Epidemiology and Infection

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Occurrence, antimicrobial susceptibility patterns and genotypic relatedness of *Salmonella* spp. isolates from captive wildlife, their caretakers, feed and water in India

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Original Paper

Cite this article: Milton AAP, Agarwal RK, Priya GB, Athira CK, Saminathan M, Reddy A, Aravind M, Kumar A (2018). Occurrence, antimicrobial susceptibility patterns and genotypic relatedness of *Salmonella* spp. isolates from captive wildlife, their caretakers, feed and water in India. *Epidemiology and Infection* **146**, 1543–1549. https://doi.org/10.1017/S0950268818001553

Received: 11 September 2017
Revised: 2 April 2018
Accepted: 18 May 2018
First published online: 14 June 2018

Key words:
Antimicrobial resistance; ERIC-PCR; India; *Salmonella enterica*; wild animals

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Abstract

Occurrence of *Salmonella* spp. in captive wild animal species in India is largely unknown. The purpose of this study was to determine the occurrence of different *Salmonella* serotypes, antimicrobial resistance patterns and genotypic relatedness of recovered isolates. A total of 370 samples including faecal ($n = 314$), feed and water ($n = 26$) and caretakers stool swabs ($n = 30$) were collected from 40 different wild animal species in captivity, their caretakers, feed and water in four zoological gardens and wildlife enclosures in India. Salmonellae were isolated using conventional culture methods and tested for antimicrobial susceptibility with the Kirby–Bauer disc diffusion method. *Salmonella* isolates were serotyped and genotyping was performed using enterobacterial repetitive intergenic consensus (ERIC) PCR and 16S rRNA sequencing. Animal faecal samples were also subjected to direct PCR assay. *Salmonella* was detected in 10 of 314 (3.1%) faecal samples by isolation and 18 of 314 (5.7%) samples by direct PCR assay; one of 26 (3.8%) feed and water samples and five of 30 (16.7%) caretakers stool swabs by isolation. *Salmonella* was more commonly isolated in faecal samples from golden pheasants (25%; 2/8) and leopard (10%; 2/20). *Salmonella enterica* serotypes of known public health significance including *S.* Typhimurium (37.5%; 6/14), *S.* Kentucky (28.5%; 4/14) and *S.* Enteritidis (14.3%; 2/14) were identified. While the majority of the *Salmonella* isolates were pan-susceptible to the commonly used antibiotics. Seven (43.7%; 7/16) of the isolates were resistant to at least one antibiotic and one isolate each among them exhibited penta and tetra multidrug-resistant types. Three *S.* Kentucky serotype were identified in a same golden pheasants cage, two from the birds and one from the feed. This serotype was also isolated from its caretaker. Similarly, one isolate each of *S.* Typhimurium were recovered from ostrich and its caretaker. These isolates were found to be clonally related suggesting that wildlife may serve as reservoir for infections to humans and vice versa. These results emphasise the transmission of *Salmonella* among hosts via environmental contamination of feces to workers, visitors and other wildlife.

Introduction

Salmonella is one of the noteworthy foodborne pathogens worldwide. *Salmonella* is ubiquitous with wide host range due to its capacity to survive in adverse environments. Most of the studies on public health implications of *Salmonella* in wildlife have an emphasis on amphibians and reptiles [1, 2]. The role of captive and free-range wildlife mammals and associated caretakers in the epidemiology of *Salmonella* is a domain that has rarely been investigated. Various serovars of public health significance including serovar Typhimurium and Newport and antimicrobial resistant strains have been reported from turtle, deer, wild birds and water samples [3–5]. Clonally related isolates of different serovars of public health significance in captive wild mammals and associated environments were recently reported in the USA [6]. Very limited studies have reported the same occurrence of *Salmonella* serovars from humans and wildlife species, supporting that wild animals serve as reservoirs for salmonellosis in humans [7, 8]. Another serious concern in *Salmonella* is the growing antimicrobial resistance and the dissemination of multidrug-resistant (MDR) strains. Enterobacterial repetitive intergenic consensus (ERIC) fingerprinting is effective than pulsed-field gel electrophoresis (PFGE) and it is useful

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for subtyping *Salmonella* serovars, where similar PFGE patterns occur [9]. ERIC-PCR was found effective over many other molecular typing methods [10, 11]. Besides, ERIC-PCR is a simple and cost-effective technique than PFGE. The aim of this study was to determine the occurrence, serovar distribution, antimicrobial susceptibility patterns and genotypic relatedness of *Salmonella* isolates recovered from faecal samples of captive wildlife, their caretakers, feed and water in India.

Materials and methods

The study was carried out in four zoological gardens and wildlife enclosures, *viz.*, Nainital Zoo, Nainital, Uttarakhand; Kanpur Zoo, Kanpur, Uttar Pradesh; Deer Park, Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh; Post Graduate Research Institute in Animal Sciences, Chennai, Tamilnadu, India. A total of 370 samples comprising 314 fresh faecal samples of apparently healthy captive animals (40 species) (Tables 1–3), 30 stool swabs from animal caretakers and 26 feed and water samples were collected. Briefly, 10 g samples were pre-enriched in 2% buffered peptone water (BPW) (HiMedia, India), at 37 °C for 16–18 h. The broth culture (100 µl) was transferred to 10 ml Tetrathionate Broth (HiMedia) and incubated at 37 °C for 24 h. A loopful of the suspension was streaked onto Hektoen Enteric Agar (HEA) (HiMedia) and incubated at 37 °C for 24 h [12]. Selected presumptive *Salmonella* colonies were inoculated onto triple sugar iron (TSI) agar (HiMedia) and Christensen's urea slants (HiMedia) and incubated at 37 °C for 24 h. All presumptive *Salmonella* isolates were submitted to the National *Salmonella* Centre (ICAR-IVRI, Bareilly, India) for serotyping. Genomic DNA was isolated directly from 314 faecal samples of captive wildlife by QIAamp DNA Stool Mini Kit (Qiagen, Germany). The extracted DNA was subjected to PCR as described by Lin and Tsen, [13]. Pearson's chi-square and Fisher exact test were employed to compare the prevalence of *Salmonella* spp. among different zoos and various sample groups *viz.*, wild ruminants, wild non-ruminants, wild birds, caretakers, feed and water (SPSS 22.0 version). The differences among various zoos and sample groups were considered significant at $P < 0.05$. The antimicrobial resistance profiles of *Salmonella enterica* isolates were tested to a panel of 23 different antibiotics (BD Diagnostics, Sparks, MD, USA) belonging to 10 classes using the Kirby–Bauer disc diffusion method. The antibiotic used were aminoglycosides-streptomycin (S, 10 µg), gentamicin (Gm, 10 µg), kanamycin (K, 30 µg), amikacin (Ak, 30 µg); colistin (Cl, 10 µg); cephalosporins-cefotaxime (Ctx, 30 µg), ceftazidime (Caz, 30 µg), ceftazidime + clavulanic acid (30/10 µg) and cefotaxime + clavulanic acid (30/10 µg); macrolides-erythromycin (E, 15 µg); fluoroquinolones-enrofloxacin (Ex, 10 µg), ciprofloxacin (Cip, 5 µg), ofloxacin (Of, 5 µg); monobactam-aztreonam (Atm, 30 µg); carbapenem antibiotics-imipenem (Ipm, 10 µg), meropenem (Mem, 10 µg), ertapenem (Etp, 10 µg); penicillins-carbenicillin (Cb, 100 µg), amoxicillin with clavulanic acid (Amc, 10 µg), ampicillin (Am, 10 µg); tetracycline (Te, 30 µg); sulphonamides (sulphamethoxazole with trimethoprim (Sxt, 10 µg)) and others-nitrofurantoin (F/M, 100 µg). Isolates showing resistance to three or more classes of antimicrobials were classified as MDR. Multiple antibiotic resistance index (MARI) for each resistance pattern was calculated.

The ERIC-PCR assay was performed as per Campioni *et al.* [14]. The oligonucleotide primers described in a previous study was used [15]. The PCR reaction mixture for amplification consisted of 12.5 µl of 2× PCR master mixtures (ThermoFisher Scientific), 1 µl (10 pmol/µl) of each primer (Eurofins, India), 2 µl of DNA template and nuclease-free water to make final volume up to 25 µl. The program used for the ERIC-PCR are as follows: initial denaturation at 94 °C for 7 min followed by 30 cycles each of denaturation at 94 °C for 30 s, annealing at 52 °C for 1 min and extension at 65 °C for 8 min followed by final extension at 65 °C for 10 min. The PCR was performed in a Thermal Cycler (Eppendorf, Germany). The ERIC-PCR reaction was repeated at least twice for each isolate to verify the reproducibility of the assay. The 16S rRNA gene of the recovered isolates was amplified employing primers of a previous study [16] and sequenced by Sanger dideoxy method using commercial sequencing services (Eurofins Ltd., Bangalore, India). The PCR reaction mixture for amplification consisted of 12.5 µl of 2× PCR master mixtures (ThermoFisher Scientific), 1 µl (10 pmol/µl) of each primer (Eurofins, India), 2 µl of DNA template and nuclease-free water to make final volume up to 25 µl. The cycling conditions for PCR consisted of 5 min initial denaturation at 95 °C followed by 35 cycles each of 1 min denaturation at 94 °C, 30 s annealing at 63 °C and 45 s extension at 72 °C and a final extension step of 5 min at 72 °C. The nucleotide sequences were deposited in GenBank using the National Centre for Biotechnology Information (NCBI, Bethesda, MD) Bankit submission tool http://www3.ncbi.nlm.nih.gov.

Results

Salmonella was detected in 10 of 314 (3.1%) wildlife faecal samples by isolation and 18 of 314 (5.7%) by direct PCR assay; one of 26 (3.8%) feed and water samples and five of 30 (16.7%) caretakers stool swabs. *Salmonella* was more commonly isolated in faecal samples from golden pheasants (25%; 2/8) and leopard (10%; 2/20). The occurrence of *Salmonella* in different species is presented in Tables 1–3. Out of 10 isolates from wildlife faecal samples 1 isolate (0.8%) was from captive wild ruminants ($n = 126$), four isolates (4.6%) were from captive wild non-ruminants ($n = 86$), five isolates (4.9%) were from captive wild birds ($n = 102$). Prevalence of *Salmonella* was further analysed as per the sample group and zoo (Tables 4 and 5). Prevalence of *Salmonella* spp. among different sample group and different zoos were statistically significant ($P < 0.05$). By serotyping, 6/16 isolates (37.5%) were found to be *S.* Typhimurium, four isolates (28.5%) were recognised as *S.* Kentucky, two isolates (14.3%) were identified as *S.* Enteritidis, two isolates (14.3%) were untypable, and one each isolate (6.2%) were *S.* Senftenberg and *S.* Lamberhurst (Table 6).

We found antimicrobial resistance among the isolates at varied frequencies. The highest frequency of resistance was found against cefotaxime (3; 18.7%) and ceftazidime (3; 18.7%), followed by carbenicillin (2; 12.5%), aztreonam (2; 12.5%), amoxiclav (2; 12.5%), sulphamethoxazole with trimethoprim (2; 12.5%), nitrofurantoin (2; 12.5%), ampicillin (2; 12.5%) and tetracycline (1; 6.2%). No resistance was found against streptomycin, gentamicin, kanamycin, amikacin, colistin, ceftazidime + clavulanic acid, cefotaxime + clavulanic acid, erythromycin, enrofloxacin, ciprofloxacin, ofloxacin, meropenem, imipenem, ertapenem. The overall multidrug resistance was low (2/16; 12.5%). Seven (7/16; 43.7%) of the isolates were resistant to one antibiotic and one isolate each exhibited penta and tetra resistance MDR with AtmCbCtxCazAmc and AtmCbCtxCaz R-types, respectively. It should be noted that these two isolates belonged to serovar Typhimurium and were recovered from the ostrich and its caretaker. Eight of the isolates (50%) were

Table 1. Prevalence of *Salmonella* spp. in faecal samples collected from captive wild ruminants

Captive wild ruminants				
Common name	Scientific name	No. of samples	Isolation (prevalence %)	Direct PCR (prevalence %)
Sambar deer	*Rusa unicolor*	5	–	–
Himalayan goral	*Naemorhedus goral*	8	–	–
Barking deer	*Muntiacus muntjak*	5	–	–
Thamin or Eld's deer	*Panolia eldii*	10	–	–
Swamp deer or Barasingha	*Cervus duvaucelii*	15	1 (6.7)	2 (13.3)
Nilgai or bluebull	*Boselaphus tragocamelus*	12	–	–
Spotted deer or Chital	*Axis axis*	32	–	–
Blackbuck	*Antilope cervicapra*	17	–	–
Indian hog deer	*Hyelaphus porcinus*	15	–	–
Sika deer or Japanese deer	*Cervus nippon*	3	–	–
Chousingha deer	*Tetracerus quadricornis*	2	–	–
Himalayan blue sheep	*Pseudois nayaur*	2	–	–
Total		126	1 (0.8)	2 (1.6)

Table 2. Prevalence of *Salmonella* spp. in faecal samples collected from captive wild non-ruminants

Captive wild non-ruminants				
Common name	Scientific name	No. of samples	Isolation (Prevalence%)	Direct PCR (Prevalence %)
Leopard	*Panthera pardus*	20	2 (10)	3 (15)
Bengal tiger (inc. one white)	*Panthera tigris tigris*	9	–	–
Hyena (striped)	*Hyaena hyaena*	10	1 (10)	1 (10)
Tibetian wolf	*Canis lupus chanco*	2	–	–
Jackal	*Canis aureus*	5	–	–
Himalayan black bear	*Ursus thibetanus laniger*	7	–	2 (28.6)
Sloth bear	*Melursus ursinus*	2	–	–
Hippopotamus	*Hippopotamus amphibious*	6	–	–
Indian rhinoceros	*Rhinoceros unicornis*	3	–	–
Gray langur	*Semnopithecus entellus*	5	1 (20)	1 (20)
Bonnet macaque	*Macaca radiate*	5	–	–
Rhesus macaque	*Macaca mulatta*	3	–	–
Japanese macaque	*Macaca fuscata*	2	–	–
Palm civet	*Paradoxurus hermaphrodites*	3	–	1 (33.3)
Red panda	*Ailurus fulgens*	2	–	–
Leopard cat	*Prionailurus bengalensis*	1	–	–
Zebra	*Equus quagga*	1	–	–
Total		86	4 (4.6)	8 (9.3)

pan-susceptible to the panel of 23 antimicrobials included in this study. These isolates belonged to Kentucky ($n = 3$), Typhimurium ($n = 1$), Senftenberg ($n = 1$), Lamberhurst ($n = 1$) and Untypable serovars ($n = 2$). MARI among the isolates ranged from 0 to 0.21. Resistance patterns and MARI of the *Salmonella* serovars are shown in Table 6.

The ERIC PCR typing of 16 *S. enterica* isolates to determine the genetic diversity and phylogenetic relationship among the

Table 3. Prevalence of *Salmonella* spp. in faecal samples collected from captive wild birds

Captive wild birds				
Common name	Scientific name	No. of samples	Isolation (prevalence %)	Direct PCR (prevalence %)
Golden pheasant	*Chrysolophus pictus*	8	2 (25)	2 (25)
Silver pheasant	*Lophura nycthemera*	8	–	–
Cockatiel	*Nymphicus hollandicus*	6	–	–
Lady Amherst pheasant	*Chrysolophus amhersitae*	12	1 (8.3)	1 (8.3)
Kalij pheasant	*Lophura leucomelanos*	8	–	–
Sun conure	*Aratinga solstitialis*	6	–	–
Red jungle fowl	*Gallus gallus*	2	–	–
Indian peafowl	*Pavo cristatus*	4	–	–
White peafowl	*Pavo cristatus mut. Alba*	3	1 (33.3)	1 (33.3)
Saras crane	*Grus antigone*	5	–	–
Emu	*Dromaius novaehollandiae*	5	–	–
Ostrich	*Struthio camelus*	35	1 (2.8)	4 (11.4)
Total		102	5 (4.9)	8 (7.8)

Table 4. Distribution of *Salmonella* spp. among the different sample groups

Group	No. of samples screened	No. positive (%)
Captive wild ruminants	126	1 (0.8%)
Captive wild non-ruminants	86	4 (4.6%)
Captive wild birds	102	5 (4.9%)
Care takers	30	5 (16.7%)
Food and water	26	1 (3.8%)
Total	370	16 (4.3%)
Pearson χ^2 value		14.961
Fishers exact test value		12.422
Asymp. Sig (two-sided) *P* value		0.005
Fishers exact test *P* value		0.007

Table 5. Distribution of *Salmonella* spp. among different zoos/enclosures

Zoo/Enclosures	No. of samples screened	No. positive (%)
Nainital zoo	118	10 (8.5%)
Kanpur zoo	167	4 (2.4%)
IVRI deer park	41	0
PGRIAS ostrich enclosure	44	2 (4.5%)
Total	370	16 (4.3%)
Pearson χ^2 value		8.273
Fishers exact test value		7.158
Asymp. Sig (two-sided) *P* value		0.041
Fishers exact test *P* value		0.036

strains yielded amplified fragments of size ranging from 150 to 2500 bp and were distributed in two clusters (A & B) with two subclusters each with a Simpson's discriminative index of 0.867 (Fig. 1). Six and ten isolates were grouped in clusters A and B, respectively. Four *S.* Kentucky isolates from two golden pheasants, feed and its caretaker of Nainital zoo grouped into the same subcluster B2 of cluster B. Similarly *S.* Typhimurium isolates from ostrich and its caretaker of Chennai grouped into same subcluster A1 of cluster A. Two untypable *Salmonella* isolates from a hyena and leopard of Kanpur zoo grouped with a *S.* Typhimurium isolate from a leopard of Nainital zoo in a same subcluster B1 of cluster B. Three *S.* Typhimurium isolates from two caretakers and a lady Amherest pheasant of Nainital zoo grouped in a same subcluster B1 of cluster B. *S.* Enteritidis from grey langur and *S.* Senftenberg from white peafowl of Nainital zoo grouped in a subcluster A2 of cluster A. Similarly *S.* Enteritidis isolated from caretaker of Kanpur zoo grouped with *S.* Lamberhurst isolated from swamp deer of Kanpur zoo in a subcluster A2 of cluster A. These results coincided with the 16S rRNA gene sequences submitted in GenBank and the accession numbers are indicated in Table 6.

Discussion

The present study documents the occurrence of *Salmonella* among captive wildlife in India for the first time and indicates the importance of captive wildlife as potential sources of human infections through occupational or other direct or indirect contact with wild animals. In this study, the prevalence varied among the host species (Tables 1–3). The number of tested animals is low for several species, because of the lesser exhibits kept in a zoo. This is the first report regarding the prevalence

Table 6. Characterisation of *Salmonella* isolated from this study

Sl. no	Isolate name	Source	Location	Serotype	Resistance profile	MARI	ERIC cluster	GenBank Accession no.
1	NLA1	Grey langur	Nainital zoo	Enteritidis	F/MSxt	0.08	A2	KT026973.1
2	NL5	Leopard	Nainital zoo	Typhimurium	Pan-susceptible	0	B1	KT026980.1
3	NWP1	White peafowl	Nainital zoo	Senftenberg	Pan-susceptible	0	A2	KT026979.1
4	NLAP12	Lady amherest pheasant	Nainital zoo	Typhimurium	F/M	0.04	B1	KT026982.1
5	NGP3	Golden pheasant	Nainital zoo	Kentucky	Pan-susceptible	0	B2	KT026975.1
6	NGP8	Golden pheasant	Nainital zoo	Kentucky	Pan-susceptible	0	B2	KT026976.1
7	NF6	Feed (leftover from cage)	Nainital zoo	Kentucky	Pan-susceptible	0	B2	KT026974.1
8	NCT11	Care taker	Nainital zoo	Kentucky	Te	0.04	B2	KT026978.1
9	NCT8	Care taker	Nainital zoo	Typhimurium	AmcAmp	0.08	B1	KT026977.1
10	NCT9	Care taker	Nainital zoo	Typhimurium	AmpSxt	0.08	B1	KT026981.1
11	KSWD12	Swamp deer	Kanpur zoo	Lamberhurst	Pan-susceptible	0	A2	KT026985.1
12	KHY1	Hyena	Kanpur zoo	Untypable	Pan-susceptible	0	B1	KT026984.1
13	KL2	Leopard	Kanpur zoo	Untypable	Pan-susceptible	0	B1	KT026986.1
14	KCT3	Caretaker	Kanpur zoo	Enteritidis	CtxCaz	0.08	A2	KT026983.1
15	PGRIASO24	Ostrich	PGRIAS, Chennai	Typhimurium	AtmCbCtxCaz	0.17	A1	KT026988.1
16	PGRIASCT1	Caretaker	PGRIAS, Chennai	Typhimurium	AtmCbCtxCazAmc	0.21	A1	KT026987.1

of *Salmonella* in healthy captive wild animals in India except for few case reports [17–19]. Previous studies from other countries like the USA have shown that free-range wildlife species such as wild pig, deer, opossum, coyote, crow, elk, etc. could be the main source of *S. enterica* contamination to water, cattle, pre-harvest lettuce and spinach [3]. To the best of our knowledge and based on available literature, isolation of *Salmonella* from swamp deer, lady Amherst pheasant and white peafowl appears to be for the first time in the world.

In previous studies *Salmonella* was isolated from grey langur [20], hyena [21], leopard [22], golden pheasants [23] and ostrich [24]. The isolation rate of *Salmonella* from the feces of all captive wildlife was 3.1% and by direct PCR assay, detection rate was found to be 5.7%. Direct PCR detection of *Salmonella* from feces has been reported in many previous studies [25–28]. Our results were in accordance with the previous studies where PCR was found more sensitive than the culture methods. Rychlik *et al.* [29] reported that the sensitivity of the culture method may be lower than that of DNA-based detection assays because of the inability of culture to detect sublethally injured or viable non-culturable cells. The predominant serovars identified in this study were Typhimurium and Kentucky. Both of these serovars were identified earlier from human infections in India [30]. Two Typhimurium strains isolated in this study were MDR with penta and tetra-type resistant patterns suggesting the significance of this serovar in animal and public health. MDR *S.* Typhimurium which is associated with invasive infections and mortality in humans has previously been reported from human, domestic and wild animals [31, 32]. In our study, majority of the isolates were pan-susceptible, which is found to be in accordance with a previous study on wildlife [6] and is probably linked to less usage of antibiotics in zoos compared with animal farms. It is clear from our study, prevalence of animal faecal carriers are low. The route of *Salmonella* transmission are multifaceted and our findings suggest that environmental contamination through indirect sources such as infected animal or human faeces and feed could play important roles. Nevertheless direct transmission of *Salmonella* from captive wildlife to visitors or caretakers is rare. Genotyping of isolates in the current study showed that most of the *Salmonella* isolates within serovar are clonally related (Fig. 1). Interestingly, clonally related isolates detected in both animals and caretakers suggest the role of wildlife in transmission, even if it is difficult to determine its direction. In addition, the presence of clonally related isolates in different species of captive wild animals from the same zoo shows that the spread could be due to fomites including caretakers, visitors, vehicles, other implements and other animal species, such as rodents and wild birds. Further we suggest intensive longitudinal studies which may also shed light on various risk factors involved. In conclusion, our study suggests that captive wildlife is asymptomatic carriers of *Salmonella.* The point to be noted is prevalence of shedding in animals was low. The occurrence of *Salmonella* and different serovars in captive wildlife species and resistance of some isolates are of public health concern. Understanding the epidemiology and transmission pattern of *Salmonella* between

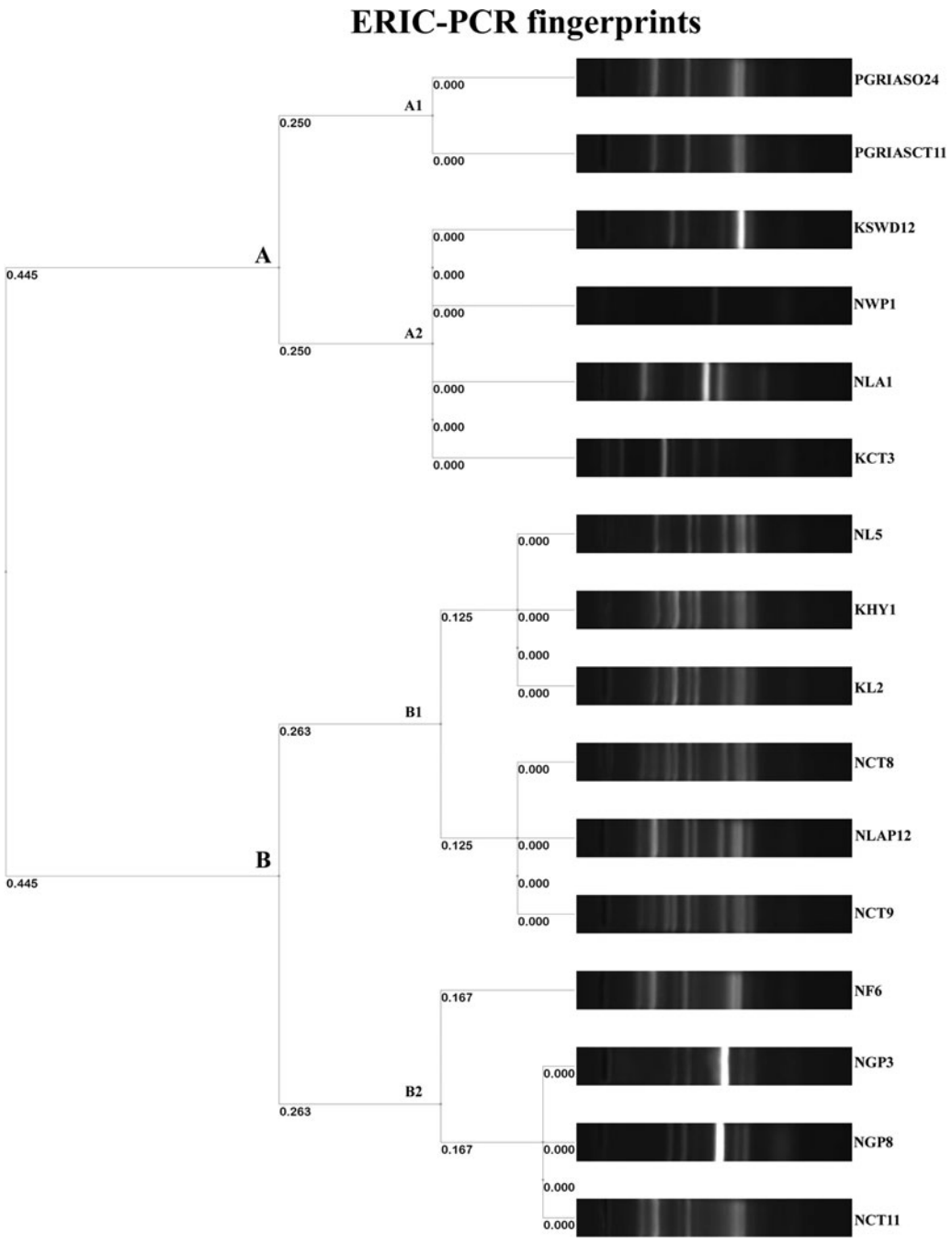

Fig. 1. Dendrogram representing genetic relationships among *Salmonella strains* based on ERIC-PCR fingerprints.

captive wildlife and caretakers could help to prevent and control the introduction and spread of infections among people.

Acknowledgements. We would like to express our gratitude to ICAR for funding the project, Outreach Programme on Zoonoses.

Conflict of interest. None.

Ethical standards. Not required.

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