Polymerase Chain Reaction Assay and Conventional Isolation of *Salmonella* spp. from Philippine Bats

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ABSTRACT

**Background:** Salmonellae are important food and waterborne pathogens and the leading causes of the most widespread acute gastrointestinal illnesses around the globe. The organism has been detected in a wide range of host species such as mites, insects, crustaceans, mussels, fish, amphibians, reptiles, birds and mammals including wildlife animals. Salmonellae have been isolated in many species of bats in other countries. In the Philippines, there are 70 species of Philippine bats reported of which nine are considered as endemic. Although human salmonellosis (typhoid, paratyphoid and other *Salmonella*-associated infections) was the primary cause of illnesses and death from the 60 reported foodborne outbreaks (1995 to 2004), no case was ever reported involving Philippine bats. Since transmission of *Salmonella* from wildlife to humans is possible, as advocated by previous reports, the present study endeavored to isolate and molecularly detect *Salmonella* spp. from Philippine bats captured from Aklan, Laguna and Quezon City using conventional isolation method and polymerase chain reaction assay respectively.

**Materials, Methods & Results:** A total of 96 apparently healthy bats were used in the study. Bats were captured using nylon mobile mist nets of 3 m long and 1.5 m high with 35 mm mesh size. Eleven species of bats were collected and identified following the reported key to the identification of Philippine bats. Majority of the collected species were insectivores under family Vespertilionidae while the largest population of the Philippine bats were frugivores belonging to family Pteropodidae. Necropsy was performed and intestines were collected and subjected to conventional culture method and PCR detection for *Salmonella* spp. Two samples (2.08%) were molecularly detected as positive for *Salmonella* spp. bacterial pathogen. The positive samples were obtained from the intestines of the adult female insect-eating bat species, *Miniopterus australis* and *M. schreibersi*, originating from Pangihan cave of Barangay Pablacion, Malay in Aklan. No *Salmonella* spp. was isolated using the conventional method.

**Discussion:** The study reports the first detection and molecular evidence of *Salmonella* spp. in Philippine bats by PCR using intestinal samples. In addition, the data strongly indicated that PCR detection appears to be more sensitive over the conventional isolation method. The successful detection was attributed to the ability of PCR to sensitively detect atypical *Salmonella* and non-viable *Salmonella* cells. Results in the present study revealed that the Philippine bats, *Miniopterus australis* and *M. schreibersi*, both adult female insect-eating bats captured in Pangihan cave of Barangay Pablacion, Malay, Aklan harbored *Salmonella* in their intestines. Since salmonellae have been detected in a large variety of environment and host species including insects, these bats may have acquired these microorganisms in water and in their diet. This finding shows that Philippine bats may serve as potential reservoir and carrier of *Salmonella* organisms. The data also strongly indicates that bats may actively contribute in the dissemination of salmonellae into the environment through fecal route. This currently makes Philippine bats as a potential threat to livestock and may pose a serious public health concern, since all serotypes of *Salmonella* are considered to be pathogenic to humans.

**Keywords:** Bats, *Miniopterus australis*, *Miniopterus schreibersi*, *Salmonella*, PCR.
INTRODUCTION

Salmonellae are important food and waterborne pathogens of the most widespread acute gastrointestinal illnesses worldwide [1,4,28]. The organism is present in the gastrointestinal tract of warm-blooded and cold-blooded animals and hence, excretion in feces results in contamination of water, food and environment [11]. The organism has been detected in a variety of host species such as mites, insects, crustaceans, mussels, fish, amphibians, reptiles, birds and mammals including wildlife animals [2,10,15,17,21]. Many studies have reported bats as natural hosts of many emerging and re-emerging infectious diseases [19].

Salmonellae have been isolated in many species of bats in other countries [2,15]. In the Philippines, there are 70 species of Philippine bats reported of which nine are endemic as listed in the 2000 IUCN Red List of Threatened Species [16]. Since Salmonella is zoonotic in nature and previous study has reported that wildlife may serve as a reservoir for Salmonella infections [26], the present study endeavored to isolate and molecularly detect Salmonella spp. from Philippine bats captured from Aklan, Laguna and Quezon City using conventional isolation and polymerase chain reaction (PCR) assay respectively.

Two PCR positive samples (2.08%) were obtained from the intestines of the adult female insectivorous bat species, Miniopterus australis and M. schreibersi, collected from Pangihan cave of Barangay Pablacion, Malay in Aklan. No Salmonella spp. was isolated using the conventional method. This finding indicates that Philippine bats are potential carrier of Salmonella spp. and may play a significant role in the dissemination of these pathogenic organisms in the environment. Furthermore, the study represents the first detection of Salmonella spp. in Philippine bats.

MATERIALS AND METHODS

Collection of bats

A total of 96 apparently healthy bats were used in the study. Forty (40) bats were collected at the Pangihan caves in Barangay Pablacion, Malay and Libertad caves in Barangay Libertad, Nabas in Aklan using nylon mobile mist nets of 3 m long and 1.5 m high with 35 mm mesh size. The mist nets were set up on the entrance and inside the caves. Nylon mist nets of 12 m long and 2 m high with 35 mm mesh size were used to capture twenty four (24) and thirty two (32) bats from the University of the Philippines Los Baños (UPLB) Hortorium in Laguna, and UP Diliman Marine Science Institute (MSI) and Protected Areas and Wildlife Bureau (PAWB) in Quezon City respectively. Seven net nights for one night placed along trails on forest gaps and across the river were set up in Laguna while 14 net nights, seven mist nets for two nights placed near swampy areas in Quezon City.

Species identification of bats

Eleven species of bats were collected and identified following the reported key to identification of Philippine bats [9]. Five species of insect-eating bats and one species of fruit-eating bat were captured from Aklan namely, Miniopterus australis, M. schreibersi, M. tristis, Hipposideros diadema, Myotis macrotarsus and Ptenochirus jagori respectively. Three species of fruit-eating bats, Ptenochirus jagori, Cynopterus brachyotis and Eonycteris spelaea, were collected from Laguna. Two species of insect-eating bats, Scotophilus kuhlii and Pipistrellus kuhlii and four species of fruit-eating bats, Ptenochirus jagori, Cynopterus brachyotis, Rousettus aplexicaudatus and Eonycteris spelaea were captured in Quezon City.

Necropsy of bats and sample collection

After the collection, the body weight of each bat was determined and the dosage for anesthetic was computed using a dose of 0.45 mL of 5% zoalazepam-tiletamine1 per 30 g body weight. The anesthetic was given intramuscularly and the bat was euthanized through intracardiac exsanguination. The body parameter measurements of each carcass were recorded to use for identification purposes.

Each bat was then placed on a necropsy board where the skin over the thorax and abdomen was reflected. The thorax was opened and the internal organs were collected by research collaborators from Japan for other investigative works. In the present study, the peritoneum was incised and the intestine was detached from its mesentery. The entire intestinal tract was cut through the rectum, ligated on both ends and placed on a sterile Petri dish with normal saline solution. The carcass was submitted to the UPLB Museum of Natural History for preservation and storage.

Conventional isolation method

The small intestines were minced and transferred to a pre-labeled tube of nutrient broth2. The samples were
incubated at 37°C for 24 h. After incubation, aliquots of the samples were transferred to tetrathionate brilliant green (TBG) broth and incubated under 42°C for 24 h.

All of the enriched samples were streaked on xylose-lysine deoxycholate (XLD) agar plates and incubated at 37°C for 24 h. Expected *Salmonella* colonies appear as pink colonies with black center on XLD media. The suspected colonies were purified, subjected to biochemical tests (triple sugar iron, indole, Methyl-Red, Voges-Proskauer, citrate, urease and lysine decarboxylase tests) and confirmed using miniaturized identification kit.

**DNA extraction**

The DNA extraction was done based on the National Institute of Molecular Biology and Biotechnology (BIOTECH) protocol. Briefly, each 1.5 mL TBG broth was placed in a 1.5 mL microcentrifuge tube and centrifuged at 8,050 x g for 10 s to remove the minced tissues. The supernatant was transferred to fresh microcentrifuge tube and the debris was discarded. The tube was then centrifuged at 8,050 x g for 5 min to collect the cell pellets. The supernatant was removed and the cell pellets were washed with 150 µL HPLC-grade water. The mixture was mixed using vortex mixer and centrifuged for another 5 min. The supernatant was again discarded and the tube was suspended in 45 µL HPLC-grade water. The mixture was boiled for 5 min. After boiling, 0.95 mL of HPLC-grade water was added in the mixture and mixed. The PCR products along side with 1kb DNA ladder and negative and positive control were resolved using 1% Tris-acetate-EDTA (TAE) agarose gel in an electrophoresis chamber containing 0.5x TAE buffer. The gel was run at 100 V for 30 min until the dye indicator reached the target lane. At the end of each run, the gel was soaked in ethidium bromide solution for 15 min, washed and viewed under an ultraviolet transilluminator machine. The machine was connected to a computer with a software program for the documentation of each run.

**DISCUSSION**

A total of 11 species of bats were collected, namely *Miniopterus australis*, *M. schreibersi*, *M. tristis*, *Hipposiderus diadema*, *Myotis macrotarsus*, *Ptenochirus jagori*, *Scotophilus kuhlii*, *Pipistrellus javanicus*, *Cynopterus brachyotis*, *Eonycteris spelaea*, and *Rousettus amplexicaudatus*. These different species are grouped into three families and based on the number of species obtained, the least falls under Rhinolophidae (1/11), followed by Pteropodidae (4/11), while majority were classified under Vespertilionidae (6/11).

Two (2.08%) of the 96 samples subjected to PCR produced the expected 450-bp band for *Salmonella* positive samples as exhibited in Figure 1. The positive samples came from *Miniopterus australis* and *M. schreibersi*. These chiropterans were both insect-eating adult female bats captured from Pangihan cave of Barangay Pablacion, Malay in Aklan. However, all of the minced small intestine samples of the bats were found negative for *Salmonella* spp. using the conventional isolation method. Suspected colonies from xylose-lysine deoxycholate agar plates were confirmed by biochemical tests (triple sugar iron, indole, methyl-red, Voges-Proskauer, citrate, urease and lysine decarboxylase tests) and further verified by a miniaturized identification kit.

Two (2.08%) of the 96 apparently healthy bats were subjected to conventional culture method and PCR detection for *Salmonella* spp. The summary of the species of bats included in the study is shown in Table 1.
Table 1. Summary of the species of bats tested for Salmonella spp. using conventional isolation and PCR methods.

<table>
<thead>
<tr>
<th>Bat Species (Common name)</th>
<th>Family</th>
<th>Place of Collection</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Miniopterus australis</em> (Little bent-winged bat)</td>
<td>Vespertilionidae</td>
<td>A</td>
</tr>
<tr>
<td><em>Miniopterus schreibersi</em> (Common bent-winged bat)</td>
<td>Vespertilionidae</td>
<td>21</td>
</tr>
<tr>
<td><em>Miniopterus tristis</em> (Greater bent-winged bat)</td>
<td>Vespertilionidae</td>
<td>15</td>
</tr>
<tr>
<td><em>Scotophilus kuhlii</em> (Lesser Asian house bat)</td>
<td>Vespertilionidae</td>
<td>1</td>
</tr>
<tr>
<td><em>Pipistrellus javanicus</em> (Javan pipistrelle)</td>
<td>Vespertilionidae</td>
<td>0</td>
</tr>
<tr>
<td><em>Myotis macrotarsus</em> (Philippine large-footed myotis)</td>
<td>Vespertilionidae</td>
<td>0</td>
</tr>
<tr>
<td><em>Ptenochirus jagori</em> (Musk fruit bat)</td>
<td>Pteropodidae</td>
<td>1</td>
</tr>
<tr>
<td><em>Cynopterus brachyotis</em> (Common short-nosed fruit bat)</td>
<td>Pteropodidae</td>
<td>0</td>
</tr>
<tr>
<td><em>Eonycteris spelaea</em> (Common nectar/dawn bat)</td>
<td>Pteropodidae</td>
<td>0</td>
</tr>
<tr>
<td><em>Rousettus amplexicaudatus</em> (Common rousette)</td>
<td>Pteropodidae</td>
<td>0</td>
</tr>
<tr>
<td><em>Hipposideros diadema</em> (Diadem roundleaf bat)</td>
<td>Rhinolophidae</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td>40</td>
</tr>
</tbody>
</table>

*A*- Aklan  
*L*- Laguna  
*QC*- Quezon City

Figure 1. Polymerase Chain Reaction Amplification of the Salmonella genus-specific 450-bp PCR products from bat samples. (M) Molecular size marker (1 kbp DNA ladder); (1) Negative control (HPLC-grade water); (2) Positive control (Salmonella Thyphimurium); 3-4 (Sample 1) & 5-6 (Sample 2) Two Salmonella positive samples showing the expected 450-bp amplicon size.
tissue samples from mesenteric lymph nodes of the kit has been used on swab samples from ileum and feeds and ingredients, and animal organs. Furthermore, government and private testing laboratories on animal previously performed [24] in collaboration with several animal feeds. Extensive validation trials of the kit were samples, artificially spiked and naturally contaminated enough to detect the presence of based detection kit was also establish to be sensitive [5,14,18,25]. Likewise, the BIOTECH more reliable than the traditional culture methods [22,23].

Local studies revealed that the combination of TBG enrichment with bacterial lysis method and capillary gel electrophoresis is suitable for a rapid Salmonella detection in chicken feces [3]. However, false-negative results can still occur and the bile salt component of TBG broth used as enrichment medium for Salmonella spp. may inhibit PCR amplification of DNA [27]. Additionally, other substances that are inhibitory to PCR include large amounts of polysaccharides, phenolic and metabolic compounds in feces [13].

The results of the study strongly indicated that PCR detection appears to be more sensitive over the conventional isolation method. No Salmonella spp. isolates were obtained from the small intestines of 96 apparently healthy bats using the conventional method of isolation. The inability to obtain a Salmonella spp. culture can be attributed to several factors. It is foremost believed that the short intestinal length and rapid transit time in bats [12] could have prevented the stasis necessary for adherence, colonization and multiplication of bacteria in the distal small intestine. Another consideration is the fact that the pre-enrichment stage which provides nutrition, promotes revival of damaged or stressed cells and multiplication of Salmonella, is non-selective, hence it favors the overgrowth of other organisms which may have overwhelmed Salmonella species due to its poor competitive nature. Therefore, it is most likely that highly competing non-Salmonella organisms may hamper the identification of Salmonella on agar plates. Lastly, the atypical appearance of Salmonella in the selective media plates may have been overlooked since only those pink colonies with black centers were considered positive on XLD media.

In general, results have shown that there is a significant degree of agreement between the conventional and PCR method, especially for the samples from Laguna and Quezon City. The samples subjected to both methods were found negative for Salmonella spp.

**CONCLUSION**

The study reports the first detection of Salmonella spp. in Philippine bats using intestinal samples. Results in the present study revealed that the Philippine bats, *Miniopterus australis* and *M. schreibersi*, both apparently healthy dogs. Atypical *Salmonella* that failed to be detected using the plate culture assay can be positively identified using the kit. Under optimized conditions, the level of PCR assay sensitivity is $10^3$ cells [22,23].

11) (Table 1). However, based on the number of collected samples, majority were frugivores (Pteropodidae). This can be due to small body size and ability to produce echolocation signals of insectivores which enable them to escape and avoid being trapped in the mist nets [7]. Vespertilionidae is the largest family within the order Chiroptera and is worldwide in distribution. Most species are known to be insectivores and roost in caves. Likewise in the Philippines, previous data have shown that the number of insectivores was almost twice as that of frugivores [7,16].

The present study was able to molecularly detect *Salmonella* species from the small intestines of two (2/96 bats) vespertilionids adult female bats (*Miniopterus australis* and *M. schreibersi*) from Panay Island (Pangihan cave, Aklan). The successful detection was attributed to the ability of PCR to detect atypical *Salmonella* spp. and non-viable *Salmonella* cells which makes it more sensitive and specific in determining the presence of the target *Salmonella* DNA. This result is in agreement with previous studies.

It was previously shown that the detection of *Salmonella* sp. in 391 fecal samples from cattle, pig and poultry in Sweden using commercial PCR-based method (BAX® system) was proven to be satisfactory [6]. In addition, a study using PCR for *Salmonella hilA* gene also successfully amplified the expected 784-bp DNA fragment in all the 33 *Salmonella* strains from 27 serotypes while none from all the non-*Salmonella* strains tested. Furthermore, it was able to detect *S. choleraesuis* subsp. *choleraesuis* serovar Typhimurium in artificially contaminated fecal samples at a concentration of $3 \times 10^2$ cfu/mL [20]. Similarly, the use of PCR for specific detection of *Salmonella* spp. in food has been documented. It was reported that PCR targeting specific gene (e.g. *fimA*, *invA*, *ssaR*) displayed high degree of diagnostic accuracy, without unspecific amplification or false signals and was found to be faster, less costly and more reliable than the traditional culture methods [5,14,18,25]. Likewise, the BIOTECH *Salmonella* PCR-based detection kit was also establish to be sensitive enough to detect the presence of *Salmonella* in fecal samples, artificially spiked and naturally contaminated animal feeds. Extensive validation trials of the kit were previously performed [24] in collaboration with several government and private testing laboratories on animal feeds and ingredients, and animal organs. Furthermore, the kit has been used on swab samples from ileum and tissue samples from mesenteric lymph nodes of...
adult female insectivorous bats captured in Pangihan cave of Barangay Pablacion, Malay, Aklan harbored *Salmonella* in their intestines. Since salmonellae have been detected in a large variety of environment and host species including insects, these bats may have acquired these microorganisms in water and in their diet.

This finding showed that Philippine bats may serve as potential reservoir and carrier of *Salmonella* organisms. The data also strongly indicates that bats may actively contribute in the dissemination of salmonellae into the environment through fecal route. This currently makes Philippine bats as a significant threat to livestock and a serious public health concern, since all serotypes of *Salmonella* are considered to be pathogenic to man [1,8].

Lastly, the results also indicate that majority of the collected species were insectivores under family Vespertilionidae while the largest population of the Philippine bats were frugivores belonging to family Pteropodidae.

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**SOURCES AND MANUFACTURERS**

1. Virbac Philippines, Inc., Pasig, Philippines
2. BBL, Maryland, USA
3. Biotest, Dreieich, Germany
4. Difco, Maryland, USA
5. HiMedia, Mumbai, India
6. Conda, Madrid, India
7. Hispanlab, S.A., Madrid, Spain
8. Difco, Michigan, USA
9. BBL Crystal™ Identification System Enteric/Nonfermenter ID kit, Difco, Maryland, USA
10. Sigma, St. Louis, USA
11. *Salmonella* DNA Amplification System™, BIOTECH, Philippines
12. AB Applied Biosystems™, California, USA
13. Promega Corporation, Wisconsin, USA
14. UVP LLC, California, USA
15. Labworks™ Analysis Software, California, USA

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