Comparative Immunology, Microbiology and Infectious Diseases 65 (2019) 154–159

Contents lists available at ScienceDirect

Comparative Immunology, Microbiology and Infectious Diseases

journal homepage: www.elsevier.com/locate/cimid

Complex bacterial flora of imported pet tortoises deceased during quarantine: Another zoonotic threat?

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ARTICLE INFO

Keywords:
Tortoises
Salmonella spp.
Escherichia coli
Antimicrobial resistance

ABSTRACT

Turtoises are a great puzzle when it comes to their bacterial flora, the composition and structure of which are still unknown in details. Its component which has been best described so far is *Salmonella* spp., presumably due to the threat of reptile-associated salmonellosis in humans. This investigation tried to assess and characterize intestinal bacterial flora of imported tortoises found dead during quarantine. Most of the animals carried various serovars of *Salmonella* showing no antimicrobial resistance. Presence of multiresistant *Escherichia coli* was possibly a result of industrial breeding and high usage of antimicrobials. Thirteen bacterial species or genera like *Citrobacter* spp., *Morganella* spp., *Pseudomonas* spp. were identified. Their commensal character is assumed, although pathogenic potential might be verified. The results indicate global tortoise trade as a source of common and exotic bacteria or antimicrobial resistance mechanisms in new geographical areas. These dangers indicate the need for a systematic survey of exotic pets and establishment of legal requirements for reptile health conditions on breeding, trade premises and in households with such pets.

1. Introduction

Chelonians, mysterious ancient creatures, with their ease of breeding, intriguing habitus, physiology, and behaviour have become common pets worldwide [1]. These long-lived "living presents for children" [2] are not convenient pets and therefore they are often released to the environment. The negative environmental effects of non-native animals have led to identification of some turtles and tortoise species as invasive and their importation is banned in the European Union (EU) [3]. Soon after the enactment of the ban, other species were introduced to the market. High population density at rearing farms contributes to colonisation by and cross-infections with multiple bacteria, and methaphylactic antimicrobial usage leads to selection of resistance. Animals from this source might therefore be considered vectors of potentially zoonotic agent introduction into a household [1,4]. To date, the few extant complex studies have merely scratched the surface of the problem.

Reptile-associated salmonellosis (RAS) is the most recognised topic in chelonian zoonoses. The real frequency of *Salmonella* spp. in turtles and tortoises is hard to estimate, due to incompatibility between the outcomes of studies [4] which are often limited to isolation with no *Salmonella* serovar identification [5,6]. Reptile-associated salmonellae were responsible for 6% of the total 1,4 million cases of salmonellosis in United States of America at the end of 20th century [7,8]. In Europe RAS cases have been described, but comprehensive assessment of the magnitude of the problem is lacking [9,10]. Special recommendations are addressed to reptile owners to protect children, pregnant women and immunocompromised people [11].

Salmonella spp. is considered a natural component of reptilian gut flora, whereas in the evolutionary history of birds and mammals it was replaced by *Escherichia coli* (*E. coli*) [12,13]. Anthropogenic *E. coli* might be found in chelonians due to intensive breeding and – if virulent – contribute to diarrhea or animal death [14–16]. Commensal *E. coli* is currently an indicator bacteria in EU antimicrobial resistance monitoring in animal production showing the magnitude and trends of antimicrobial use [17].

Multiple mostly Gram-negative bacteria have been reported in chelonians. *Aeromonas* spp., *Pseudomonas* spp., *Citrobacter* spp., and

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https://doi.org/10.1016/j.cimid.2019.05.007
Received 4 April 2018; Received in revised form 12 April 2019; Accepted 6 May 2019

Vibrio spp. were reported both from clinical cases (bronchopneumonia, ulcerations, stomatitis, septicaemia etc.) and carriers [13,15,16,18–20]. *Klebsiella* spp., *Enterobacter* spp., *Shewanella* spp., *Acinetobacter* spp., and numerous others were reported as opportunistic bacteria found in Testudines [6,13,15,16,18,19]. Both pathogenic and opportunistic bacteria might cause infections in other poikilothermic and homoeothermic animals as well as humans, particularly those with immune deficiency [21–23].

Considering these factors, we characterised bacterial flora from imported chelonians deceased during quarantine. In the absence of legal requirements for bacteriological monitoring of healthy pet reptiles, we applied convenience sampling related to massive deaths reported by a tortoise importer in a quarantine site. For possible clinical relevance, attention was paid to internal organs isolates suspected of being the cause of death. The other focus was to identify Gram-negative flora, namely *Salmonella* and cephalosporin-resistant *E. coli*, since both are of zoonotic character and included in official antimicrobial resistance monitoring in the EU [17]. Other bacteria were targeted to identify putative pathogens for tortoises or their possible owners.

2. Materials and methods

2.1. Study subjects

Sixteen dead tortoises were obtained from an importer reporting severe animal losses during quarantine: Horsefield tortoise (*Testudo horsfieldii*; n = 9) imported from Uzbekistan, leopard tortoise (*Psammobates pardalis*; n = 6) imported from Tanzania, and pancake tortoise (*Malacochersus tornieri*; n = 1) originating from Togo. Pinworm treatment with Fenbendazol and Profender after transportation was reported in *T. horsfieldii*. At necropsy the body weight was recorded (28 ÷ 168 g, Table 1). Nasal exudate was noted in few cases. Carapaces were correctly curved, plastrons were soft, and in several cases petechiae in groins, armpits and carapaces were noted under macroscopic examination. In most cases bladder was filled with urine containing a large amount of uric acid, occasionally coloured green.

2.2. Sampling procedures

During the necropsy the internal organs of each animal were aseptically pooled into two samples: intestines (n = 15) (small, large bowel, and cloaca) and organs (n = 13) (lungs, kidneys, liver, and ovaries in the case of females).

2.3. Isolation of bacteria

Sample processing: Each sample was weighed (Table 1), homogenized and immersed in buffered peptone water (BPW, 1:10 w/v, Oxoid). After 18 ± 2 h incubation at 37 °C a loopful of culture was inoculated onto Modified Semi-Solid Rappaport-Vassiliadis Agar (MSRV, Merck), Rappaport-Vassiliadis Soya Peptone Broth (RVS, Bio-Rad), and simultaneously streaked onto Rapid *Salmonella* Agar (RSA, Bio-Rad). Following incubation (44 ± 1 °C for MSRV and RVS and 37 ± 1 °C for RSA; 24 ± 2 h) the suspected colonies on RSA as well as RVS and MSRV cultures were streaked on XLD Agar (Xylose Lysine Deoxycholate Agar, Oxoid). Additionally, MSRV culture was streaked onto BxLH Agar (Brilliant green – Xylose – Lysine – P-Aminobenzenesulfonamide – Hoszowski, homemade). The plates were cultured for 24 ± 2 h in 37 ± 1 °C. Additionally, BPW cultures were inoculated on MacConkey Agar (Oxoid) supplemented with 2 mg/L cefotaxime (CTX) and CHROMAgar Orientation (BioMaxima). The media were incubated in 37 ± 1 °C for 24 ± 2 h.

2.3.1. Salmonella identification

Colonies showing typical morphology on *Salmonella*-selective media were further identified biochemically and serotyped according to White-Kaufmann-Le Minor scheme [24], with polyvalent and monovalent somatic and flagellar antisera (Sifin, Immunolab, SSI, Biomed). *Salmonella* isolates were tested with multiplex PCR to identify *Salmonella* species and subspecies [25].

2.3.2. E. coli identification

Colonies showing typical *E. coli* morphology on MacConkey Agar supplemented with 2 mg/L cefotaxime were confirmed with PCR targeting *uspA* gene [26].

2.3.3. CHROMAgar cultures

Bacterial growth was recorded by differences in colony morphology (color, size, shape, shine, presence and colour of colony centre, smoothness of colony edge, color of surrounding medium). The isolates (Table 1) were stored (Cryobank, Mast) until required. A subset of available isolates was identified with matrix-assisted laser desorption ionisation-time-of-flight mass spectrometry (MALDI-TOF). The selection was based on the following criteria: 1) cephalosporin resistant isolates from cefotaxime-supplemented MacConkey agar (n = 29, originated from 10 internal organs and 12 intestine samples) and 2) Gram-negatively stained bacteria (n = 17) obtained from organs (n = 11) on CHROMagar Orientation. Selected isolates were tested for oxidase activity and according to the result, oxidase-positives were further identified by API20NE (BioMerieux) and oxidase-negatives – by API20E (BioMerieux). To avoid presumptive duplicate isolates of the same microorganism only a single isolate from each animal was included in further analysis and presented in the result section (Table 1).

2.3.4. Antimicrobial resistance

A subset of 21 isolates (12 *Salmonella spp.* and 9 *E. coli*) was tested for minimal inhibitory concentration (MIC) determination with a microbroth dilution method using Sensititre EUMVS2 plates (Table 2) and – if cephalosporin resistance was observed – with Sensititre EUVSEC2 panels (Trek D.S.). MIC values were interpreted according to EUCAST epidemiological criteria (www.eucast.eu). Isolates resistant to critically important antimicrobials such as cephalosporins, quinolones and polymyxins were further tested in a way previously described [27] with a number of PCR assays for resistance gene identification.

2.3.5. Isolate storage and Sanger sequencing

The isolates were saved in Lysogeny Broth with 15% glycerol at < −70 °C until required. A total of 65 relevant PCR amplicons of resistance genes of 12 *E. coli* isolates were sequenced (Genomed) and nucleotide sequences were aligned and analysed with MEGA7 software (Center for Evolutionary Medicine and Informatics). The obtained sequences were deposited in GenBank (accession numbers listed in Table 2).

3. Results

Twenty-eight samples (15 intestines, 13 organs) were obtained. Bacteriological examination yielded a total of 143 distinguishable cultures considered as non-repetitive and unique isolates. A summary of bacteriological findings was shown in Table 1.

3.1. Salmonella spp

Salmonella spp. was found in 12 samples (eight intestines and four organs) originating from nine individuals: *T. horsfieldii* (n = 6), *P. pardalis* (n = 2) and *M. tornieri*. The isolates represented five serovars belonging to *Salmonella enterica* subsp. *enterica*: serovars Gaminara, Oslo, Kimberley, Lindern, and *S. enterica* subsp. *salamae* 13,22:z$_{29}$:1,5. Two *T. horsfieldii* (nos. 4 and 6, Table 1) harbored the same serovar (Gaminara or *S. enterica* subsp. *salamae* 13,22:z$_{29}$:1,5) in both intestines and organs. *T. horsfieldii* (no. 7, Table 1) hosted two serovars simultaneously, namely Gaminara and Kimberley, isolated from intestines and

Table 1
Description of tested samples and summary microbiological findings.

Turtle		Sample		*Salmonella* serovar	Number of isolates[1]		MALDI-TOF identification	No of isolates[2]	
species (origin)	No. (weight)	type (weight [g])	ID		MacConkey + cefotaxime (2 mg/L)	ChromAgar		A	B
T. horsfieldii (Uzbekistan)	1 [144 g]	int. [6,5 g]	20B	neg.	G- [1]*	nn [1]	*E. coli*	1	2
		org. [21,6 g]	21B	neg.	neg.	G- coryneforms [1]		0	1
	2 [86 g]	int. [4,2 g]	22B	*S.* Gaminara	*E. coli*	nn [4]		2	6
					G- [1]*		*E. coli*		
	3 [138 g]	int. [18,4 g]	23B	II 13,22:z29:1,5	*E. coli*	nn [3]	*E. coli, Enterobacter cloacae*	3	6
					G- [2]*				
		org. [6,3 g]	24B	neg.	nn [1]*	G+ cocci [3]		4	7
						G- coccobacilli [1]*, [3]	*Myroides odoratimimus* [2][3]		
						G- bacilli [2]*, [4]	*Enterobacter cloacae* [2][4]		
	4 [148 g]	int. [7,4 g]	25B	*S.* Gaminara	G- [2]*	nn [4]	*Pseudomonas mendocina, Enterobacter cloacae*	2	6
		org. [1,3 g]	26B		nn [2]*		*Morganella morganii*[5]	3	4
						nn [2]*,[5]	*Acinetobacter pittii, Myroides odoratimimus*		
	5 [158 g]	int. [12,6 g]	27B	neg.	*E. coli*	nn [4]		1	6
					nn [1]				
		org. [4,3 g]	28B	neg.	nn [1]*	G- coccobacilli [1]	*Morganella morganii*	1	4
						G+ cocci [1]			
						nn [1]			
	6 [80 g]	int. [6,7 g]	29B	II 13,22:z29:1,5	*E. coli*	nn [6]		1	7
		org. [3,3 g]	30B		*E. coli*	G- coccobacilli [1]*, [6]	*Citrobacter spp*[6]	2	5
						G- cocci [1]			
						G+ cocci [1]			
						nn [1]			
	7 [168 g]	int. [20,5 g]	31B	*S.* Gaminara	*E. coli*	nn [3]		1	4
		org. [2,4 g]	32B	*S.* Kimberley	nn [1]*	G- coccobacilli [1]*	*Citrobacter* spp	2	4
						G- coryneforms [1]	*Acinetobacter pittii*		
						G+ cocci [1]			
	8 [162 g]	int. [15,1 g]	33B	*S.* Lindern	*E. coli*	nn [4]		1	5
		org. [2,5 g]	34B	neg.	nn [2]*	G- coccobacilli [2]*, [7]	*Citrobacter* spp[7]	2	5
						G- cocci [1]	*Citrobacter* spp		
	9 [80 g]	int. [5,0 g]	35B	neg.	nn [2]*	nn [3]	*E. coli, Comamonas aquatica*	2	5
		org. [1,5 g]	36B	neg.	*E. coli*	G- bacilli [1]*, [6]	*Citrobacter* spp[6]	2	5
					nn [1]	nn [2]			
P. pardalis (Tanzania)	10 [50 g]	int. [3,0 g]	37B	neg.	nn [1]*	nn [2]	*Pseudomonas aeruginosa*	1	3
		org. [0,3 g]	38B	neg.	nn [1]*	G- bacilli [2]*	*Enterobacter cloacae,*	3	4
						G- cocci [1]	*Pseudomonas aeruginosa*		
							Enterobacter cloacae		
	11 [58 g]	int. [3,5 g]	39B	neg.	nn [1]*	nn [3]	*Pseudomonas mendocina*	1	4
	12 [28 g]	int. [3,8 g]	40B	*S.* Gaminara	nn [1]*	nn [3]	*Pseudomonas aeruginosa*	1	4
		org. [0,3 g]	41B	neg.	nn [1]*	G- bacilli [2]*	*Pseudomonas aeruginosa* [3]	3	3
	13 [92 g]	int. [4,0 g]	42B	neg.	nn [2]*	nn [3]	*Pseudomonas putida,*	2	5
							Stenotrophomonas maltophila		
		org. [0,1 g]	43B	*S.* Gaminara	nn [1]*	G- bacilli [3]*	*Acinetobacter calcoaceticus,*	3	6
						G+ cocci [2]	*Enterobacter cloacae*		
							Pseudomonas aeruginosa		
	14 [38 g]	int. [3,6 g]	44B	neg.	nn [2]*	nn [3]	*Citrobacter* spp	1	5
		org. [0,3 g]	45B	neg.	*E. coli*	G- bacilli [1]*	*Morganella morganii*	3	5
					nn [1]*	G- coryneforms [1]	*Pseudomonas aeruginosa*		
						nn [1]			
	15 [40 g]	org. [0,2 g]	46B	neg.	nn [2]*	G- bacilli [3]*	*Citrobacter* spp,	4	5
							Aeromonas hydrophila		
							Citrobacter spp [2]		
M. tornieri (Togo)	16 [34 g]	int. [3,2 g]	47B	*S.* Oslo	nn [1]*	nn [4]	*Aeromonas hydrophila*	1	5

[1]isolates differentiated by different colony morphology and Gram-staining; asterisk (*) designates isolates identified further with MALDI-TOF, nn – not known.
[2](A) number of isolates identified with PCR (*E. coli* cultured on MacConkey supplemented with cefotaxime) and MALDI-TOF (cultures from CHROM Agar and MacConkey supplemented with cefotaxime) and (B) total number of cultures (colony morphologies) on both media.
[3÷7]identified with API32E® (bioMerieux) as: ([3]) *Myroides* spp./*Bergeyella zooheicum*, ([4]) *Enterobacter cloacae*, ([5]) *Morganella morganii*, ([6]) *Citrobacter freundii*, ([7]) *Salmonella enterica* subsp. *Arizonae.*
G- - Gram-negative.
G+ - Gram-positive.

organs, respectively. The remaining serovars were identified in single samples and individuals. All *Salmonella* isolates were susceptible to 14 antimicrobials representing 10 classes (Table 2).

3.2. Antimicrobial resistant E. coli

Thirteen *E. coli* were isolated from samples collected from eight *T. horsfieldii* and one *P. pardalis*. Nine strains showed typical colony morphology on selective agar. Characteristic *E. coli* growth was observed in all but four cultures, which were further identified as *E. coli* following Gram-staining and detail identification with API and/or MALDI-TOF. MIC testing revealed multidrug resistant profiles comprising up to nine compounds representing all tested antimicrobial classes (Table 2). Synergy between cephalosporins and clavulanic acid (Sensititre EUVSEC2 MIC panel) was observed in all tested *E. coli*, these therefore being regarded as extended-spectrum betalactamase-producers (ESBL). No cefoxitin and meropenem resistance excluded AmpC-type cephalosporin and carbapenem resistance mechanisms. Resistance profiles were used to exclude duplicates of the same strain from a sample or an individual.

3.2.1. Cephalosporin resistance mechanisms

PCR and sequencing identified cephalosporin resistance due to the $bla_{\text{CTX-M-15}}$ gene present in all 13 *E. coli*. Nine of them simultaneously carried betalactamase TEM-1B genes. Due to PCR products length the genes might have been misidentified (see Table 2 footnote for details).

3.2.2. Quinolone resistance mechanisms

All the above *E. coli* were resistant to ciprofloxacin (MIC range: 0.25 − > 8 mg/L) and seven of them simultaneously demonstrated resistance to nalidixic acid. Resistance to both compounds indicated chromosomal mutations in the quinolone resistance determining region (QRDR), while resistance to ciprofloxacin alone signalled plasmid-mediated quinolone resistance (PMQR). Relevant mutations resulting in aminoacid substitutions were observed in *gyr*A (Ser83Leu and Asp87Asn in six isolates and Ser83Leu alone in single *E. coli* strain), *par*C (Ser80Ile in six *E. coli*), and *par*E (Ser458Ala in four *E. coli*, and single isolates with Ala426Gly, Lys427Gln, and Ser458Ala). Four strains harboured four mutations in three genes, and two genes were affected in two other *E. coli*. A single strain showed six mutations in three genes (CTX/29B Table 2).

Three different plasmid-mediated quinolone resistance mechanisms were found in 12 *E. coli*. Nine of them, including four from the same samples and animals, carried *qnr*S1 gene (or *qnr*S3 – both proteins differ by a single amino acid at His11Arg and cannot be differentiated with common PCR assays). Additionally, one of *qnr*S1-positive isolates simultaneously carried the *qep*A gene responsible for quinolone efflux pump. Three isolates carried the *aac(6)Ib-cr* gene encoding plasmid-mediated ciprofloxacin-modifying enzyme that differed from the common aminoglycoside acetyltransferase by two amino acid substitutions (Trp102Arg and Asp179Tyr) [27].

The ciprofloxacin MIC value in *E. coli* carrying PMQR mechanisms, but no QRDR mutations ranged between 0.25 and 0.5 mg/L. Strains with chromosomal mutations mostly showed MIC ≥ 8 mg/L.

3.2.3. Other resistance

Colistin resistance was noted in four *E. coli*, but none of them harbored plasmid-mediated *mcr*-1, −2, −3, −4 or *mcr* − 5 genes detected according to PCR method described by Rebelo et al. [28]. Other resistance included folate path inhibitors (sulphonamides and trimethoprim), chloramphenicol, tetracycline, and aminoglycosides (streptomycin, gentamycin), but the mechanisms were left unidentified.

3.3. Other bacterial cultures

3.3.1. Cefotaxime-supplemented MacConkey Agar and CHROMAgar orientation cultures

Cefotaxime-supplemented MacConkey Agar yield 40 distinctive cultures. Nine isolates were initially identified as *E. coli* (described above). The remaining were isolated from intestines (n = 17) and internal organs (n = 14). Ninety-one isolates were obtained from CHROMAgar Orientation (50 from intestines and 41 from internal organs).

3.3.2. MALDI-TOF identification

Forty-four isolates, mostly Gram-negative cephalosporin resistant bacilli and cocobacilli were classified with Compass 4.1.70. MALDI Biotyper (with MBT 6903 MPS Library, Bruker) to 13 bacterial species or genera. Bacteria growing on cefotaxime-supplemented MacConkey Agar were identified mostly as *E. coli* (n = 4), *Pseudomonas* (*P.*) *aeruginosa* (n = 3), *P. mendocina* (n = 2), and *Acinetobacter pittii* (n = 2). The most strongly represented CHROMagar Orientation cultures were: *Enterobacter* (*E.*) *cloacae* (n = 4), *Citrobacter freundii* (n = 3), *P. aeruginosa* (n = 3) and *Morganella morganii* (n = 2). A summary of bacteriological findings was given in Table 1.

4. Discussion

The current study provides an insight into reptile bacteria and microbial threats related to tortoise pets. Commercial breeding of trade reptiles carried out on crowded farms along with the long-distance transport of animals favour bacterial transmission and disease transmissions. To protect animal welfare and prevent economical losses antimicrobials are widely used [29]. Although no data on antibiotic treatment was available for the current study, isolation of multiresistant *E. coli* proves enormously high selective pressure in tortoise and turtle husbandry. Twelve isolates originating from *T. horsfieldii* raised in Uzbekistan were resistant to multiple antimicrobials, including broad spectrum cephalosporins and quinolones. Numerous mutations within QRDR and variable PMQR genes indicate constant quinolone usage on the tortoise farm. All isolates yielded $bla_{\text{CTX-M-15}}$, which suggests horizontal transmission of plasmid mediated ESBL resistance. Animals from the same batch that survived transportation were most probably also colonised by multidrug- resistant bacteria and, if placed in a household, they would pose a health risk to their owners. Resistance genes located on mobile genetic elements could be transferred in a tortoise to pathogens like *Salmonella*, or to some antimicrobial-susceptible flora in humans following direct contact with carrier animal. Previously we described a similar case: the $bla_{\text{CTX-M-25}}$ gene located on a rare plasmid was acquired from an environmental source by multidrug, high ciprofloxacin-resistant *S*. Kentucky [30]. Similarly, the $bla_{\text{CTX-M-15}}$ gene was observed in wild boars, but not in food animals and neither *aac(6)Ib*-cr nor *qep*A were previously described in animal sources in Poland [27,31,32]. Not only resistance genes, but also some of the *Salmonella* serovars found in the currently tested tortoises were not previously noted in Poland [33]. The finding supports reports on a salmonellosis threat to exotic pet reptile [34]. Both exotic *Salmonella* and other bacteria of zoonotic potential (i.e. *Pseudomonas*, *Aeromonas*) demand our awareness of them as new and emerging threats.

It is hard to link any of the identified bacterial species with a possible cause of tortoises' deaths. A few nonspecific anatomopathological findings might support the presumption that the animals died owing to transport conditions or stress. However a long list of identified bacteria includes possible chelonian pathogens. *Aeromonas, Pseudomonas* and *Citrobacter* can lead to septicaemia, ulcers, internal organs infection, sepsis, endocarditis and brain abscesses [13,16,18,35]. Other pathogenic or opportunistic bacteria such as *Enterobacter cloacae*, *Shewanella putrefaciens*, *Aeromonas hydrophila, Morganella morganii, Myroides* and *Acinetobacter* were also detected in chelonians [6,13,15,16,19,36]. The

Table 2
Antimicrobial resistance profiles and resistance gene identification in *E. coli*.

Source (Turtle No.)	*E. coli* No.	Resistance profile[2]	Resistance genes (Genebank No.)			
			betalactams[3]	quinolones		colistin[1]
				chromosomal gene mutations[4]	plasmid mediated[5]	
1	P/15/CTX/20B	AmpCtxCazNalCipSmxTetCol	$bla_{\text{CTX-M-15}}$ (MG742328)	*gyr*A[6]: Ser83Leu, Asp87Asn *gyrB* (MG742342) *par*C: Ser80Ile (awaiting accession number) *par*E: Ser458Ala (MG757172)	*aac(6)Ib-cr* (awaiting accession number)	neg
2	P/15/CTX/22B/1	AmpCtxCazCipCol	$bla_{\text{CTX-M-15}}$ (MG742329)	–	*qnr*S1 (awaiting accession number)	neg
	CTX/22B	AmpCtxCazCipStrSmxTetTmp	$bla_{\text{CTX-M-15}}$ (MG742330) $bla_{\text{TEM-1b}}$ (awaiting accession number)	–	*qnr*S1 (awaiting accession number)	–
3	P/15/CTX/23B/2	AmpCtxCazNalCipSmxTetTmp	$bla_{\text{CTX-M-15}}$ (MG742332) $bla_{\text{TEM-1b}}$ (awaiting accession number)	*gyrA*[6]: Ser83Leu, Asp87Asn *gyrB* (MG742344) *par*C: Ser80Ile (awaiting accession number) *par*E (MG757174)	*qnr*S1 (awaiting accession number)	–
	CTX/23B	AmpCtxCazChlNalCipStrSmxTetTmp	$bla_{\text{CTX-M-15}}$ (MG742331) $bla_{\text{TEM-1b}}$ (awaiting accession number)	*gyrA*[6]: Ser83Leu, Asp87Asn *gyrB* (MG742343) *par*C: Ser80Ile (awaiting accession number) *par*E: Ser458Ala (MG757173)	*qnr*S1 (awaiting accession number) *qep*A (MG742341)	–
5	CTX/27B	AmpCtxCazNalCipGenTetTmp	$bla_{\text{CTX-M-15}}$ (MG742333) $bla_{\text{TEM-1b}}$ (awaiting accession number)	*gyrA*[6]: Ser83Leu *gyrB* (MG742345) *par*C: Ser80Ile (awaiting accession number) *par*E (MG757175)	*qnr*S1 (awaiting accession number)	–
6	CTX/29B	AmpCtxCazFoxNalCipSmxTetTmp	$bla_{\text{CTX-M-15}}$ (MG742334)	*gyrA*[6]: Ser83Leu, Asp87Asn *gyrB* (MG742346) *par*C: Ser80Ile (awaiting accession number) *par*E: Ala426Gly, Lys427Gln Ser458Ala (MG757176)	neg	–
	CTX/30B	AmpCtxCazCipStrSmxTetTmp	$bla_{\text{CTX-M-15}}$ (MG742335) $bla_{\text{TEM-1b}}$ (awaiting accession number)	–	*qnr*S1 (awaiting accession number)	–
7	CTX/31B	AmpCtxCazCipStrSmxTetTmp	$bla_{\text{CTX-M-15}}$ (MG742336) $bla_{\text{TEM-1b}}$ (awaiting accession number)	–	*qnr*S1 (awaiting accession number)	–
8	CTX/33B	AmpCtxCazFoxNalCipStrGenSmxTetTmpCol	$bla_{\text{CTX-M-15}}$ (MG742337) $bla_{\text{TEM-1b}}$ (awaiting accession number)	*gyrB* (MG742347) *parE* (MG757177)	*qnr*S1 (awaiting accession number)	neg
9	P/15/CTX/35B/1	AmpCtxCazNalCipSmxTetCol	$bla_{\text{CTX-M-15}}$ (MG742338)	*gyrA*[6]: Ser83Leu, Asp87Asn *gyrB* (MG742348) *par*C: Ser80Ile (awaiting accession number) *par*E: Ser458Ala (MG757178)	*aac(6)Ib-cr* (awaiting accession number)	neg
	CTX/36B	AmpCtxCazNalCipStrGenSmxTet	$bla_{\text{CTX-M-15}}$ (MG742339) $bla_{\text{TEM-1b}}$ (awaiting accession number)	*gyrA*[6]: Ser83Leu, Asp87Asn *gyrB* (MG742349) *par*C: Ser80Ile (awaiting accession number) *par*E: Ser458Ala (MG757179)	*aac(6)Ib-cr* (awaiting accession number)	–
14	CTX/45B	AmpCtxCazCipStrSmxTetTmp	$bla_{\text{CTX-M-15}}$ (MG742340) $bla_{\text{TEM-1b}}$ (awaiting accession number)	–	*qnr*S1 (awaiting accession number)	–

[1] *mcr-1-5* were tested if colistin resistance was found.
[2] Interpretation criteria of antimicrobials tested: Amp – ampicillin, Ctx – cefotaxime, Caz – ceftazidime, Fox – cefoxitin, Chl – chloramphenicol, Nal – nalidixic acid, Cip – ciprofloxacin, Str – streptomycin, Gen – gentamycin, Smx – sulfamethoxazole, Tet – tetracycline, Tmp – trimethoprim, Col – colistin.
[3] Only positive results (resistance genes were shown; due to short PCR amplicons it was not possible to differentiate $bla_{\text{TEM-1b}}$ from $bla_{\text{TEM-104}}$, and $bla_{\text{TEM-206}}$ and $bla_{\text{CTX-M-15}}$ from $bla_{\text{CTX-M-}}$28 and blaCTX$_{\text{-M-88}}$.
[4] *gyr*A, *gyr*B, *par*C, and *par*E gene amplicons were tested if *E. coli* was resistant to both Nalidixic acid and Ciprofloxacin; only relevant aminoacid substitutions in QRDR region of the genes were shown; wild-type genes were not listed.
[5] *qnr*A, *qnr*B, *qnr*C, *qnr*D, *qnr*S, *qep*A and *aac(6)Ib* were tested; only detected PMQR genes were listed; due to short PCR amplicon it was not possible to differentiate *qnr*S1 from *qnr*S3.
[6] Sequence too short for GeneBank submission.

cited reports refer to different chelonians and possible species vulnerability should be considered. Current data only draw attention to possible pet tortoise and public health risks. The other limitations are the differences that might occur in healthy and diseased tortoises living in natural and controlled environments. Further, we have focussed on a fraction of culturable, selectively enriched bacteria. The others and the general structure of the chelonian bacterial microbiome might be targeted with a metagenomic approach [37]. Those knowledge gaps, involving also other variabilities related to diet or age specificity, among other factors still need filling.

5. Conclusions

The current study neither allows for unambiguous and definite identification of cause of death of the examined tortoises nor indicates threats to pets or their owners. The observed multiple and complex flora simply show that the global turtle trade should be considered a source and vector of common and exotic bacteria or antimicrobial resistance mechanisms for new geographical areas and individual households. It indicates the need for systematic survey of exotic pets and establishment of legal requirements for reptile welfare as well as health conditions on breeding and trade premises and in households which own such pets.

Acknowledgement

The results were obtained during a pilot study for National Science Centre, Poland, Grant No. 2013/11/B/NZ7/01690. Peter Pristas and Ľudmila Hamarová acknowledge the support in the form of grant APVV-16-0171 from the Slovak Research and Development Agency

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