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Enteropathogenic bacteria isolated in *Sturnira hondurensis* from central México

Aislamiento de bacterias enteropatógenas en *Sturnira hondurensis* del centro de México

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About 45 % of human deaths from infectious diseases are caused by enteropathogens, among them *Salmonella*, *Shigella* and *Escherichia* stand out. This group of bacteria has been poorly studied in bats and their ecosystem. The aim of this work was to evaluate the presence of enterobacteria in bats associated with a contaminated body of water in Xalapa, México. Two mist nets were placed along the stream bank of the Honduras River. The bat species were identified, and fecal samples were obtained from them, which were grown in triplicate in *Salmonella-Shigella* medium, incubated at 35 °C for 48 hr. Additionally, a sample of water from the stream was isolated, also in triplicate. The bacterial species were identified by colorimetry. Four specimens of *Sturnira hondurensis* were collected. Strains from *Salmonella enterica* (2 individuals), *Shigella flexneri* (2 individuals), and colony-forming unit (CFU) suspected of *Escherichia coli* (3 individuals) were isolated. Two coinfections of *S. enterica* - CFU suspected of *E. coli* was reported. All these enterobacteria were isolated from the stream water. This study represents the first report of the isolation of *S. enterica* and *S. flexneri* in Mexican bats, specifically in *S. hondurensis*. Finally, the importance of good wastewater management is highlighted to prevent bodies of water from being potential sources of infection for wildlife, as well as the need for multidisciplinary studies with the "One Health" approach.

Key words: Anthropozoonosis; contaminated water bodies; *Escherichia coli*; *Salmonella*; *Shigella*.

Cerca del 45 % de las muertes humanas causadas por enfermedades infecciosas son causadas por enteropatógenos, entre ellos destacan los géneros *Salmonella*, *Shigella* y *Escherichia*. Este grupo de bacterias han sido poco estudiadas en murciélagos y sus ecosistemas. El objetivo de este trabajo fue evaluar la presencia de enterobacterias en murciélagos asociados a un cuerpo de agua contaminado en Xalapa, México. Se colocaron 2 redes de niebla en el riachuelo Honduras, Xalapa, México. Los murciélagos se identificaron a nivel especie y de ellos se obtuvieron muestras de heces, las cuales se inocularon por triplicado en medio *Salmonella-Shigella*, se incubaron a 35 °C por 48 hr, adicionalmente se aisló, también por triplicado, una muestra de agua del riachuelo. Las especies bacterianas se identificaron mediante colorimetría. Se colectaron 4 individuos de *Sturnira hondurensis*. Dos individuos fueron positivos para *Salmonella enterica*, 2 positivos para *Shigella flexneri* y unidades formadoras de colonia (UFC) sospechosas a *Escherichia coli* se detectaron en 3 individuos. En 2 individuos se observó la coinfección de *S. enterica* - UFC sospechosas a *E. coli*. Todas estas especies se obtuvieron del cultivo del agua del riachuelo. Este estudio representa el primer reporte de aislamiento de *S. enterica* y *S. flexneri* en murciélagos mexicanos, específicamente en *S. hondurensis*. Finalmente, se resalta la importancia del buen manejo de aguas residuales, para evitar que cuerpos de agua sean potenciales fuente de infección para la fauna silvestre, así como la necesidad de estudios multidisciplinarios con el enfoque de "Una Salud".

Palabras clave: Antropozoonosis; cuerpos de agua contaminados; *Escherichia coli*; *Salmonella*; *Shigella*.

In recent decades there has been an increase in the study of zoonotic diseases from different perspectives, such as prevention (Quaresma *et al.* 2023), their effect on biological conservation (Galindo-González 2023), the role of wildlife as reservoirs (Wood *et al.* 2012), epidemiological studies (van der Westhuizen *et al.* 2023), evaluation of transmission routes between hosts (Colunga-Salas *et al.* 2022), among other aspects. However, since the transmission cycles of zoonotic pathogens include animals, humans, and ecosystems, the "One Health" approach has been widely proposed and used in multidisciplinary studies (Cunningham *et al.* 2017). This approach seeks to study the emergence of zoonotic pathogens from a holistic point of view, bringing together various areas aiming to assess the veterinary, human and ecosystem health together to address and prevent among other emergencies, those caused by zoonotic pathogens (Lerner and Berg 2015; Cunningham *et al.* 2017; Colunga-Salas *et al.* 2021).

One of the groups of zoonotic pathogens that cause ~ 45 % of human deaths due to infectious diseases are enteropathogens, mainly bacteria and viruses, that causes diarrhea (Ecker *et al.* 2005). Particularly, enterobacteria stand

out, a family of Gram-negative bacilli of medical importance, according to the World Health Organization (WHO 2023). The species of genus *Salmonella*, *Shigella,* and *Escherichia* are noteworthy due to the number of pathogenic strains and the presence of drugs-resistant strains; their transmission is mainly carried out through infected drinks and foods (Pérez-Guerrero *et al.* 2014). Paradoxically, this group of enterobacteria has been widely studied in the human population given the high mortality, but poorly studied in terms of its presence and effect on wild mammals' health, as well as ecosystem health (Colunga-Salas *et al.* 2021). Currently, the genus *Salmonella* comprises only 2 species, *S. enterica* and *S. bongori*, both with several serovars. Of this group, *S. enterica* serovar Typhi stands out, whom natural reservoirs are only humans, with an annual estimate of ~ 9 million typhoid cases, of which, about 110,000 are fatal (WHO 2023). On the other hand, *S. enterica* serovar Typhimurium also highlight among the rest of the serotypes for its high morbidity and mortality in humans, which are its only natural reservoirs. The symptoms appear between 6 and 72 hr after ingestion of contaminated food or water and the infection persists between 2 to 7 days (Mellado-Ferreiro *et al.* 2016). On the other hand, the genus *Shigella* comprises 4 species, *S. dysenteriae*, *S. flexneri*, *S. boydii*, and *S. sonnei*. Around, ~164,000 annual deaths are attributable to *Shigella* species (Kotloff *et al.* 2018; Bennish *et al.* 2020). The most important species are *S. flexneri* and *S. sonnei*, which are characterized by fulminant dysenteries with a wide range of intestinal complications. Like *Salmonella*, this genus only has humans as its natural reservoirs (Kotloff *et al.* 2018). *Escherichia coli* stands out as the agent that causes the most infections and diarrhea in humans. It is part of the normal intestinal microbiota of several mammalian species, but due to its great capacity for mutation, it can generate resistance to immune mechanisms, causing various episodes of reinfection, reaching the circulatory system and urinary tract (Mueller and Tainter 2023). Within this species, several serotypes have been described with a wide human pathogenesis such as enterohaemorragic diarrhea (Campos *et al.* 2004) or hemolytic-uremic syndrome (Freedman *et al.* 2023).

A total 12 of the 18 families of bats throughout all continents have been associated with these enterobacteria (Colunga-Salas *et al.* 2021). The alimentary guild most affected are frugivores, due to the spread of bacteria through contaminated fruits (Colunga-Salas *et al.* 2021). Also, there is a report of antibiotic-resistant enterobacteria in bats, which could provide evidence that humans and these organisms coexist closely, which can favor the spread of these pathogens (Cláudio *et al.* 2018). Recently, the presence of antibiotic-resistant strains of *Salmonella*, *Staphylococcus*, and *E. coli* in the digestive tract of fruit bats which were captured near a contaminated river in Bangladesh, were reported (Uddin *et al.* 2020). For this reason, the objective of this work was to record and isolate enterobacteria from the digestive tract of bats associated with a water body that receives wastewater discharges in eastern México.

During a 2-day sampling (June 29 and 30, 2023), 2 12-meter mist nets were placed from 18:30 to 23:00 hr along the stream bank of the Honduras River (19° 30' 36" N, 96° 55' 12" W; and 19° 30' 0" N, 96° 55' 12" W) in the central area of Xalapa - Enríquez, Veracruz, México (Figure 1a, b). The mist nets were checked every 30 min, all individuals were manually releasing and morphologically identified using the field guide of Medellín *et al.* (2008) and then were released at the same sampling site. Handling of vertebrate hosts was done following all requirements specified in the General Wildlife Federal Law of México (Ley General de Vida Silvestre) under collection permit SGPA/DGVS/03821/22, issued to P. F. Colunga Salas. From each released bat, a feces sample was taken directly from the anus and immediately collected in a 1.5 µl low-adherence tubes (LoBind®, Eppendorf AG, Hamburg, Germany) previously sterilized and with 500 µl of isotonic sodium chloride (NaCl) saline solution at 0.9 % NaCl (PiSA, Lot P23E701). Previously to isolation, fecal samples were homogenized using a vortex and incubated at 25 °C for 12 hr as previously reported (Dusch and Altwegg 1995; Mikoleit 2010). Additionally, in a previously sterilized 50 ml tube, a water sample from the Honduras River in the adjacent site where the nets were placed was taken.

As the gold standard test for detection of *Salmonella* and *Shigella* in mammalian feces still being the isolation and culture in selective media (Andrews and Ryan 2015; Tai *et al.* 2016; CDC 2019; CDC 2024), the isolation and identification of enteropathogens from the fecal samples was carried out by triplicate in Petri dishes with *Salmonella-Shigella* agar (SS) (MCD LAB, Lot 716122E002, a certificate culture medium for clinical testing; de la Garza-Garza *et al.* 2020; Flores-Alfonso *et al.* 2021; Parra-Flores *et al.* 2022) following the manufacturer's instructions, incubating the culture media at 35 °C for 48 hr. This culture medium reports a recovery percentage greater than 80 % for *S. flexneri*, *S.*

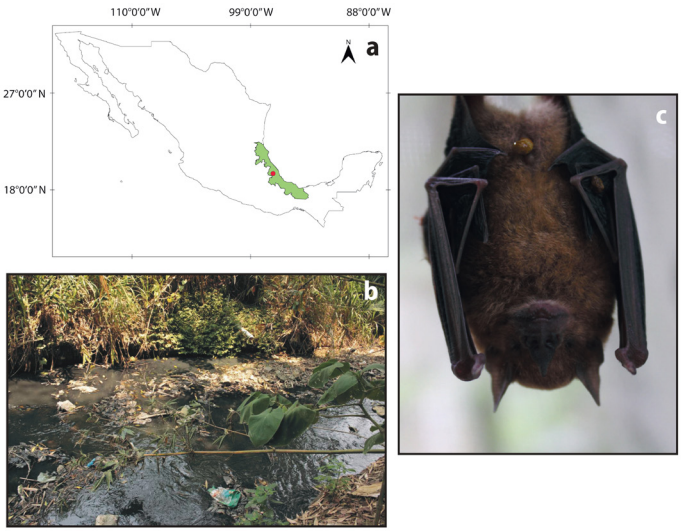

Figure 1. a) Sampling site marked with a red dot within the state of Veracruz, México; b) Honduras River in Xalapa, Veracruz, México; c) *Sturnira hondurensis* collected and sampled. Image Figure 1c available at pcolunga@uv.mx.

enterica serovar Typhi, and *S. enterica* serovar Typhimurium, as well as 25 % for *E. coli* and the inhibition of *Enterococcus faecalis* and *Staphylococcus aureus* (data sheet available: https://mcd.com.mx/medios-de-cultivo/45-158-agar-salmonella-shigella.html). This identification of the colony-forming units was carried out based on the color, as mentioned by the manufacturer, with colorless or transparent colonies for *S. flexneri*, colorless or transparent colonies with black center for *S. enterica* serovar Typhimurium and serovar Typhi and pink colonies with pink precipitate zone for CFU suspected of *E. coli* (see data sheet of the culture medium previously mentioned). As a positive control, the stream water sample was also cultured in triplicate. Finally, 2 negative controls were included. The first was a triplicate culture of the stock of saline solution used as a collection buffer and the second was a Petri dish with SS medium open throughout the entire sample cultivation process. All cultures were performed in a laminar flow hood previously disinfected with 0.01 % NaClO followed by 70 % ethanol.

Four female individuals were captured (1 juvenile and 3 adults), all identified as *Sturnira hondurensis* and without signs of reproductive activity (Figure 1c). The presence of transparent-slightly beige colonies with a black dot characteristic of *S. enterica* was identified in 2 samples (Figure 2a, 2b). Similarly, the presence of *S. flexneri* colonies was identified in 2 samples (Figure 2c, 2d), given the transparent-slightly beige color and no black dot, as well as the presence of CFU suspected of *E. coli*, given the pinky color in 3 individuals (Figure 2a, 2b). Coinfection with *S. enterica* and CFU suspected of *E. coli* was recorded in 2 individuals

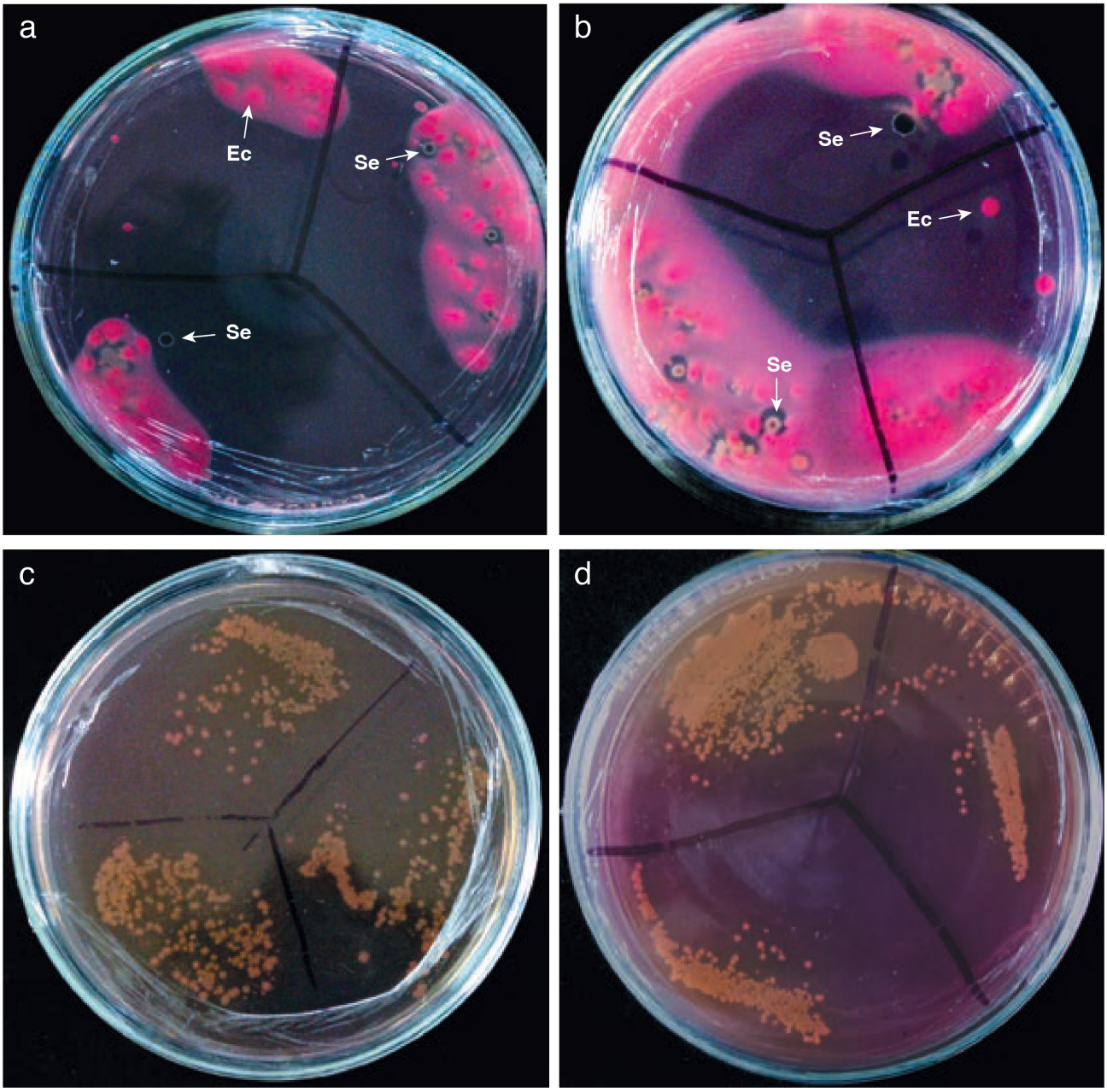

Figure 2. Cultures of the 4 individuals collected from *Sturnira hondurensis*. a, b) Colonies of *Salmonella* sp. (Se) and *Escherichia coli* (Ec). c, d) Colonies of *Shigella flexneri*.

(Figure 2b). No coinfections of the 3 bacteria were found in the same bat. No colonies were observed in any of the negative controls (Figure 3a, 3b). On the other hand, the presence of *S. enterica*, CFU suspected of *E. coli*, and *S. flexneri* was confirmed in the sample from the stream (Figure 3c). The total infection frequency of *S. enterica* and *S. flexneri* was 0.5 for both cases, while for CFU suspected of *E. coli* it was 0.75.

This study represents the first attempt to identify and isolate enteropathogens, specifically *S. enterica* and *S. flexneri* from Mexican bats. However, a limitation of this work is the impossibility of identifying the *S. enterica* serovar, because the 2 serovars that can be isolated in the culture medium have similar morphology (de la Garza-Garza *et al.* 2020). Therefore, it is important to consider additional methods in the future, whether serological or molecular, for the complete identification. On the other hand, it represents the third report of *E. coli* in Mexican bats (Souza *et al.* 1999; Sandner *et al.* 2001; Galicia *et al.* 2014), and the first report of *E. coli*, *Salmonella* sp., and *S. flexneri* in *S. hondurensis*.

It is possible that the source of infection of these bacteria occurs at the bats' feeding sites. The above considering that some species of bats defecate near their roosts (Twente 1955; Klite 1965; Staliński 1994). For instance, there is a report of *Glossophaga mutica* individuals infected with *S. enterica* serovar Copenhagen, possibly through the consumption of food contaminated with feces from other

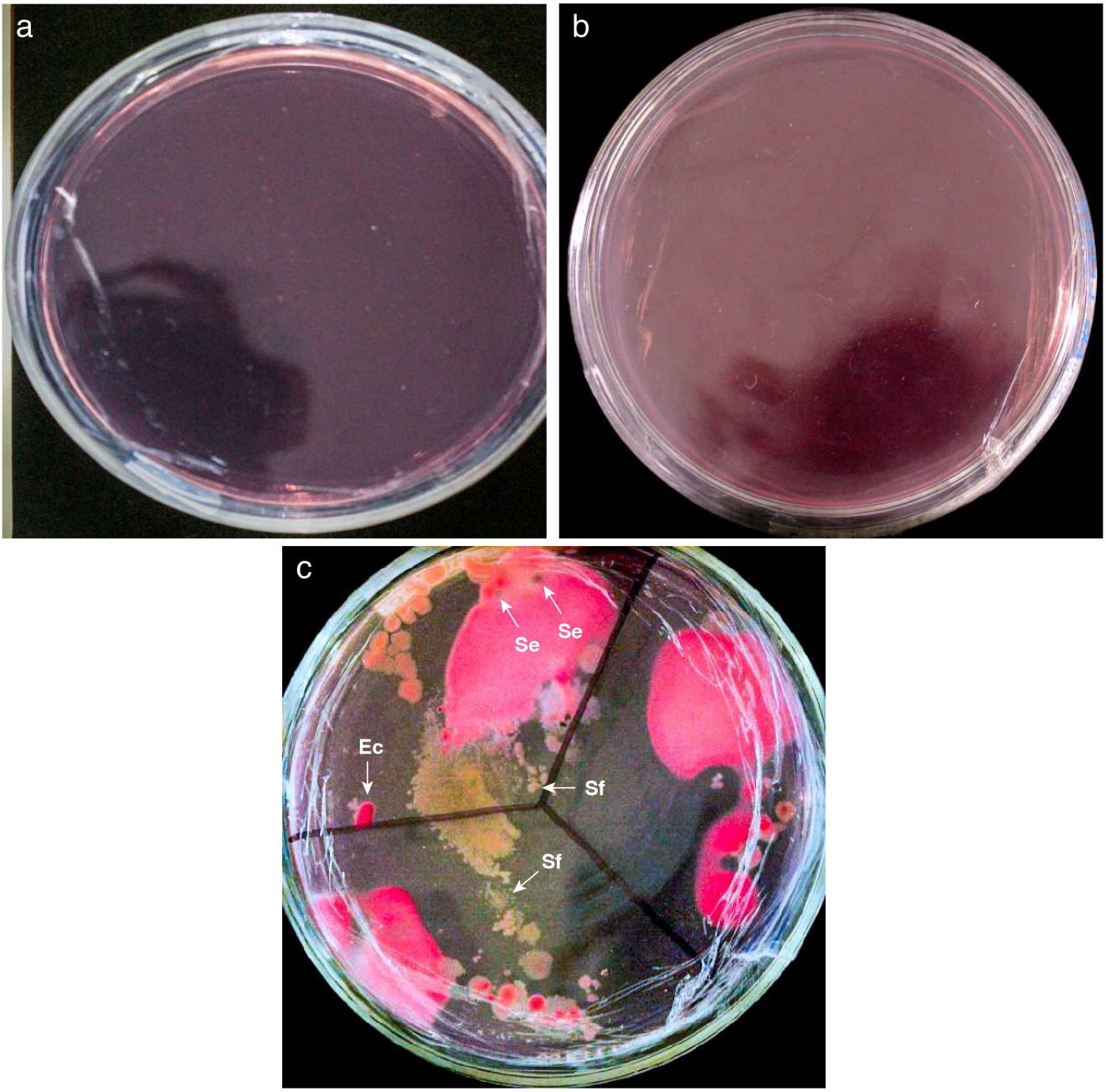

Figure 3. a, b) Cultures of the 2 negative controls and c) for the positive corresponding to a water sample of Honduras River. Ec: *Escherichia coli*; Se: *Salmonella* sp.; Sf: *Shigella flexneri*.

individuals of the same species, as they tend to defecate around foraging sites (Klite 1965). Therefore, it is possible that a similar transmission mechanism may occur in *S. hondurensis*, considering their behavior of roosting and foraging in groups (Cortés-Delgado and Sosa 2014; García-García *et al.* 2014).

Regarding the initial focus of infection of the isolated enteropathogens in *S. hondurensis*, although it is difficult to infer with our results, it cannot be ruled out that because the Honduras River receives daily discharges of residential wastewater, this could act as a source of a continuous infection. A possible indication of this is that the same bacterial species recovered form fecal samples were also recovered from the stream water, as a previous study suggests may have occurred with fruit bats from Bangladesh (Uddin *et al.* 2020). However, to corroborate this hypothesis, it is necessary to carry out a study whose methodology allows us to verify if this happens in the sampling area. It is important to highlight that human activities can affect the health of bats. Therefore, promoting good ecosystem health – throughout proper wastewater management, taking actions to prevent the emergence of drug-resistant strains and the responsibly disposing of medical drugs – can positively support the conservation of not only bats but also wildlife associated with human settlements.

Finally, it is important to highlight the necessity of conducting multidisciplinary studies focused on recognizing infection routes and potential factors that may favor the transmission of zoonotic pathogens between humans and wild mammals, as well as among wild mammals themselves. These studies are crucial for developing sanitary and veterinary management plans aimed at promoting optimal ecosystem health and preventing future zoonotic outbreaks (Cunningham *et al.* 2017).

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