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Conventional isolation and polymerase chain reaction for detection of *Escherichia coli* O157:H7 from intestines of Philippine bats

Jomalyn T. Italia[1], Hope G. Rovira[1]*, Joseph S. Masangkay[1], Yasuhiro Yoshikawa[2], Maria Theresa M. Perez[3], Alisha Wehdnesday B. Reyes[1], and Waren N. Baticados[1]

[1]*Department of Veterinary Paraclinical Sciences, College of Veterinary Medicine, University of the Philippines Los Baños, College Laguna, Philippines*

[2]*University of Tokyo, Graduate School of Agricultural and Life Sciences, Japan*

[3]*National Institute of Molecular Biology and Biotechnology, University of the Philippines Los Baños, College Laguna, Philippines*

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ABSTRACT

It is currently reported that bats in the Philippines harbor bacterial organism (*Salmonella* spp.) with pathogenic potential. The paper describes the conventional isolation and polymerase chain reaction (PCR) assay for the detection of another bacterium, *Escherichia coli,* from a sample population of 56 apparently healthy bats collected from Laguna and Quezon City, Philippines. Nineteen of the samples were positive for *E. coli* using the conventional method of isolation, while PCR molecularly detected the bacteria in 15 samples. The presence of hemolysin among the isolates was not observed. The isolates were subjected to *E. coli* O157:H7 serotype detection using the latex agglutination test and another PCR assay specific for this serotype. The data revealed that none of the isolates was positive for *E. coli* O157:H7 using serological and molecular diagnostic methods, which indicates that bats from Laguna and Quezon City, Philippines were not carriers of the pathogenic strain, *E. coli* O157:H7. The study also presents the first local report of conventional isolation and molecular detection of *E. coli* from Philippine bats.

Key words: *Escherichia coli*, latex agglutination test, Philippine bats, polymerase chain reaction

*Corresponding author:

Dr. Hope G. Rovira, Asst. Prof., Department of Veterinary Paraclinical Sciences, College of Veterinary Medicine, University of the Philippines Los Baños, College Laguna, Philippines, Phone/Fax: +63 49 536 2730; E-mail: hgrovira@gmail.com

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Introduction

Escherichia coli is considered a commensal of the large intestines of warm-blooded animals, including man. Some strains of *E. coli* are associated with diseases and can be subdivided into several pathological groups. One of these pathogroups is the diarrheagenic *E. coli* (DEC) which includes strains that cause gastroenteritis in humans and animals. This pathogroup is further categorized into specific groups based on virulence properties, mechanisms of pathogenicity, clinical syndromes and distinct O:H antigenic serotypes (MONTVILLE and MATTHEWS, 2008). The most important category of DEC is *E. coli* O157:H7 or the enterohemorrhagic *E. coli* (EHEC), which is also the most severe (ACHA and SZYFRES, 2001).

Collibacillosis caused by EHEC has been a public health problem in Europe and USA since it was first recognized in 1982 (RANGEL et al., 2005). The etiologic agent can survive at low temperatures and acidic conditions. In addition, a small dose of 10 to 100 cells is sufficient to initiate serious complications, such as the life-threatening hemolytic uremic syndrome.

As a pathogen, EHEC is transmitted to humans via contaminated food. Farm animals are known to serve as important reservoirs for some DEC main pathotypes, hence the spread of these pathogens is possible, which provides an entry into the food chain and subsequently, transmission to humans (BOLTON et al., 2009).

The prevalence of *E. coli* in bats has been reported in several countries. However, the presence of EHEC in these animals has not been proven. Since bats have been associated with many zoonotic diseases (OMATSU et al., 2007) and recently *Salmonella* spp. have been detected on these animals in the Philippines (REYES et al., 2011), the present study was conducted to isolate and detect *E. coli* from the intestines of Philippine bats and to confirm if the isolates were of the *E. coli* O157:H7 serotype. The study aims to determine if bats can serve as carriers of this pathogenic serotype and, therefore, to identify their role in disease transmission.

Materials and methods

Animals. A total of 56 apparently healthy bats were captured using nylon mist nets, 12 m long and 2 m high, with 35 mm mesh size in Laguna (University of the Philippines Los Baños Hortorium) and in Quezon City (Protected Areas and Wildlife Bureau, and U.P. Diliman Marine Science Institute). Twenty-four bats were captured from Laguna using seven net nights placed along trails in forest gaps and across the river for one night, while 32 bats were captured from Quezon City using seven net nights placed near a river and water lodge for two nights.

After the collection, the body mass of each bat was taken and the anesthetic (5% zolazepam-tiletamine) was given intramuscularly with a dose of 0.45 mL per 30 g

body mass. The bats were euthanized through intracardiac exsanguination and the body parameters were recorded. The carcasses were identified by a biologist following the reported key to identification of Philippine bats (INGLE and HEANEY, 1992).

Conventional isolation method. Each bat was placed in a necropsy board and the skin near the thorax and abdomen was reflected. The thorax and abdomen were opened and other internal organs were collected by research collaborators for other investigative work. In the present study, the entire intestinal tract was detached from its mesentery and ligated on both ends. The ligated intestinal tract was cut and placed in a sterile Petri dish with normal saline solution. The carcasses were submitted to the UPLB Museum of Natural History for preservation.

The intestinal tract was minced and an adequate amount was transferred into a pre-labeled test tube of buffered peptone water (BPW) and incubated at 37 °C for 24 h. After incubation, a loopful of the sample was streaked in MacConkey agar (MCA) plate and incubated at 37 °C for 24 h. The BPW tubes were stored at -40 °C prior to PCR.

Pink round isolated colonies in MCA were streaked in eosin methylene blue (EMB) agar incubated at 37 °C for 24 h. The characteristic green metallic sheen growth of colonies is a presumptive identification for *E. coli*. Purification of the isolates was performed and after Gram staining, biochemical tests were done for confirmation. The biochemical tests include the following: IMViC (Indole, Methyl Red, Voges-Proskauer and Simmons Citrate) and sugar (xylose, inositol, maltose, trehalose, lactose, arabinose, glucose, sorbitol and mannitol) tests (QUINN et al., 1994; TIMONEY, 1988).

DNA extraction from bacterial culture. Cultures that exhibited typical *E. coli* reactions were streaked in nutrient slants and incubated at 37 °C for 24 h. The growth of each isolate was scraped and suspended in a tube with 100 µL HPLC-grade water and mixed. Subsequently, the tubes were placed in a boiling water bath for ten minutes and mixed. The samples were diluted ten-fold in HPLC-grade water.

Detection of E. coli O157:H7 serotype. Twenty-four hour cultures of *E. coli* confirmed by PCR were streaked in sorbitol MacConkey (SMAC) agar and fluorocult agar (FA) incubated at 37 °C for 24 hr. Colorless to yellowish colonies in SMAC agar and colonies with the absence of fluorescence under UV light (365 nm) in FA were presumptive identification for *E. coli* O157:H7. The confirmation was done using latex slide agglutination test (*E. coli* O157:H7 Latex Test Kit, Oxoid®, Missouri, USA) and PCR in accordance with the manufacturers' instructions.

The *E. coli* isolates were also observed for the presence of hemolysin via inoculation in 5% blood agar plates (BAP) incubated at 37 °C for 24 hr.

DNA extraction from tissue samples. Each 0.5 mL of BPW inoculated with intestinal sample was transferred into a sterile 1.5 mL microcentrifuge tube and centrifuged for 10

min at 8,050 x g. The supernatant was discarded and 0.5 mL sterile HPLC-grade water was added and mixed to suspend the cells. After boiling in a water bath for 10 min and mixed for 30 s, a final dilution (1:10) was done using HPLC-grade water.

PCR and agarose gel electrophoresis analysis. The protocol for PCR assay was performed as described by the manufacturer's instructions (*E. coli* DAS ™ Biotech, Philippines). Five microliters of the diluted DNA samples from tissues and bacterial cultures were added separately in each reaction tube with PCR kit mixtures. The samples together with positive and negative controls were loaded in the thermal cycler (AB Applied Biosystems™, California, USA) subjected to the following conditions: initial denaturation at 95 °C for 5 min, denaturation at 94 °C for 2 min, annealing at 50 °C for 2 min, extension at 72 °C for 2 min and final extension at 72 °C for 10 min. These were repeated for 30 cycles.

The PCR products alongside with a 1 kb DNA ladder (Promega Corporation, Wisconsin, USA), together with negative and positive controls, were resolved using 1% Tris-acetate-EDTA (TAE) agarose gel in an electrophoresis chamber (Mupid®, Japan) 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 30 min, washed and viewed under an ultraviolet transilluminator machine (UVP LLC, California, USA) connected to a computer. Visualization and documentation of results were performed using a computer software program (Labworks™ Analysis Software, California, USA).

Statistical analysis of difference and correlation between the conventional isolation and PCR methods. The difference and the correlation between the conventional isolation method and PCR assay were analyzed statistically using the Student's T test and Pearson Correlation test respectively, at alpha 5% level of significance (Statistical Analysis Software, SAS version 9.1).

Results

The 56 bats used in this study were comprised of six species: two of which were insectivores (family Vespertilionidae), *Pipistrellus javanicus* and *Scotophilus kuhlii* while four were frugivores (family Pteropodidae), *Cynopterus brachyotis*, *Ptenochirus jagori*, *Eonycteris spelaea*, and *Rousettus amplexicaudatus*.

Three species of frugivores (*Cynopterus brachyotis*, *Ptenochirus jagori* and *Eonycteris spelaea*) were captured in Laguna while four species of frugivores (*Cynopterus brachyotis*, *Ptenochirus jagori*, *Eonycteris spelaea* and *Rousettus amplexicaudatus*) and two species of insectivores (*Pipistrellus javanicus* and *Scotophilus kuhlii*) were collected in Quezon City. The majority of the species captured in Laguna and Quezon City were *Ptenochirus jagori* (17/24) and *Cynopterus brachyotis* (20/32) respectively. In

combination, the majority of the collected bats were of the *Cynopterus brachyotis* species (24/56) as shown in Table 1.

Table 1. Total population and number of bats according to species tested positive for *E. coli* using Conventional isolation and PCR methods

Bat Species	Family	Collection site						Total
		Laguna			Quezon City			
		CIM	PCR	N	CIM	PCR	N	
Ptenochirus jagori (Musky fruit bat)	P	6	4	17	1	0	1	18
Cynopterus brachyotis (Common short-nosed fruit bat)	P	2	3	4	5	5	20	24
Eonycteris spelaea (Common nectar/dawn bat)	P	3	2	3	0	0	1	4
Rousettus amplexicaudatus (Common rousette)	P	0	0	0	1	0	2	2
Scotophilus kuhlii (Lesser Asian house bat)	V	0	0	0	1	1	5	5
Pipistrellus javanicus (Javan pipistrelle)	V	0	0	0	0	0	3	3
Total		11	9	24	8	6	32	56

P - Pteropodidae; V - Vespertilionidae; N - Sample population. CIM - Conventional isolation method; PCR - Polymerase chain reaction

Nineteen (33.93%) intestinal samples were positive for *E. coli* based on the conventional isolation method as shown in Table 1. The positive samples came from seven *Cynopterus brachyotis* (36.84%), seven *Ptenochirus jagori* (36.84%), three *Eonycteris spelaea* (15.80%), one *Scotophilus kuhlii* (5.26%) and one *Rousettus amplexicaudatus* (5.26%). No *E. coli* was isolated from any of the three *Pipistrellus javanicus* captured. On the other hand, PCR assay detected *E. coli* in 15 (26.8%) enriched samples in BPW from eight *Cynopterus brachyotis* (53.33%), four *Ptenochirus jagori* (26.67%), two *Eonycteris spelaea* (13.33%), one *Scotophilus kuhlii* (6.67%) and no *E. coli* DNA was detected from three *Pipistrellus javanicus*. In general, the data gathered showed that *E. coli* was present in all of the species of bats collected, except for *Pipistrellus javanicus* and more *E. coli*-positive samples were detected using the conventional isolation method.

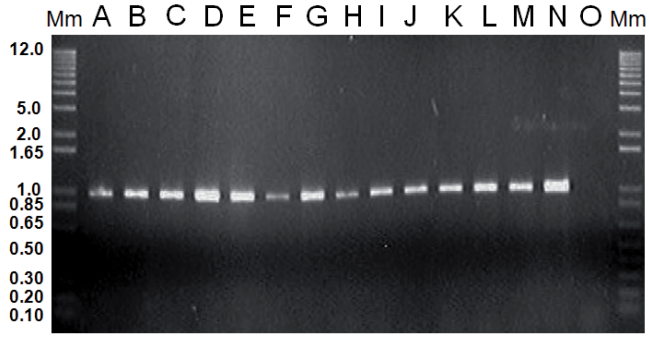

Fig. 1. Polymerase Chain Reaction Amplification of *Escherichia coli.*
Lane (Mm) Molecular size marker (1kb DNA ladder). Lanes (N) and (O) denote positive control and negative control (HPLC-grade water) respectively. Lanes A-M indicates *E. coli* positive samples showing the expected 1000-bp amplicon size.

All of the *E. coli* isolates were confirmed using PCR, with the exception of one sample, a *Ptenochirus jagori* captured in Laguna, which yielded a negative result using this method. These confirmed *E. coli* isolates were processed for *E. coli* O157: H7 screening. Four isolates yielded colorless to yellowish colonies in SMAC agar, which came from three *Cynopterus brachyotis,* one from *Eonycteris spelaea* and one from *Ptenochirus jagori*. All of these bats were captured from Laguna except one, the *Cynopterus brachyotis*. One isolate from a *Eonycteris spelaea* captured in Laguna showed no characteristic fluorescence in FA when viewed under UV light. These characteristic growths in SMAC agar and FA were suggestive of *E. coli* O157:H7. However, none of the suspected isolates were found positive using the latex agglutination test and PCR for *E. coli* O157:H7 confirmation. These isolates were also inoculated in 5% BAP to observe for the presence of hemolysin. However, none of the samples produced complete hemolysis.

Table 2. Results of culture and PCR of enriched samples of bats from Laguna and Quezon City

Detection methods	Culture positive	Culture negative	Total	%
PCR Positive	8	8	16	28.6
PCR Negative	11	29	40	71.4
Total	19	37	56	
%	33.9	66.1		

On the other hand, thirty-seven (66.1 %) out of 56 samples showed the same results for *E. coli* detection using conventional isolation and PCR methods, while 19 (33.9%)

were not in agreement, as shown in Table 2. The difference using Student's T test and the correlation using Pearson Correlation test between the two methods were analyzed at alpha 5% level of significance. In the former test, a p-value of 0.3127 was determined, which is greater than 0.05, while a p-value of 0.0121 was obtained in the latter test, which is less than 0.05, indicating that the two methods have no significant difference and were related respectively.

Discussion

A total of 56 apparently healthy bats were used in the study. Twenty-four bats were captured from Laguna and 32 bats from Quezon City. The bats collected belonged to two families, Pteropodidae and Vespertilionidae. The former family comprised of four frugivore species, *Cynopterus brachyotis, Ptenochirus jagori, Eonycteris spelaea* and *Rousettus amplexicaudatus* while the latter family comprised of two insectivore species, *Scotophilus kuhlii* and *Pipistrellus javanicus*. The majority of the bats collected were *Cynopterus brachyotis* (24/56) followed by *Ptenochirus jagori* (18/56), *Scotophilus kuhlii* (5/56), *Eonycteris spelaea* (4/56), *Pipistrellus javanicus* (3/56) and *Rousettus amplexicaudatus* (2/56).

The species of bats captured in Laguna were all frugivores, *Ptenochirus jagori, Cynopterus brachyotis* and *Eonycteris spelaea.* Four species of frugivores, *Ptenochirus jagori, Cynopterus brachyotis, Eonycteris spelaea* and *Rousettus amplexicaudatus*, and two species of insectivores, *Pipistrellus javanicus* and *Scotophilus kuhlii,* were captured in Quezon City.

Most of the species of bats collected were frugivores (48/56). This may be due to their larger size, the location (open field) and the time (late in the afternoon until early in the morning) the nets were placed in the collection sites, so that in spite of having comprehensive visual perception, these bats were unable to avoid the nets. On the other hand, insectivores have the ability to produce echolocation signals which enable them to detect the nets, thus preventing them from being trapped.

In the present study, the isolation of *E. coli* from 56 intestinal samples of bats yielded 19 (33.93%) isolates, which came from seven *Cynopterus brachyotis* (36.84%), seven *Ptenochirus jagori* (36.84%), three *Eonycteris spelaea* (15.80%), one *Scotophilus kuhlii* (5.26%) and one *Rousettus amplexicaudatus* (5.26%). No *E. coli* was recovered from the intestines of three *Pipistrellus javanicus* which conforms to a study done by DI BELLA et al. (2003) where no *E. coli* was isolated from *Pipistrellus kuhlii*. The factors involved are intestinal length and transit time essential for adherence, colonization and multiplication of bacteria (KLITE, 1965).

The Biotech *E. coli* DAS™ PCR-based detection kit used in the study was able to detect *E. coli* from 15 (26.8%) enriched intestinal samples in BPW. These positive samples came from eight *Cynopterus brachyotis* (53.33%), four *Ptenochirus jagori* (26.67%), two *Eonycteris spelaea* (13.33%), one *Scotophilus kuhlii* (6.67%) and no *E. coli* DNA was detected from three *Pipistrellus javanicus.* The kit claimed the ability to detect 5 ng/μL DNA templates and 10^4 cfu/mL *E. coli* (MERCADO, 2002). This PCR method was able to detect *E. coli* in enriched intestinal bat samples that were negative by the conventional isolation method, since PCR can detect non-viable cells, which cannot be cultured in the laboratory. However, there were samples identified as *E. coli* using the conventional isolation method but not detected in PCR. This is possible if the final concentrations of *E. coli* cells in BPW when tested were lower than the concentration detectable by the kit. Although in lower concentrations, viable *E. coli* cells are able to replicate when grown in suitable media.

Generally, the data gathered revealed that a higher percentage of *E. coli*-positive samples was obtained using the conventional isolation method, which may be due to several factors. First, the enriched samples used for PCR contain *E. coli* cells less than the minimum concentration required by the kit. The samples were stored at freezing temperature (-40 °C) which ceased replication and eventually killed the bacterial cells. HOLLAND et al. (2000) reported the possibility of false-negative results in PCR due to small number of target organisms present in the sample and the decreased stability of cells with storage. Second, the BPW broth used to enrich *E. coli* cells increases the concentration of substrate inhibitors, such as organic and phenolic compounds, heavy metals, lipids, certain cations, hemoglobin and urea present in the sample, yielding a negative result (MALORNY and HOORFAR, 2005). Third, one of the most common endogenous inhibitors of the polymerase enzyme in PCR is insufficiently purified template DNA, which can be due to unoptimized extraction methods (VILJOEN et al., 2005). Fourth, the PCR tubes, powder from gloves and nylon are all common materials in the molecular technique, which may inhibit PCR detection (VILJOEN et al., 2005). Last, the presence of large amounts of other bacteria relative to the amount of the *E. coli* target result in lower sensitivities of assays (HOLLAND et al., 2000).

The two methods, conventional isolation and PCR assay showed same results in 37 (66.07%) of the 56 samples, while 19 (33.93%) were not in agreement. Using the Students T test and Pearson Correlation test at alpha 5% level of significance, the difference and the correlation of the two methods were determined respectively. The tests showed that the two methods have no significant difference and were related. Hence, both methods can be used equally for the detection of *E. coli* from intestinal samples however, conventional isolation is still the gold standard.

The prevalence of *E. coli* in bats reported in other countries ranged from 25 to 85.7% of the studied bat sample population (ADESIYUN, 1999; CHAVERRI, 2006; GORDON and COWLING, 2003). In the present study, the isolation rate of *E. coli* was 33.9% and within the reported range. GORDON and COWLING (2003) explained that the likelihood of isolating *E. coli* in mammals depends on their diet and proximity to human settlements.

The *E. coli* isolates were further confirmed using the same PCR kit. All the isolates were confirmed as *E. coli* except for one sample, which came from a *Ptenochirus jagori* captured in Laguna. These confirmed isolates were inoculated into SMAC agar and FA to screen for *E. coli* O157:H7. In the SMAC agar, five isolates showed characteristic yellowish colonies, of which three came from *Cynopterus brachyotis,* one from *Eonycteris spelaea* and one from *Ptenochirus jagori*. All these bats were from Laguna except for one, the *Cynopterus brachyotis,* which came from Quezon City. One isolate showed no characteristic fluorescence in FA when viewed under UV light. This sample was isolated from a *Eonycteris spelaea* collected in Laguna. These characteristic growths in SMAC agar and FA were suggestive of *E. coli* O157:H7.

The use of SMAC agar to detect *E. coli* O157 has been reported to have a sensitivity of 100%, a specificity of 85% and an accuracy of 86% (ADESIYUN, 1999). Using this medium, MULLER and EHLERS (2005) reported false-positive results in identifying non-sorbitol fermenting bacteria. Aside from *E. coli* O157:H7, other non-sorbitol-fermenting bacteria are *Burkholderia, Pseudomonas, Vibrio* and *Aeromonas* spp. On the other hand, FA has been reported to have a 99.6 to 99.8% specificity (HEIZMANN et al., 1988).

The suspected *E. coli* O157:H7 isolates in SMAC agar and FA were tested for confirmation using the latex agglutination test (Oxoid® *E. coli* O157 Latex Test Kit) and PCR (Biotech *E. coli* O157:H7 DAS™ detection kit) specific for this pathogen. However, none of the suspected isolates revealed positive results using both methods. Furthermore, all of the PCR-confirmed *E. coli* isolates were inoculated in 5% BAP to observe for the presence of hemolysin but none of the samples produced complete hemolysis.

The latex agglutination kit designed to detect *E. coli* O157 has been claimed to have 100% sensitivity and 100% specificity (MARCH and RATMAN, 1989). In their study, the kit identified 230 isolates of *E. coli* O157 which included 30 clinical isolates during the 1988 outbreak in Canada. Additionally, the kit showed negative reactions to 50 non-*E. coli* O157 isolates and to 150 non-sorbitol-fermenting cultures other than *E. coli* O157.

HOLLAND et al. (2000) evaluated commercial extraction methods for the detection of *E. coli* O157:H7 from stool samples using PCR. Seventy-three out of 107 culture-positive samples revealed consistent results using four different DNA extraction methods, of which 67 were PCR-positive and six were PCR-negative. However, the remaining 34 culture-positive samples yielded different PCR results. The study showed that not all

culture-positive samples of *E. coli* can be detected by PCR assay since the extraction method and the number of cells present in the samples can affect sensitivity of the assay.

The present study revealed that the *E. coli* isolates from Philippine bats captured in Laguna and Quezon City were not *E. coli* O157 serotype. Similar conclusions were reported by ADESIYUN (1999) in Trinidad-Tobago, where, out of 377 bats tested, 49 were positive for *E. coli,* seven of which were non-sorbitol fermenters but no *E. coli* O157 was identified.

Conclusion

The study reports the first local isolation and PCR detection of *E. coli* from the intestines of Philippine bats. The two methods when compared revealed no significant difference and were related. The data gathered revealed that *Cynopterus brachyotis, Ptenochirus jagori*, *Scotophilus kuhlii*, *Eonycteris spelaea* and *Rousettus amplexicaudatus* harbor *E. coli* in their intestinal tract while no *E. coli* was recovered and detected in all the three *Pipistrellus javanicus* captured. All of the isolates were confirmed to be not of the *E. coli* O157:H7 serotype, using the latex agglutination test and PCR assay. Furthermore, all the confirmed *E. coli* isolates were observed to be negative for hemolysin production. Generally, the data indicates that Philippine bats were not carriers of the pathogenic *E. coli* O157:H7 serotype.

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SAŽETAK

Poznato je da šišmiši na Filipinima mogu biti nositelji potencijalno patogenih bakterija, npr. salmonela. U radu je opisano izdvajanje i dokaz lančanom reakcijom polimerazom bakterije *Escherichia coli* iz 56 naizgled zdravih šišmiša uhvaćenih na području Laguna i Quezon City na Filipinima. *E. coli* bila je izdvojena iz 19 uzoraka, dok je lančanom reakcijom polimerazom ona bila dokazana u 15 uzoraka. Izolati nisu tvorili hemolizu, a lateks aglutinacija i specifični PCR rabljeni su za dokaz serovara O157:H7 bakterije *E. coli*. Nijedan izolat nije pripadao serovaru O157:H7, na osnovi čega se može zaključiti da šišmiši na području Laguna i Quezon City na Filipinima nisu nositelji patogenog soja *E. coli* O157:H7. Istraživanje je ujedno prvi dokaz o izdvajanju i molekularnoj identifikaciji bakterije *E. coli* u šišmiša na Filipinima.

Ključne riječi: *Escherichia coli*, lateks aglutinacija, šišmiši, Filipini, lančana reakcija polimerazom