humans, and microbiological agents. Therefore, different epidemiological situations and the related risk to introduce and spread infectious diseases, especially zoonotic agents, have to be considered. The aim of this study was to investigate the microbiological and parasitological situation in 2 chelonian facilities (a private breeding of tortoises and a shelter for turtles) collecting oral/cloacal swabs and cloacal flushes to research viruses, bacteria, and parasites. No Chelonian Herperviruses, *Cryptosporidium* spp., and *Giardia* spp. infections were found. *Salmonella* spp. were detected in 8% of tortoises and in 37.5% of turtles and oxyurid eggs in 23.7% of tortoises and 15% of turtles; ascarid eggs were present only in tortoises. Moreover, 6 turtles showed cutaneous lesions, where *Aeromonas sobria* was isolated as main pathogen. Further studies should be performed to understand the zoonotic and infectious risk in each chelonian facility and to characterize the variables that could influence the microbiological patterns.

Indagine microbiologica e parassitologica su tartarughe allevate in Italia

Parole chiave
Aeromonas spp.,
Herpesvirus,
Parassita,
Salmonella spp.,
Tartaruga,
Zoonosi.

Riassunto
Gli animali esotici stanno acquisendo, in Italia, un'importanza sempre maggiore come animali da affezione. Tra questi, le tartarughe possono rappresentare un rischio per l'introduzione e la diffusione di malattie infettive e/o parassitarie per la mancanza di adeguati controlli riguardo al loro commercio e all'abitudine sempre più consolidata dei cittadini di abbandonare soggetti adulti in aree pubbliche urbane. Lo studio ha avuto l'obiettivo di indagare la situazione microbiologica e parassitologica in due diverse strutture ospitanti tartarughe (un allevamento privato di testuggini e una struttura pubblica per tartarughe acquatiche), ponendo particolare attenzione agli agenti responsabili di zoonosi. Tamponi orali e cloacali e lavaggi cloacali, raccolti individualmente, sono stati destinati alla ricerca di virus, batteri e parassiti. *Salmonella* spp. sono state isolate nell'8% delle testuggini e nel 37,5% delle tartarughe d'acqua. Il 23,7% delle testuggini e il 15% delle tartarughe acquatiche sono risultate positive per Ossiuridi. Uova di Ascaridi sono state rinvenute esclusivamente nelle testuggini. Inoltre, in sei tartarughe d'acqua è stata isolata, da lesioni cutanee, la specie *Aeromonas sobria*. Tutti i campioni sono risultati negativi per Herpervirus (ChHV), *Cryptosporidium* spp. e *Giardia* spp. Ulteriori indagini epidemiologiche saranno improntate al fine di comprendere meglio le variabili che influenzano gli aspetti microbiologici e i rischi di zoonosi connessi ai Cheloni.

Introduction

The increasing habit of having exotic animals as pets is generating new ecosystems, that must be examined to evaluate the related risks. The rapid rise in the number of pet chelonians and their illegal trade imbues both the phenomenon of abandonment by owners in public urban areas and the mixing of animals of different origins together. Accordingly, a modified ecology involving exotic pets, humans, and microbiological agents, has been created, about which limited knowledge is available to date.

Understanding the ecology of this new system is the first step towards the risk analysis and its management (OIE 2012). Two main aspects emerge: the risk of disease introduction in a captive collection by new animals, and the zoonotic risk due to new transmission pathways (Pasmans *et al.* 2008).

Most microbiological agents are able to overgrow and cause severe diseases in the host, especially in captive conditions, if no appropriate temperature and humidity, limited space, high density and poor sanitary conditions exist. Many viruses, bacteria, protozoans, helminths, and arthropods can take advantage of this situation, threaten the health status and the welfare of the animals and cause the death of the most susceptible animals (Papini *et al.* 2011, Pasmans *et al.* 2008). Furthermore, the human-animal interaction can represent a key factor in emerging infectious diseases considering that chelonians have already been recognized as reservoirs or carriers of some zoonotic agents, such as *Salmonella* spp., and *Cryptosporidium* spp. (Harris *et al.* 2010, Pasmans *et al.* 2000, Traversa *et al.* 2008).

Salmonella spp. are ubiquitous enteric bacteria; the broad genus contains many serovars, and some of them can cause enteric and multisystem diseases in humans and animals. Reptiles are generally considered carriers of *Salmonella* spp. and numerous outbreaks and zoonotic infections have been attributed to contact with turtles, especially in young children (Harris *et al.* 2010, Van Meervenne *et al.* 2009).

Cryptosporidium are cosmopolitan Apicomplexa parasites that affect the gastrointestinal tract of humans and a wide range of animals (Traversa *et al.* 2008); *Cryptosporidium* infections are common in reptiles. Although *Cryptosporidium varanii* and *Cryptosporidium serpentis* are the most common species recovered (Graczyk *et al.* 1999, Griffin *et al.* 2010); *Cryptosporidium pestis* (*Cryptosporidium parvum* 'bovine genotype'), which is the main species responsible for zoonotic transmission, has also been found in reptiles including tortoises (Fayer 2010, Rinaldi *et al.* 2012, Traversa *et al.* 2008).

A further protozoan parasite that might cause public health concern is *Giardia* spp., an enteric polyflagellate, globally widespread in a wide range of vertebrate hosts, including humans, pets, livestock, and wildlife (Thompson 2004). Among the 6 different species recognized, *Giardia duodenalis* is the most important due to its potential zoonotic transmission (Lalle *et al.* 2005). Several studies have been conducted to determine whether *G. duodenalis* can infect humans through a zoonotic route and to identify animal species acting as source of infection (Traub *et al.* 2004, Trout *et al.* 2004). Nevertheless, no information on the relationship between this protozoan and chelonians is currently available.

The aim of this study is to investigate the microbiological situation occurring in a private facility of tortoises and a shelter of abandoned water turtles, paying particular attention to chelonian Herpesviruses, different types of parasites and 3 potential zoonotic agents, such as *Salmonella* spp., *Cryptosporidium* spp., and *Giardia* spp.

Materials and methods

Between April and May 2011, 2 kinds of chelonian centres, a private facility of tortoises and a public shelter for abandoned turtles, were sampled. All animals were housed outdoors and were long-term inhabitants of the respective breedings. In the private facility the tortoises were divided into groups (5-8 animals each) and were kept in fenced areas, while in the shelter all turtles lived in a single artificial pond (15 x 15 m). Both facilities had a quarantine station. The tortoises were fed with local vegetables, whereas commercial pellet was used in the shelter.

Oral/cloacal swabs and cloacal flushes were individually collected from 38 tortoises (27 *Testudo hermanni*, 7 *Testudo marginata*, 3 *Testudo graeca*, 1 *Testudo horsfieldii*), which represented the whole adult population reared in the breeding, and 40 turtles (25 *Trachemys scripta scripta*, 15 *Trachemys scripta elegans*) of approximately 250 turtles kept in the shelter. At the time of the sampling, a complete clinical examination was also performed for each animal.

The swabs were collected through sterile polyester swabs, dipped in 500 µL of phosphate buffered saline (PBS, pH 7.2) and stored at 4 °C in Amies transport medium (Oxoid, Milan, Italy). The cloacal flushes were performed by a 2-3 mm diameter catheter connected with a syringe containing 2-4 mL of physiological solution (Klingenberg 2007). Swabs from the clinical specimens were collected when skin disease or nasal or ocular discharge were present.

As for the virological examination, DNA was extracted from 200 µL of each oral swab using a commercial kit (DNeasy Tissue kit, Qiagen, Milan,

Italy), in accordance with the manufacturer's instructions. A consensus nested Polymerace Chain Reaction (PCR) protocol was performed to amplify a conserved fragment of the DNA polymerase of the herpesviruses (VanDevanter *et al.* 1996), and previously applied for chelonians (Bicknese *et al.* 2010, Marschang *et al.* 2006). Three positive controls (2 for the *alphaherpesvirinae* subfamily, which were the American Type Culture Collection reference strains of Equid Herpesvirus type 1 and Canine Herpesvirus type 1, and 1 for the *gammaherpesvirinae* subfamily, which was a previously sequenced sample of EHV-5) and 2 negative controls (a negative DNA and water without DNA) were included in each set of reactions.

For the bacteriological and fungal examination, the swabs from the clinical specimens were inoculated onto blood (5% sheep blood), McConkey and Sabouraud dextrose (with chloramphenicol selective supplement) agars (Oxoid, Milan, Italy) and incubated aerobically at 37 °C and 25 °C to determine any bacteria or fungi that might be present. The bacteria were identified by Gram staining, oxidase, and catalase tests and miniaturized biochemical tests (Api, bioMérieux, Florence, Italy). In case of fungal growth, filamentous fungi were identified on the basis of macroscopic and microscopic morphological characteristics. Yeasts were identified by morphology, and physiological and biochemical characteristics were assessed using the API ID 32C system (bioMérieux, Florence, Italy).

The cloacal swab was inoculated in the Rappaport-Vassiliadis broth, the enrichment medium selective for *Salmonella*, and incubated at 41.5 °C for 24-48 hours. The samples were subcultured at 37 °C on xylose lysine desoxycholate agar (XLD, Oxoid, Milan, Italy) and brilliant green agar (Oxoid, Milan, Italy) solid selective media. The results were read after 24 and 48 hours of incubation. Identification of suspect colonies was performed by composite biochemical media and commercial biochemical tests, such as API RAPID 20E (bioMérieux, Florence, Italy). *Salmonella* spp. isolates were serotyped by direct slide agglutination using specific antisera (Statens Serum Institut, Copenhagen, Denmark), according to the Kaufmann-White scheme (Grimont & Weill 2007).

For parasitic research, the cloacal flush was firstly macroscopically examined for the presence of helminths and then divided into 2 aliquots: the first one was submitted to routine flotation methods, using the modified Sheather's sugar solution (specific gravity 1.3) and the potassium mercury iodine solution (specific gravity 1.45), to detect parasite eggs/cysts/oocysts. The second aliquot was submitted to a sucrose gradient purification (Lebbad *et al.* 2008) and then examined for the detection of *Cryptosporidium* and *Giardia* cyst/oocyst respectively by a commercial Direct Fluorescent Assay (DFA, MERIFLUOR® *Cryptosporidium/Giardia*, Meridian Diagnostic, Cincinnati, USA), according to the manufacturer's instructions.

Results

All oral swabs were negative for DNA of chelonian herpesviruses.

No tortoises showed clinical signs in the facility, whereas 5 turtles out of 40 had erosive/ulcerative lesions in their feet and 1 in the carapace. Swabs were taken from these lesions. *Aeromonas sobria* was identified in 4 cases out of 5 in ulcerative lesions and *Pasteurella aerogenes* was isolated from the lesions in the carapace and in 2 cases of ulcerative cutaneous lesions. One case had mixed infections with *Pasteurella aerogenes* and *Aeromonas sobria*. No fungal growth was observed.

Salmonella enterica subsp. *enterica* was isolated from 3 of the 38 tortoises in the facility (8%) and the serovars were identified as Hermannswerder (28:c:1,5), Langford (28:b:e,n,z_{15}) and Abony (4:b:e,n,x). Fifteen turtles were positive for isolation of *Salmonella* in the shelter (15/40, 37.5%) and all the isolates were *Salmonella enterica* subsp. *diarizonae* serovar 47:k:1,5,7.

Oxyurid eggs were identified in 9/38 tortoises (23.7%) and in 6/40 turtles (15%); 3/38 tortoises (7.9%) were also infested with ascarids. All cloacal flushes were negative for *Cryptosporidium* spp. and *Giardia* spp.

Discussion

The present study describes the different microbiological state existing in 2 types of chelonian situations. The tortoises in the private facility had good clinical conditions, good management. An adequate relative density of animals was also found in this facility, given that the animals were divided into little fenced facilities. In the public shelter, that hosted many animals in the same pond, the turtles sometimes reached a high relative density and this could cause stress and immunodepression. Moreover, the admission of abandoned chelonians with a wide range of origins in the shelter increases the risk of introducing new pathogens. Beside the private or public nature of the facility, the management and the use of the habitat, diet, season or age are determinant in the transmission of pathogens. Accordingly, the factors observed in the shelter during the present study, associated with aquatic environment (Edgreen *et al.* 1953, Frye 1991, Hulse 1976), could have favoured the emergence of cutaneous clinical signs in 6 animals, in which the

main pathogen was *Aeromonas sobria*, a bacterium previously associated with cutaneous infections in turtles and considered agent of severe zoonotic infections by reptile bites or contaminated water (Janda & Abbott 2010, Sun & Su 2002).

At least 6 CHV have been identified in chelonians, responsible for rhinitis-stomatitis and cutaneous lesions until death in tortoises and fibropapillomas in marine turtles (Davison 2010). To detect different types of CHV a PCR consensus protocol was performed, but no active herpesvirus infection was detected. Latent infection, not detectable by PCR on oral swabs, however, could not be excluded and precautions should always be taken to avoid introducing CHV infection, especially in naïve populations because of its high morbility and mortality.

This study shows a different *Salmonella* prevalence of 8% in the private facility and 37.5% in the shelter. However, a single sample could have underestimated *Salmonella* prevalence for both samplings, because there is no identification of intermittent shedding. At the same time, a wide variety of *Salmonella* prevalence was previously reported in chelonians (0-100%), probably depending on differences in host species, feeding habits (carnivours *vs* herbivours), environment (terrestrial *vs* aquatic), management, location, or *Salmonella* serotypes involved (Chen *et al.* 2010, Hidalgo-Vila *et al.* 2007, Percipalle *et al.* 2011, Saelinger *et al.* 2006). A seasonal variation of the prevalence could also be hypothesized and the time of the sampling of the present study was quite favourable for the multiplication of the bacterium. Moreover, the different living conditions between tortoises and turtles could be a reason for higher prevalence in the shelter. Water is considered a good medium for the growth of *Salmonella*, especially if high-protein feed is administered to turtles (as in this case, where a commercial pellet was used) and the ingestion of faeces or contaminated water represents a probable way of colonization of *Salmonella* (Chen *et al.* 2010, Hidalgo-Vila *et al.* 2007). However, previous studies detected lower prevalences, ranging from 6.38% to 15%, in free-living exotic turtles from natural ponds (Hidalgo-Vila *et al.* 2007, Hidalgo-Vila *et al.* 2008). Although the artificial pond in the present study was external, an effect on the *Salmonella* prevalence by artificial and natural pond can be hypothesized.

All sampled animals in the shelter resulted positive with the same serotype, so cross-contamination among turtles cannot be excluded. However, precautions taken at sampling should have limited contamination. Hidalgo-Vila and colleagues also found lower diversity of *Salmonella* serotypes in aquatic turtles compared to terrestrial ones (Hidalgo-Vila *et al.* 2007). Two serotypes, Abony and the 47:k:1,5,7 subsp. *diarizonae*, isolated in the breeding and in the shelter respectively, were previously detected in tortoises and turtles and recognized responsible for human salmonellosis outbreaks (Bertrand *et al.* 2008, Schröter *et al.* 2004, Van Meervenne *et al.* 2009). The aquatic environment could be a further source of transmission to humans not only for *Salmonella*, but also for other zoonotic agents, like *Aeromonas*, isolated in this study. Unfortunately, no water samples were collected in the present study to confirm this hypothesis.

In the parasitological survey, the prevalence of helminth infestation was higher in tortoises than in turtles. This is in contrast with the prevalence of bacteria observed in the same animals: probably bacteria spread easily in water, while the helminth eggs may sink to the bottom of the pond. Both oxyurids and ascarids are direct life cycle parasites and the main source of contamination is the soil where the animals usually move and feed; this could be expected, since it is very difficult for chelonians kept in captivity to have access to intermediate hosts. The tortoises sampled in the present survey were kept in small fenced facilities where parasites may increase gradually, due to the high persistence of eggs, and contaminate the vegetables, that represent the main source of feed for the animals.

Moreover, in turtles, the type of feed, diluted with the water of the pond, may allow a dilution of parasitic burden, reducing the risk of infestation. Nevertheless, the oxyurids are generally considered symbionts, especially in herbivorous species: they can be indicators of poor sanitary conditions and become responsible for clinical manifestations (Papini *et al.* 2011).

All samples resulted negative for enteric protozoa. *Cryptosporidium* is common in reptiles and different chelonians have been considered shedders of potentially zoonotic species, e.g. *C. pestis* (Traversa *et al.* 2008). With regards *Giardia* spp., the most important species for its potential zoonotic transmission is *G. duodenalis*, but no data are currently available on *Giardia* infection in chelonians. The animals sampled may not have been infected or infected with a low burden of protozoa, not detectable by DFA. In the future an ELISA identifying specific metabolic coproantigen of the organisms could be used as screening test to increase this performance, considering that some authors found a very weak reaction for oocysts with immunofluorescence antibody test for *Cryptosporidium* in some reptiles, such as lizards (Graczyk *et al.* 1999, Pasmans *et al.* 2008).

This study shows that every breeding represents a different epidemiological situation and probably a unique ecosystem. Accordingly, a different microbiological risk is present in each breeding or

shelter and it probably increases when new animals are introduced. Furthermore, the epidemiology of infections in sheltered chelonians could be of interest for the practitioner. In this environment, zoonotic agents such as *Salmonella* spp., *Aeromonas* spp., and *Cryptosporidium* spp. can be present. Accordingly, the management of chelonian breedings becomes very important, especially in the case of colonies of abandoned turtles in public urban areas, in which the mixing of different animals is the rule, and the risk of transmission of zoonotic agents can become dangerous. A regular clinical monitoring, a parasitological and microbiological survey, the application of the quarantine as well as a check-up of new animals during the quarantine should be encouraged. A professional and informed management of these breedings is essential to control and reduce this risk, also considering the new conditions created by the popular habit of keeping reptiles as pets, especially when owners are not expert.

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