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Research Article

Isolation of *Salmonella* from reptiles in pet shop and its susceptibility to antibiotics in Indonesia

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Abstract. Aim: The prevalence of *Salmonella* carriage in reptiles, from pet shops in Bogor, Indonesia, on January 2016 was studied to assess the risk of disease exposure for humans once these animals were traded as pets. Antibiotic susceptibility patterns were also analyzed to estimate the emergence of antibiotic-resistant *Salmonella* strains. Methods: Samples (n=40) were obtained by touching the skin around cloaca with sterile cotton swabs. Identification of *Salmonella* was conducted by a series of biochemical and genetic tests using polymerase chain reaction (PCR) techniques. Resistance to antibiotics was tested by the disc diffusion methods. Results: The prevalence of *Salmonella* in healthy reptiles in this study was 10%. Resistance was most commonly observed against streptomycin (75%) and norfloxacin (75%), followed by gentamicin (25%), nalidixic acid (25%) and tetracycline (25%). A total of 75% strains showed resistance to two or more antibacterial drugs. All *Salmonella* isolates proved to be susceptible to trimethoprim/sulfamethoxazole and ampicillin.

Key Words: *Salmonella*, reptiles, antibiotic resistance.

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Introduction

Salmonella is a zoonotic pathogen agent so that it can cause disease in both animals and humans (Pasquali et al 2014). The potential transmission of *Salmonella* from captive animals and wild animals cannot be ignored (Pees et al 2013; Kikillus et al 2011; Hernández et al 2012). Reptiles are one of *Salmonella* transmitting agents (Bertelloni et al 2016). Reptiles are carriers of *Salmonella* in their digestive tract and digestive organs (Harris et al 2010). These animals do not show symptoms of diseases and shed *Salmonella* through feces (Jiménez et al 2015; Goupil et al 2012). Septicemia, severe diarrhea, and fatal salmonellosis caused by contamination from reptiles have been reported in several countries (Prapasarakul et al 2012). Since the increased use of reptile as a pet, there has also been an increased in the incidence of reptile-associated salmonellosis (Kocianová et al 2010). Reptile-associated salmonellosis has been increased from 0.3% in 1988 to 9.3% in 2013, in Netherlands (Mughini-Gras et al 2016). Reptile collections with purchased animals had a significantly higher prevalence of *Salmonella* than collections from pure breeders. Furthermore, animals from pet shops were more frequently affected (89%) than wild caught animals (59%) (Scheelings et al 2011; Marin et al 2016). Reptiles pose a significant zoonotic risk to pet owners, zookeepers, and veterinarians (Lukac et al 2015; Scheelings et al 2011). *Salmonella* can be transmitted from reptiles to humans both by direct contact or indirect contact with surfaces contaminated with reptile faeces (Hale et al 2012). Even if less frequent than foodborne related salmonellosis, outbreaks of reptile-associated salmonellosis in humans have been widely reported, in particular affecting young children, the elderly, and immunocompromized people, but also healthy persons (Bosch et al 2016; Aiken et al 2010). The common symptom experienced by patients with salmonellosis is gastrointestinal disorder, but in a severe incidence, sepsis and even death can occur (Kuroki et al 2015).

The aim of this study was to analyse the prevalence of *Salmonella* and antibiotic susceptibility patterns in healthy reptiles from pet shops in Bogor, Indonesia, on January 2016.

Material and methods

Samples

Forty samples were collected from pet shops in Bogor, Indonesia, on January 2016. Samples were obtained by touching the skin around cloaca with sterile cotton swabs. All the reptiles were healthy. Table 1 show the samples code, the species of reptiles, and the number of investigated animals.

Salmonella isolation and identification

Samples were cultured and identified according to Corrente et al 2004 with minor modification. Swabs were inoculated into 10 mL of buffered peptone buffer (BPW) 0.1% followed by incubation at 37°C for 18 h. Incubated swab (0.1 mL of BPW) was added to 10 mL Rappaport-Vassiliadis broth and incubated for 24 h. Samples were plate on both *Salmonella-Shigella* agar and bismuth sulphite agar for 24-48 h. Suspected *Salmonella* spp. base on colony morphology from each selective plating medium were inoculated into triple sugar iron agar (TSIA) and lysine iron agar (LIA) and then incubated at 37°C for 24 h. Presumptive *Salmonella* colonies submitted to biochemical

Table 1. The samples code, the species of reptiles, and the number of investigated animals

Sample Code	Species
1	*Gonyosoma oxycephala* (n=1)
2, 3 ,4 ,5	*Ptyas mucosa* (n=4)
6, 7, 8	*Varanus salvator* (n=3)
9, 10, 11	*Pyton curtus* (n=3)
12, 13, 14	*Pyton reticulatus* (n=3)
15, 16, 17	*Panana gigas* (n=3)
18, 19, 20, 21, 22	*Eublepharis macularius* (n=5)
23, 24, 25, 26, 27	*Ahaetulla prasina* (n=5)
28	*Eublepharis macularius* (n=1)
29	*Pyton curtus* (n=1)
30, 31	*Boiga dendrophila* (n=2)
32, 33	*Morelia trancyae* (n=2)
34	*Acrochordus granulatus* (n=1)
35	*Boa contactor* (n=1)
36	*Varanus salvadorii* (n=1)
37	*Pyton molurus* (n=1)
38	*Amyda catilaginea* (n=1)
39, 40	*Hydrosaurus amboinensis* (n=2)

identification. *Salmonella* Typhimurium ATCC 14028 was used as positive control.

PCR for identification of *Salmonella* spp

Confirmation of *Salmonella* isolates were conducted by polymerase chain reaction (PCR) according to de Freitas et al 2010 with minor modification. Fresh overnight cultures in brain heart infusion (BHI) were used to nucleic acid extraction using PureLink® Genomic DNA Kits. PCR was conducted to detection ompC gene (204 bp) using a 20-bp forward primer (5′-ATC GCT GAC TTA TGC AAT CG-3′) and a 20-bp reverse primer (5′-CGG GTT GCG TTA TAG GTC TG-3′). Amplification reaction were carried out with 5 µL 5X PCR buffer (Kapa®), 0.5 µL dNTP (Kapa®, 10 mM), 1 µL primer (XIDT®, 10 pmol µL-1), 0.15 µL Taq Polymerase (Kapa®, 5 U µL-1), and 3.0 µL DNA template. Distilled water (DNAse, RNAse free) was added to bring the final volume to 25 µL. PCR protocol consisted of an initial denaturation step for 3 min at 95 °C, followed by 35 cycles, with 1 cycle for 15 sec at 95 °C, 15 sec at 52 °C, and 10 sec at 72 °C, and a final elongation step for 5 min at 72 °C. In each PCR run, a non-template control was included to detect possible external DNA contamination and control positive (S. Typhi ATCC 35250) were used for confirmation. Aliquot of PCR product was taken 10 µL each and electrophoresed on a 1.5% agarose gel, stained with ethidium bromide (0.5 µg mL-1) and visualized and photographed under UV illumination.

Antibiotic susceptibility

All *Salmonella* isolates were tested for susceptibility to antimicrobial based on disc diffusion methods described by Clinical and Laboratory Standards Institute (CLSI 2014). Fresh overnight cultures were prepared and used for antibiotic sensitivity tests. An aliquot (100 µL) of each isolate suspension equivalent to a 0.5% Mc Farland Standard was spread plated on Mueller Hinton Agar (MHA). There were eight types from seven groups of antibiotics used in this research. β-lactams antibiotics used was ampicillin 10 µg (AMP; OXOID® CT0003B). Aminoglycosides antibiotics used was gentamicin 10 µg (CN; OXOID® CT0024B) and streptomycin 10 µg (S; OXOID® CT0047B). Cephalosporins antibiotics used was cephalothin 30 µg (KF; OXOID® CT0010B). Quinolones antibiotics used was nalidixic acid 30 µg (NA; OXOID® CT0031B). Fluoroquinolones antibiotics used was norfloxacin 10 µg (NOR; OXOID® CT0434B). Tetracycline antibiotics used was tetracycline 30 µg (T; OXOID® CT0031B). Trimethoprim/sulfamethoxazole 25 µg (STX; OXOID® CT0052B) was used in this research. Blank disc (OXOID® CT0998B) was used in the testing as a negative control. Plate was then incubated for 24 hours at 37°C and antibiotics inhibition zone then measured. Justification of susceptible (S), Intermediate (I) and resistant (R) reaction was done based on the size of antibiotics inhibition zone measured (CLSI 2014).

Results

Isolation and identification of *Salmonella* spp. in 40 swab samples of reptile skin using the PCR method (Figure 1) found four isolates confirmed as *Salmonella* spp. The number and origin of the isolates positive as *Salmonella* spp. were the isolate number 4 from *Ptyas mucosa*, number 25 from *Ahaetulla prasina*, number 35 from *Boa contactor*, and number 40 from *Hydrosaurus amboinensis*. The four isolates were then tested for resistance to antibiotics. Table 2 presents the results of resistance testing of *Salmonella* spp. isolates to some antibiotics.

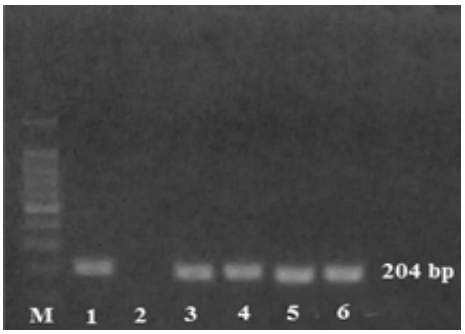

Fig. 1. Agarose gel electrophoresis of PCR product of *Salmonella* spp. isolates (M: marker, 1: positive control, 2: negative control, 3: sample no. 4, 4: sample no. 25, 5: sample no. 35, 6: sample no. 40).

Table 2. Susceptibility (percent of resistant, intermediate, and susceptible) of *Salmonella* spp. isolates to a panel of antimicrobial drugs

Antimicrobial agent	Susceptible (%)	Intermediate (%)	Resistant (%)
Ampicillin	100	0	0
Gentamicin	75	0	25
Streptomycin	25	0	75
Cephalothin	75	25	0
Nalidixic acid	75	0	25
Norfloxacin	0	25	75
Tetracycline	75	0	25
Trimethoprim/ Sulfamethoxazole	100	0	0

Resistance was most commonly observed against streptomycin (75%) and norfloxacin (75%), followed by gentamicin (25%), nalidixic acid (25%) and tetracycline (25%). A total 75% showed resistance to two or more antibacterial drugs. One isolate was resistant to two antibiotics, namely streptomycin and norfloxacin. Two isolates were resistant to two combination of three antibiotics, namely streptomycin-norfloxacin-tetracycline and gentamicin-streptomycin-nalidixic acid. One isolate was intermediate to two antibiotics, namely cephalothin and norfloxacin. All *Salmonella* isolates were susceptible to a large proportion of the antimicrobial drugs tested. All *Salmonella* isolates proved to be susceptible to ampicilin and trimethoprim/sulfamethoxazole. *Salmonella* isolates showed a high sensitivity to gentamicin (75%), Cephalotin (75%), nalidixic acid (75%), and tertrasycline (75%).

Discussion

Salmonella is a bacterium belonging to the family of *Enteribactericeae* that inhabit the gastrointestinal tract of animals (Drumo et al 2016) and humans (Ahmer and Gunn 2011; Hallstrom and McCormick 2011). This bacterium is a zoonotic pathogen (Jang et al 2008) that can cause disease in both animals and humans (Hoelzer et al 2011). This disease can be transmitted from animals to humans or vice versa (Hoelzer et al 2011; Hendriksen et al 2004). The sample in this study is a reptile skin swab. Reptiles used for the sampling purposes are healthy reptile pets. Sampling was done in pet shops as these have a relatively high population of reptiles as pets.

The results obtained during the present study confirm that captive reptiles are important reservoirs of *Salmonella* and represent a source of infection for humans. The results of this study showed that the prevalence of *Salmonella* spp. in reptiles in pet shops in Bogor, Indonesia is 10%. Most *Salmonella* was identified in snakes. Previously researches report several levels of infection *Salmonella* in reptiles. Scheelings et al 2011 found that *Salmonella* was identified in 28% of reptiles in Australia; the study was conducted on wild reptiles and captive reptiles. In a recent study of Bertelloni et al 2016 was determined the prevalence of *Salmonella enterica* infection in captive reptiles in pet shop. The *Salmonella enterica* prevalence rates were 13.61%. In this study, Chelonians were infected most frequently than the other cold-blooded animals.

The presence of *Salmonella* in reptiles as pet can act as a source of infection for their owners. Interaction and intense contact between reptiles and their owners can lead to infection and a severe disease, especially in children. In addition, infected reptiles can spread *Salmonella* to other animals such as dogs or cats, thus further increasing the risk of infection in humans. Reptiles have the role as a reservoir of *Salmonella* that can not only cause intestinal tract infections, but also can produce pathogenic strains resulting in extra-intestinal diseases. The most optimal prevention of *Salmonella* infections originating from reptiles mainly used as pets is to improve personal hygiene and sanitation of the reptile cages.

Tests of antibiotic resistance showed that the highest resistance occur against the antibiotic group of aminoglycoside (namely streptomycin) and fluoroquinolone (namely norfloxacin). Resistance to streptomycin has been largely documented. Aminoglycoside resistance occurs through three main mechanisms namely modification of the ribosomal binding sites by mutation of the 16S rRNA or ribosomal proteins (Galiman et al 2005), reduced uptake and increased efflux (Koskiniemi et al 2011), and deactivation of aminoglycosides by aminoglycoside-modifying enzymes (Gad et al 2011). Its extensive use, in particular in veterinary medicine, has contributed to the successful spread of resistance genes (Pezzella et al 2004). Fluoroquinolone antibiotic is an antibiotic that works by inhibiting DNA gyrase of bacterium, thereby inhibiting the DNA synthesis of the bacterium. Norfloxacin are active against Gram-negative enteric bacteria (Ferric FC 2015). Resistance to this group of antibiotics will result in the reduced choice of antibiotics for the treatment of *Salmonella* infections, especially in severe cases.

Intermediate resistance was showed against cephalothin (25%) and norfloxacin (25%). Intermediate resistance indicates that the antibiotics used are not optimal in clinical use. Thus requiring the antibiotics with higher doses to obtain optimal results. In addition, this intermediate condition is an indication of early development in the resistance ability.

All isolates identified in this study are already resistant to one or more antibiotics. Only one isolate is resistant to one antibiotic, while the other isolates show resistance to more than two types of antibiotics. Resistance to various antibiotics is a great concern in treatment. The choice of antibiotics in case of bacterial infection will decrease. This condition can be made worse if the infection becomes more widespread and systemic, resulting in more severe diseases.

Salmonella isolates show a high sensitivity to trimethoprim/sulfamethoxazole (100%), ampicillin (100%), gentamicin (75%), cephalothin (75%), nalidixic acid (75%), and tetracycline (75%). This indicates that the use of these antibiotics is effective to treat *Salmonella* infections. The use of these antibiotics, which are still susceptible, should be controlled so as not to induce resistance.

Conclusion

The *Salmonella* prevalence in healthy reptile in a pet shop in Bogor, Indonesia is 10%. Resistance was most commonly observed against streptomycin (75%) and norfloxacin (75%), followed by gentamicin (25%), nalidixic acid (25%) and tetracycline (25%). A total of 75% strains showed resistance to two or more antibacterial drugs. All *Salmonella* isolates proved to be susceptible to trimethoprim/sulfamethoxazole and ampicilin.

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