UNIVERSIDAD SAN FRANCISCO DE QUITO USFQ

Colegio de Posgrados

Exploring child exposure to animal feces and zoonotic pathogens in northern coastal Ecuador: A mixed methods study

Tesis en torno a una hipótesis o problema de investigación y su contrastación

Viviana Atalía Albán Marañón

Gabriel Trueba, Ph.D.
Director de Trabajo de Titulación

Trabajo de titulación de posgrado presentado como requisito
para la obtención del título de Magíster en Microbiología

Quito, noviembre 2021

UNIVERSIDAD SAN FRANCISCO DE QUITO USFQ

COLEGIO DE POSGRADOS

HOJA DE APROBACIÓN DE TRABAJO DE TITULACIÓN

Exploring child exposure to animal feces and zoonotic pathogens in northern coastal Ecuador: A mixed methods study.

Viviana Atalía Albán Marañón

Nombre del Director del Trabajo de Titulación:	Gabriel Trueba
Título académico:	Ph.D.
Nombre del Miembro de Comité de Titulación:	Karen Levy
Título académico:	Ph.D.
Nombre del Miembro de Comité de Titulación:	Daniel Garzón
Título académico:	Ph.D.
Nombre del Director del Programa:	Patricio Rojas
Título académico:	Ph.D.
Director del programa de:	Maestría en Microbiología
Nombre del Decano del colegio Académico:	Carlos Valle
Título académico:	Ph.D.
Decano del Colegio:	COCIBA
Nombre del Decano del Colegio de Posgrados:	Hugo Burgos
Título académico:	Ph.D.

Quito, noviembre 2021

© DERECHOS DE AUTOR

Nombre del estudiante:	Viviana Atalía Albán Marañón
Código de estudiante:	00210301
C.I.:	1804385233
Lugar y fecha:	Quito, 09 de noviembre de 2021.

ACLARACIÓN PARA PUBLICACIÓN

Nota: El presente trabajo, en su totalidad o cualquiera de sus partes, no debe ser considerado como una publicación, incluso a pesar de estar disponible sin restricciones a través de un repositorio institucional. Esta declaración se alinea con las prácticas y recomendaciones presentadas por el Committee on Publication Ethics COPE descritas por Barbour et al. (2017) Discussion document on best practice for issues around theses publishing, disponible en http://bit.ly/COPETheses.

UNPUBLISHED DOCUMENT

Note: The following graduation project is available through Universidad San Francisco de Quito USFQ institutional repository. Nonetheless, this project – in whole or in part – should not be considered a publication. This statement follows the recommendations presented by the Committee on Publication Ethics COPE described by Barbour et al. (2017) Discussion document on best practice for issues around theses publishing available on http://bit.ly/COPETheses.

DEDICATORIA

A mi familia.

AGRADECIMIENTOS

Al Instituto de Microbiología de la Universidad San Francisco de Quito. Al proyecto EcoMiD y a todos sus colaboradores de la Universidad de Washington, Universidad de Emory y Universidad de Michigan, en especial a Karen Levy, Joseph Eisenberg y Gabriel Trueba -investigadores principales y Kelsey Jesser y April Ballard, a quienes pertenece gran parte de la propiedad intelectual de esta investigación.

A todos los asistentes de campo, sobre todo a Denys y Mauricio, por facilitar la ejecución del proyecto y a los habitantes de las comunidades de Borbón, Maldonado, Colón, Santo Domingo y la ciudad de Esmeraldas, por su colaboración.

RESUMEN

Los animales domésticos son ubicuos en los países de ingresos bajos y medios, siendo utilizados con fines económicos, alimentación, transporte y como animales de compañía. Sin embargo, la falta de separación de las heces de los animales de los entornos domésticos representa un alto riesgo para la salud de los niños, ya que su exposición persistente y las infecciones entéricas recurrentes se asocian con diarrea, disfunción entérica ambiental y déficit de crecimiento infantil. Este estudio de métodos mixtos convergentes aplicó un enfoque cualitativo utilizando entrevistas semiestructuradas y de acompañamiento y un enfoque microbiológico a través de ensayos de qPCR tiempo real y ELISA para identificar las dinámicas y rutas de transmisión de patógenos zoonóticos entre niños menores de dos años en el noroeste de Ecuador a lo largo de un gradiente urbano-rural. Se encontró que, los niños están expuestos a diferentes tipos de animales y sus heces a través de numerosas vías directas e indirectas y a diferentes, especialmente en comunidades rurales y semi-rurales donde la prevalencia y concentraciones de patógenos fueron altas. Es probable que, los perros y los pollos representen un mayor riesgo para los niños dada su prominencia de heces observada cerca de las áreas de juego infantil y la presencia de múltiples enteropatógenos humanos en concentraciones altas. En conclusión, los datos cualitativos y microbiológicos obtenidos, nos permiten comprender mejor cómo los niños están expuestos a los animales y a los enteropatógenos asociados a los animales en un entorno de recursos bajos y medios.

ABSTRACT

Domestic animals are ubiquitous throughout low- and middle-income settings for income, food, transportation, and companionship. However, insufficient separation of animal feces from domestic environments poses serious health risks for children as persistent exposure and recurrent enteric infections are associated with diarrhea, environmental enteric dysfunction, and child growth deficits. This convergent mixed methods study applied qualitative methods using semi-structured and go-along interviews and a microbiological approach via real-time qPCR and ELISA assays to identify zoonotic pathogen transmission dynamics and routes among children under two in northwestern Ecuador along an urban-rural gradient. We found that children are exposed to different animal types and their feces through numerous direct and indirect pathways, especially in rural and semi-rural communities where pathogen prevalence and concentrations were high. Dogs and chickens likely pose the highest risk to children given the observed prominence of their feces near child play areas and their carriage of multiple human enteropathogens at high concentrations. In conclusion, the qualitative and microbiological data types enabled us to better understand how children are exposed to animals and animal-associated enteropathogens in a LMIC setting

TABLA DE CONTENIDOS

INTRODUCTION

Gastrointestinal zoonotic diseases – overview.

Zoonoses are infectious diseases transmitted from vertebrates to humans under natural conditions (World Health Organization., 2019). These transmissions can occur either directly through exposure to infected animals and its derivate products or indirectly through intermediate vectors (Enriquez, C., et al., 2001). Human exposure to zoonotic pathogens has been described as a consequence of a human-animal-environment interface (World Health Organization., 2020). During the last 30 years, it has been documented a rise in emerging and re-emerging infectious diseases, and more that 70% of them had been originated from animals (Wang, L. & Crameri, G., 2014).

Zoonoses research has focused on vector borne and respiratory pathogens, while those associated with acute gastrointestinal illness have been given less attention (Penakalapati, G., et al., 2017). According to the National Institute of Allergy and Infectious Diseases (NIH), the emerging infectious pathogens classified as B category including *Cryptosporidium* spp., *Giardia lamblia*, *Campylobacter* spp., *Salmonella* spp., diarrheagenic *Escherichia coli* and *Yersinia enterocolitica* are considered gastrointestinal zoonotic microorganisms with a moderately easy capacity to disseminate among animals including humans, causing moderate morbidity rates which need specific diagnostic capacity and surveillance programs (National Institute of Allergy and Infectious Diseases, 2016).

The association between gastrointestinal pathogens and children morbidity and mortality has been well established (Black, R., et al., 2010). In 2017, nearly 424,000 deaths caused by diarrheal diseases in under 5 years old children were reported to the World Health Organization (WHO). Approximately, 7,644 of these deaths occurred in the Americas region from which, 5,224 deaths corresponded to Latin America, being Brazil, Mexico, Venezuela,

Bolivia, and Peru the countries with the highest mortality rates (World Health Organization, 2017). However, the etiology of these gastrointestinal cases has been under the scope of clinicians, investigators and government, causing neglected data record which represents the major barrier to understand the epidemiological situation and the economic burden of this disease (Rodriguez-Morales, A. &. Delgado-López, C., 2012).

Zoonotic enteric pathogens in domestic animals.

Domestic animals, could be infected or colonized by a wide variety of pathogens, including those responsible of gastrointestinal diseases in humans (Damborg. P., et al., 2016). *Salmonella* spp. and *Campylobacter* spp. commonly colonize the gastrointestinal tract of poultry leading to a contamination of its derived products during food chain (Centers for Disease Control and Prevention, 2021). Birds and wild animals may have enteric zoonotic pathogens in its feces such as *Salmonella* spp., *Campylobacter* spp. (Bolton, D., O'Neill, C., & Fanning, S., 2012), *Eschericha coli* aEPEC and STEC (Sanches, L., et al., 2017). In contrast, *Cryptosporidium* spp, *Eimera* spp, *Salmonella* spp and some viruses have been described as diarrhea-causing pathogens in humans and domestic animals (Mawatari, T., et al., 2014). Finally, companion animals such as dogs and cats could also present *Salmonella* spp. *Campylobacter* spp. and *Cryptosporidium* spp. in its gastrointestinal tract (Vasco, G., et al., 2014).

Compared with foodborne and waterborne zoonoses, the risk of pathogen transmission by close contact with animals or animal feces is usually ignored (Penakalapati, G., et al., 2017). Approximately 85% of fecal waste of animals around the world comes from domestic animals such as pigs, cattle, and poultry, reaching a fecal production rate of 2.62 x 10^{13} kg per year (Food and Agricultural Organization, 2021), suggesting that fecal exposure

could be a major transmission route, specifically in places where animal and human coexist in the same environment as a part of their culture.

Salmonella spp.

Salmonella spp. is one of the most frequent foodborne pathogens, being eggs, fresh fruit, vegetables, swine, poultry, and cattle uncooked meat the main sources of *Salmonella* infections (Pui, C., et al., 2011). *S.* enterica subsp. enterica is predominantly found in warm-blooded animals (Brenner, W., et al., 2000) and the non-typhoid *Salmonella* serovars are distributed among humans and animals (Connor B.A., et al., 2005) but just a few of them cause salmonellosis (LeLièvre, V., et al., 2019). *Salmonella* spp. is present in the intestine of different animal types as a common microorganism and normally does not cause infections (Chlebicz, A., et al., 2018).

Poultry has been considered an important source for salmonellosis given that these animals are asymptomatic and that horizontal and vertical transmission routes are recognized (Antunes, P., et al., 2016). In central Ecuador, several studies have identified different *Salmonella* serotypes related to human diseases from poultry such as, *S.* Thyphimurium, *S.* Kentucky, *S.* Enteritidis and, *S.* Infantis (Sánchez-Salazar, E., et al., 2020; Calero-Cáceres, W., et al., 2020; Vinueza-Burgos, C., et al., 2019, Mejia, L., et al., 2020), being this last one, the most prevalent serotype found in all these reports. *S.* Infantis isolated from chicken carcasses and human stool samples and its association with a betalactamase $bla_{CTX\text{-}M}$ production has been described in Ecuador (Mejia, L., et al., 2020).

Disease severity depends on the serovar involved in the infection and the host's characteristics, being children under 5 years old, elderly people, and immunocompromised patients the most susceptible people (Shu-Kee Eng, et al., 2015). Once *Salmonella* enters the digestive tract, these bacteria exceed the initial barrier made of gastric acidity by its acid-

tolerance response (Garcia-del Portillo F., et al., 1993). When entering the small bowel, *Salmonella* goes through the intestinal mucus barriers and begins to express several fimbriae to adhere to the intestinal epithelium (Baumler A.J., et al., 1996). To invade cells, *Salmonella* induces a specific process called bacterial-mediated endocytosis through the expression of gene clusters found in Salmonella pathogenicity islands (SPI) (Lou, L., et al., 2019).

***Campylobacter* spp.**

More than 700 Campylobacter serotypes have been reported, and among these, there are just a few thermotolerant species with clinical significance in animal and human health (Mikulic, M., et al., 2016). *Campylobacter jejuni,* and *Campylobacter coli* are responsible of 90% of bacterial gastroenteritis cases in humans (Mikulic. M., et al., 2016). However, *Campylobacter* infections are still considered underdiagnosed due to the biased methods of detection used on clinical and veterinary laboratories (Acke, E., 2018) and, one concern physiological characteristic of this genus is its coccoid form, which is viable but non culturable (VBNC), and able to survive in hostile conditions outside the host for long periods (Bolton, D., 2015).

Campylobacter spp. can colonize almost all animal's gastrointestinal tract; thus, animals are considered the most common source for human campylobacteriosis, particularly, poultry, causing almost 80% of campylobacteriosis cases in humans (Young, K. T., 2007; Bolton D., 2015). It has been established that owning poultry and maintaining poultry in household´s patios (where children are around), is a risk factor for campylobacteriosis (El-Tras, W., et al., 2015). In Ecuador, MLST analyses of *C. jejuni* and *C. coli* isolates from domestic animals and humans, showed shared Sequence Types (ST) (Vasco, K., et al., 2016).

The major transmission routes of *Campylobacter* are ingestion of contaminated food or water and animal contact (Zenebe, T., et al., 2020). After its ingestion, this species adheres

to fibronectin through the expression of the cadF outer membrane protein to trigger a signaling process which induces *Campylobacter* invasion. This invasion occurs through flagella, which has the function to serve as a secretion apparatus (T3SS) besides motility. *Campylobacter* is capable to produce a cytolethal distending toxin (CDT) composed of three subunits, coding in the genes: *cdtA*, *cdtB* and *cdtC*, where CdtB (product) is enzymatically active and plays a key role in cell transition from G2 phase (Bolton, D., 2015).

Escherichia coli **pathotypes (aEPEC and STEC)**

E. coli aEPEC has been defined as an *E. coli* strain that may or may not belong to a classical EPEC serogroup and that is capable to produce histopathological lesions on intestinal cells without expressing the bundle-forming pilus (bfp) or Shiga-toxin genes (Hernandes, R., et al., 2009). In contrast with typical enteropathogenic *E. coli* (tEPEC), for which its major reservoir are humans, for atypical EPEC, both humans and healthy or diseased animals normally act as reservoirs, but this is not a definite statement, since the transmission dynamics among reservoirs are not yet fully understood (Trabulsi, L., et al., 2002).

Atypical EPEC has been identified in several animal types in previous reports, (Morato, E., et al., 2009; Blanco, M., et al., 2005; Nakazato, G., et al., 2004; García-Meniño, I., et al., 2018; Farooq, S., et al., 2009; Vasco, K., et al., 2016), however, even when the linkage between typical EPEC and diarrhea is well described in developing countries (Clarke, S., et al., 2003; Ochoa. T., et al., 20011), atypical EPEC data regarding its association with illness is still controversial since some studies propose its implication in persistent diarrhea (Afset, J., et al., 2004; Nguyen, R., et al., 2006) while others link this pathogen with acute disease or described it as a non-emerging pathogen (Araujo, J., et al., 2007).

Certain *E. coli* strains had acquired Shiga toxin (*stx*) genes via bacteriophage infection and some of them are able to produce more than one toxin since they can possess several stx-phages as part of their genomes (Herold, S., et al., 2004). These toxins are composed of two subunits, the B subunit are responsible for the toxin-binding to the target cell receptor while the A subunit is the one with the highest enzymatic activity and inhibits protein synthesis which ultimately cause cell death (O'Brien, A., & Holmes, R., 1987).

Cryptosporidium spp.

Cryptosporidium spp. is an intracellular parasite that belongs to the phylum Apicomplexa (Ryan, U., et al., 2015). *C. hominis* and *C. parvum* are the most common species recognized to cause 90% of cryptosporidiosis cases in humans. *Cryptosporidium* oocysts are resistant to chlorine and conventional disinfectants, show resistance to environmental factors (remain viable for over 140 days), and can be transmitted by animals (high range of reservoirs), which leads to significant contamination of water sources and soil (Hassan, E., et al., 2021). Food contamination has been described throughout the entire food chain process (Ryan, U., et al., 2018).

The life cycle of this pathogen begins once the sporulated oocysts have been digested. Motile sporozoites are released to the gastrointestinal system by excystation and they start to release attachment proteins that help the subsequent invasion process through the formation of an extra cytoplasmatic parasitophorous vacuole membrane (PVM) derived from the host to protect itself from the gut environment and to ensure nutrients (Hassan, E., et al., 2021). At the end of its life cycle, infective thick-walls oocysts are released through host’s feces (Power, M., et al., 2005).

References

1. World Health Organization. Global Health Observatory data repository. 2017. Available online: https://apps.who.int/gho/data/view.main.ghe1002015-CH3?lang=en. Accessed on 17 September 2021.
2. Enriquez, C., Nwachuku, N., & Gerba, C. P. (2001). Direct exposure to animal enteric pathogens. Reviews on environmental health, 16(2), 117-132.
3. World Health Organization, Food and Agriculture Organization of the United Nations and World Organization for Animal Health. (2020). Joint Risk Assessment Operational Tool (JRA OT) An Operational Tool of the Tripartite Zoonoses Guide Taking a Multisectoral, One Health Approach: A Tripartite Guide to Addressing Zoonotic Diseases in Countries. Available online: https://www.fao.org/documents/card/ar/c/cb1520en/. Accessed on 21 September 2021.
4. Wang, L. F., & Crameri, G. (2014). Emerging zoonotic viral diseases. Revue scientifique et technique (International Office of Epizootics), 33(2), 569–581. https://doi.org/10.20506/rst.33.2.2311
5. Penakalapati, G., Swarthout, J., Delahoy, M. J., McAliley, L., Wodnik, B., Levy, K., & Freeman, M. C. (2017). Exposure to Animal Feces and Human Health: A Systematic Review and Proposed Research Priorities. Environmental science & technology, 51(20), 11537–11552. https://doi.org/10.1021/acs.est.7b02811
6. National Institute of Allergy and Infectious Diseases. (2016). NIAID Emerging Infectious Diseases/ Pathogens. Available online: https://www.niaid.nih.gov/research/emerging-infectious-diseases-pathogens. Accessed on 22 September 2021.
7. Black, R. E., Cousens, S., Johnson, H. L., Lawn, J. E., Rudan, I., Bassani, D. G., Jha, P., Campbell, H., Walker, C. F., Cibulskis, R., Eisele, T., Liu, L., Mathers, C., & Child Health Epidemiology Reference Group of WHO and UNICEF (2010). Global, regional, and national causes of child mortality in 2008: a systematic analysis. Lancet (London, England), 375(9730), 1969–1987. https://doi.org/10.1016/S0140-6736(10)60549-1
8. Rodriguez-Morales, A. &. Delgado-López, C. (2012). Impact of Climate Change on Zoonotic Diseases in Latin America. Human and Social Dimensions of Climate Change, May. https://doi.org/10.5772/50605
9. Damborg, P., Broens, E. M., Chomel, B. B., Guenther, S., Pasmans, F., Wagenaar, J. A., Weese, J. S., Wieler, L. H., Windahl, U., Vanrompay, D., & Guardabassi, L.

(2016). Bacterial Zoonoses Transmitted by Household Pets: State-of-the-Art and Future Perspectives for Targeted Research and Policy Actions. Journal of Comparative Pathology, 155(1), S27–S40. https://doi.org/10.1016/j.jcpa.2015.03.004

10. Centers for Disease Control and Prevention (2021). CDC and Food Safety. Available online: https://www.cdc.gov/foodsafety/cdc-and-food-safety.html. Accessed on 27 September 2021.
11. Bolton, D. J., O'Neill, C. J., & Fanning, S. (2012). A preliminary study of Salmonella, verocytotoxigenic Escherichia coli/Escherichia coli O157 and Campylobacter on four mixed farms. Zoonoses and Public Health, 59(3), 217-228.
12. Sanches, L. A., Gomes, M. da S., Teixeira, R. H. F., Cunha, M. P. V., Oliveira, M. G. X. de, Vieira, M. A. M., Gomes, T. A. T., & Knobl, T. (2017). Captive wild birds as reservoirs of enteropathogenic E. coli (EPEC) and Shiga-toxin producing E. coli (STEC). Brazilian Journal of Microbiology, 48(4), 760–763. https://doi.org/10.1016/j.bjm.2017.03.003
13. Mawatari, T., Hirano, K., Ikeda, H., Tsunemitsu, H., & Suzuki, T. (2014). Surveillance of diarrhea-causing pathogens in dairy and beef cows in Yamagata Prefecture, Japan from 2002 to 2011.
14. Vasco, G., Trueba, G., Atherton, R., Calvopiña, M., Cevallos, W., Andrade, T., Eguiguren, M., & Eisenberg, J. N. S. (2014). Identifying etiological agents causing diarrhea in low income Ecuadorian communities. American Journal of Tropical Medicine and Hygiene, 91(3), 563–569. https://doi.org/10.4269/ajtmh.13-0744
15. Food and Agricultural Organization FAOSTAT. Food and agriculture data. Available online: https://www.fao.org/faostat/en/#home. Accessed on: 02 October 2021.
16. Pui, C. F., Wong, W. C., Chai, L. C., Tunung, R., Jeyaletchumi, P., Noor Hidayah, M. S., Ubong, A., Farinazleen, M. G., Cheah, Y.K. and Son, R. (2011). Salmonella: A foodborne pathogen. International Food Research Journal 18: 465-473.
17. Brenner F.W., Villar R.G., Angulo F.J., Tauxe R., Swaminathan B. (2000). Salmonella nomenclature. J Clin Microbiol. 38:2465–2467.
18. Connor B.A., Schwartz E. 2005. Typhoid and paratyphoid fever in travellers. The Lancet Infectious Diseases. 5:623–628.
19. LeLièvre, V., Besnard, A., Schlusselhuber, M., Desmasures, N., and Dalmasso, M. (2019). Phages for biocontrol in foods: What opportunities for Salmonella sp. control along the dairy food chain? Food Microbiol. 78, 89–98. doi: 10.1016/j.fm.2018.10.009

20. Chlebicz, A., & Śliżewska, K. (2018). Campylobacteriosis, Salmonellosis, Yersiniosis, and Listeriosis as Zoonotic Foodborne Diseases: A Review. International journal of environmental research and public health, 15(5), 863. https://doi.org/10.3390/ijerph15050863
21. Antunes, P., Mourão, J., Campos, J., & Peixe, L. (2016). Salmonellosis: the role of poultry meat. Clinical microbiology and infection: the official publication of the European Society of Clinical Microbiology and Infectious Diseases, 22(2), 110–121. https://doi.org/10.1016/j.cmi.2015.12.004
22. Sánchez-Salazar, E., Gudiño, M. E., Sevillano, G., Zurita, J., Guerrero-López, R., Jaramillo, K., & Calero-Cáceres, W. (2020). Antibiotic resistance of Salmonella strains from layer poultry farms in central Ecuador. Journal of applied microbiology, 128(5), 1347–1354. https://doi.org/10.1111/jam.14562
23. Calero-Cáceres, W., Villacís, J., Ishida, M., Burnett, E., & Vinueza-Burgos, C. (2020). Whole-Genome Sequencing of Salmonella enterica Serovar Infantis and Kentucky Isolates Obtained from Layer Poultry Farms in Ecuador. Microbiology resource announcements, 9(13), e00091-20. https://doi.org/10.1128/MRA.00091-20
24. Vinueza-Burgos, C., Baquero, M., Medina, J., & De Zutter, L. (2019). Occurrence, genotypes and antimicrobial susceptibility of Salmonella collected from the broiler production chain within an integrated poultry company. International journal of food microbiology, 299, 1–7. https://doi.org/10.1016/j.ijfoodmicro.2019.03.014
25. Mejía L, Medina JL, Bayas R, Salazar CS, Villavicencio F, Zapata S, Matheu J, Wagenaar JA, González-Candelas F, Vinueza-Burgos C. Genomic Epidemiology of Salmonella Infantis in Ecuador: From Poultry Farms to Human Infections. Front Vet Sci. 2020 Sep 29;7:547891. doi: 10.3389/fvets.2020.547891. PMID: 33134346; PMCID: PMC7550756.
26. Shu-Kee Eng, Priyia Pusparajah, Nurul-Syakima Ab Mutalib, Hooi-Leng Ser, Kok-Gan Chan & Learn-Han Lee. (2015). Salmonella: A review on pathogenesis, epidemiology and antibiotic resistance, Frontiers in Life Science, 8:3, 284-293, DOI: 10.1080/21553769.2015.1051243
27. Garcia-del Portillo F., Foster J.W., Finlay B.B.(1993). Role of acid tolerance response genes in Salmonella typhimurium virulence. Infect. Immun. 61:4489–92
28. Baumler A.J., Tsolis R.M., Heffron F. (1996). Contribution of fimbrial operons to attachment to and invasion of epithelial cell lines by Salmonella typhimurium. Infect. Immun. 64:1862–65

29. Lou, L., Zhang, P., Piao, R., & Wang, Y. (2019). Salmonella Pathogenicity Island 1 (SPI-1) and Its Complex Regulatory Network. Frontiers in cellular and infection microbiology, 9, 270. doi:10.3389/fcimb.2019.00270
30. Mikulić, M., Humski, A., Njari, B., Ostović, M., Duvnjak, S., & Cvetnić, Ž. (2016). Prevalence of Thermotolerant Campylobacter spp. in Chicken Meat in Croatia and Multilocus Sequence Typing of a Small Subset of Campylobacter jejuni and Campylobacter coli Isolates. Food technology and biotechnology, 54(4), 475–481. https://doi.org/10.17113/ftb.54.04.16.4647
31. Acke E. (2018). Campylobacteriosis in dogs and cats: a review. New Zealand veterinary journal, 66(5), 221–228. https://doi.org/10.1080/00480169.2018.1475268
32. Bolton D. J. (2015). Campylobacter virulence and survival factors. Food microbiology, 48, 99–108. https://doi.org/10.1016/j.fm.2014.11.017
33. Young, K. T., Davis, L. M., & Dirita, V. J. (2007). Campylobacter jejuni: molecular biology and pathogenesis. Nature reviews. Microbiology, 5(9), 665–679. https://doi.org/10.1038/nrmicro1718
34. El-Tras, W. F., Holt, H. R., Tayel, A. A., & El-Kady, N. N. (2015). Campylobacter infections in children exposed to infected backyard poultry in Egypt. Epidemiology and infection, 143(2), 308–315. https://doi.org/10.1017/S095026881400096X.
35. Vasco, K., Graham, J. P., & Trueba, G. (2016). Detection of Zoonotic Enteropathogens in Children and Domestic Animals in a Semirural Community in Ecuador. Applied and environmental microbiology, 82(14), 4218–4224. https://doi.org/10.1128/AEM.00795-16.
36. Zenebe, T., Zegeye, N., & Eguale, T. (2020). Prevalence of Campylobacter species in human, animal and food of animal origin and their antimicrobial susceptibility in Ethiopia: a systematic review and meta-analysis. Annals of clinical microbiology and antimicrobials, 19(1), 61. https://doi.org/10.1186/s12941-020-00405-8
37. Trabulsi, L. R., Keller, R., & Tardelli Gomes, T. A. (2002). Typical and atypical enteropathogenic Escherichia coli. Emerging infectious diseases, 8(5), 508–513. https://doi.org/10.3201/eid0805.010385
38. Morato, E. P., Leomil, L., Beutin, L., Krause, G., Moura, R. A., & Pestana de Castro, A. F. (2009). Domestic cats constitute a natural reservoir of human enteropathogenic Escherichia coli types. Zoonoses and public health, 56(5), 229–237. https://doi.org/10.1111/j.1863-2378.2008.01190.x

39. Blanco, M., Schumacher, S., Tasara, T., Zweifel, C., Blanco, J. E., Dahbi, G., Blanco, J., & Stephan, R. (2005). Serotypes, intimin variants and other virulence factors of eae positive Escherichia coli strains isolated from healthy cattle in Switzerland. Identification of a new intimin variant gene (eae-eta2). BMC microbiology, 5, 23. https://doi.org/10.1186/1471-2180-5-23
40. Nakazato, G., Gyles, C., Ziebell, K., Keller, R., Trabulsi, L. R., Gomes, T. A., Irino, K., Da Silveira, W. D., & Pestana De Castro, A. F. (2004). Attaching and effacing Escherichia coli isolated from dogs in Brazil: characteristics and serotypic relationship to human enteropathogenic E. coli (EPEC). Veterinary microbiology, 101(4), 269–277. https://doi.org/10.1016/j.vetmic.2004.04.009
41. García-Meniño, I., García, V., Mora, A., Díaz-Jiménez, D., Flament-Simon, S. C., Alonso, M. P., Blanco, J. E., Blanco, M., & Blanco, J. (2018). Swine Enteric Colibacillosis in Spain: Pathogenic Potential of mcr-1 ST10 and ST131 E. coli Isolates. Frontiers in microbiology, 9, 2659. https://doi.org/10.3389/fmicb.2018.02659
42. Farooq, S., Hussain, I., Mir, M. A., Bhat, M. A., & Wani, S. A. (2009). Isolation of atypical enteropathogenic Escherichia coli and Shiga toxin 1 and 2f-producing Escherichia coli from avian species in India. Letters in applied microbiology, 48(6), 692–697. https://doi.org/10.1111/j.1472-765X.2009.02594.x
43. Clarke, S. C., Haigh, R. D., Freestone, P. P., & Williams, P. H. (2003). Virulence of enteropathogenic Escherichia coli, a global pathogen. Clinical microbiology reviews, 16(3), 365–378. https://doi.org/10.1128/CMR.16.3.365-378.2003
44. Ochoa, T. J., & Contreras, C. A. (2011). Enteropathogenic escherichia coli infection in children. Current opinion in infectious diseases, 24(5), 478–483. https://doi.org/10.1097/QCO.0b013e32834a8b8b
45. Afset, J. E., Bevanger, L., Romundstad, P., & Bergh, K. (2004). Association of atypical enteropathogenic Escherichia coli (EPEC) with prolonged diarrhoea. Journal of medical microbiology, 53(Pt 11), 1137–1144. https://doi.org/10.1099/jmm.0.45719-0
46. Nguyen, R. N., Taylor, L. S., Tauschek, M., & Robins-Browne, R. M. (2006). Atypical enteropathogenic Escherichia coli infection and prolonged diarrhea in children. Emerging infectious diseases, 12(4), 597–603. https://doi.org/10.3201/eid1204.051112
47. Araujo, J. M., Tabarelli, G. F., Aranda, K. R., Fabbricotti, S. H., Fagundes-Neto, U., Mendes, C. M., & Scaletsky, I. C. (2007). Typical enteroaggregative and atypical

enteropathogenic types of Escherichia coli are the most prevalent diarrhea-associated pathotypes among Brazilian children. Journal of clinical microbiology, 45(10), 3396–3399. https://doi.org/10.1128/JCM.00084-07

48. Herold, S., Karch, H., & Schmidt, H. (2004). Shiga toxin-encoding bacteriophages--genomes in motion. International journal of medical microbiology: IJMM, 294(2-3), 115–121. https://doi.org/10.1016/j.ijmm.2004.06.023
49. O'Brien, A. D., & Holmes, R. K. (1987). Shiga and Shiga-like toxins. Microbiological reviews, 51(2), 206–220. https://doi.org/10.1128/mr.51.2.206-220.1987
50. Ryan, U., & Hijjawi, N. (2015). New developments in Cryptosporidium research. International journal for parasitology, 45(6), 367–373. https://doi.org/10.1016/j.ijpara.2015.01.009
51. Hassan, E. M., Örmeci, B., DeRosa, M. C., Dixon, B. R., Sattar, S. A., & Iqbal, A. (2021). A review of Cryptosporidium spp. and their detection in water. Water science and technology: a journal of the International Association on Water Pollution Research, 83(1), 1–25. https://doi.org/10.2166/wst.2020.515
52. Ryan, U., Hijjawi, N., & Xiao, L. (2018). Foodborne cryptosporidiosis. International journal for parasitology, 48(1), 1–12. https://doi.org/10.1016/j.ijpara.2017.09.004
53. Power, M. L., Sangster, N. C., Slade, M. B., & Veal, D. A. (2005). Patterns of Cryptosporidium oocyst shedding by eastern grey kangaroos inhabiting an Australian watershed. Applied and environmental microbiology, 71(10), 6159–6164. https://doi.org/10.1128/AEM.71.10.6159-6164.2005

ORIGINAL ARTICLE

Exploring child exposure to animal feces and zoonotic pathogens in northern Coastal Ecuador: A mixed methods study.

Viviana Albán M.[1†], April M. Ballard[2†], Kelsey Jesser[3], Gwenyth Lee[4], Joseph N.S. Eisenberg[4], Gabriel Trueba[1], Bethany A. Caruso[2] and Karen Levy[3*]

[1] Instituto de Microbiología, Universidad San Francisco de Quito, Quito, Ecuador.

[2] Emory University Rollins School of Public Health, Atlanta, GA, USA.

[3] Department of Environmental and Occupational Health Sciences, University of Washington, Seattle, Washington, USA.

[4] Department of Epidemiology, University of Michigan School of Public Health, Ann Arbor, MA.

† These authors contributed equally to this work.

Keywords: children; animal exposure; gastrointestinal diseases; zoonotic pathogens; qPCR.

Abstract's word count: 180 words.

Text's word count: 4572 words.

Running head: Child exposure to zoonotic pathogens in Ecuador.

*** Corresponding author:** e-mail: klevyx@uw.edu. Tel.: 206.543.4341

ABSTRACT

Domestic animals are ubiquitous throughout low- and middle-income settings for income, food, transportation, and companionship. However, insufficient separation of animal feces from domestic environments poses serious health risks for children as persistent exposure and recurrent enteric infections are associated with diarrhea, environmental enteric dysfunction, and child growth deficits. This convergent mixed methods study applied qualitative methods using semi-structured and go-along interviews and a microbiological approach via real-time qPCR and ELISA assays to identify zoonotic pathogen transmission dynamics and routes among children under two in northwestern Ecuador along an urban-rural gradient. We found that children are exposed to animals and their feces through numerous direct and indirect pathways and animal types, especially in rural and semi-rural communities where pathogen prevalence and concentrations were high. Dogs and chickens likely pose the highest risk to children given the observed prominence of their feces near child play areas and their carriage of multiple human enteropathogens at high concentrations. In conclusion, the qualitative and microbiological data types enabled us to better understand how children are exposed to animals and animal-associated enteropathogens in a LMIC setting

INTRODUCTION

Domestic animals are ubiquitous in low- and middle-income (LMIC) countries where they are important sources of income, food, transportation, and companionship. However, insufficient separation of animals and their feces from domestic environments poses health risks for children, as persistent and recurrent exposure to zoonotic enteropathogens is associated with diarrhea, environmental enteric dysfunction, and child growth deficits.[1,2,3] Nearly 424,000 deaths caused by enteric diseases were reported in children under five globally in 2017; 5,224 of these deaths occurred in Latin America.[4]

Recent water, sanitation, and hygiene (WaSH) trials in LMIC settings have not resulted in the anticipated decrease in enteropathogen-related infections or diarrhea.[5,6] This may be due in part to the prevalence of animal fecal contamination of the environment, which may be more extensive than human contamination in these settings.[7] Major transmission routes for enteropathogens in animal feces to humans are summarized by the modified F-diagram. These include: animal feces running off into water sources, animals defecating in fields or using animal feces as field fertilizer, management of animal products, unsafe disposal of animal feces, household surfaces contaminated with animal feces, and direct contact with animal fecal matter.[2] Studies are needed to better understand how people are exposed to animal feces and the risks associated with these exposures in LMICs. Specifically, it is important to define both direct and indirect pathways for contact with animal fecal material, behaviors related to animal husbandry practices, and the prevalence of human-associated enteropathogens in animal feces.[3]

Among the most frequently described enteropathogens transmitted to humans via animal feces are members of the bacterial genera *Campylobacter* and *Salmonella*, the *E. coli* pathotypes Shiga toxin-producing *E. coli* (STEC) and atypical Enteropathogenic *E. coli* (aEPEC), and *Cryptosporidium* parasites.[8] These pathogens are considered Rank 2 zoonotic pathogens (except for *Cryptosporidium* spp. which is considered Rank 1 given that it is not inactivated by chlorination) as they meet the following criteria: (1) strong evidence of its zoonoses, (2) waterborne transmission, (3) outbreak-causing, and (4) responsible for severe human illness.[9,10]

To understand zoonotic enteropathogen exposure routes among infants and young children in an LMIC setting, we conducted a convergent mixed methods study in five communities across an urban-rural gradient in northwestern coastal Ecuador. This study was designed to answer four research questions: (1) How are children exposed to animals and

their feces? (2) What pathogens are present in animal feces, and at what concentrations? (3) Which animals pose a risk to child exposure and health? And (4) Do animal-related exposure pathways vary along an urban-rural gradient? We conducted qualitative go along interviews and observations, and quantitative microbiological measurements. The qualitative and microbiological data were collected independently but interpreted together to address our research questions.

METHODS

Study settings. This study was conducted between June and August 2019 in the northwestern Ecuadorian province of Esmeraldas. Our research team collected data in five communities along an urban-rural gradient, including (1) the region's urban hub, Esmeraldas which is the largest community in the study area (population of approximately 155,000), as well as in the province of Esmeraldas, serving as the capital and principal trading center for agriculture and lumber (2) the semi-rural community of Borbón (population of approximately 8,000) that connects remote villages to resources and (3) the rural villages of Maldonado (population of approximately 2,000), Santo Domingo (population of approximately 500) and Colón (population of approximately 1,000) that lie along the Cayapas, Santiago and Onzole rivers. Santo Domingo and Colón are approximately 3.5 hours by boat from Borbón and are inaccessible by road. According to a local community researcher, the rural communities have more transient inhabitants as they need to access the trading center (Borbón, which sits at the confluence of the three rivers) and are more socially fragmented.

Ethics approval and ethical considerations. Ethics approval was obtained from the Emory University, Atlanta, USA (STUDY00010353) and Universidad San Francisco de Quito,

Quito, Ecuador (2018-022M) Institutional Review Boards. Before data collection, the study aims were explained to the participants, confidentiality was guaranteed, and a consent form was signed. Interviews were conducted and recorded with the participant's permission.

Data collection methods

***Household interviews*.** Go-along semi-structured interviews, a hybrid between participant observation and interviewing,[11] and traditional semi-structured interviews (n=35) were conducted among Afro-Ecuadorian mothers of children under two-years of age that owned at least one animal. Prior to beginning data collection in each community, walkabouts were conducted to identify households with children under age two that owned animals. Purposive sampling was then used to ensure households with varying animal types were included that are typical of each study community.

Go-along interviews explored household animal ownership and child exposure to animals, animal feces, and feces-contaminated soil, and captured data on potential exposure pathways, key behaviors that facilitate or deter child exposure, and maternal perceptions around child-animal interactions. Participants were asked to respond a ten-question survey that captured child demographics, household water and sanitation characteristics, and animal ownership. Interviews lasted 30-60 minutes and were audio recorded when permitted by participants. Traditional in-depth interviews were conducted when go-along methods were not possible.

Detailed observational and interview content notes were taken during and after interviews. Photographs of animals and the environment, without human faces, were also taken. After each interview, a profile and summary were written including contextual information about the household and family structure, observations, animal ownership and interactions, behaviors of children, and perceptions and norms as conveyed by participants.

123

124 **Laboratory methods**

125 ***Sample collection for microbiological analyses*.** Animal feces in and around interviewee
126 households were sampled opportunistically. A total of 120 fecal samples were collected from
127 the following domestic animals: cats (n=6), cows (n=14), dogs (n=21), chickens (n=28), other
128 birds such as ducks and parrots (n=14), horses (n=13), and pigs (n=21). Samples were
129 obtained at all three studied zones. For each sample, 5-10 g of fecal material was collected in
130 plastic sterile containers, preserved on ice, and transported to the field lab. All samples were
131 then aliquoted into four separate cryovials, typically within 6 hours of collection and flash
132 frozen and stored in a liquid nitrogen dewar for about during transport to USFQ where they
133 were maintained at -80°C.

134

135 ***Identification and quantification of enteropathogens in animal stool.*** Genomic DNA was
136 extracted using the PowerSoil® DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA,
137 US) according to the manufacturer's recommendations. Quantitative PCR (qPCR) was used
138 to measure the abundance of enteropathogen DNA in animal stools. Assay gene targets were
139 as follows: *Salmonella* spp.: *invA, Campylobacter jejuni/coli*: *cadF, E. coli* aEPEC: *eae*
140 without *bfpA, stx1* and *stx2* (*bfpA* is only present in tEPEC which is not zoonotic),[12] *E. coli*
141 STEC: *eae* without *bfpA* and with *stx1* and/or *stx2*.[13] All primer sets are listed in Table S1. A
142 gblock standard with all target sequences was constructed for this study (Integrated DNA
143 Technologies, Coralville, CA, USA). Seven serial 10-fold gblock dilutions with
144 concentrations ranging from 10^6 to 10^0 gene copies were prepared each day to generate a
145 standard curve on each plate (Figure S1).

146 Standards and samples were run in duplicate on a real-time PCR system (CFX96, Bio-
147 Rad, USA) under the following conditions: final volume reaction of 20 ul, containing 10 ul of

148 Taqman® Universal PCR Master Mix (Applied biosystems, Life Technologies Corporation,
149 Carlsband, CA, US), 1 uM of each forward and reverse primer, 0.1 uM of probe, and 4 ul of
150 DNA template. Cycling conditions were as follows: 50°C for 2 min, 95°C for 10 min, and 40
151 cycles of 95°C for 15 sec, and 55°C for 1 min (for *invA, eae, stx1* and *stx2*) / 60°C for 1 min
152 (for *bfpA* and *cadF*). Presence/absence determination of *Cryptosporidium parvum* was
153 performed using the enzyme immunoassay RIDASCREEN® *Cryptosporidium* following
154 manufacturer's recommendations.
155
156 ***Quality control*.** To assess inhibition of amplification in qPCR assays, a 220-bp artificial
157 Internal Amplification Control (IAC) gblock was synthesized by Integrated DNA
158 Technologies (Coralville, IA, USA).[14] An amount of 1 x 10^6 copies of the IAC were spiked in
159 each DNA sample and amplified using a SYBR qPCR assay. A melt curve analysis was
160 assessed to assay specificity on each plate. No template controls (NTC) and negative
161 extraction controls were included in each run to verify reagent's contamination.
162 *Cryptosporidium parvum* ELISA assays, positive and negative controls from the kit were
163 used in each plate.
164
165 ***Qualitative data analyses*.** Observational and interview notes, audio recordings, photographs,
166 and interview profiles and summaries were used to develop profiles for each community type.
167 Profiles included information about animal ownership, animal husbandry and health,
168 community and household animal exposure dynamics, behaviors of children, and maternal
169 perceptions and norms. Themes were then identified across communities using profiles, with
170 particular attention to how children under two are exposed to animals and their feces.
171

***Laboratory data analyses*.** Gene target abundance was quantified relative to a mean standard curve, where curve slope, and y-intercept were averaged across four standard curves that were run alongside unknown samples in four separate runs (Table 1). Standard curves were analyzed according to published Minimum Information for Publication of Quantitiative Real-Time PCR Experiments (MIQE) guidelines.[15] The Limit of Detection (LoD) was defined as the lowest standard concentration that met two criteria: a standard deviation <1 for replicates and >95% replicate detection. The Limit of Quantification (LoQ) was calculated using the LoD Cq value and its standard deviation (σ) as follows: $CtLoQ = CtLoD - 2(\sigma LoD)$.[16] (Assay LoD, efficiency, linear dynamic range, and standard curve R^2 values and slopes for each assay are summarized in Table 1. NTCs run on each plate showed no amplification at 40 cycles.

A sample was considered detectable and quantifiable (DQ) if both duplicate reactions were amplified and the mean Cq value was between the highest and lowest dilutions on the standard curve. If a sample had a mean Cq value below the LoQ and both duplicate reactions were amplified, it was considered as detectable but not quantifiable (DNQ) and the value of the limit of detection was assigned. A sample was considered not detected (ND) if one or more out of two reactions had no detectable amplification and a value of half the limit of detection was assigned. For the *Cryptosporidium* ELISA assay, a cut-off value was established by adding 0.15 extinction units to the negative control measurement and a sample was considered positive if its extinction rate was >10% higher than the established cut-off value.

R v. 3.6.2 and RStudio v. 1.3.959 software was used for statistical analyses. Kruskall-Wallis and Wilcoxon rank-sum tests were run using the R package dplyr to compare mean concentrations of the microorganisms between the three zones. Significance was defined as *p*

196 < 0.05. Graphical summaries of results, including box plots, bar plots, and pie charts were
197 produced using the ggplot2 R package.
198

199 **RESULTS**

200 **Qualitative findings**

201 **WaSH characteristics of households.** In total, 35 interviews were conducted among the
202 three zones, 10 from urban zones, 10 from semi-rural zones, and 15 from rural zones.
203 Regarding drinking water access, 50% of households from urban zones used household tap
204 water as their primary drinking water source and the other half used purchased bottled water,
205 while approximately 73.3% of homes from semi-rural zone used purchased bottled water for
206 their primary drinking water and the rest (36.7%) used tap water, tube well water, or
207 rainwater. In contrast, 70% of households from rural zones used rainwater and 30% used
208 river water as their primary drinking water source. For sanitation access, 100% and 93.3% of
209 the interviewed household from the urban and semi-rural zone had access to a household
210 toilet or latrine, while in the rural zone, 50% of household had access to a private toilet or
211 latrine, 20% of them used public or community latrines and 30% used their neighbor's toilet
212 or latrine. Household WaSH conditions and demographics are summarized in Table 1.
213

214 ***Presence of animals in and around households*.** Dogs, cats, and chickens were the most
215 prevalent animal types owned by participants across all three zones. Most (54.5%) dog
216 owners had two or more dogs and 66.7% of cat owners had only one cat, while chicken
217 owners had an average of 11 chickens. Households owning pigs had from one to seven
218 animals and households that owned cows had approximately 30 (Table 1). We found that
219 animals are free to roam throughout rural and semi-rural communities, including dogs, cats,

chickens, and ducks, as reported by mothers, and observed during interviews. Fewer free-roam animals were seen in the urban zone, and most animals were observed on patios or terraces of households. In rural and semi-rural communities, doors are open, and patios are not enclosed which leads to encounters with animals in and around households, even in those where the family did not own a particular animal. In all zones, larger animals were kept outside of communities on farms, except for pigs which were often kept in the back yard, especially in the semi-rural zone.

Animal habitats for chickens and pigs were near households in rural and semi-rural zones, either attached to the side of homes, underneath homes, or behind them. Areas for chickens and pigs were often intermingled with other animals such as ducks and dogs, and animals were frequently observed in spaces for laundry, dishwashing, and other household activities specially in the semi-rural communities. In the urban zone, the living spaces of animals did not overlap as much with spaces for other animals or areas for household activities. Also, we observed and heard interviewees across neighborhoods from the urban zone explain that chickens live and sleep in mango trees at night near households. Having the chickens sleep in trees was said to prevent them from fighting and to protect them from foxes and other predators.

***Fecal contamination of the environment*.** Because animals are free to roam throughout communities, animal fecal contamination of the environment can be observed near households regardless of animal ownership, especially in the rural and semi-rural zones. Specifically, dog and chicken feces were observed to be ubiquitous in the environment, especially in Borbón (semi-rural community) and Colón (rural community). Animal feeding practices where chickens tend to crowd, demonstrated in Colón by caregivers as entertainment for children, may contribute to and concentrate fecal contamination in some

spaces. Colón had the greatest amount of observed fecal contamination while Santo Domingo (another rural community) had the least evident animal feces. This may be because Santo Domingo had fewer animals than Colón or the other rural communities, and the majority of animals in Santo Domingo were kept on farms across the river.

Child behavior and interpersonal interactions that contribute to exposure. Child exposure to animals and animal feces is both direct and indirect. Direct exposures included hand-to-mouth consumption of soil and feces, as children were observed frequently putting their hands in their mouths after crawling on floors. Indirect exposures included object mouthing behaviors and siblings or other children in the household playing in the same areas where animals and animal feces were observed and then playing with or putting their hands in the mouths of children under age two. Observed contact with animals and their feces in the urban zone was largely related to interaction with the environment and mouthing. In contrast, in semi-rural and rural zones, even when the mothers stated that their children had no contact with animals, observations during several interviews demonstrated that children do play with animals very often. In addition, family members, usually fathers or other males, in semi-rural and rural zones frequently worked on farms, which was identified as a potential indirect exposure pathway for young children.

Microbiological findings

Prevalence of enteropathogens in animal fecal samples. Enteropathogen frequencies by zone and animal type are summarized in Figure 1. The prevalence of each enteropathogen was calculated as the number of positive samples for that pathogen divided by the total number of samples tested in a determined zone (Table 1). aEPEC was the most common enteropathogen detected in domestic animal feces (44.17%). It was almost homogeneously

distributed among the three zones, and was detected in 38.98%, 51.72%, and 46.88% of animal feces collected in rural, semi-rural, and urban zones, respectively. These high prevalence rates for aEPEC were primarily driven by horses and ducks in the rural zones and chickens, pigs, and dogs in the semi-rural zone. In the urban zone, we observed an equivalent prevalence contribution in terms of animal type for aEPEC. *Salmonella* was present in 36.67% of animal fecal samples, with the highest prevalence in the semi-rural zone (41.38%), followed by the rural zone (40.68%), and urban zone (25.00%) (Table 2). All five pig samples (100.00%) from the semi-rural zone and nine (75.00%) from the rural zone were positive for *Salmonella* (Table S2). STEC was identified in 35% of animal samples, and cows were the predominant carriers across all zones. *Campylobacter jenuni/coli* had lower prevalence in our study but was most often identified in chickens (50%) and dogs (62.5%) from rural zones. Finally, *Cryptosporidium parvum* had the lowest prevalence among the five enteropathogens we assayed but was identified in 100% of horses from the rural zone, 50% of horses from the urban zone and 62.5% of ducks from the rural zone (Table S2).

aEPEC, STEC, and *Salmonella* were present in all animal types sampled in this study, except for other birds (ducks and parrots) and horses, none of which were positive for *E. coli* STEC or *Salmonella* spp. *Campylobacter jenuni/coli* was more prevalent in chickens and dogs than other animal types, whereas *Cryptosporidium parvum* was associated with horses and other birds (Figure 1). Interestingly, 1 cat, 2 chickens, 2 cows, 2 dogs, 2 horses and 1 pig were *eae*+, *stx1*+, and *stx2*+, a pattern that suggests carriage of an enterohemorrhagic (EHEC) serotype. *E. coli* O157:H7 serotype. We also found an *eae*+ and *bfpA*+ pattern in 1 cat, 11 chickens, 9 dogs, 4 ducks, 1 horse, and 1 pig which could be consistent with the pattern of typical EPEC carriage.

***Co-occurrence of multiple enteropathogens*.** Figure 2 summarizes the detection of zero, one, or more enteropathogens in a given fecal sample by animal type. In 14 samples (11.7%), no enteropathogens were detected, in 49 samples (40.8%) one pathogen was present, and in 57 samples (47.5%) more than one enteropathogen was detected (Figure 2); two and three enteropathogens were present in 50 (87.71%) and 7 (18.83%) samples showing co-infection patterns, respectively. Chickens constituted the major animal group carrying multiple enteropathogens (28.07%), followed by pigs (17.54%) and dogs (15.79%). We determined 11 different enteropathogen co-occurrence patterns, with 10 unique co-occurrence patterns present in chickens, 6 in dogs, 4 in pigs, 3 in cows, ducks, and cats, and 2 in horses. The most predominant patterns were *Salmonella* + STEC (21.05% of co-occurring enteropathogens), followed by aEPEC + *Salmonella* (19.30%), aEPEC + *C. parvum* (15.79%) and aEPEC + *C.* jejuni/coli (14.04%). (Table S4).

Concentration of zoonotic enteropathogens in animal fecal samples. To estimate a microbial risk associated with animal fecal contamination, we used qPCR data to measure concentrations of enteropathogen virulence genes. In the rural zone, aEPEC and *C. jejuni/coli*-associated gene targets were found at higher concentrations compared to concentrations in semi-rural and urban zones, although these differences were not significant. In contrast, *Salmonella* and STEC showed higher concentrations in the semi-rural zone than in rural and urban zones (Figure 3). Kruskal-Wallis testing showed that STEC concentrations were significantly different among the zones (Chi-square = 3.71, df = 2, p = 0.04088). To calculate pairwise comparisons between zones levels, we applied the Wilcoxon rank-sum test which showed that STEC concentrations were different between semi-rural and urban zones only (p = 0.022).

DISCUSSION

This mixed study demonstrates that children are exposed to animals and their feces through numerous pathways and animal types, especially in rural and semi-rural communities where pathogen prevalence and concentrations were high. Dogs and chickens likely pose the highest risk to children given the observed prominence of their feces near child play areas and their carriage of multiple human enteropathogens at bacterial concentrations on par with defined human infective doses (based on the assumption of one virulence gene copy per enteropathogen genome). In addition, animal ownership is not the sole predictor exposure to animals and their feces as animals roamed freely throughout communities and were often near and even inside of households with small children. Thus, animal husbandry practices and community norms related to free range animals likely contribute to child exposure. Child mouthing and siblings' behaviors were also noted as potential pathways for exposure to animal-derived enteropathogens.

Children in rural and semi-rural communities were more likely to be exposed to animal feces and enteric pathogens compared to their urban counterparts due to free-range husbandry practices, community norms (e.g., open household doors), indoor and outdoor fecal contamination, and high prevalence and concentrations of all the investigated enteropathogens. More than a half of our animal samples from rural and semi-rural zones were positive for aEPEC (Table 1). Although the link between aEPEC and diarrhea is still controversial,[17,18,19] the transmission of this bacterium between animals and humans has been confirmed.[20,21] *Salmonella* was also commonly found in animals from the rural and semi-rural zones, particularly in chickens and pigs. Of concern, poultry has been considered an important source for salmonellosis in central Ecuador[22,23,24,25] and although pigs are not frequently associated with salmonellosis, they can certainly act as reservoirs.[26] In the same way, 20-30% of animal samples taken from rural and semi-rural zones were positive for

344 STEC either by *stx-1+* or *stx-2+* or both, and cows were considered dominant carriers of this
345 pathogen. In Ecuador, a report has proved the presence of O157:H7 *E. coli* STEC strains in
346 cattle,[27] however, there were no human illness reports caused by this serotype in this country.
347 Finally, *Campylobacter jejuni/coli* and *Cryptosporidium parvum* were less prevalent in these
348 communities as they were only found in chickens and horses, respectively. These patterns
349 have been described by others research groups.[21,28]

350 We conclude that dogs and chickens are the highest risk animals for children in rural
351 and semi-rural zones due to the prominence of their feces and their association with high
352 concentrations of enteropathogens. High prevalence of chicken feces has also been described
353 in other LMIC settings. For example, chicken feces were described as the most prevalent type
354 of animal feces encountered in households in rural Zambia,[29,37] and Bangladesh[30] where they
355 are typically left in place because they are small and odorless. In the semi-rural zone, dog
356 samples reached a concentration of 10^{8} cells/g for aEPEC; in the rural communities,
357 concentrations of 10^{7}-10^{9} copies/g for *Campylobacter* spp. were identified in dogs and
358 chickens, while chickens from the rural zone showed loads of 10^{8} cells/g and dogs had loads
359 of 10^{7} cells/g for *Salmonella* spp. (Figure 4). Importantly, all these concentrations are
360 considerable higher than the human infective doses reported in the literature, assuming one
361 gene copy per cell.[31,32,33] Chickens and dogs were also notable in terms of enteropathogen co-
362 occurrence, which we defined as the presence of more than one pathogen in a single stool
363 sample. Approximately 15.87% of dog samples and 25.40% of chicken samples demonstrated
364 co-occurrence of two or more enteropathogens. aEPEC was present in a large portion of co-
365 occurrence patterns, and aEPEC in our study. Previous work in Ecuador and elsewhere have
366 also found that aEPEC commonly co-occurrs in child and animal stools with other
367 enteropathogens. A study in central Ecuador found co-ocurrences of aEPEC +
368 *Campylobacter* spp. and aEPEC + *Giardia* spp. and *Campylobacter* spp. + *Giardia* spp. in

children and animal stool samples.[21] Co-infections with aEPEC and other *E. coli* pathotypes as ETEC, EAEC, *Shigella flexneri*, rotavirus, norovirus, and adenovirus in children less than 5 years of age have also been described in South Africa.[34] Relative to dogs and chickens, other animal types likely pose less risk regardless of enteropathogen infections since cows, horses and some pigs were physically separated from households. Nevertheless, our laboratory results revealed that these animals were not pathogen-free (Table S2). Considering that some household members frequently work on farms, especially in rural zones, direct or indirect contact with these animals could represent another potential exposure pathway for young children (Supplementary Table 3).

We found that child exposure to animal feces and associated enteropathogens does not solely depend on one animal or on animal ownership per se. Different community behaviors related to free roaming animals on streets, animals being near households, and even entering homes were identified in the study zones. In the semi-rural area, it was common for families to have direct and/or indirect contact with animals from the street including dogs, cats, and chickens given that front and back doors of houses were often kept open in this community, and patios were not fenced or blocked in. For this reason, it was not uncommon to encounter animal feces of various types in the environment in and around the household. This was also observed in the rural communities, though to a lesser extent. In contrast, less free roaming animals were encountered in the urban zone as most were observed on patios or terraces of households. Similar findings about animal free-roaming in and around households have been documented in previous studies which explored animal exposure and potential risk of fecal-oral microbial transmission in different LMIC such as Burkina Faso,[35] Bangladesh[36] and Zambia.[29]

Children often played outdoors or on the floor of the home, which often was where animals spent most of their time. Child behaviors such as hand mouthing after crawling or

394 touching objects around the home was observed in all three zones. Siblings were also
395 identified as an indirect exposure pathway since they usually have extensive contact with
396 animals and dirt outside the home and would very likely have contact with the furniture and
397 child afterwards. A previous study in Perú, demonstrated that children had direct contact with
398 poultry feces 2.9 times/12 hours and that in 3.9 opportunities, episodes of hand-to-mouth and
399 object-to-mouth per household/ 12 hours occurred, showing a strong correlation (R=0.94)
400 between feces-to-hand and feces-to-mouth contamination.[37] Further, soil ingestion can act as
401 a direct pathway of exposure as reported by George, C., et al., 2015 in Bangladesh, where
402 geophagy events were observed in 18% of participants, *E. coli* was identified in 97% of soil
403 samples, and 14% of enrolled children carried diarrheagenic *E. coli*.[38]

404 Taken together, the qualitative and microbiological data types enabled us to better
405 understand how children are exposed to animals and animal-associated enteropathogens in a
406 LMIC setting. Our results suggest that direct and indirect pathways are involved in child
407 exposure to animal feces and associated enteropathogens in Coastal Ecuador. As over half of
408 our animal fecal samples were taken from households where we also conducted interviews,
409 our microbiological results could reflect a real enteropathogen exposure in many cases. These
410 findings add to a growing body of evidence that children are exposed to enteropathogens
411 from animal feces, and that community, household, and child behaviors and norms enable
412 these exposure pathways. Further investigation is necessary to characterize risks and animal
413 sources associated with fecal contamination of specific household locations and sample types,
414 including water sources, soil, food, and other objects. Finally, future studies are needed to
415 identify interventions approaches to prevent child exposure to enteric pathogens from
416 animals.

417 This study has some limitations. We were not able to quantify the load for
418 *Cryptosporidium parvum* using qPCR since our DNA extraction protocols were not sufficient

to obtain genetic material from parasite oocysts. In addition, since we performed only molecular analysis by qPCR, the results for the aEPEC should be carefully analyzed given that the *eae intimin* gene is also a virulence factor for other bacterial species such as *E. albertii*, *Citrobacter rodentium* and other *E. coli* strains[39] that may have been present in our samples.

Acknowledgments:

Financial Support: This study was funded by the EcOMiD project.

Disclosures: The authors declare no conflict of interest.

References

1. Delahoy MJ, Wodnik B, McAliley L, Penakalapati G, Swarthout J, Freeman MC, & Levy K, 2018. Pathogens transmitted in animal feces in low- and middle-income countries. International journal of hygiene and environmental health 221: 661–676. https://doi.org/10.1016/j.ijheh.2018.03.005
2. Penakalapati G, Swarthout J, Delahoy MJ, McAliley L, Wodnik B, Levy K, & Freeman MC, 2017. Exposure to Animal Feces and Human Health: A Systematic Review and Proposed Research Priorities. Environmental science & technology 51: 11537–11552. https://doi.org/10.1021/acs.est.7b02811
3. Zambrano LD, Levy K, Menezes N, & Freeman MC, 2014. Human diarrhea infections associated with domestic animal husbandry: a systematic review and meta-analysis. Transactions of the Royal Society of Tropical Medicine and Hygiene 108: 313–325. https://doi.org/10.1093/trstmh/tru056

4. World Health Organization. Global Health Observatory data repository, 2017. Available at: https://apps.who.int/gho/data/view.main.ghe1002015-CH3?lang=en. Accessed September 17, 2021.
5. Rogawski ET, Platts-Mills JA, Gratz J, Zhang J, Moulton LH, Mutasa K, Majo FD, Tavengwa N, Ntozini R, Prendergast AJ, Humphrey JH, Liu J, & Houpt ER, 2020. Impact of Water Quality, Sanitation, Handwashing, and Nutritional Interventions on Enteric Infections in Rural Zimbabwe: The Sanitation Hygiene Infant Nutrition Efficacy (SHINE) Trial. The Journal of infectious diseases 221: 1379–1386. https://doi.org/10.1093/infdis/jiz179
6. Pickering AJ, Null C, Winch PJ, Mangwadu G, Arnold BF, Prendergast AJ, Njenga SM, Rahman M, Ntozini R, Benjamin-Chung J, Stewart CP, Huda T, Moulton LH, Colford JM, Luby SP, & Humphrey JH, 2019. The WASH Benefits and SHINE trials: interpretation of WASH intervention effects on linear growth and diarrhoea. The Lancet. Global health 7: e1139–e1146. https://doi.org/10.1016/S2214-109X(19)30268-2
7. Schriewer A, Odagiri M, Wuertz S, Misra PR, Panigrahi P, Clasen T, & Jenkins MW, 2015. Human and Animal Fecal Contamination of Community Water Sources, Stored Drinking Water and Hands in Rural India Measured with Validated Microbial Source Tracking Assays. The American journal of tropical medicine and hygiene 93: 509–516. https://doi.org/10.4269/ajtmh.14-0824
8. Dufour A, & Bartram J, 2012. Animal waste, water quality and human health. IWA publishing.
9. Havelaar AH, Van Rosse F, Bucura C, Toetenel MA, Haagsma JA, Kurowicka D, Heesterbeek JH, Speybroeck N, Langelaar MF, Van der Giessen JW, Cooke RM, &

466 Braks MA, 2010. Prioritizing emerging zoonoses in the Netherlands. PLoS ONE 5:
467 e13965.
468 10. Craun GF, Brunkard JM, Yoder JS, Roberts VA, Carpenter J, Wade T, Calderon RL,
469 Roberts JM, Beach MJ & Roy SL, 2010. Causes of outbreaks associated with drinking
470 water in the United States from 1971 to 2006. Clin. Microbiol. Rev. 2: 507–528
471 11. Kusenbach M. (2003). Street Phenomenology: The go-along as ethnographic research
472 tool. Ethnography 4: 455–485. doi:10.1177/146613810343007.
473 12. Arais LR, Barbosa AV, Andrade J, Gomes T, Asensi MD, Aires C, & Cerqueira A,
474 2018. Zoonotic potential of atypical enteropathogenic Escherichia coli (aEPEC)
475 isolated from puppies with diarrhoea in Brazil. Veterinary microbiology 227: 45–51.
476 https://doi.org/10.1016/j.vetmic.2018.10.023
477 13. Rogawski ET, Liu J, Platts-Mills JA, Kabir F, Lertsethtakarn P, Siguas M, Khan SS,
478 Praharaj I, Murei A, Nshama R, Mujaga B, Havt A, Maciel IA, Operario DJ, Taniuchi
479 M, Gratz J, Stroup SE, Roberts JH, Kalam A, Aziz F, MAL-ED Network
480 Investigators, 2018. Use of quantitative molecular diagnostic methods to investigate
481 the effect of enteropathogen infections on linear growth in children in low-resource
482 settings: longitudinal analysis of results from the MAL-ED cohort study. The Lancet.
483 Global health 6: e1319–e1328. https://doi.org/10.1016/S2214-109X(18)30351-6
484 14. Deer DM, Lampel KA, & González-Escalona N, 2010. A versatile internal control for
485 use as DNA in real-time PCR and as RNA in real-time reverse transcription PCR
486 assays. Letters in applied microbiology 50: 366–372. https://doi.org/10.1111/j.1472-
487 765X.2010.02804.x
488 15. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R,
489 Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, & Wittwer CT, 2009. The MIQE
490 guidelines: minimum information for publication of quantitative real-time PCR

491 experiments. Clinical chemistry 55: 611–622.
492 https://doi.org/10.1373/clinchem.2008.112797
493 16. Forootan A, Sjöback R, Björkman J, Sjögreen B, Linz L, & Kubista M, 2017.
494 Methods to determine limit of detection and limit of quantification in quantitative
495 real-time PCR (qPCR). Biomolecular detection and quantification 12: 1–6.
496 https://doi.org/10.1016/j.bdq.2017.04.001
497 17. Afset JE, Bevanger L, Romundstad P, & Bergh K, 2004. Association of atypical
498 enteropathogenic Escherichia coli (EPEC) with prolonged diarrhoea. Journal of
499 medical microbiology 53: 1137–1144. https://doi.org/10.1099/jmm.0.45719-0
500 18. Nguyen RN, Taylor LS, Tauschek M, & Robins-Browne RM, 2006. Atypical
501 enteropathogenic Escherichia coli infection and prolonged diarrhea in children.
502 Emerging infectious diseases 12: 597–603. https://doi.org/10.3201/eid1204.051112
503 19. Araujo JM, Tabarelli GF, Aranda KR, Fabbricotti SH, Fagundes-Neto U, Mendes C.
504 M, & Scaletsky IC, 2007. Typical enteroaggregative and atypical enteropathogenic
505 types of Escherichia coli are the most prevalent diarrhea-associated pathotypes among
506 Brazilian children. Journal of clinical microbiology 45: 3396–3399.
507 https://doi.org/10.1128/JCM.00084-07
508 20. Morato EP, Leomil L, Beutin L, Krause G, Moura RA, & Pestana de Castro AF,
509 2009. Domestic cats constitute a natural reservoir of human enteropathogenic
510 Escherichia coli types. Zoonoses and public health 56: 229–237.
511 https://doi.org/10.1111/j.1863-2378.2008.01190.x
512 21. Vasco K, Graham JP, & Trueba G, 2016. Detection of Zoonotic Enteropathogens in
513 Children and Domestic Animals in a Semirural Community in Ecuador. Applied and
514 environmental microbiology 82: 4218–4224. https://doi.org/10.1128/AEM.00795-16

515 22. Sánchez-Salazar E, Gudiño ME, Sevillano G, Zurita J, Guerrero-López R, Jaramillo
516 K, & Calero-Cáceres W, 2020. Antibiotic resistance of Salmonella strains from layer
517 poultry farms in central Ecuador. Journal of applied microbiology 128: 1347–1354.
518 https://doi.org/10.1111/jam.14562
519 23. Calero-Cáceres W, Villacís J, Ishida M, Burnett E, & Vinueza-Burgos C, 2020.
520 Whole-Genome Sequencing of Salmonella enterica Serovar Infantis and Kentucky
521 Isolates Obtained from Layer Poultry Farms in Ecuador. Microbiology resource
522 announcements 9: e00091-20. https://doi.org/10.1128/MRA.00091-20
523 24. Vinueza-Burgos C, Baquero M, Medina J, & De Zutter L, 2019. Occurrence,
524 genotypes and antimicrobial susceptibility of Salmonella collected from the broiler
525 production chain within an integrated poultry company. International journal of food
526 microbiology 299: 1–7. https://doi.org/10.1016/j.ijfoodmicro.2019.03.014
527 25. Mejía L, Medina JL, Bayas R, Salazar CS, Villavicencio F, Zapata S, Matheu J,
528 Wagenaar JA, González-Candelas F, Vinueza-Burgos C, 2020. Genomic
529 Epidemiology of Salmonella Infantis in Ecuador: From Poultry Farms to Human
530 Infections. Frontiers in veterinary science 7:547891.
531 https://doi.org/10.3389/fvets.2020.547891
532 26. Chlebicz A & Śliżewska K, 2018. Campylobacteriosis, Salmonellosis, Yersiniosis,
533 and Listeriosis as Zoonotic Foodborne Diseases: A Review. International journal of
534 environmental research and public health 15: 863.
535 https://doi.org/10.3390/ijerph15050863
536 27. Trueba G, Garcés V, Barragan V, Colman RE., Seymour, M., Vogler, A. J., & Keim,
537 P. (2013). Escherichia coli O157:H7 in Ecuador: animal reservoirs, yet no human
538 disease. Vector borne and zoonotic diseases (Larchmont, N.Y.), 13(5), 295–298.
539 https://doi.org/10.1089/vbz.2012.1128

28. Li F, Su J, Chahan B, Guo Q, Wang T, Yu Z, Xiao L, 2019. Different distribution of Cryptosporidium species between horses and donkeys. Infection, Genetics and Evolution 75: 103954. doi:10.1016/j.meegid.2019.103954
29. Reid B, Seu R, Orgle J, Roy K, Pongolani C, Chileshe M, Fundira D, & Stoltzfus R, 2018. A Community-Designed Play-Yard Intervention to Prevent Microbial Ingestion: A Baby Water, Sanitation, and Hygiene Pilot Study in Rural Zambia. The American journal of tropical medicine and hygiene 99: 513–525. https://doi.org/10.4269/ajtmh.17-0780
30. Ercumen A, Pickering AJ, Kwong LH, Arnold BF, Parvez SM, Alam M, Sen D, Islam S, Kullmann C, Chase C, Ahmed R, Unicomb L, Luby SP, & Colford JM, 2017. Animal Feces Contribute to Domestic Fecal Contamination: Evidence from E. coli Measured in Water, Hands, Food, Flies, and Soil in Bangladesh. Environmental science & technology 51: 8725–8734. https://doi.org/10.1021/acs.est.7b01710
31. Mellies JL, Barron AM, & Carmona AM, 2007. Enteropathogenic and enterohemorrhagic Escherichia coli virulence gene regulation. Infection and immunity 75: 4199–4210. https://doi.org/10.1128/IAI.01927-06
32. Kothary M, & Babu U, 2001. Infective dose of foodborne pathogens in volunteers: A review. Journal of Food Safety 21: 49–68. doi:10.1111/j.1745-4565.2001.tb00307.x
33. Borges KA, Martelo EB, Dos Santos LA, Furian TQ, Cisco IC, Manto L, & Dos Santos LR, 2019. Detection and quantification of Salmonella spp. in poultry slaughterhouses of southern Brazil. Journal of infection in developing countries 13: 455–460. https://doi.org/10.3855/jidc.11107
34. Ledwaba SE, Kabue JP, Barnard TG, Traore AN, & Potgieter N, 2018. Enteric pathogen co-infections in the paediatric population from rural communities in the

564 Vhembe District, South Africa. South African Journal of Child Health 12: 170.
565 doi:10.7196/sajch.2018.v12i4.155
566 35. Ngure F, Gelli A, Becquey E, Ganaba R, Headey D, Huybregts L, Pedehombga A,
567 Sanou A, Traore A, Zongo F, & Zongrone A, 2019. Exposure to Livestock Feces and
568 Water Quality, Sanitation, and Hygiene (WASH) Conditions among Caregivers and
569 Young Children: Formative Research in Rural Burkina Faso. The American journal of
570 tropical medicine and hygiene 100: 998–1004. https://doi.org/10.4269/ajtmh.18-0333
571 36. George CM, Oldja L, Biswas SK, Perin J, Lee GO, Ahmed S, Haque R, Sack RB,
572 Parvin T, Azmi IJ, Bhuyian SI, Talukder KA, & Faruque AG, 2015. Fecal Markers of
573 Environmental Enteropathy are Associated with Animal Exposure and Caregiver
574 Hygiene in Bangladesh. The American journal of tropical medicine and hygiene 93:
575 269–275. https://doi.org/10.4269/ajtmh.14-0694
576 37. Marquis GS, Ventura G, Gilman RH, Porras, E, Miranda E, Carbajal L, & Pentafiel
577 M, 1990. Fecal contamination of shanty town toddlers in households with non-
578 corralled poultry, Lima, Peru. American journal of public health 80: 146–149.
579 https://doi.org/10.2105/ajph.80.2.146
580 38. Guerrero T, Calderón D, Zapata S, & Trueba G, 2020. Salmonella grows massively
581 and aerobically in chicken faecal matter. Microbial biotechnology 13: 1678–1684.
582 https://doi.org/10.1111/1751-7915.13624
583 39. Yang X, Sun H, Fan R, Fu S, Zhang J, Matussek A, Xiong Y, & Bai X, 2020. Genetic
584 diversity of the intimin gene (eae) in non-O157 Shiga toxin-producing Escherichia
585 coli strains in China. Scientific reports 10: 3275. https://doi.org/10.1038/s41598-020-
586 60225-

TABLES AND FIGURES

Table 1: Household demographic data

	Total (n=35)	**Rural (n=10)**	**Semi-rural (n=15)**	**Urban (n=10)**
Maternal age (years)	26 (6)	24 (6)	27 (6)	26 (5)
Child age (months)	13 (6)	14 (5)	12 (6)	13 (7)
Child sex				
Male	13 (37.1)	3 (30.0)	6 (40.0)	4 (40.0)
Female	22 (62.9)	7 (70.0)	9 (60.0)	6 (60.0)
Drinking water source				
Purchased water	16 (45.7)	0 (0.0)	11 (73.3)	5 (50.0)
Well or tubewell water	1 (2.9)	0 (0.0)	1 (6.7)	0 (0.0)
Piped water	7 (20.0)	0 (0.0)	2 (13.3)	5 (50.0)
Rainwater	8 (22.9)	7 (70.0)	1 (6.7)	0 (0.0)
River water	3 (8.6)	3 (30.0)	0 (0.0)	0 (0.0)
Bathroom access				
Household toilet or latrine	29 (82.9)	5 (50.0)	14 (93.3)	10 (100.0)
Public or community latrine	2 (5.7)	2 (20.0)	0 (0.0)	0 (0.0)
Neighbor's toilet or latrine	3 (8.6)	3 (30.0)	0 (0.0)	0 (0.0)
Hole or pit	1 (2.9)	0 (0.0)	1 (6.7)	0 (0.0)
Animal ownership				
Chickens (production and creole)	13 (31.1)	3 (30.0)	6 (40.0)	4 (40.0)
Dogs	22 (62.9)	5 (50.0)	11 (73.3)	6 (60.0)
Cats	21 (60.0)	6 (60.0)	9 (60.0)	6 (60.0)
Pigs	4 (11.4)	1 (10.0)	3 (20.0)	0 (0.0)
Horses, donkeys, or cows	2 (5.7)	2 (20.0)	0 (0.0)	0 (0.0)
Other birds (e.g., ducks, parrots)	2 (5.7)	0 (0.0)	2 (13.3)	0 (0.0)

Table 2: Analytical performance of qPCR assays

Indicator*	***bfpA***	***cadF***	***eae***	***invA***	***stx1***	***stx2***
Efficiency (%)[1]	96.72	93.46	95.02	94.57	93.3	92.24
R2[1]	0.999	0.999	0.999	0.999	1.000	1.000
Slope	-3.403	-3.489	-3.447	-3.459	-3.494	-3.520
Y-intercept	44.713	48.019	47.264	46.420	48.006	46.210
LOD[2] *(equiv. no. of copies)*	10^5 (100)	10^5 (100)	10^5 (100)	10^5 (100)	10^5 (100)	10^5 (100)
Reproducibility[3] *(Low- and high-concn. CV (%))*	0.39-2.93	0.54-1.05	0.72-2.90	0.99-1.42	0.49-1.00	0.22-1.26

*****All analyses are based on four standard curves per target
[1] The linearity range was 10 3to 10 6 copy numbers per reaction for all targets
[2] LOD, copy number of the artificial template per gram of stool, equiv. no. of copies (equivalent copy numbers per 1 µL of volume.
[3] Coefficients of variance (CVs) at both low and high concentrations are shown.

Table 3: Frequency of zoonotic enteropathogens identified in animal fecal samples.

Site	n	*Salmonella spp. (%)*	*Campylobacter spp. (%)*[1]	*E. coli aEPEC (%)*	*E. coli STEC (%)*[2]	*Cryptosporidium spp. (%)*[3]
Rural	59	24 (40.68)	12 (20.34)	23 (38.98)	15 (25.42)	13 (22.03)
Semi-rural	29	12 (41.38)	7 (24.14)	15 (51.72)	8 (27.59)	4 (13.79)
Urban	32	8 (25.00)	4 (12.50)	15 (46.88)	7 (21.88)	4 (12.50)
TOTAL	**120**	**44 (36.67)**	**23 (19.17)**	**53 (44.17)**	**30 (35.00)**	**21 (17.50)**

[1] *Campylobacter* spp. includes: *Campylobacter jejuni* and/or *Campylobacter coli.*
[2] *stx-1 E. coli* positives, *stx-2 E. coli* positives or both were classified as *E. coli* STEC.
[3] *Cryptosporidium* spp. includes: *Cryptosporidium hominis* and/or *Cryptosporidium parvum*.

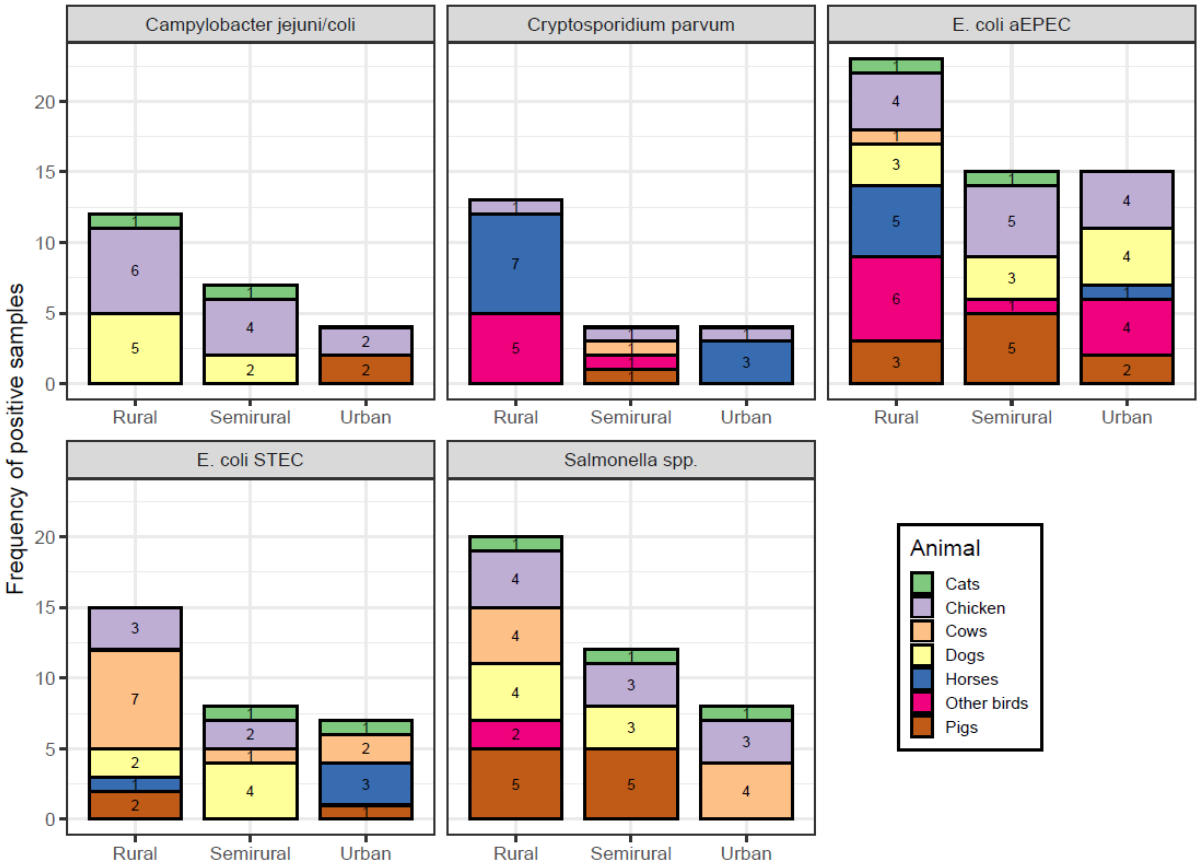

Figure 1: Frequency of zoonotic enteropathogen detection by rural, semi-rural and urban zones and animal types.

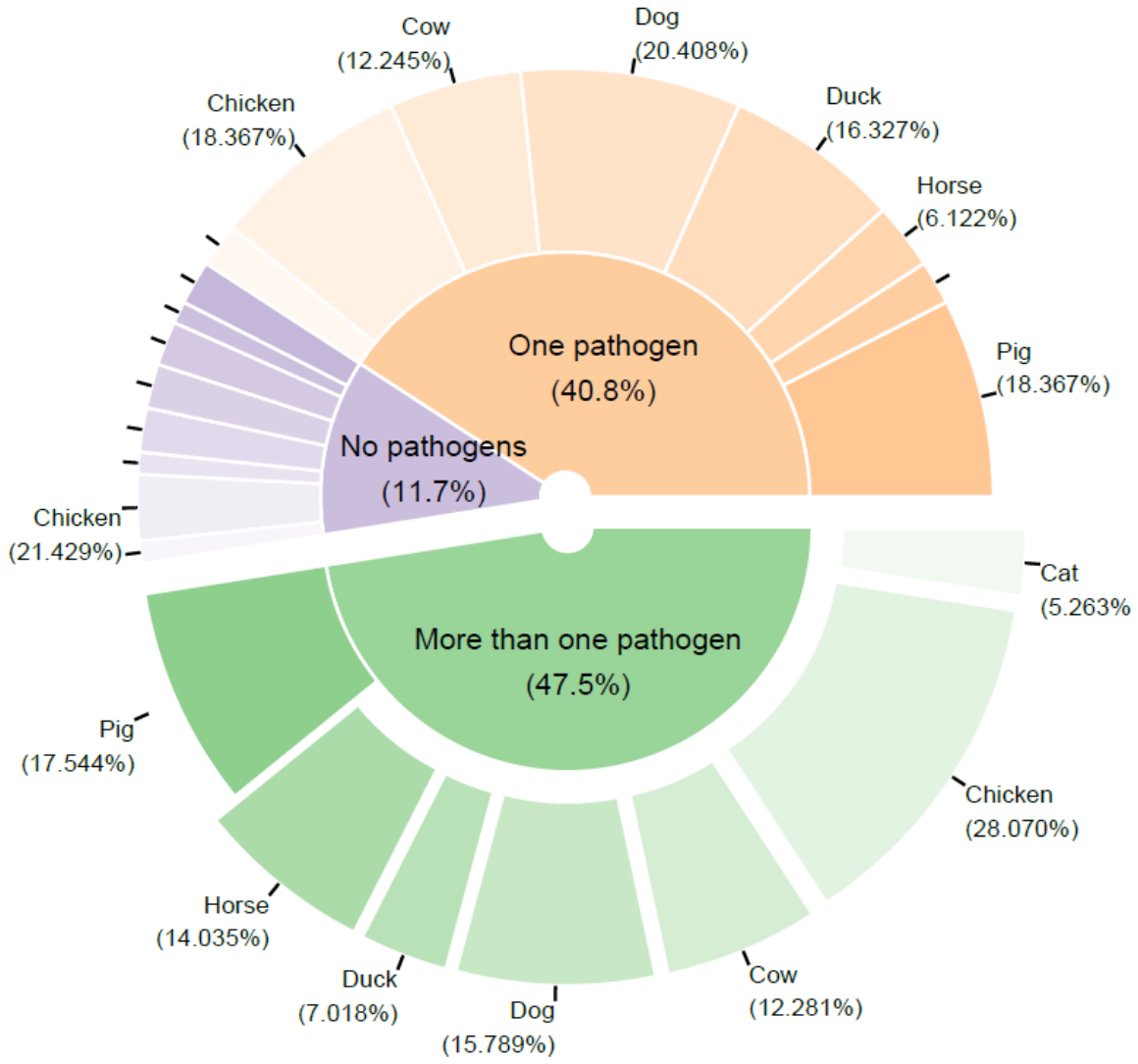

Figure 2: Percent of animal samples where no pathogens were detected, only one pathogen was detected, or more than two pathogens were detected. Percentages for animal types were calculated as the number of positive samples for that animal divided by the total number of samples tested for that animal.

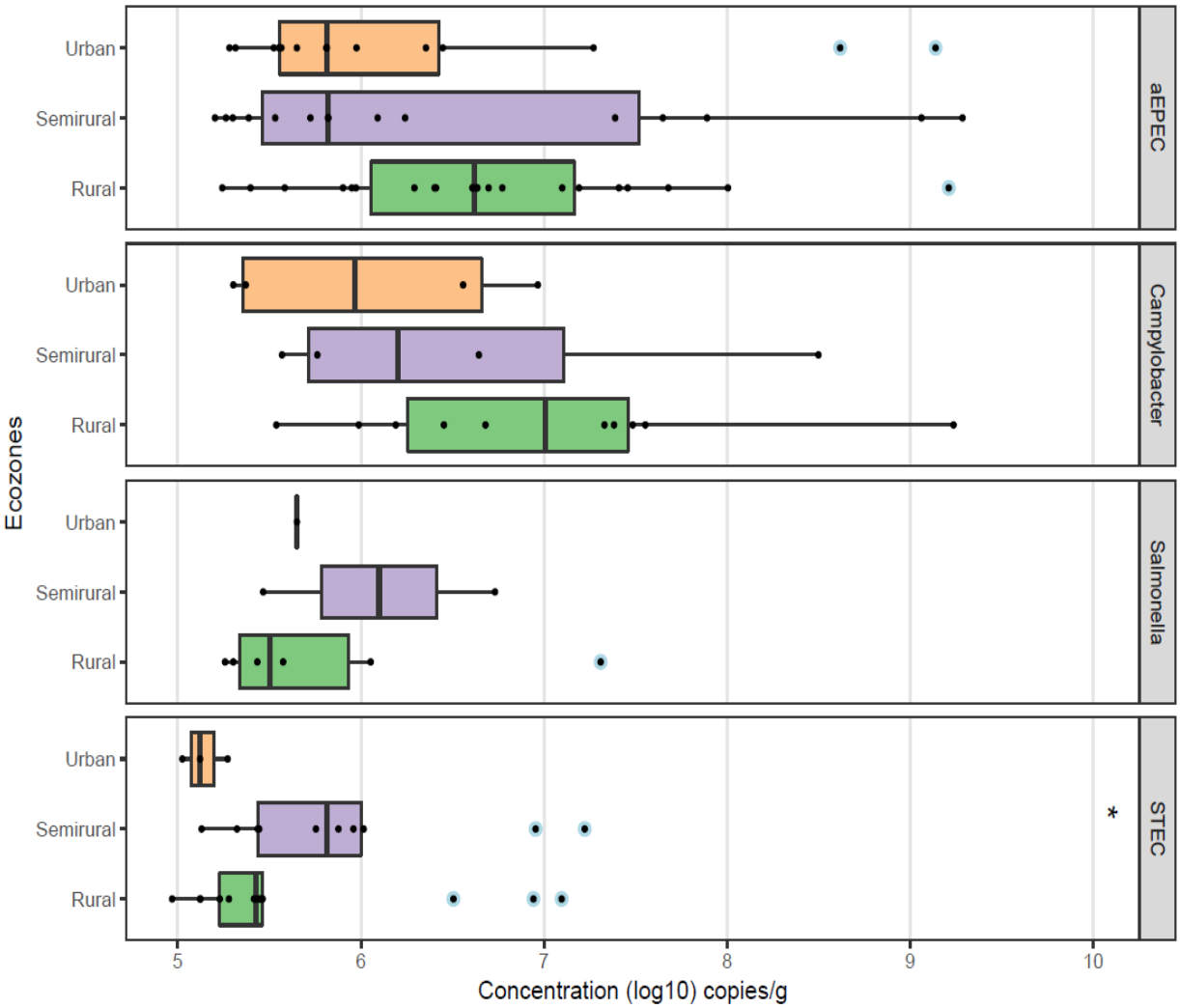

Figure 3: Enteropathogen concentrations in each zone. Data is represented as gene copy number (log10) per gram of feces. Box plots indicate the median, lower and upper quartiles. Black circles represent the concentration of a single sample, and outliers are highlighted in blue

SUPPLEMENTARY MATERIAL

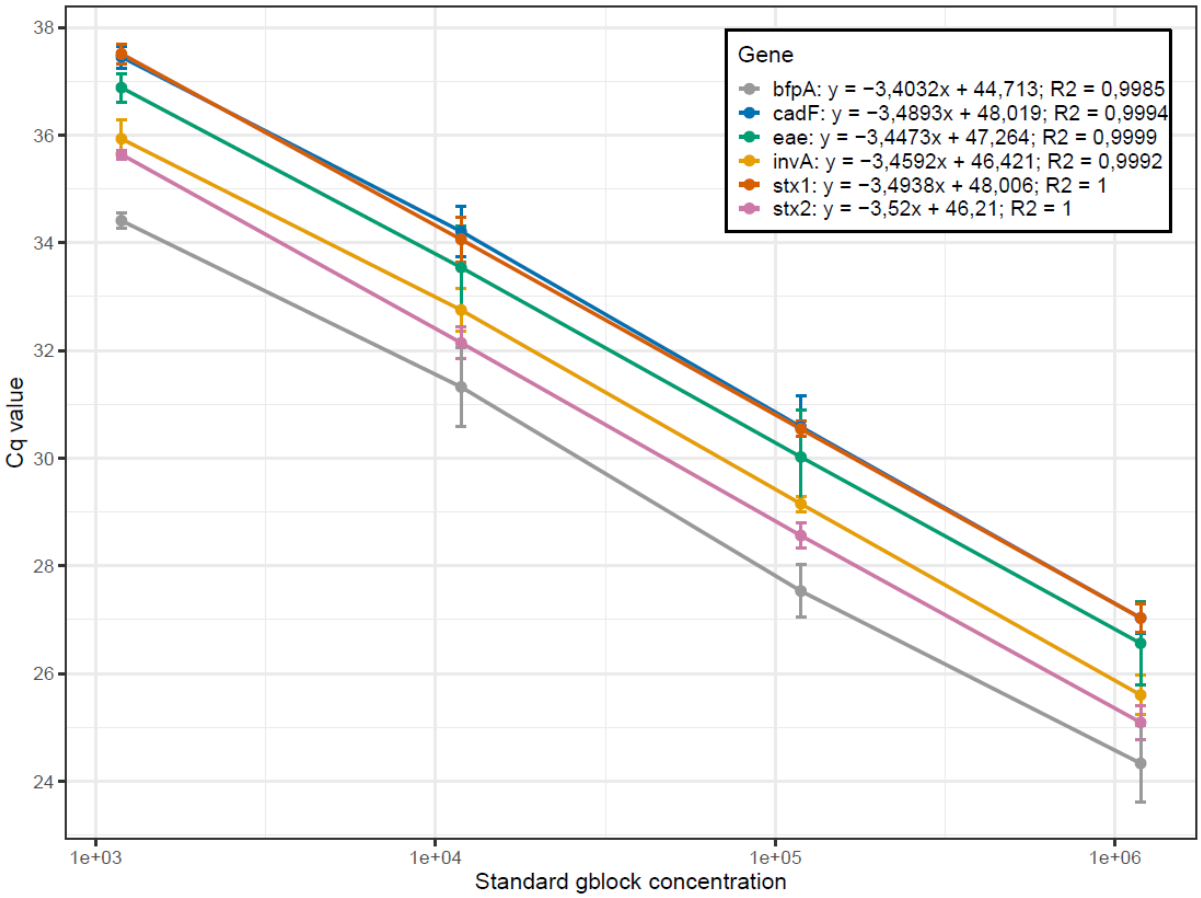

Fig. S1: Mean standard curves of gblock 10-fold serial dilutions generated from four individual standard curves per gene. concentrations ranged from 10 3 to 10 6 gene copies. error bars represent the standard deviation (sd) of cq values at each concentration.

Table S1: Primers and probes sequences.

Gene	Primers	Reference
invA	**F:** GCTGCTTTCTCTACTTAAC **R:** GTAATGGAATGACGAACAT **P:** FAM-CATCACCATTAGTACCAGAATCAGT-BHQ1	Heymans, R, et al., 2018
cadF	**F:** CTGCTAAACCATAGAAATAAAATTTCTCAC **R:** CTTTGAAGGTAATTTAGATATGGATAATCG **P:** FAM-CATTTTGACGATTTTTGGCTTGA-BHQ1	Liu, J., et al., 2013
eae	**F:** CATTGATCAGGATTTTTCTGGTGATA **R:** CTCATGCGGAAATAGCCGTTA **P:** FAM-ATACTGGCGAGACTATTTCAA-BHQ1	Liu, J., et al., 2013
bfpA	**F:** TGGTGCTTGCGCTTGCT **R:** CGTTGCGCTCATTACTTCTG **P:** FAM-CAGTCTGCGTCTGATTCCAA-BHQ1	Liu, J., et al., 2013
sxt1	**F:** ACTTCTCGACTGCAAAGACGTATG **R:** ACAAATTATCCCCTGWGCCACTATC **P:** FAM-CTCTGCAATAGGTACTCCA-BHQ1	Liu, J., et al., 2013
stx2	**F:** CCACATCGGTGTCTGTTATTAACC **R:** GGTCAAAACGCGCCTGATAG **P:** FAM-TTGCTGTGGATATACGAGG-BHQ1	Liu, J., et al., 2013
IAC	**F:** CTAACCTTCGTGATGAGCAATCG **R:** GATCAGCTACGTGAGGTCCTAC	Deer, D., et al., 2010

Table S2: Frequency of zoonotic enteropathogens identified in different animal types**.**

Site	n	*Salmonella spp. (%)*	*C. jejuni/coli (%)*	*aEPEC (%)*	*STEC (%)*[1]	*Cryptosporidium parvum (%)*
Rural	59	24 (40.68)	12 (20.34)	23 (38.98)	15 (25.42)	13 (22.03)
Cats	2	1	1	1	0	0
Chickens	12	4	6	4	3	1
Cows	8	4	0	1	7	0
Dogs	8	4	5	3	2	0
Ducks	8	2	0	5	0	5
Horses	7	0	0	5	1	7
Parrots	2	0	0	1	0	0
Pigs	12	9	0	3	2	0
Semi-rural	29	12 (41.38)	7 (24.14)	15 (51.72)	8 (27.59)	4 (13.79)
Cats	3	1	1	1	1	0
Chickens	10	3	4	5	2	1
Cows	1	0	0	0	1	1
Dogs	8	3	2	3	4	0
Ducks	2	0	0	1	0	1
Pigs	5	5	0	5	0	1
Urban	32	8 (25.00)	4 (12.50)	15 (46.88)	7 (21.88)	4 (12.50)
Cats	1	1	0	0	1	0
Chickens	6	3	2	4	0	1
Cows	5	4	0	0	2	0
Dogs	5	0	0	4	0	0
Ducks	4	0	0	3	0	0
Horses	6	0	0	1	3	3
Parrots	1	0	0	1	0	0
Pigs	4	0	2	2	1	0
TOTAL	120	44 (36.67)	23 (19.17)	53 (44.17)	30 (35.00)	21 (17.50)

[1] *stx-1 E. coli* positives, *stx-2 E. coli* positives or both were classified as *E. coli* STEC.

Table S3: Means and maximum pathogen's abundances reported as gene copies per gram of feces segmented by rural, semi-rural and urban zones and by animal types.

Site	*Salmonella spp.*			*C. jejuni/coli*			*aEPEC*			*STEC (stx-1)*			*STEC (stx-2)*	
	n	***Mean***	***Maximum***	***n***	***Mean***	***Maximum***	***n***	***Mean***	***Maximum***	***n***	***Mean***	***Maximum***	***n***	***Mean***	***Maximum***
Rural															
Cats	**1**	1.19×10^5	1.19×10^5	**1**	2.41×10^7	2.41×10^7	**1**	5.91×10^6	5.91×10^6	**0**	ND	ND	**0**	ND	ND
Chickens	**4**	1.19×10^5	1.19×10^5	**6**	7.46×10^6	3.57×10^7	**4**	2.51×10^5	3.84×10^5	**1**	1.33×10^5	1.33×10^5	**3**	1.67×10^5	2.90×10^5
Cows	**4**	2.32×10^5	3.76×10^5	**0**	ND	ND	**0**	ND	ND	**3**	7.14×10^6	1.25×10^7	**7**	2.24×10^5	2.83×10^5
Dogs	**4**	7.20×10^6	2.04×10^7	**5**	3.54×10^8	1.72×10^9	**3**	1.95×10^7	2.87×10^7	**1**	1.19×10^5	1.19×10^5	**1**	1.19×10^5	1.19×10^5
Ducks	**2**	1.19×10^5	1.19×10^5	**0**	ND	ND	**5**	1.16×10^6	2.57×10^6	**0**	ND	ND	**0**	ND	ND
Horses	**0**	ND	ND	**0**	ND	ND	**5**	2.61×10^7	1.01×10^8	**0**	ND	ND	**1**	1.19×10^5	1.19×10^5
Parrots	**0**	ND	ND	**0**	ND	ND	**1**	4.27×10^6	4.27×10^6	**0**	ND	ND	**0**	ND	ND
Pigs	**9**	1.58×10^5	2.72×10^5	**0**	ND	ND	**3**	1.97×10^6	4.98×10^6	**1**	3.20×10^6	3.20×10^6	**2**	1.44×10^5	1.70×10^5
Semi-rural															
Cats	**1**	5.39×10^6	5.39×10^6	**1**	1.19×10^5	1.19×10^5	**1**	1.60×10^5	1.60×10^5	**1**	1.67×10^7	1.67×10^7	**1**	8.99×10^6	8.99×10^6
Chickens	**3**	1.19×10^4	1.19×10^5	**4**	1.37×10^6	4.41×10^6	**5**	9.37×10^6	4.46×10^7	**1**	1.04×10^6	1.04×10^6	**2**	5.15×10^5	9.11×10^5
Cows	**0**	ND	ND	**0**	ND	ND	**0**	ND	ND	**0**	ND	ND	**1**	2.78×10^5	2.78×10^5
Dogs	**3**	2.06×10^5	2.93×10^5	**2**	1.58×10^8	3.15×10^8	**3**	2.97×10^6	7.56×10^6	**4**	3.20×10^5	7.53×10^5	**3**	2.99×10^5	5.68×10^5
Ducks	**0**	ND	ND	**0**	ND	ND	**1**	6.61×10^5	6.61×10^5	**0**	ND	ND	**0**	ND	ND
Pigs	**5**	1.19×10^5	1.19×10^5	**0**	ND	ND	**5**	6.16×10^8	1.93×10^9	**0**	ND	ND	**0**	ND	ND
Urban															
Cats	**1**	1.19×10^5	1.19×10^5	**0**	ND	ND	**0**	ND	ND	**1**	1.19×10^5	1.19×10^5	**0**	ND	ND
Chickens	**3**	2.83×10^5	4.47×10^5	**2**	4.72×10^6	9.25×10^6	**4**	1.04×10^8	4.14×10^8	**0**	ND	ND	**0**	ND	ND
Cows	**4**	1.19 x 105	1.19×10^5	**0**	ND	ND	**0**	ND	ND	**1**	1.87×10^5	1.87×10^5	**2**	1.12×10^5	1.19×10^5
Dogs	**0**	ND	ND	**0**	ND	ND	**4**	6.32×10^6	1.86×10^7	**0**	ND	ND	**0**	ND	ND
Ducks	**0**	ND	ND	**0**	ND	ND	**2**	4.93×10^5	6.48×10^5	**0**	ND	ND	**0**	ND	ND
Horses	**0**	ND	ND	**0**	ND	ND	**1**	2.80×10^6	2.80×10^6	**2**	1.25×10^5	1.32×10^5	**3**	1.19×10^5	1.19×10^5
Parrots	**0**	ND	ND	**0**	ND	ND	**1**	1.37×10^9	1.37×10^9	**0**	ND	ND	**0**	ND	ND
Pigs	**0**	ND	ND	**2**	1.92×10^6	3.61×10^6	**2**	1.46×10^6	2.26×10^6	**0**	ND	ND	**1**	1.19×10^5	1.19×10^5

ND: Non-detectable

Table S4: Enteropathogen co-occurrence patterns in different animal types**.**

Co-ocurrence patterns	Animal type								
	Cats	**Chickens**	**Cows**	**Dogs**	**Ducks**	**Horses**	**Parrots**	**Pigs**	**Total**
aEPEC + *C. parvum.*	0	1	0	0	2	6	0	0	9
aEPEC + *Salmonella spp.*	0	2	1	1	1	0	0	6	11
aEPEC + *C. jenuni/coli*	0	4	0	3	0	0	0	1	8
Salmonella spp. + *C. jejuni/coli*	1	2	0	1	0	0	0	0	4
Salmonella spp. + *C. parvum*	0	1	0	0	0	0	0	0	1
Salmonella spp. + STEC	1	2	5	2	0	0	0	2	12
C. jejuni/coli + STEC	0	1	0	0	0	0	0	0	1
C. parvum + STEC	0	1	1	0	0	2	0	0	4
Salmonella spp. + aEPEC + *C. parvum*	0	0	0	0	1	0	0	1	2
Salmonella spp. + *C. jejuni/coli* + aEPEC	0	1	0	1	0	0	0	0	2
Salmonella spp. + *C. jejuni/coli* + STEC	1	1	0	1	0	0	0	0	3
Total	3	16	7	9	4	8	0	10	57