Universidad Austral de Chile

Facultad de Ciencias Veterinarias

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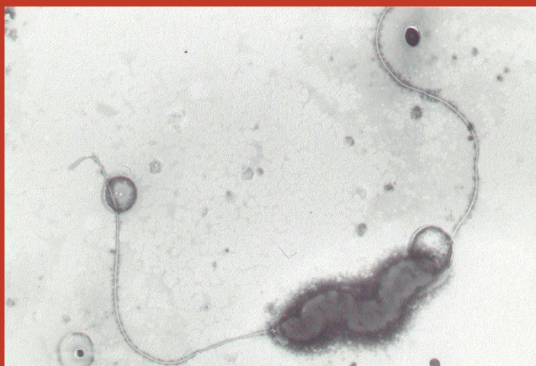

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ORIGINAL ARTICLE

Non-O157 Shiga toxin-producing *Escherichia coli* with potential harmful profiles to humans are isolated from the faeces of calves in Uruguay

Ana Umpiérrez[a*], Déborah Ernst[a], Andrea Cardozo[a], Alexia Torres[b], Magalí Fernández[a], Martín Fraga[c], Rafael Vignoli[d], Inés Bado[d], Roberto Vidal[b,e], Pablo Zunino[a]

ABSTRACT. Shiga toxin-producing *Escherichia coli* (STEC) infections are responsible for acute illnesses and deaths in humans. Cattle and humans are exposed to STEC through faeces and contaminated food and water. The big six and O157 STEC serogroups are important food and water-borne human pathogens. Additionally, Stx1a, Stx2a and Stx2c subtypes are highly associated with the haemolytic uremic syndrome. This study aimed to determine Shiga toxin-subtypes, the presence of antigen 43 families, the genotypic and phenotypic antimicrobial susceptibility profiles, O-serogrouping, phylotypes and phylogenetic relatedness of STEC of calf origin. Sixteen STEC isolates from calf origin were analysed. PCR was performed to determine Stx subtypes, serogroups, the presence of *ag43* I and II and phylotypes. The antimicrobial profile was evaluated and the presence of PMQR and fosfomycin genes was determined by PCR. The clonal relatedness of STEC was studied by PFGE. The genotypes *stx1a+c, stx1a+*, *stx1a+/stx2e+*, *stx1a+c/stx2e* and *stx2a* were detected. Ag43 II was the most prevalent among subfamilies. STEC isolates were serotyped as O103 (*n*=5) and O111 (*n*=6). Fifty per cent of the isolates were classified as B1 phylogroup, 4/16 as E, 1/16 as C, and 1/16 as F. Non-O157 STEC isolates showed a high level of diversity, independent of the geographical and farm-origin. Isolates were resistant to ampicillin, ciprofloxacin, gentamicin, and fosfomycin-trometamol. The gene *fosA7* was detected in 1 isolate. The virulence profiles, including Shiga toxin-subtypes and serogroups, denote the potential harm of non-O157 STEC isolates to humans. We also confirmed that circulating non-O157 STEC from cattle present genetic heterogeneity and are susceptible to antibiotics.

Key words: Non-O157 STEC, Shiga toxin subtypes, antimicrobial resistance.

INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) is a bacterial pathogen with a defined zoonotic potential (Gyles & Fairbrother, 2010). The main natural reservoir of STEC is the bovine intestine, although it can be isolated from other domestic animals (Gyles & Fairbrother, 2010). Infections in humans are usually caused by the consumption of undercooked meat, contaminated vegetables, dairy products and contact with contaminated water. Some infections are also caused by the contact with the environment of animals and ruminants in the farm and person-to-person contact (Kintz *et al.*, 2017). Even though some individuals infected with STEC recover without significant complications, some STEC strains are highly virulent to humans (Majowicz *et al.*, 2014). STEC infections cause over 2.8 million illnesses annually, including haemorrhagic colitis (HC), haemolytic uraemic syndrome (HUS), renal failure and even in some cases haemorrhagic cystitis around the world (Gadea *et al.*, 2012, Majowicz *et al.*, 2014). The incidence of STEC infections differs between countries alongside South America. In Uruguay, cases of HUS and HC are sporadic and have an incidence of 4 to 5 per 100,000 children, whereas Argentina, considered the country with the highest incidence of HUS in children under 5 years, has 300 to 400 HUS cases per year (Blanco *et al.*, 2004, Pérez *et al.*, 2014).

STEC main virulence factors are Shiga toxin type 1 and type 2 (Stx1 and Stx2, respectively). They are encoded in the genome of tempered double-stranded lambdoid prophages (Scheutz, 2014). The number of Stx1 and Stx2 subtypes is continuously upgrading. The Stx1 group is conserved and has four subtypes, a, c, d, and e, while Stx2 is more heterogeneous, and 11 subtypes have been distinguished so far: a-k, with some of them been reported in severe disease in humans (Probert *et al.*, 2014, Scheutz, 2014, Bai *et al.*, 2018, Yang *et al.*, 2020a). In addition, some STEC isolates possess the locus of enterocyte effacement (LEE) pathogenicity island. STEC LEE+ strains are defined by the expression of Intimin, and the translocated intimin receptor (Tir), among other virulence factors. Together, they are responsible for the attaching and effacing (A/E) lesions induced in intestinal epithelial cells (Torres *et al.*, 2018).

Despite the great diversity of phenotypes and combinations of virulence factors, *E. coli* presents a clonal structure. So far, commensal *E. coli* strains, diarrheagenic *E. coli* from the diarrheagenic *E. coli* group (DEC), and those causing extraintestinal infections (ExPEC) can be grouped into eight phylogenetic groups: A, B1, B2, C, D, E, F, and clade I (Clermont *et al.*, 2013). ExPEC strains

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have been mainly assigned to group B2 and a lesser extent to groups D and F, while commensals and diarrheagenic *E. coli* strains have been assigned mostly to groups A, B1, and E (Tenaillon *et al.*, 2010). At the same time, *E. coli* strains are distinguished by their lipopolysaccharide (O) and flagellum (H) composition and antigenicity (Rivas *et al.*, 2014). Currently, in addition to *E. coli* O157:H7, other serogroups have been associated with severe HUS outbreaks. Big six serotypes O26, O45, O103, O111, O121, and O145, together with *E. coli* O157, are the most prevalent within STEC LEE+ strains (Rivas *et al.*, 2014).

As aforementioned, bovines are the main reservoir of STEC and besides the severe damage caused to humans, it can survive imperceptibly in both the bovine intestinal epithelium and in the environment. STEC abundance is low in cattle intestine, however, it can manage to be viably transmitted to the farm environment and from there, even in low abundances, to infect other animals or contaminate water courses (Sapountzis *et al.*, 2020). Persistence in such different places is associated with the phenotypes and plasticity to adapt to the ecological niches and usually involves the ability to form biofilms or have thigh adhesion conditions. Autoaggregation, cell-cell adhesion to the host and biofilm formation of STEC have been associated with the presence of autotransporter proteins like Antigen 43 (Ag43). This adhesin has been more frequently linked to pathogenic STEC LEE+ strains like *E. coli* O157:H7 than to commensals ones (Kjaergaard *et al.*, 2000).

The emergence of multidrug-resistant bacteria has been recognised as a global health issue. Antimicrobial misuse in humans and animals over the decades has determined the occurrence of non-effective treatments for several infectious diseases (Marshall & Levy, 2011). Even antibiotic treatment in STEC infections is not recommended, it is important to keep in mind the association of resistance mechanisms to mobile genetic elements, such as transposons, integrons and plasmids, that give bacteria the capability to rapidly transfer resistance genes (Marshall & Levy, 2011).

In Uruguay, the proportion of STEC in the faeces of calves is low, according to previous studies of our group (Umpiérrez *et al.*, 2017, 2021). However, given the severity and outcome of the illness, the regional context of HUS cases in children, and the ecology of STEC transmission and persistence in the environment it is required to find out the potentially harmful effects of circulating STEC isolates.

This study aimed to determine the Shiga toxin subtypes and the presence of the Antigen 43 gene, the antimicrobial phenotype, O-serogrouping, phylotypes and phylogenetic relatedness of STEC isolates of calf origin.

MATERIAL AND METHODS

ISOLATES AND GROWTH CONDITIONS

Sixteen STEC strains isolated from bovine faeces were collected between 2014 and 2017. As previously described, faeces collection and biochemical and molecular characterisation of *E. coli* were performed (Umpiérrez *et al.*, 2017, 2021). Briefly, all isolates except one were collected from faeces of seven dairy calves with signs of neonatal calf diarrhoea (NCD) and from faeces of dairy calves without NCD signs under 35 days old (table 1), whereas one isolate was collected from an ileum sample of a dead calf affected with NCD (table 1). All isolates were previously characterised by PCR, regarding the presence of *stx1*, *stx2*, and *eae* among other *E. coli* virulence genes, and classified as STEC LEE+ (*stx*+/*eae*+) (Umpiérrez *et al.*, 2017, 2021). For the routine cultivation of STEC, isolates were grown on trypticase soy agar (TSA) plates (OXOID) for 18-24 h at 37 ºC.

SHIGA TOXIN GENES SUBTYPING

Subtyping of Stx was performed according to the protocol described by Scheutz *et al.*, (2012). Three *stx1*subtypes (*stx1a*, *stx1c*, *stx1d*) and 7 *stx2* subtypes (*stx2a, stx2b, stx2c, stx2d, stx2e, stx2f*, *stx2g*) were evaluated by multiplex PCR. When required, the whole *stx* operon was amplified and sequencing analysis was performed using the free software BioEdit (version7.2.5) to assign the Stx subtype to each isolate (Scheutz *et al.*, 2012).

ANTIGEN 43 GENE DETECTION

The presence of Ag43 was evaluated by PCR. Partial amplification of the *ag43* gene was performed as previously described (Kjaergaard *et al.*, 2000). Amplicon size of the two Ag43 subfamilies was determined by agarose gel electrophoresis (subfamily I amplicon size: 1569pb; subfamily II amplicon size: 1839pb) (Kjaergaard *et al.*, 2000).

SEROGROUP

The O26, O45, O103, O111, O113, O121, O145, and O157 serogroups were determined by PCR according to the procedure described by Paddock *et al.*, (2012). PCR positive controls were included in each amplified serogroup. *E. coli* O157:H7 EDL933 strain was used as a positive control for O157 serogroup. The rest of the DNA controls were extracted from clinical isolates from the "Pathogenic *Escherichia coli* Laboratory" pathogenic strains collection at Universidad de Chile.

PHYLOGENETIC ANALYSIS

To assign STEC isolates into any of the phylogroups, partial amplifications of *chuA, yjaA*, *tspE4.C2* and *arpA* genes by PCR was performed according to Clermont *et al.*, (2013). The multiplex PCR method uses the amplification profile of these genes to assign isolates to eight different phylogroups: A, B1, B2, C, D, E, F, and Clade-I.

Table 1. Virulence profile, antimicrobial phenotype, and resistance genes, O-serogrouping and phylotypes of STEC isolates recovered from animals with signs of NCD and from healthy calves.

Animal	Isolate	Year of isolation	Animal signs	Virulence profile	Antibiotic resistance profiles	Resistance genes	Serogroup	Phylogroup
1	1 (74.2)	2014	NCD signs	*stx1a, ag43(II)*	---		O111	B1
2	2 (16.16)	2015	NCD signs[n]	*stx1a+c*	AMP / CN / FOT	*fosA7*	n/d	A
3	3 (AG2.1)	2016	NCD signs	*stx2a*	FOT		n/d	A
4	4(AD1.5)	2016	NCD signs	*stx1a+c, ag43(I)*	AMP/CIP		n/d	E
	5 (AD1.6)			*stx1a, ag43(I)*	AMP/CIP		O111	F
	6 (AD1.7)			*stx1a+c*	AMP		n/d	B1
	7 (AD1.9)			*stx1a+c*	AMP		O111	E
5	8 (AD3.2)	2016	NCD signs	*stx1a+c, ag43(II)*	AMP		O111	B1
6	9 (AD7.2)	2016	NCD signs	*stx1a*	AMP		O111	B1
	10 (AD7.5)			*stx1a*	AMP		O111	C
	11 (AD7.10)			*stx1a, ag43(II)*	---		n/d	E
7	12 (AC3.1)	2016	Calf without signs	*stx1a+c, ag43(II)*	CIP		O103	B1
	13 (AC3.10)			*stx1a, ag43(II)*	---		O103	B1
8	14 (BJ1.3)	2017	NCD signs	*stx1a/stx2e, ag43(II)*	---		O103	E
	15 (BJ1.5)			*stx1a+c, stx2e, ag43(II)*	AMP		O103	B1
	16 (BJ1.10)			*stx1a, stx2e, ag43(II)*	AMP		O103	B1

[n] *E. coli* isolated from an ileum sample of a dead calf with NCD. n/d: none determined serogroup. *ag43(I)* corresponds to Ag43 subfamily I, and *ag43(II)* corresponds to Ag43 subfamily II. AMP, Ampicillin; FOT, fosfomycin-trometamol; CN, gentamicin.

PULSED-FIELD GEL ELECTROPHORESIS

Clonality of the isolates was evaluated by XbaI Pulsed-Field Gel Electrophoresis (PFGE) according to PulseNet protocol. *Salmonella* Braenderup H9812 and *Staphylococcus aureus* subsp. *aureus* (strain NCTC 8325) were used as reference strains. Band patterns were analysed with BioNumerics v.6.6 software (Applied Maths, Sint-Martens-Latem, Belgium). A dendrogram was generated by the UPGMA method, using the Dice coefficient with a 1.0% of band position tolerance.

ANTIMICROBIAL SUSCEPTIBILITY ANALYSES

The Kirby-Bauer disc-diffusion method was used to analyse antimicrobial susceptibility, according to the Clinical Laboratory Standard Institute (2017). Grown isolates in Mueller-Hinton (MH) agar plates (OXOID) for 18-24 h at 37 ºC were tested for 11 different antibiotics: ampicillin (AMP), cefuroxime (CXM), ceftazidime (CAZ), ceftriaxone (CRO), nalidixic acid (NA), ciprofloxacin CIP, enrofloxacin (ENR), gentamicin (CN), amikacin (AK), trimethoprim-sulfamethoxazole (SXT), and fosfomycin trometamol (FOT). All antibiotic discs were purchased from Oxoid. Quality control was performed with *E. coli* ATCC 25922. The interpretation of results was performed according to CLSI 2017, except for ENR, which was interpreted using Veterinary Antimicrobial Susceptibility Testing (VAST) (Patel, 2014). Considering the One Health concept and the zoonotic potential of these isolates, all antibiotics were selected based on the frequency with which they are employed in the medical practice of human infectious diseases.

FOSFOMYCIN AND PLASMID MEDIATED QUINOLONE RESISTANCE GENES

With regard to resistance genes, we searched for the main mechanisms of transferable resistance to antibiotics considered critical by the World Health Organization and previously detected in our country. The presence of PMQR and fosfomycin genes was evaluated by PCR. PMQR genes *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, and *qepA* were partially amplified as previously described (Umpiérrez *et al.*, 2017). On the other hand, the search for fosfomycin resistance genes *fosA*, *fosB*, *fomA*, *fomB*, *fosA3*, *fosC2*, and *fosA7* was performed following protocols and using primers from literature (Cóppola *et al.*, 2020). Primers to detect *fosA7* gene were designed in this study: fosA7F 5'- ATGCTTCAATCTCTGAACCAC -3', fosA7R 5'- CCGAAACGCATTCCAGAGTA -3'. All PCR products were confirmed by direct sequencing.

RESULTS

A collection of 16 STEC isolates (13 isolates from calves with NCD signs, two isolates from a calf without signs of NCD and one isolate from an ileum sample of a dead calf with NCD) were characterised.

SUBTYPES OF STX1 AND STX2

Four Shiga toxins subtypes were detected by PCR. Some STEC isolates presented more than one variant of subtypes simultaneously. All *stx1*+ isolates were typed as *stx1a*+ (15 isolates), 6 of them were also *stx1c*+ and one presented a *stx1a+/stx1c+/stx2e+* genotype (table 1). In addition, 2 isolates were *stx1a+/stx2e+* simultaneously and 1 isolate was *stx2a*+ (table 1). Gene variants *stx1d*, *stx2b*, *stx2c*, *stx2d*, *stx2f* and *stx2g* were not detected.

PRESENCE OF ANTIGEN 43 GENE

Ag43 subfamily II, was predominant among STEC isolates (*n*=8), while only 2 isolates were assigned to Ag43 subfamily I (table 1). The rest of the isolates (*n*=6) were *ag43* negative (table 1).

O-SEROGROUPING AND PHYLOTYPES

Two *E. coli* serogroups were detected by PCR. Five STEC isolates were ascribed to O103 serogroup, whereas 6 isolates were ascribed to O111 (table 1). The remaining 5 isolates were not assigned to any of the 8 serogroups evaluated (31.3% of the isolates) (table 1).

On the other hand, STEC phylotyping showed high diversity. Fifty per cent of the isolates were classified as B1 (*n*=8) (table 1), and the rest were classified as follows: 4 belonged to E phylogroup, 2 to A phylogroup, 1 to F phylogroup and one to C phylogroup (table 1).

CLONAL RELATEDNESS OF STEC ISOLATES

A total of 13 distinct restriction patterns of the 16 STEC isolates were detected using ≥85% of similarity of the Dice coefficient (figure 1). A high level of diversity amongst isolates was observed, however, isolates with the same herd-origin were more genetically similar to each other and as a consequence showed more similar restriction patterns. Two isolates from 1 animal with symptoms of NCD (isolates AD1.5 and AD1.6) were considered indistinguishable from each other (figure 1). STEC isolates AC3.1 and AC3.10 from an asymptomatic animal were considered indistinguishable from each other (figure 1). Additionally, isolates AD1.7 and AD 7.2 coming from different animals but from the same farm, were considered identical (figure 1).

PHENOTYPIC AND GENOTYPIC ANTIMICROBIAL RESISTANCE OF STEC

STEC isolates were mostly susceptible to antibiotics. Ten out of 16 (62.5%) of the isolates were resistant to AMP, whereas 4/16 (25%) were susceptible to all antibiotics tested. Ten STEC isolates were resistant to beta-lactams (10 isolates were resistant to ampicillin), 3 STEC isolates were resistant to ciprofloxacin and 2 STEC isolates were resistant to fosfomycin trometamol (table 1). The isolate from the ileum sample was the only one resistant to 3 antibiotics: ampicillin, gentamicin and fosfomycin trometamol, whereas 2 STEC isolates were ciprofloxacin- ampicillin-resistant (table 1).

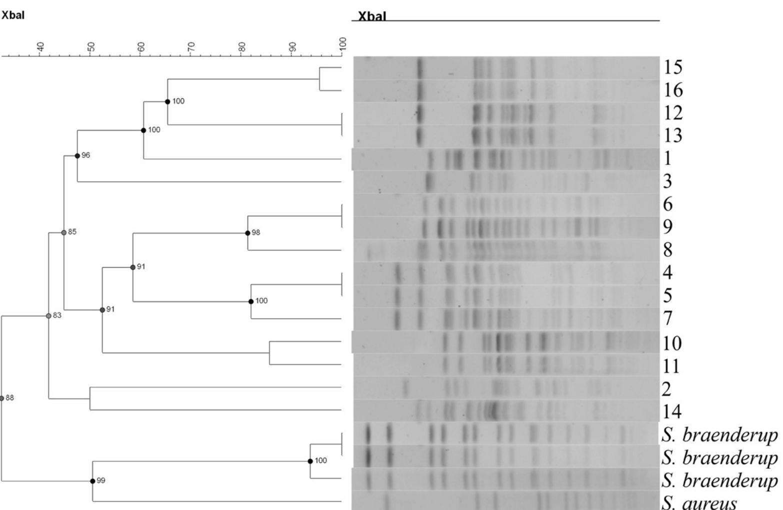
PMQR genes were evaluated in 3 isolates that showed resistance or intermediate susceptibility to CIP: AD1.5, AD1.6 and AC3.1 (table 1). None of the 7 PMQR genes was detected. Otherwise, FOT gene *fosA7* was detected in 1 STEC isolate: 16.16 (table 1).

DISCUSSION

STEC infections in humans constitute a global health concern and are endemic in Latin America, accounting for almost 2% of acute diarrhoea cases and 20%-30% of bloody diarrhoea (Torres *et al.*, 2018). STEC colonises the gastrointestinal tract of cattle, which are mainly asymptomatic carriers, and only under specific circumstances can develop diarrhoea. It can adapt and survive in different environments such as soil and water and contaminates food, meat, and dairy products, through which it can reach and infect other animals and humans (Daly & Hill, 2016). Non-O157 STEC isolates have increasingly been reported associated with human outbreaks (Bettelheim & Goldwater, 2014).

The severity of STEC infections is determined by the interaction of both host and microorganism factors. Regarding the bacterial virulence profile, all detected Stx subtypes in the present study are linked to illness in humans (EFSA BIOHAZ Panel, 2020). We detected *stx1a+c* (*n*=8) and *stx1a* (*n*=6) genotypes in high proportion amongst isolates, which were similar to previous reports in non-O157:H7 STEC isolates from cattle faeces in the United States (Shridhar *et al.*, 2017) and the occurrence of *stx1a* together with *stx2a*, *stx2c*, or *stx2d* have also been described in STEC isolates from bovine origin in China (Fan *et al.*, 2019). In this study, *stx1a/stx2e* and *stx1a+c/stx2e* genotypes were detected. Stx2e is associated with mild gastroenteritis in humans, while it is the most frequent Stx subtype in pigs, wild boars, and their meat products (Beutin *et al.*, 2008). Additionally, *stx2a* gene variant, widely associated with HUS, was detected in one isolate from an animal with signs of NCD. This is the first report of the presence of this variant in non-O157 STEC isolates from calf origin in our country.

Autotransporter protein Ag43 has been linked to pathogenic O157 and non-O157 *E. coli* strains and is associated with autoaggregation, cell-cell interaction with the host and biofilm formation (Matheus-Guimarães *et al.*, 2014). It has been observed that the main role of Ag43 depends on the presence of other virulence factors like adhesins, and the genetic background of the strain (Carter *et al.*, 2017). In this work, both Ag43 subfamilies were detected. Subfamily II (the Calcium-binding Antigen43



Isolate	Signs	Virotyping	Phylogroup	Serogroup	Antibiotic resistance profiles	Resistance genes
15 (BJ 1.5)	NCD	*stx1a+c, stx2e, ag43(II)*	B1	O103	AMP	---
16 (BJ 1.10)	NCD	*stx1a, stx2e, ag43(II)*	B1	O103	AMP	---
12 (AC 3.1)	No NCD signs	*stx1a+c, ag43(II)*	B1	O103	Sensitive	CIP
13 (AC 3.10)	No NCD signs	*stx1a, ag43(II)*	B1	O103	---	---
1 (74.2)	NCD	*stx1a, ag43(II)*	B1	O111	---	---
3 (AG 2.1)	NCD	*stx2a*	A	N/D	FOT	*fosA7*
6 (AD 1.7)	NCD	*stx1a+c*	B1	N/D	AMP	---
9 (AD 7.2)	NCD	*stx1a*	B1	O111	AMP	---
8 (AD 3.2)	NCD	*stx1a+c, ag43(II)*	B1	O111	AMP	---
4 (AD 1.5)	NCD	*stx1a+c, ag43(I)*	E	N/D	AMP/CIP	---
5 (AD 1.6)	NCD	*stx1a, ag43(I)*	F	O111	AMP/CIP	---
7 (AD 1.9)	NCD	*stx1a+c*	E	O111	AMP	---
10 (AD 7.5)	NCD	*stx1a*	C	O111	AMP	---
11 (AD 7.10)	NCD	*stx1a, ag43(II)*	E	N/D	---	---
2 (16.16)	NCD_n	*stx1a+c*	A	N/D	AMP/CN/FOT	---
14 (BJ 1.3)	NCD	*stx1a, stx2e, ag43(II)*	E	O103	---	---
S. braenderup						
S. braenderup						
S. braenderup						
S. aureus						

Figure 1. Phylogenetic relationships of STEC isolates. The phylogenetic tree was generated using the UPGMA method, using the Dice coefficient with a 1.0% of band position tolerance (BioNumerics v.6.6, Applied Maths, Sint-Martens-Latem, Belgium). *Salmonella* Braenderup and *Staphylococcus aureus* were used as reference strains.

Homologue, Cah) was detected in 14 isolates, while two STEC isolates were Ag43 subfamily I, positive. It has been observed that the gene that codes for subfamily II (*cah*) has a high mutation rate, which is associated with the adaptability of STEC to different environments (Carter *et al.*, 2017). On the other hand, Ag43 subfamily I, is not present in LEE+ strains since it is encoded in LAA pathogenicity island (Locus of Adhesion and Autoaggregation) (Montero *et al.*, 2017). Primers to detect both subfamilies used in this work were designed based on a difference of 270bp between them, and subfamily I could not be found in STEC LEE+ isolates. Therefore, it is probable that our STEC LEE+ isolates present heterogenicity among this gene, which may be due to distinct functions that could favour an in-host or environmental status.

The major serogroup associated with STEC infections in humans still is *E. coli* O157:H7, the first serogroup recognised causing enteric bloody diarrhoea (EFSA BIOHAZ Panel, 2020). However, other STEC serogroups are now recognised as important food and water-borne pathogens. Among them, the “big six” group of strains is frequently detected in HUS cases (Shridhar *et al.*, 2017). In this work, O103 and O111 were the only serogroups detected. Five STEC isolates were ascribed to the O103 serogroup (three isolates from a bovine with NCD signs and two isolates from a calf without signs) whereas six isolates were ascribed to O111. The remaining five isolates could not be assigned to any of the evaluated serogroups (31.3% of the isolates). Considering that there are over 1150 published STEC serotypes, these isolates probably could be assigned to other than the “big six”. O103 and O111 serogroups have been previously determined in cattle (Thomas *et al.*, 2012, Bibbal *et al.*, 2015, Jajarmi *et al.*, 2017, Rivelli Zea *et al.*, 2020), their faeces (Blanco *et al.*, 2004, Cernicchiaro *et al.*, 2013) and carcasses (Cap *et al.*, 2019), in countries from South America and other regions. On the other hand, the isolation of STEC O111 in bloody diarrhoea cases has been reported in our country (Varela *et al.*, 2008), which demonstrates a previous circulation of this serogroup. Also, a positive correlation between the presence of the virulence-marker gene *eae* in non-O157 STEC and the occurrence of HUS have been established (Yang *et al.*, 2020b), an affirmation that agrees with the assumption that non-O157 STEC LEE+ isolates from this study could be harmful to human.

It is well known that *E. coli* isolates from different sources of isolation usually belong to different phylogroups (Clermont *et al.*, 2013). Therefore, it would be expected that the STEC isolates of bovine origin of this work all belong to the same phylogroup. However, five different phylogroups were determined. According to the molecular assignment, 50% (8/16) of the isolates were classified as B1. This observation is consistent with reports which indicate that B1 is mainly present in the microbiota of domestic animals, often associated with intestinal commensal and pathogenic *E. coli* (Tenaillon *et al.*, 2010). The second most frequently detected phylogroup was E (4/16). It has recently been reported that this phylogroup predominantly includes O157:H7 strains (Tenaillon *et al.*, 2010). The A phylogroup has been also associated with commensal/ intestinal pathogens, and in this work was assigned to 2/16 STEC isolates. Finally, C and F phylogroups were each represented with 1/16 of the isolates. Both phylogroups have been proposed to be sister groups of B1 and B2, respectively (Clermont *et al.*, 2013).

When the clonal relatedness of the isolates was analysed, a high level of diversity amongst isolates was observed. PFGE profiles showed 13 distinct restriction patterns out of 16 STEC isolates. We only found two indistinguishable band patterns within animals (the pair of isolates: AD1.5 and AD1.6 / AC3.1 and AC3.10 came each from one animal), and within a bovine herd (isolates AD1.7 and AD 7.2 came from different animals but from the same herd). Further, more than one band pattern was detected within bovine herds. When we looked out serogroups, we detected that STEC isolates ascribed to the O103 were closely related; and those ascribed to the O111 serogroup were closely related too. Similar results from PFGE analyses were previously determined in STEC isolates from cattle origin (Bibbal *et al.*, 2015, Bumunang *et al.*, 2019), which reaffirms the role of bovine as non-O157 STEC strains reservoirs.

It has been observed that antibiotic resistance in non-O157 STEC isolates from animal origin is higher than in O157 STEC strains (Mir & Kudva, 2019). Likewise, multidrug resistance is frequently associated with non-O157 STEC strains with *eae*+/*stx1*+ virulence profiles (Mora *et al.*, 2015). In the present study, 11 antibiotics were evaluated using the Kirby-Bauer method. AMP resistance was the most frequent amongst isolates (62.5%), and it was determined in the following arrangements: AMP (*n*=7), AMP/CIP (*n*=2), and AMP/CN/FOT (*n*=1) being the last one the only isolate considered MDR. Finally, one isolate was only FOT^R and 1 isolate was only CIP^R. The high percentage of resistance to AMP agrees with the fact that β-lactams are the most used antibiotics in animals and with previous reports by our group, although the antibiotic resistance profiles then were considerably higher in numbers and included resistance to cephalosporins and trimethoprim-sulfamethoxazole among others (Umpiérrez *et al.*, 2017, 2021). Even though isolates were in general susceptible to the tested antibiotics, 5/12 of the resistant isolates were also classified as O111 and 3/12 were classified as O103. In previous works, O111 serogroup has been associated with multidrug-resistant non-O157, *eae*+ STEC isolates (Mora *et al.*, 2015). Particularly it has been associated with resistance levels higher than 25% to ampicillin, amoxicillin-clavulanic acid, cephalothin, trimethoprim-sulfamethoxazole, tetracycline, chloramphenicol and streptomycin. Meanwhile, O103 STEC isolates have been associated with low resistance levels, except for trimethoprim-sulfamethoxazole, tetracycline and streptomycin (Schroeder *et al.*, 2002, Amézquita-López *et al.*, 2016). Even though non-O157 STEC isolates of this

study were mainly susceptible to antibiotics, it is important to state that they were screened from a large collection of *E. coli* isolates, in which the percentage of antibiotic resistance is significant (Umpiérrez *et al.*, 2017). In that collection, MDR isolates represented a substantial source for antibiotic resistance genes. In this study, we also detected the presence of the *fosA7* gene (STEC isolate 16.16), which confers resistance to fosfomycin. The presence of this gene has been reported in environmental *E. coli* isolates and *Salmonella* spp. from animal and human origin (Rehman *et al.*, 2017, Balbin *et al.*, 2020). Resistance to fosfomycin is a major concern in human health. In our country, on the one hand, it is within the scarce therapeutic resources available for infections of multi-resistant microorganisms (Seija *et al.*, 2015) and, on the other, it is one of the first therapeutic options for urinary tract infections (García-Fulgueiras *et al.*, 2021). Recently, we have reported the presence of *fosA3* in animals belonging to the food-production chains (Coppola *et al.*, 2020). With this scenario, the detection of *fosA7* in production chain animals reinforces the need to monitor the presence of this mechanism with a One Health concept. To the best of our knowledge, this is the first study to report on the *fosA7* gene in STEC from bovines in Uruguay. Considering the critically important antimicrobials for human medicine list published periodically by WHO (World Health Organization & WHO Advisory Group on Integrated Surveillance of Antimicrobial Resistance, 2017), dissemination of resistance to such antibiotics through non-O157 STEC isolates of calf origin did not seem to be of human or animal health concern.

It is concluded that studies encompassing molecular characterisation of non-O157 STEC of calf origin are on the rise, due to an increase in detecting these serogroups associated with severe disease in humans. Although in this study the number of evaluated isolates from cattle was low, the virulence profiles, including the confirmation of Shiga toxin subtypes *stx1a* and *stx2a*, and serogroups O103 and O111 (the last one, has been reported in human infections in our country), denote the harmful potential of them for humans. We also observed some genetic heterogeneity among the Ag43 gene, which could be associated with adaptation to different niches. Finally, circulating non-O157 STEC LEE+ from cattle faeces were not from one cluster only and showed high genetic heterogeneity, being in general susceptible to antibiotics. Further investigations with a higher number of STEC are needed to confirm these observations.

The spread of STEC isolates to the environment through bovine faeces is always a source of concern to human health, but also represents a way to contaminate the dairy environment, which increases the probability of bovine infections within a herd. Results from this study call for attention regarding the virulence profile and transferable resistance genes of non-O157 STEC LEE+ isolates, that could cause severe disease to humans and denote the essentiality of determining how they persist and transmit in the dairy environment.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing interests.

ETHICS STATEMENT

No ethical approval was required in this work, as this is an original article with only bacterial isolates and data. No animal or human samples were employed.

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REFERENCES

Amézquita-López, B.A., Quiñones, B., Soto-Beltrán, M., Lee, B.G., Yambao, J.C., Lugo-Melchor, O.Y., & Chaidez, C. (2016). Antimicrobial resistance profiles of Shiga toxin-producing *Escherichia coli* O157 and non-O157 recovered from domestic farm animals in rural communities in Northwestern Mexico. *Antimicrobial Resistance & Infection Control, 5*(1). https://doi.org/10.1186/s13756-015-0100-5

Bai, X., Fu, S., Zhang, J., Fan, R., Xu, Y., Sun, H., He, X., Xu, J., & Xiong, Y. (2018). Identification and pathogenomic analysis of an *Escherichia coli* strain producing a novel Shiga toxin 2 subtype. *Scientific Reports, 8*, 6756. 10.1038/s41598-018-25233-x

Balbin, M.M., Hull, D., Guest, C., Nichols, L., Dunn, R., Hull, D., & Siddhartha, T. (2020). Antimicrobial resistance and virulence factors profile of *Salmonella* spp. and *Escherichia coli* isolated from different environments exposed to anthropogenic activity. *Journal of Global Antimicrobial Resistance*, *22*, 578-583. https://doi.org/10.1016/j.jgar.2020.05.016

Bettelheim, K., & Goldwater P. 2014. Serotypes of Non-O157 Shigatoxigenic *Escherichia coli* (STEC). *Advances in Microbiology*, *4*, 377-389. https://doi.org/10.4236/aim.2014.47045

Beutin, L., Krüger, U., Krause, G., Miko, A., Martin, A., & Strauch, E. (2008). Evaluation of major types of Shiga toxin 2E-producing *Escherichia coli* bacteria present in food, pigs, and the environment as potential pathogens for humans. *Applied and Environmental Microbiology*, *74*(15), 4806-4816. https://doi.org/10.1128/AEM.00623-08

Bibbal, D., Loukiadis, E., Kérourédan, M., Ferré, F., Dilasser, F., Peytavin de Garam, C., Cartier, P., Oswald, E., Gay, E., Auvray, F., & Brugère, H. (2015). Prevalence of carriage of Shiga toxin-producing *Escherichia coli* serotypes O157:H7, O26:H11, O103:H2, O111:H8, and O145:H28 among slaughtered adult cattle in France. *Applied and Environmental Microbiology*, *81*(4), 1397-1405. https://doi.org/10.1128/AEM.03315-14

Blanco, M., Padola, N.L., Krüger, A., Sanz, M.E., Blanco, J.E., González, E.A., Ghizlane, D., Azucena, M., Bernárdez, M.I., Etcheverría, A.I., Arroyo, G.H., Lucchesi, P.M.A., Parma, A.E., & Blanco, J. (2004). Virulence genes and intimin types of Shiga-toxin-producing

Escherichia coli isolated from cattle and beef products in Argentina. *International Microbiology*, *7*(4), 269-276.

Bumunang, E.W., McAllister, T.A., Zaheer, R., Polo, R.O., Stanford, K., King, R., Niu, Y.D., & Ateba, C.N. (2019). Characterization of non-O157 *Escherichia coli* from cattle faecal samples in the North-West Province of South Africa. *Microorganisms*, *7*(8), 272. https://doi.org/10.3390/microorganisms7080272

Cap, M., Carbonari, C.C., D'Astek, B.A., Zolezzi, G., Deza, N., Palladino, M.P., Masana, M., Chinen, I., & Rivas, M. (2019). Frequency, characterization and genotypic analysis of Shiga toxin-producing *Escherichia coli* in beef slaughterhouses of Argentina. *Revista Argentina de Microbiología*, *51*(1), 32-38. https://doi.org/10.1016/j.ram.2018.03.005

Carter, M.Q., Brandl, M.T., Kudva, I.T., Katani, R., Moreau, M.R., & Kapur, V. (2017). Conditional function of autoaggregative protein cah and common *cah* mutations in Shiga toxin-producing *Escherichia coli*. *Applied and Environmental Microbiology*, *84*(1), e01739-17. https://doi.org/10.1128/AEM.01739-17

Cernicchiaro, N., Cull, C.A., Paddock, Z.D., Shi, X., Bai, J., Nagaraja, T.G., & Renter, D.G. (2013). Prevalence of Shiga toxin-producing *Escherichia coli* and associated virulence genes in feces of commercial feedlot cattle. *Foodborne Pathogens and Disease*, *10*(10), 835-841. https://doi.org/10.1089/fpd.2013.1526

Clermont, O., Christenson, J.K., Denamur, E., & Gordon, D.M. (2013). The Clermont *Escherichia coli* phylo-typing method revisited: improvement of specificity and detection of new phylo-groups. *Environmental Microbiology Reports*, *5*(1), 58-65. https://doi.org/10.1111/1758-2229.12019

Clinical and Laboratory Standards Institute. (2017). *Performance Standards for Antimicrobial Susceptibility Testing*. CLSI supplement M100.

Cóppola, N., Freire, B., Umpiérrez, A., Cordeiro, N.F., Ávila, P., Trenchi, G., Castro, G., Casaux, M.L., Fraga, M., Zunino, P., Bado, I., & Vignoli, R. (2020). Transferable resistance to highest priority critically important antibiotics for human health in *Escherichia coli* strains obtained from livestock feces in Uruguay. *Frontiers in Veterinary Science*, *7*, 588919. https://doi.org/10.3389/fvets.2020.588919

Daly, R.F., & Hill, N.T. (2016). Characterizing the role of animal exposures in cryptosporidiosis and Shiga toxin-producing *Escherichia coli* infections: South Dakota, 2012. *Zoonoses and Public Health*, *63*(6), 467-476.

EFSA BIOHAZ Panel. (2020). Pathogenicity assessment of Shiga toxin-producing *Escherichia coli* (STEC) and the public health risk posed by contamination of food with STEC. *EFSA Journal 18*, e05967. https://doi.org/10.2903/j.efsa.2020.5967

Fan, R., Shao, K., Yang, X., Bai, X., Fu, S., Sun, H., Xu, Y., Wang, H., Li, Q., Hu, B., Zhang, J., & Xiong, Y. (2019). High prevalence of non-O157 Shiga toxin-producing *Escherichia coli* in beef cattle detected by combining four selective agars. *BMC Microbiology*, *19*(1), 213. https://doi.org/10.1186/s12866-019-1582-8

Gadea, M., Deza, N., Mota, M.I., Carbonari, C., Robatto, M., D`Astek, B., Balseiro, V., Bazet, C., Rügnitz, E., Livrelli, V., Schelotto, F., Rivas, M., & Varela, G. (2012). Two cases of urinary tract infection caused by Shiga toxin-producing *Escherichia coli* O157:H7 strains. *Revista Argentina de Microbiología*, *44*(2), 94-96.

García-Fulgueiras, V., Caiata, L., Bado, I., Giachetto, G., Robino, L. (2021). Antibiotic susceptibility and fosfomycin resistance characterization in a cohort of children older than 6 years of age with urinary tract infection. *Revista Argentina de Microbiología*. https://doi.org/10.1016/j.ram.2021.04.002

Gyles, J.M., & Fairbrother, F.C.L. (2010). *Escherichia coli*. In C.L. Gyles, J.F. Prescott, J.G. Songer, & C.O. Thoen, (Eds.), *Pathogenesis of Bacterial Infections in Animals* (pp. 276-308). Wiley-Blackwell.

Jajarmi, M., Imanifooladi, A.A., Badouei, M.A., & Ahmadi, A. (2017). Virulence genes, Shiga toxin subtypes, major O-serogroups, and phylogenetic background of Shiga toxin-producing *Escherichia coli* strains isolated from cattle in Iran. *Microbial Pathogenesis*, *109*, 274-279. https://doi.org/10.1016/j.micpath.2017.05.041

Kintz, E., Brainard, J., Hooper, L., & Hunter, P. (2017). Transmission pathways for sporadic Shiga-toxin producing *E. coli* infections: A systematic review and meta-analysis. *International Journal of Hygiene and Environmental Health*, *220*(1), 57-67. https://doi.org/10.1016/j.ijheh.2016.10.011

Kjaergaard, K., Schembri, M.A., Hasman, H., & Klemm, P. (2000). Antigen 43 from *Escherichia coli* induces inter- and intraspecies cell aggregation and changes in colony morphology of *Pseudomonas fluorescens*. *Journal of Bacteriology*, *182*(17), 4789-4796. https://doi.org/10.1128/JB.182.17.4789-4796.2000

Majowicz, S.E., Scallan, E., Jones-Bitton, A., Sargeant, J.M., Stapleton, J., Angulo, F.J., Yeung, D.H., & Krik, M.D. (2014). Global incidence of human Shiga toxin-producing *Escherichia coli* infections and deaths: a systematic review and knowledge synthesis. *Foodborne Pathogens and Disease*, *1*, 447-455.

Marshall, B.M., & Levy, S.B. (2011). Food Animals and Antimicrobials: Impacts on Human Health. *Clinical Microbiology Reviews, 24*(4), 718-733. https://doi.org/10.1128/CMR.00002-11

Matheus-Guimarães, C., Gonçalves, E.M., & Guth, B.E.C. (2014). Interactions of O157 and non-O157 Shiga toxin-producing *Escherichia coli* (STEC) recovered from bovine hide and carcass with human cells and abiotic surfaces. *Foodborne Pathogens and Disease*, *11*(3), 248-255. https://doi.org/10.1089/fpd.2013.1653

Mir, R.A., & Kudva, I.T. (2019). Antibiotic-resistant Shiga toxin-producing *Escherichia coli*: An overview of prevalence and intervention strategies. *Zoonoses and Public Health*, *66*(1), 1-13.https://doi.org/10.1111/zph.12533

Montero, D.A., Velasco, J., Del Canto, F., Puente, J.L., Padola, N.L., Rasko, D.A., Farfán, M., Salazar, J.C., & Vidal, R. (2017). Locus of Adhesion and Autoaggregation (LAA), a pathogenicity island present in emerging Shiga Toxin-producing *Escherichia coli strains*. *Scientific Reports*, *7*, 7011. https://doi.org/10.1038/s41598-017-06999-y

Mora, A., Blanco, J.E., Blanco, M., Alonso, M.P., Dhabi, G., Echeita, A., González, E.A., Bernárdez, M.I., & Blanco, J. (2005). Antimicrobial resistance of Shiga toxin (verotoxin)-producing *Escherichia coli* O157:H7 and non-O157 strains isolated from humans, cattle, sheep and food in Spain. *Research in Microbiology*, *156*(7), 793-806. https://doi.org/10.1016/j.resmic.2005.03.006

Paddock, Z., Shi, X., Bai, J., & Nagaraja, T.G. (2012). Applicability of a multiplex PCR to detect O26, O45, O103, O111, O121, O145, and O157 serogroups of *Escherichia coli* in cattle feces. *Veterinary Microbiology*, *156*(3-4), 381-388. https://doi.org/10.1016/j.vetmic.2011.11.017

Patel, J.B. (2014). *Performance Standards for Antimicrobial Susceptibility Testing* (24th ed). Clinical and Laboratory Standards Institute.

Pérez, L., Apezteguía, L., Piñeyrúa, C., Dabezies, A., Bianco, M.N., Schelotto, F., & Varela, G. (2014). Hemolytic uremic syndrome with mild renal involvement due to Shiga toxin-producing *Escherichia coli* (STEC) O145 strain. *Revista Argentina de Microbiología*, *46*(2), 103-106. https://doi.org/10.1016/S0325-7541(14)70056-2

Rehman, M.A., Yin, X., Persaud-Lachhman, M.G., & Diarra, M.S. (2017). First detection of a fosfomycin resistance gene, *fosA7*, in *Salmonella enterica* serovar Heidelberg isolated from broiler chickens. *Antimicrobial Agents and Chemotherapy*, *61*(8), e00410-17. https://doi.org/10.1128/AAC.00410-17

Rivas, M., Chinen, I., Miliwebsky, E., & Masana, M. (2014). Risk Factors for Shiga Toxin-Producing *Escherichia coli*-Associated Human Diseases. *Microbiology Spectrum*, *2*(5). https://doi.org/10.1128/microbiolspec.EHEC-0002-2013

Rivelli Zea, S.M., Padola, N.L., Etcheverría, A.I, Florentín, M., Acuña, P., Rodríguez, F., Colello, R., & Guillén Fretes, R.M. (2020). Caracterización molecular de aislamientos de *Escherichia coli* productores de toxina Shiga obtenidos en 2 establecimientos ganaderos del Paraguay. *Revista Argentina de Microbiología*, *52*(2), 131-135. https://doi.org/10.1016/j.ram.2019.07.001

Sapountzis, P., Segura, A., Desvaux, M., & Forano, E. (2020). An overview of the elusive passenger in the gastrointestinal tract of cattle: The

Shiga toxin producing *Escherichia coli*. *Microorganisms*, *8*(6), 877. https://doi.org/10.3390/microorganisms8060877

Scheutz, F., Teel, L.D., Beutin, L., Piérard, D., Buvens, G., Karch, H., Mellmann, A., Caprioli, A., Tozzoli, R., Morabito, S., Strockbine, N.A., Melton-Celsa, A.R., Sanchez, M., Persson, S., & O'Brien, A.D. (2012). Multicenter evaluation of a sequence-based protocol for subtyping Shiga toxins and standardizing Stx nomenclature. *Journal of Clinical Microbiology*, *50*(9), 2951-2963. https://doi.org/10.1128/JCM.00860-12

Scheutz, F. (2014). Taxonomy meets public health: The case of Shiga toxin-producing *Escherichia coli*. *Microbiology Spectrum*, *2*. https://doi.org/0.1128/microbiolspec.EHEC-0019-2013

Schroeder, C.M., Meng, J., Zhao, S., DebRoy, C., Torcolini, J., Zhao, C., McDermott, P.F., Wagner, D.D., Walker, R.D., & White, D.G. (2002). Antimicrobial resistance of *Escherichia coli* O26, O103, O111, O128, and O145 from animals and humans. *Emerging Infectious Diseases*, *8*(12), 1409-1414. https://doi.org/10.3201/eid0812.0200770

Seija, V., Medina Presentado, J.C., Bado, I., Papa Ezdra, R., Batista, N., Gutiérrez, C., Guirado, M., Vidal, M., Nin, M., & Vignoli, R. (2015). Sepsis caused by New Delhi metallo-β-lactamase (bla_{NDM-1}) and *qnrD*-producing *Morganella morganii*, treated successfully with fosfomycin and meropenem: case report and literature review. *International Journal of Infectious Diseases*, *30*, 20-26. https://doi.org/10.1016/j.ijid.2014.09.010

Shridhar, P.B., Siepker, C., Noll, L.W., Shi, X., Nagaraja, T.G., & Bai, J. (2017). Shiga toxin subtypes of non-O157 *Escherichia coli* serogroups isolated from cattle feces. *Frontiers in Cellular and Infection Microbiology*, *7*. https://doi.org/10.3389/fcimb.2017.00121

Tenaillon, O., Skurnik, D., Picard, B., & Denamur, E. (2010). The population genetics of commensal *Escherichia coli*. *Nature Reviews Microbiology*, *8*(3), 207-217. https://doi.org/10.1038/nrmicro2298

Thomas, K.M., McCann, M.S., Collery, M.M., Logan, A., Whyte, P., McDowell, D.A., & Duffy, G. (2012). Tracking verocytotoxigenic *Escherichia coli* O157, O26, O111, O103 and O145 in Irish cattle. *International Journal of Food Microbiology 153*(3), 288-296. https://doi.org/10.1016/j.ijfoodmicro.2011.11.012

Torres, A.G., Amaral, M.M., Bentancor, L., Galli, L., Goldstein, J., Krüger, A., & Rojas-López, M. (2018). Recent advances in Shiga toxin-producing research in Latin America. *Microorganisms*, *6*(4), 100. https://doi.org/10.3390/microorganisms6040100

Umpiérrez, A., Bado, I., Oliver, M., Acquistapace, S., Etcheverría, A.L., Padola, N.L., Vignoli, R., & Zunino, P. (2017). Zoonotic Potential and Antibiotic Resistance of *Escherichia coli* in Neonatal Calves in Uruguay. *Microbes Environ*, *32*(3), 275-282. https://doi.org/10.1264/jsme2.ME17046

Umpiérrez, A., Ernst, D., Fernández, M., Oliver, M., Casaux, M.L., Caffarena, R.D., Schild, C., Giannitti, F., Fraga, M., & Zunino, P. (2021). Virulence genes of *Escherichia coli* in diarrheic and healthy calves. *Revista Argentina de Microbiología*, *53*(1), 34-38. https://doi.org/10.1016/j.ram.2020.04.004

Varela, G., Chinen, I., Gadea, M.P., Miliwebsky, E., Mota, M.I., González, S., González, G., Gugliada, M.J., Carbonari, C.C., Algorta, G., Bernadá, M., Sabelli, R., Pardo, L., Rivas, M., & Schelotto, F. (2008). Detección y caracterización de *Escherichia coli* productor de toxina Shiga a partir de casos clínicos y de alimentos en Uruguay. *Revista Argentina de Microbiología*, *40*(2), 93-100.

World Health Organization & WHO Advisory Group on Integrated Surveillance of Antimicrobial Resistance. (2017). Critically important antimicrobials for human medicine: ranking of antimicrobial agents for risk management of antimicrobial resistance due to non-human use, 5th rev. World Health Organization. https://apps.who.int/iris/handle/10665/255027.

Yang, X., Bai, X., Zhang, J., Sun, H., Fu, S., Fan, R., He, X., Scheutz, F., & Matussek, A. (2020a). *Escherichia coli* strains producing a novel Shiga toxin 2 subtype circulate in China. *International Journal of Medical Microbiology*, *310*(1), 151377. https://doi.org/10.1016/j.ijmm.2019.151377

Yang, X., Sun, H., Fan, R., Fu, S., Zhang, J., Matussek, A., Xiong, Y., & Bai, X. (2020b). Genetic diversity of the intimin gene (*eae*) in non-O157 Shiga toxin-producing *Escherichia coli* strains in China. *Scientific Reports*, *10*, 3275. https://doi.org/10.1038/s41598-020-60225-w

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ORIGINAL ARTICLE

Captive collared peccary carries ESBL-producing diarrheagenic *Escherichia coli* pathotypes

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ABSTRACT. Collared peccaries (*Pecari tajacu*) roam the forests of natural areas in America. Wild collared peccary appears to be a carrier for bacteria associated with infections in humans and animals, however, the presence of diarrheagenic *E. coli* (DEC) pathotypes has not been studied in the captive collared peccary. This study aimed to study the prevalence of DEC, the susceptibility to antibiotics, and the frequency of β-lactamase genes (ESBL) in captive collared peccary faeces. DEC strains were identified in 44.4% (N=56) of the *E. coli*-carrying peccaries under study. The following DEC strains were identified: ETEC (35.7%), EAEC (28.6%), STEC (21.4%) and EPEC (14.3%). Most of the identified DEC strains belonged to clade I (58.9%). The genetic marker *rfbO157* was not found in any STEC strain. Of the DEC strains, 67.9% (N=38) were considered multidrug resistant and were not susceptible to ampicillin (75%) nor to carbenicillin (51.8%). The combination of the genes blaTEM + blaCTX and blaTEM + blaSHV (6 strains respectively) was the most frequent among the DEC strains. It is concluded that captive collared peccaries are carriers of DEC strains that carry β-lactamase blaTEM, blaCTX and blaSHV genes and are not susceptible to ampicillin. Given the current efforts of the Wildlife Management Units (WMU) to reintroduce the collared peccary into natural areas, these captive collared peccaries act as carriers of diarrheagenic *E. coli* strains and therefore a potential source of gastrointestinal disease in humans and animals.

Key words: β-lactamase, carrier, diarrheagenic pathotypes, *Pecari tajacu*, wildlife management Unit.

INTRODUCTION

The collared peccary *Pecari tajacu* is a gregarious species distributed from the southeast of the United States to Argentina. It is considered a predator and a seed disperser in tropical forests, deserts, and deforested areas (Marinho *et al.*, 2019). Currently, the International Union for the Conservation of Nature (IUCN2019-1) and the Official Mexican Standard NOM-059-SEMARNAT-2010 (PROFEPA 2010) classify peccaries as a species of least concern (LC).

Collared peccaries roam the jungles and forests of Mexico. They are thus vulnerable to hunting and habitat destruction. Wildlife Management Units (WMU) have developed several strategies for the sustainable management of the species for the purpose of conservation, recovery, reproduction, and reintroduction (Sisk *et al.*, 2007). Previous studies have shown that wild collared peccaries can carry bacteria and viruses associated with infections in humans and domestic animals, such as *Clostridium perfringens* type A and multiple serotypes of *Salmonella* spp. (Shender *et al.*, 2009), shiga toxin-producing *Escherichia coli* (Jay-Russell *et al.*, 2014), *Mycobacterium hyopneumoniae*, *Pasteurella multocida*, circovirus type 2 (PCV2) and herpesvirus type 1 (SuHV) (de Castro *et al.*, 2014). The prevalence of *Leptospira* spp. (78%) in collared peccaries was recently reported, the most frequent serovars are *bratislava*, *grippotyphosa*, *icterohaemorrhagiae* and *pomona* (Montenegro *et al.*, 2018). Collared peccaries have also been found to harbour classical swine fever virus (5%), porcine circovirus type 2 (7%) and vesicular stomatitis (33%). They also share pathogens with domestic and wild pigs (*Sus scrofa*) (Montenegro *et al.*, 2018; Molina-Barrios *et al.*, 2018).

Although some efforts have been made to understand the role of captive collared peccaries as pathogen carriers (de Carvalho *et al.*, 2011), their role as carriers of diarrheagenic *Escherichia coli* (DEC) pathotypes is still unknown. The DEC group includes enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), Shiga toxin-producing *E. coli* (STEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC), DEC pathotypes are associated with gastrointestinal infections in humans and animals (Croxen *et al.*, 2013; Shabana *et al.*, 2013; Tamayo *et al.*, 2021). Enterohaemorrhagic *E. coli* (EHEC) is a subgroup of STEC strains associated with diarrhoea and haemorrhagic colitis, occasionally progressing to haemolytic uremic syndrome (HUS), which can have serious consequences in humans, including death (Oh *et al.*, 2016). *Escherichia coli* can contain several antibiotic resistance genes, including those that encode extended spectrum β-lactamases (ESBL) (Faridah

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et al., 2020). Therefore, understanding the mechanisms of *E. coli* dissemination is a necessary step in finding a solution to the growing worldwide incidence of multidrug resistant (MDR) and extensively resistant (XDR) bacterial strains (Taghadosi *et al.*, 2019).

This study aimed to identify the role of captive collared peccaries as a carrier of ESBL-producing diarrheagenic *Escherichia coli* pathotypes. This would allow designing sanitary measures to mitigate the potential of collared peccaries to spread pathogens of public health importance as a consequence of their management and a possible reintroduction to natural areas. The objective of this research was to study the prevalence of diarrheagenic *Escherichia coli* pathotypes that colonise the intestine of collared peccaries (*Pecari tajacu*) in captivity. In addition, this study aimed to find out if the DEC strains identified in captive collared peccaries are associated with antibiotic resistance and, if so, the genes involved. To do this, the susceptibility of DEC strains to commonly used antibiotics and the frequency of genes that encode extended spectrum β-lactamases (ESBL) were determined.

MATERIAL AND METHODS

PECCARY SAMPLING

Between the autumn of 2017 and the summer of 2019, 140 clinically healthy collared peccaries with no record of antibiotic therapy were selected among a group kept in 11 intensive wildlife management units (WMU) in the state of Chiapas, Mexico. The specimens of these WMU were obtained by purchase, donation, under protection or loan, and their origin may be wild or captivity. Animal studies were approved by the Ethics Committee of the University of Sciences and Arts of the state of Chiapas (approval #049/02-2018).

In the WMU, an average of 25 peccaries were kept in roofless cages averaging 8 x 24 meters in size, with floors made of dirt, logs or twigs, and walls commonly made of stone and steel mesh. Their daily diet consisted mainly of seasonal agricultural waste and food waste from homes, restaurants and markets: ears of corn, plant waste such as rhizomes (ginger, sugar cane, bamboo, among others), tubers (potato, cassava, sweet potato, carrot, jicama, radishes, among others), bulbs (onion, leek, garlic, among others) and fruits (cucumber, tomato, papaya, watermelon, among others); water was supplied *ad libitum* in concrete or wood drinking troughs.

ISOLATION OF *E. COLI* AND IDENTIFICATION OF DEC STRAINS

The peccaries were transferred to a wooden corridor (average measurements: 1 m high, 1.20 m long and 60 cm wide) with a liftgate at each end to carry out the sampling. Faeces samples were collected by introducing a swab into the rectum of the animal, and then into agar gel medium or Stuart transport medium (Copan Diagnostics, Inc.) for transfer to the laboratory. The swabs were inoculated onto Eosin Agar and Methylene Blue (BD-BBI™) plates and vatted at 37 °C for 24 hours. Up to 10 lactose fermenting colonies were selected and analysed by standard biochemical tests. Plates of MacConkey Agar with sorbitol were used simultaneously to promote the growth of EHEC serotype O157: H7. The genetic identification of the *E. coli* strains was carried out by amplifying the *uid*A gene (Tsai *et al.*, 1993).

IDENTIFICATION OF DIARRHEAGENIC PATHOTYPES OF *E. COLI* BY POLYMERASE CHAIN REACTION (PCR)

In the PCR, the *E. coli* strain ATCC® 25922 TM was used as a negative control. The following strains were used as positive control: STEC EDL933 (O157: H7), EAEC 042 (044: H18), ETEC H10407 (O78: H11), EPEC E2348-69 (O127: H6) and EIEC E11 (O124NM). The strains were provided by Dr. Teresa Estrada García from CINVESTAV, Mexico, and were kept in the bacteriological collection of the University of Sciences and Arts of Chiapas (Universidad de Ciencias y Artes) (Gutiérrez-Jiménez *et al.*, 2015). To obtain the DNA, the selected colonies were suspended in 1 mL of deionized water and the preparation was boiled for 10 min. The suspension was centrifuged at 10,000 rpm for 5 min and the supernatant containing DNA was removed and stored at -80 °C. Specific primers were used to amplify the Enteroaggregative *E. coli* (EAEC) *aap*, *agg*R, and *AAprobe* genes using the technique described by Cerna (Cerna *et al.*, 2003).

For Shiga toxin-producing *E. coli* (STEC) (*stx*1 and *stx*2 genes), Enteropathogenic *E. coli* (EPEC) (*bfp*A and *eae*A genes), Enterotoxigenic *E. coli* (ETEC) (*lt* and *st* genes), and Enteroinvasive *E. coli* (EIEC) (*ial* gene), the targets and conditions used were those previously described (López-Saucedo *et al.*, 2003). Shiga toxin-producing *E. coli* O157 was studied by amplifying the *rfb* gene (specific O-polysaccharide) (Paton & Paton, 1998).

The *E. coli* strains were classified by quadruplex PCR into seven phylogroups: A, B1, B2, C, D, E, F, and the crypto Clado I, based on the presence or absence of the genes *chuA*, *yjaA*, *arpA* and *trpA*, as well as the TSPE4 C2 DNA fragment (Clermont *et al.*, 2013). The amplification of the extended spectrum β-lactamase genes *blaTEM*, *blaSHV*, *blaCTXM*, *blaOXA* and *blaCMY* was carried out using the primers and conditions previously reported (Ahmed *et al.*, 2007). Table 1 shows the sequences of the primers and the PCR conditions used in this study.

ANALYSIS OF ANTIMICROBIAL SUSCEPTIBILITY

The following antimicrobial susceptibility discs (BD BBL™ Sensi-Disc™) were used with first-line antibiotics commonly used of different antimicrobial categories: β-lactam: ampicillin (AMP; 10 μg), carbenicillin (CAR;

Table1. Primers used in this study.

Primer pair	Sequence (5'-3')	Target	Encoded protein	Size (bp)	Reference
uidAF uidAR	AAAACGGCAAGAAAAAGCAG ACGCGTGGTTAACAGTCTTGCG	*uid*A	β-glucuronidase	147	(Tsai *et al.*, 1993)
aapF aapR	CTTGGGTATCAGCCTGAATG AACCCATTCGGTTAGAGCAC	aap	Aggregative adherence fimbria	310	(Cerna *et al.*, 2003)
aggF aggR	CTAATTGTACAATCGATGTA AGAGTCCATCTCTTTGATAAG	*agg*R	Transcriptional activator	457	(Cerna *et al.*, 2003)
AA probeF AA probeR	CTGGCGAAAGACTGTATCAT CAATGTATAGAAATCCGCTGTT	AA probe	Anti-aggregation protein transporter	629	(Cerna *et al.*, 2003)
ltFf ltRr	GGCGACAGATTATACCGTGC CGGTCTCTATATTCCCTGTT	lt	Heat-labile enterotoxin	450	(López-Saucedo *et al.*, 2003)
stFf stRr	ATT TTTCTTTCTGTATTGTCTT CACCCGGTACAAGCAGGATT	st	Heat-stable enterotoxin	190	(López-Saucedo *et al.*, 2003)
bfpAf bfpAr	AATGGTGCTTGCGCTTGCTGC GCCGCTTTATCCAACCTGGTA	*bfp*A	Bundle-forming pili	324	(López-Saucedo *et al.*, 2003)
eaeAf eaeAr	GACCCGGCACAAGCATAAGC CCACCTGCAGCA ACA AGA GG	*eae*A	Structural gene for intimin	384	(López-Saucedo *et al.*, 2003)
Ialf Ialr	GGT ATG ATG ATG ATG AGT CCA GGA GGC CAA CAA TTA TTT CC	ial	Invasion-associated locus	650	(López-Saucedo *et al.*, 2003)
Stx1F Stx1R	CTGGATTTAATGTCGCATAGTG AGAACGCCCACTGAGATCATC	*stx*1	Shiga toxin 1	150	(López-Saucedo *et al.*, 2003)
Stx2F Stx2R	GGCACTGTCTGAAACTGCTCC TCGCCAGTTATCTGACATTCTG	*stx*2	Shiga toxin 2	255	(López-Saucedo *et al.*, 2003)
O157F O157R	CGGACATCCATGTGATATGG TTGCCTATGTACAGCTAATCC	rfbO157	Polisacárido específico O	259	(Paton & Paton, 1998)
chuA.1b chuA.2	ATGGTACCGGACGAACCAAC TGCCGCCAGTACCAAAGACA	*chu*A	Membrane hemin receptor	288	(Clermont *et al.*, 2013)
yja.1b yja.2b	CAAACGTGAAGTGTCAGGAG AATGCGTTCCTCAACCTGTG	*yja*A	Stress response protein	211	(Clermont *et al.*, 2013)
ArpAgpE.f ArpAgpE.r	GATTCCATCTTGTCAAAATATGCC GAAAAGAAAAAGAATTCCCAAGAG	*arp*A	Regulator of acetyl CoA synthetase	301	(Clermont *et al.*, 2013)
trpAgpC.1 trpAgpC.2	AGTTTTATGCCCAGTGCGAG TCTGCGCCGGTCACGCCC	*trp*A	Operon leader peptide	219	(Clermont *et al.*, 2013)
trpBA.f trpBA.r	CGGCGATAAAGACATCTTCAC GCAACGCGGCCTGGCGGAAG	*trp*A (control interno)	Operon leader peptide	489	(Clermont *et al.*, 2013)
TSPE4C2 f TSPE4C2 r	CACTATTCGTAAGGTCATCC AGTTTATCGCTGCGGGTCGC	TspE4.C2	Anonymous DNA fragment	152	(Clermont *et al.*, 2013)
blaTEMf blaTEMr	ATAAAATTCTTGAAGACGAAA GACAGTTACCAATGCTTAATC	*bla*TEM	beta-lactamase TEM	1080	(Ahmed *et al.*, 2007)
blaSHVf blaSHVr	TTATCTCCCTGTTAGCCACC GATTTGCTGATTTCGCTCGG	*bla*SHV	beta-lactamase SHV	795	(Ahmed *et al.*, 2007)
blaCTXM f blaCTXM r	CGCTTTGCGATGTGCAG ACCGCGATATCGTTGGT	*bla*CTXM	beta-lactamase CTX M	550	(Ahmed *et al.*, 2007)
blaOXAf blaOXAr	TCAACTTTCAAGATCGCA GTGTGTTTAGAATGGTGA	*bla*OXA	beta-lactamase OXA	591	(Ahmed *et al.*, 2007)
blaCMYf blaCMYr	GACAGCCTCTTTCTCCACA TGGAACGAAGGCTACGTA	*bla*CMY	beta-lactamase CMY	1000	(Ahmed *et al.*, 2007)

100 µg) and oxacillin (OXA; 1 µg); aminoglycosides: amikacin (AMK; 30 µg), netilmicin (NET; 30 µg) and gentamicin (GEN; 10 µg); cephalosporins: cephalothin (CEF; 30 µg) and cefotaxime (CTX; 30 µg); quinolones: ciprofloxacin (CIP; 5 µg) and norfloxacin (NOR; 10 µg); phenicols: chloramphenicol (CHL; 30 µg); folate inhibitors: trimethoprim-sulfamethoxazole (SXT; 25 µg); furans: nitrofurantoin (NIT; 300 µg); Tetracyclines: Tetracycline (TET; 30 µg). β-lactam-resistant strains were subsequently analysed using the disc diffusion method with amoxicillin-clavulanic acid discs (AMC; 20/10 µg). The antibiotics and the disk diffusion method were used following the recommendations of the Clinical and Laboratory Standards Institute (Wayne, 2020). The *E. coli* strain ATCC® 25922 TM was used as a negative control and STEC EDL933 (O157: H7) as the positive control. *Escherichia coli* strains (including intermediate and resistant phenotypes) not susceptible to at least three antibiotics and from different antimicrobial categories were classified as multidrug resistant strains (MDR), while strains not susceptible to at least one antibiotic and belonging to each of the tested antimicrobial categories were classified as Extensively Drug-Resistant (XDR) (Magiorakos *et al.*, 2012).

STATISTICAL ANALYSIS

The prevalence of *E. coli* pathotypes, identified phylogenetic groups, resistance to antibiotics and the frequency of ESBL genes were analysed using descriptive statistics. The proportion of molecular markers among DEC strains was determined by binomial test. The two-tailed Fisher's exact test was used to test for associations between the categorical variables (when the expected frequencies were under 5), with a significance level of $P<0.05$. The statistical analysis was carried out using the IBM SPSS statistical package (Chicago SPSS Inc).

RESULTS

IDENTIFICATION OF DIARRHEAGENIC *E. COLI*

One hundred and twenty-six (90%) strains of *E. coli* were isolated from faecal samples of 140 captive collared peccaries. DEC strains were identified in 44.4% (N = 56) of the peccaries carrying *E. coli*. Among the DEC strains, ETEC (35.7%) was the category with the highest prevalence, followed by EAEC (28.6%), STEC (21.4%) and EPEC (14.3%). The PCR analysis showed a statistically significant prevalence for the ETEC and EAEC categories. No EHEC strain was detected on MacConkey/sorbitol agar plates, these strains did not carry the *eae* and *hlyA* genes, and the genetic marker *rfbO157* was not amplified by PCR in any of the STEC strains (table 2).

PCR was used to determine the phylogenetic group of DEC strains isolated in captive collared peccary. Among the identified strains of *E. coli* (N= 56), the highest number of DEC strains were grouped in Clade I (58.9%), followed by the phylogroup B2 (10.7%) and A (8.9%). Some DEC strains could not be assigned to any known phylogroup (21.4%).

Table 2. DEC strains and their virulence genes isolated in *Pecari tajacu* from Chiapas, Mexico.

Faeces samples with DEC pathotype: % (N)	Virulence gene: % (N)	P
ETEC: 35.7 (20)	*lt*, *st*: 30.3 (17)	0.001
	st: 3.6 (2)	
	lt: 1.8 (1)	
EAEC: 28.6 (16)	*aap*, AA probe: 25 (14)	0.01
	AA probe: 3.6 (2)	
STEC: 21.4 (12)	*stx*1: 12.5 (7)	0.08
	*stx*1 *stx*2: 8.9 (5)	
EPEC: 14.3 (8)	*bfpA*, *eaeA*: 10.7 (6)	0.15
	eaeA: 3.6 (2)	
Total: 100 (56)		

ANTIMICROBIAL SUSCEPTIBILITY OF DEC STRAINS

Three-thirds of the DEC strains were not susceptible to the antibiotic ampicillin (75%), while half of the strains were not susceptible to carbenicillin (51.8%). Resistance to ampicillin was also found in EAEC (81.3%), ETEC (80%), EPEC (62.5%) and STEC (50%) (table 3). Among the DEC strains not susceptible to ampicillin, 88.1% (N= 37) were susceptible to the antibiotic amoxicillin-clavulanic acid. Sixty-seven-point nine per cent (67.9%; N = 38) of the DEC strains were not susceptible to at least one antibiotic in three different categories of antimicrobials; thereby, these strains were considered Multidrug Resistant (MDR). No DEC strain was classified as Extensively Drug-Resistant (XDR). In general, most of the strains were susceptible mainly to chloramphenicol (94.6%), gentamicin (92.9%), amoxicillin-clavulanic acid (91.1%), sulfamethoxazole trimethoprim (89.3%) and tetracycline (87.5%).

The PCR was used to determine the frequency of genes encoding β-lactamase in the 42 DEC strains not susceptible to ampicillin. The combination of the genes blaTEM + blaCTX and blaTEM + blaSHV (6 strains respectively) was the most frequent among the DEC strains, followed by the combination of the blaTEM and blaCTX genes (4 strains respectively). The blaTEM (N=3) and blaCTX (N=3) genes were amplified more frequently among ETEC strains not susceptible to ampicillin. In the EAEC strains, both blaTEM + blaCTX genes were amplified (N=3). In STEC strains susceptible to ampicillin, both blaTEM + blaCTX and blaTEM + blaSHV genes were amplified (2 strains, respectively) (table 4).

Table 3. Antimicrobial non-susceptibility of DEC strains (56).

Antimicrobial	Diarrheagenic *Escherichia coli* pathotypes % non-susceptibility (n)				
	DEC	ETEC	EAEC	STEC	EPEC
	(N=56)	(N=20)	(N=16)	(N=12)	(N=8)
AMP	75 (42)	80 (16)	81.3 (13)	50 (6)	62.5 (5)
AMC	8.9 (5)	10 (2)	0	16.7 (2)	12.5 (1)
CAR	51.8 (29)	60 (12)	37.5 (6)	50 (6)	62.5 (5)
OXA	10.7 (6)	15 (3)	6.3 (1)	16.7 (2)	0
AMK	12.5 (7)	5 (1)	12.5 (2)	25 (3)	12.5 (1)
GEN	5.4 (3)	5 (1)	6.3 (1)	8.3 (1)	0
NET	33.9 (19)	45 (9)	31.3 (5)	25 (3)	25 (2)
CEF	10.7 (6)	5 (1)	12.5 (2)	16.7 (2)	25 (2)
CTX	32.1 (18)	30 (6)	31.3 (5)	25 (3)	50 (4)
CIP	37.5 (21)	40 (8)	50 (8)	33.3 (4)	12.5 (1)
NOR	30.4 (17)	35 (7)	37.5 (6)	25 (3)	12.5 (1)
CHL	3.6 (2)	10 (2)	0	8.3 (1)	12.5 (1)
SXT	5.4 (3)	5 (1)	0	8.3 (1)	12.5 (1)
NIT	12.5 (7)	5 (1)	12.5 (2)	25 (3)	12.5 (1)
TET	8.9 (5)	5 (1)	6.3 (1)	25 (3)	0

AMP; Ampicillin, AMC; Amoxicillin-clavulanic acid, CAR; Carbenicillin, OXA; Oxacillin, AMK; Amikacin, GEN; Gentamicin, NET; Netilmicin, CEF; Cephalotin, CTX; Cefotaxime, CIP; Ciprofloxacin, NOR; Norfloxacin, CHL; Chloramphenicol, SXT; Trimethoprim-sulfamethoxazole, NIT; Nitrofurantoin, TET; Tetracycline.

Table 4. Genes of extended spectrum β-lactamase producing DEC strains isolated in collared peccary.

DEC groups (n)	Non susceptible profile	β-lactamase gene	
	AMP	Gene	Number of isolates
DEC (56)	42	blaTEM	4
		blaCTX	4
		blaTEM+blaCTX	6
		blaTEM+blaSHV	6
ETEC (20)	16	blaTEM	3
		blaCTX	3
		blaTEM+blaSHV	2
EAEC (16)	13	blaTEM+blaCTX	3
		blaTEM	1
		blaTEM+blaSHV	1
STEC (12)	6	blaTEM+blaCTX	2
		blaTEM+blaSHV	2
EPEC (8)	5	blaCTX	1
		blaTEM+blaCTX	1
		blaTEM+blaSHV	1

DISCUSSION

In several countries of the Americas, sustainable strategies for the conservation, reproduction, and reintroduction of collared peccary have been proposed through Wildlife Management Units (WMU) (Sisk *et al.*, 2007). The present study showed that 44.4% of the *E. coli* strains isolated in collared peccary faeces kept in WMU are carriers of genes that encode virulence factors of diarrheagenic pathotypes, mainly ETEC (*lt* and *st*) and EAEC (*aap* and AA probe), followed by STEC non-O157 (*stx*1 and *stx*2) and EPEC (*bfp*A and *eae*A). The DEC virulence is mainly based on the ability to produce isoforms of the encoded protein related to infections in humans (Taghadosi *et al.*, 2018; Angulo *et al.*, 2021; Alfinete *et al.*, 2022). These results are consistent with previous studies demonstrating that the wild collared peccary is a carrier of bacteria associated with humans and domestic animals. In the southern areas of the United States, isolates of *Clostridium perfringens* type A and multiple Salmonella serotypes were reported in wild *Pecari tajacu* (Shender *et al.*, 2009), in addition to other possible Salmonella and STEC non-O157 serotypes (Jay-Russell *et al.*, 2014). Other bacteria have been reported in the collared peccary, such as *Mycoplasma hyopneumoniae*, *P. multocida* (de Castro *et al.*, 2014), *Brucella* and different serovars of *Leptospira* (Mendoza *et al.*, 2007; Montenegro *et al.*, 2018). However, to date there are no reports on the prevalence of diarrheagenic pathotypes of *E. coli* in captive collared peccary.

The present study demonstrated a higher prevalence of virulence genes of DEC pathotypes in captive collared peccaries compared with the prevalence of *stx1*, *eaeA* and *hlyA* showed in a study conducted on wild collared peccaries (Jay-Russell *et al.*, 2014). This suggests that captive peccaries are more exposed to these pathogens. Wild peccaries may have suitable environmental conditions such as the type of habitat and availability of food resources (Hernández-Pérez, 2019). The population density of wild peccaries is also much lower. In the WMU containing collared peccary, food and vegetables are frequently introduced and it cannot be ruled out that plant material can spread diarrheagenic *E. coli* strains; there is evidence that these products can be a reservoir of multidrug resistant bacteria harbouring antibiotic resistance and virulence genes (Richter *et al.*, 2021).

The presence of livestock also plays an important role in the dissemination of this bacteria to the environment and could be the main link in the contamination route to collared peccaries. There is evidence of domestic animals acting as carriers of strains of DEC mainly cattle (Gutema *et al.*, 2021), sheep, and goats (Shabana & Al-Enazi, 2020), birds (Kimura *et al.*, 2021) and pigs (Misumi *et al.*, 2021). In Mexico, DEC strains have been reported in humans and animals (Rivas-Ruiz *et al.*, 2020; Tamayo-Legorreta *et al.*, 2020). In southeastern Mexico, where the WMU of the collared peccaries under study are located, this type of DEC strains have been reported in captive green iguanas (Bautista-Trujillo *et al.*, 2020).

A PCR-based phylogenetic group assay was performed as proposed by Clermont *et al.* (2013). The highest frequencies of the ETEC, EAEC, STEC stx1 and STEC stx2 pathotypes, as well as EPEC, were found in Clade I, with lower frequencies in phylogroups B2 and A. Some strains could not be assigned to a known clade. A previous study on the characterisation of *E. coli* isolated in wastewater found the highest frequency of EPEC in phylogroup B2, followed by B1, C and A, while STEC strains were grouped in phylogroup B1 (Cho *et al.*, 2018). Clonal lineage strains in diarrheic poultry, in Tunisia, which could constitute a risk of their transfer to healthy animals and humans, enteropathogenic (EPEC) and extraintestinal (ExPEC) infections mainly belonging to the phylogroups B2 (Jouini *et al.*, 2021). The concentration of environmental strains into different groups could be explained by the phylogenetic variability of *E. coli*, based on their genetic content and diverse lineage. A consequence of this is the difficulty to associate pathogenic strains and commensals to a specific phylogroup, which is why the use of molecular techniques with greater clonal discrimination power for DEC strains such as MLST and PFGE is suggested (Su *et al.*, 2021).

In the present study, most (75%) of the DEC strains isolated in captive collared peccary were not susceptible to ampicillin, while 51.8% were not susceptible to carbenicillin. Overall, more than half of the DEC strains were not susceptible to at least one antibiotic from three different categories tested (MDR), mainly beta-lactams, aminoglycosides, cephalosporins, fluoroquinolones and nitrofurantoin. Resistance to beta-lactams such as ampicillin was observed in DEC isolated from canaries (Kimura *et al.* 2021), pigs (Misumi *et al.*, 2021) and bovines, cow meat and humans (Gutema *et al.*, 2021). In Mexico, multidrug resistant *E. coli* infections are a common occurrence, and the incidence of DEC strains resistant mainly to ampicillin and tetracycline has been reported in bovine faeces (Navarro *et al.*, 2018), beef and pork (Martínez-Vázquez *et al.*, 2018), as well as in humans (Castro *et al.*, 2019). There is evidence that the constant oral-faecal transmission of antibiotic-resistant *E. coli* between animals, humans, and the environment, favours the horizontal transfer of resistance genes between different microorganisms sharing the same niche (Abdel-Rhman *et al.*, 2021; Puvača & Frutos, 2021). This could be the hypothesis of a possible route of dissemination of multidrug resistant DEC strains among captive collared peccaries; however, further research is required.

Other antibiotic resistance genes have been reported in *E. coli* from livestock such as those from the polymyxins family (Ilbeigi *et al.*, 2021), however, efficacy is poorly understood in the veterinary practice in Mexico (Martínez *et al.*, 2020). In the present study, most of the DEC strains isolated in the captive collared peccary were not susceptible to beta-lactams. The presence of genes coding

for β-lactamase was evidenced in DEC strains isolated in captive collared peccary, mainly blaTEM, blaCTX and blaSHV. These findings are consistent with those reported by other authors who found the blaTEM, blaCTX and blaSHV genes in multidrug-resistant diarrheagenic *E. coli* strains in humans and animals (Quino *et al.*, 2020; Shafiq *et al.*, 2021). The results confirm that captive collared peccaries can act as carriers of multiresistant *Escherichia coli* containing genes associated with ESBL. Further research is required to make a genetic characterization of *bla* profiles and carbenicillin-hydrolyzing ß-lactamases.

This study reported the presence of DEC in captive collared peccaries. The ETEC, EAEC, STEC stx1, STECstx1 stx2 and EPEC pathotypes, carrying ESBL genes, were identified in captive collared peccary faeces, with resistance characteristics against beta-lactam antibiotics. The information from this work contribute to develop sanitary strategies for the management of collared peccary in captivity. Further research on the risk factors associated with the prevalence of DEC in captive peccary would allow to better understand how bacteria spread among captive animals and to reduce their capacity to act as carriers of DEC strains.

COMPETING INTERESTS STATEMENT

The authors declare that this study was carried out in the absence of commercial or financial relationships that could be interpreted as a potential conflict of interest and all persons gave their informed consent prior to their inclusion in the study.

AUTHOR CONTRIBUTIONS

CQ-B, CT-C and GUB-T conceived and design of the study. CQ-B, CI-M, MO-Ll, MR-S and GUB-T obtained faecal samples from peccaries, as well laboratory work. CAC-G and JG-J determine virulence genes and phylogenetic analyses. GB-T, CAC-G, SM-G and JG-J performed the statistical analysis and wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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REFERENCES

Abdel-Rhman, S. H., Elbargisy, R. M., & Rizk, D. E. (2021). Characterization of integrons and quinolone resistance in clinical *Escherichia coli* isolates in Mansoura City, Egypt. *International Journal of Microbiology, 2021*. https://doi.org/10.1155/2021/6468942

Ahmed, A. M., Motoi, Y., Sato, M., Maruyama, A., Watanabe, H., Fukumoto, Y., & Shimamoto, T. (2007). Zoo animals as reservoirs of gram-negative bacteria harboring integrons and antimicrobial resistance genes. *Applied and Environmental Microbiology*, *73*(20). https://doi.org/10.1128/AEM.01054-07

Alfinete, N., Bolukaoto, J. Y., & Barnard, T. G. (2022). Virulence and phylogenetic analysis of enteric pathogenic *Escherichia coli* isolated from children with diarrhoea in South Africa. *International Journal of Infectious Diseases, 114*. https://doi.org/10.1016/j.ijid.2021.11.017

Angulo, U. A., Gutiérrez, J. J., & Canizalez, R. A. (2021). Non-diarrheagenic and diarrheagenic *E. coli* carrying supplementary virulence genes (SVG) are associated with diarrhea in children from Mexico. Microbial pathogenesis, *157*. https://doi.org/10.1016/j.micpath.2021.104994

Bautista-Trujillo, G. U., Gutiérrez-Miceli, F. A., Mandujano-García, L., Oliva-Llaven, M. A., Ibarra-Martínez, C., Mendoza-Nazar, P., Ruiz-Sesma, B., Tejeda-Cruz, C., Pérez-Vázquez, L. C., Pérez-Batrez, J. E., Vidal, J. E., & Gutiérrez-Jiménez, J. (2020). Captive green iguana carries diarrheagenic *Escherichia coli* pathotypes. *Frontiers in Veterinary Science*, *7*. https://doi.org/10.3389/fvets.2020.00099

Castro, A. M., Mercado-Alquicira, C., Cervantes-Olmos, R. A., & Arzate-Barbosa, P. (2019). Comparative study between diarrheagenic *Escherichia coli* virotypes from Mexico City and San Luis Potosí. *Revista Médica Del Hospital General de México*, *82*(2). https://doi.org/10.24875/hgmx.m19000022

Cerna, J. F., Nataro, J. P., & Estrada-Garcia, T. (2003). Multiplex PCR for detection of three plasmid-borne genes of enteroaggregative *Escherichia coli* strains. *Journal of Clinical Microbiology*, *41*(5), 2138-2140. https://doi.org/10.1128/JCM.41.5.2138-2140.2003

Cho, S., Hiott, L. M., Barrett, J. B., McMillan, E. A., House, S. L., Humayoun, S. B., Adams, E. S., Jackson, C. R., & Frye, J. G. (2018). Prevalence and characterization of *Escherichia coli* isolated from the upper oconee watershed in Northeast Georgia. *PLoS ONE*, *13*(5). https://doi.org/10.1371/journal.pone.0197005

Clermont, O., Christenson, J. K., Denamur, E., & Gordon, D. M. (2013). The Clermont *Escherichia coli* phylo-typing method revisited: Improvement of specificity and detection of new phylo-groups. *Environmental Microbiology Reports*, *5*(1), 58-65. https://doi.org/10.1111/1758-2229.12019

Croxen, M. A., Law, R. J., Scholz, R., Keeney, K. M., Wlodarska, M., & Finlay, B. B. (2013). Recent advances in understanding enteric pathogenic *Escherichia coli*. In *Clinical Microbiology Reviews* *26*(4). https://doi.org/10.1128/CMR.00022-13

de Carvalho, M. A. G., Santos, P. S., da Cunha Nogueira, S. S., Lessa, F., Almeida, V. D. A., Leça-Junior, N. F., Salvarani, F. M., Lobato, F. C. F., & Nogueira-Filho, S. L. G. (2011). Necrotic enteritis in collared (*Pecari tajacu*) and white-lipped (*Tayassu pecari*) peccaries. *Journal of Zoo and Wildlife Medicine*, *42*(4). https://doi.org/10.1638/2010-0212.1

de Castro, A. M. M. G., Brombila, T., Bersano, J. G., Soares, H. S., de Souza Silva, S. O., Minervino, A. H. H., Ogata, R. A., Gennari, S. M., & Richtzenhain, L. J. (2014). Swine infectious agents in

Tayassu pecari and *Pecari tajacu* tissue samples from Brazil. *Journal of Wildlife Diseases*, *50*(2). https://doi.org/10.7589/2013-01-021

Faridah-Hayyun, D., Dewi, E. K., Fatimah, M. H. E., & Plumeriastuti, H. (2020). A review of antimicrobial resistance (AMR) of *Escherichia coli* on livestock and animal products: Public health importance. *Systematic Reviews in Pharmacy*, *11*(11), 1210-1218.

Gutema, F. D., Rasschaert, G., Agga, G. E., Jufare, A., Duguma, A. B., Abdi, R. D., Duchateau, L., Crombe, F., Gabriël, S., & de Zutter, L. (2021). Occurrence, molecular characteristics, and antimicrobial resistance of *Escherichia coli* O157 in cattle, beef, and humans in Bishoftu Town, Central Ethiopia. *Foodborne Pathogens and Disease*, *18*(1). https://doi.org/10.1089/fpd.2020.2830

Gutiérrez-Jiménez, J., Luna-Cazáres, L. M., Mendoza-Orozco, M. I., Díaz-Marina, G. de J., Burguete-Gutiérrez, J. C., & Feliciano-Guzmán, J. M. (2015). Organización, mantenimiento y preservación de la Colección de Cultivos Bacterianos del Instituto de Ciencias Biológicas de la Universidad de Ciencias y Artes de Chiapas (UNICACH), México. *Revista de La Sociedad Venezolana de Microbiología*, *35*(2).

Hernández-Pérez, E. L. (2019). Distribución potencial del cerdo asilvestrado, *Sus scrofa* (Artiodactyla: Suidae) y el pecarí de collar, *Pecari tajacu* (Artiodactyla: Tayassuidae) en la región de Laguna de Términos, México. *Revista de Biología Tropical*, *67*(6). https://doi.org/10.15517/rbt.v67i6.37006

Ilbeigi, K., Askari Badouei, M., Vaezi, H. & Khatereh Kafshdouzan. (2021). Molecular survey of mcr1 and mcr2 plasmid mediated colistin resistance genes in *Escherichia coli* isolates of animal origin in Iran. *BMC Research Notes*. *14*(107). https://doi.org/10.1186/s13104-021-05519-6

Jay-Russell, M. T., Hake, A. F., Rivadeneira, P., Virchow, D. R., & Bergman, D. L. (2014). Enteric human pathogens of wild boar, feral swine, and javelina (Order: Artiodactyla). *Proceedings of the Vertebrate Pest Conference*, *26*. https://doi.org/10.5070/v426110701

Jouini, A., Klibi, A., & Maaroufi, A. (2021). First Detection of Human ST131-CTX-M-15-O25-B2 Clone and High-Risk Clonal Lineages of ESBL/pAmpC-Producing E. coli Isolates from Diarrheic Poultry in Tunisia. *Antibiotics*, *10* (670). https://doi.org/10.3390/antibiotics10060670

Kimura, A. H., Koga, V. L., de Souza Gazal, L. E., de Brito, B. G., de Brito, K. C. T., Navarro-Ocaña, A., Nakazato, G., & Kobayashi, R. K. T. (2021). Characterization of multidrug-resistant avian pathogenic *Escherichia coli*: an outbreak in canaries. *Brazilian Journal of Microbiology*, 52(2). https://doi.org/10.1007/s42770-021-00443-0

López-Saucedo, C., Cerna, J. F., Villegas-Sepulveda, N., Thompson, R., Velazquez, F. R., Torres, J., Tarr, P. I., & Estrada-Garcia, T. (2003). Single multiplex polymerase chain reaction to detect diverse loci associated with diarrheagenic *Escherichia coli*. *Emerging Infectious Diseases*, *9*(1), 127-131. https://doi.org/10.3201/eid0901.01-0507

Magiorakos, A. P., Srinivasan, A., Carey, R. B., Carmeli, Y., Falagas, M. E., Giske, C. G., Harbarth, S., Hindler, J. F., Kahlmeter, G., Olsson-Liljequist, B., Paterson, D. L., Rice, L. B., Stelling, J., Struelens, M. J., Vatopoulos, A., Weber, J. T., & Monnet, D. L. (2012). Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: An international expert proposal for interim standard definitions for acquired resistance. *Clinical Microbiology and Infection*, *18*(3). https://doi.org/10.1111/j.1469-0691.2011.03570.x

Marinho, P. H., da Silva, M. D., & Lisboa, C. M. C. A. (2019). Presence of the collared peccary *Pecari tajacu* (Artiodactyla, Tayassuidae) in the far Northeast of its Brazilian distribution. *Neotropical Biology and Conservation*, *14*(4). https://doi.org/10.3897/neotropical.14.e48716

Martínez, M, R., Gastélum-Acosta, M., & Osuna-Álvarez, L. E. (2020). Actividad antimicrobiana de ceftolozano-tazobactam y ceftazidima-avibactam contra bacilos gramnegativos clínicamente relevantes aislados en México. *Gaceta médica de México*. *156*(6). https://doi.org/10.24875/gmm.19005335

Martínez-Vázquez, A. V., Rivera-Sánchez, G., Lira-Méndez, K., Reyes-López, M. Á., & Bocanegra-García, V. (2018). Prevalence, antimicrobial resistance and virulence genes of *Escherichia coli* isolated from retail meat in Tamaulipas, Mexico. *Journal of Global Antimicrobial Resistance*, *14*. https://doi.org/10.1016/j.jgar.2018.02.016

Mendoza, P., Mayor, P., Gálvez, H. A., Céspedes, M. J., & Jori, F. (2007). Antibodies against Leptospira spp. in captive collared peccaries, Peru. In *Emerging Infectious Diseases 13*(5). https://doi.org/10.3201/eid1305.060027

Misumi, W., Funamori, T., Hamada, K., Iwamoto, J., Fujisono, S., Chitose, K., & Kusumoto, M. (2021). Association between antimicrobial treatment and resistance of pathogenic escherichia coli isolated from diseased swine in kagoshima prefecture, Japan. *Journal of Veterinary Medical Science*, *83*(3). https://doi.org/10.1292/jvms.20-0338

Molina-Barrios, R., Luevano-Adame, J., Henao-Díaz, Y. A., Giménez-Lirola, L., Piñeyro, P., Magtoto, R., Cedillo-Cobián, J., Díaz-Rayo, C., Hernández, J., & Zimmerman, J. (2018). Collared peccary (*Pecari tajacu*) are susceptible to porcine reproductive and respiratory syndrome virus (PRRSV). *Transboundary and Emerging Diseases*, *65*(6). https://doi.org/10.1111/tbed.12944

Montenegro, O. L., Roncancio, N., Soler-Tovar, D., Cortés-Duque, J., Contreras-Herrera, J., Sabogal, S., Acevedo, L. D., & Navas-Suárez, P. E. (2018). Serologic survey for selected viral and bacterial swine pathogens in colombian collared peccaries (*Pecari tajacu*) and feral pigs (*Sus scrofa*). *Journal of Wildlife Diseases*, *54*(4). https://doi.org/10.7589/2017-09-236

Navarro, A., Cauich-Sánchez, P. I., Trejo, A., Gutirrez, A., Díaz, S. P., Martha, D. C., Cravioto, A., & Eslava, C. (2018). Characterization of diarrheagenic strains of *Escherichia coli* isolated from cattle raised in three regions of Mexico. *Frontiers in Microbiology*, *9*(OCT). https://doi.org/10.3389/fmicb.2018.02373

Oh, K.-H., Shin, E., Jung, S.-M., Im, J., Cho, S.-H., Hong, S., Yoo, C.-K., & Chung, G. T. (2016). First isolation of hybrid Shigatoxigenic and Enterotoxigenic *Escherichia coli* (STEC/ETEC) strain harboring stx2 and elt genes in Republic of Korea. *Japanese Journal of Infectious Diseases*, JJID-2016.

Paton, A. W., & Paton, J. C. (1998). Detection and characterization of Shiga toxigenic *Escherichia coli* by using multiplex PCR assays for stx 1, stx 2, eaeA, enterohemorrhagic *E. coli* hlyA, rfb O111, and rfb O157. *Journal of Clinical Microbiology*, *36*(2), 598-602.

Puvača, N., & Frutos, R. de L. (2021). Antimicrobial resistance in escherichia coli strains isolated from humans and pet animals. *Antibiotics, 10*(1). https://doi.org/10.3390/antibiotics10010069

Quino, W., Mestanza, O., Caro-Castro, J., Hurtado, C. V., & Gavilán, R. G. (2020). Resistoma y genómica comparativa de aislados clínicos de *Escherichia coli* diarreogénica en Lima, Perú. *Revista Peruana de Medicina Experimental y Salud Pública*, *37*(4). https://doi.org/10.17843/rpmesp.2020.374.5240

Richter, L., Plessis, E. du, Duvenage, S., & Korsten, L. (2021). High prevalence of multidrug resistant *Escherichia coli* isolated from fresh vegetables sold by selected formal and informal traders in the most densely populated Province of South Africa. *Journal of Food Science*, *86*(1). https://doi.org/10.1111/1750-3841.15534

Rivas-Ruiz, C. M., Cantú-Soto, E. U., Maldonado-Mendoza, I. E., Figueroa-López, A. M., Anduro-Jordan, J. A., Luna-Nevarez, P., & López-Castro, P. A. (2020). Detection of Shiga toxin-producing *Escherichia coli* in asymptomatic cattle in the southern Sonora, Mexico. *Ecosistemas y Recursos Agropecuarios*, *7*(2). https://doi.org/10.19136/era.a7n2.2240

Shabana, I. I., Zaraket, H., & Suzuki, H. (2013). Molecular studies on diarrhea-associated *Escherichia coli* isolated from humans and animals in Egypt. *Veterinary Microbiology*, *167*(3-4). https://doi.org/10.1016/j.vetmic.2013.08.014

Shabana, I. I., & Al-Enazi, A. T. (2020). Investigation of plasmid-mediated resistance in *E. coli* isolated from healthy and diarrheic sheep and goats. *Saudi Journal of Biological Sciences*, *27*(3). https://doi.org/10.1016/j.sjbs.2020.01.009

Shafiq, M., Huang, J., Shah, J. M., Ali, I., Rahman, S. U., & Wang, L. (2021). Characterization and resistant determinants linked to mobile elements of ESBL-producing and mcr-1-positive *Escherichia coli*

recovered from the chicken origin. *Microbial Pathogenesis*, *150*. https://doi.org/10.1016/j.micpath.2020.104722

Shender, L. A., Glock, R. D., & Spraker, T. R. (2009). Salmonellosis in a free-ranging population of javelinas (*Pecari tajacu*) in south Central Arizona. *Journal of Wildlife Diseases*, *45*(4). https://doi.org/10.7589/0090-3558-45.4.941

Sisk, T. D., Castellanos V, A. E., & Koch, G. W. (2007). Ecological impacts of wildlife conservation units policy in Mexico. *Frontiers in Ecology and the Environment*, *5*(4). https://doi.org/10.1890/1540-9295(2007)5[209:EIOWCU]2.0.CO;2

Su, Z., Zhang, L., Sun, H., Hu, Y., Fanning, S., Du, P., Cui, S., & Bai, L. (2021). Characterization of non-O157 Shiga toxin-producing *Escherichia coli* cultured from cattle farms in Xinjiang Uygur Autonomous Region, China, During 2016-2017. *Foodborne Pathogens and Disease*. https://doi.org/10.1089/fpd.2020.2843

Taghadosi, R., Shakibaie, M.R., & Ghanbarpour R. (2018). Serogroups, subtypes and virulence factors of shiga toxin producing *Escherichia coli* isolated from human, calves and goats in Kerman, Iran. *Gastroenteroloy Hepatology Bed Bench, 11*(1). PMID: 29564067; PMCID: PMC5849120.

Taghadosi, R., Shakibaie, M. R., & Hosseini-Nave, H. (2019). Antibiotic resistance, ESBL genes, integrons, phylogenetic groups and MLVA profiles of *Escherichia coli* pathotypes isolated from patients with diarrhea and farm animals in south-east of Iran. *Comparative Immunology, Microbiology and Infectious Diseases*, *63*. https://doi.org/10.1016/j.cimid.2019.01.004

Tamayo, E. M., García-Radilla., & Alpuche-Aranda, C. M. (2021). Diarrheagenic *Escherichia coli* pathotypes isolated from a swine farm in a region of Morelos state, Mexico. *Salud Pública de México*, *63*(1). https://doi.org/10.21149/11268

Tsai, Y. L., Palmer, C. J., & Sangermano, L. R. (1993). Detection of *Escherichia coli* in sewage and sludge by polymerase chain reaction. *Applied and Environmental Microbiology*, *59*(2). https://doi.org/10.1128/aem.59.2.353-357.1993

Wayne, P. A. (2020). Clinical and Laboratory Standards Institute (CLSI). Performance Standards for Antimicrobial Susceptibility Testing. 30th ed. *CLSI Supplement M100*.

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ORIGINAL ARTICLE

Domestic dog and alien North American mink as reservoirs of infectious diseases in the endangered Southern river otter

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ABSTRACT. Introduced alien carnivores are host to infectious diseases that may become an important threat for native carnivore species conservation. Canine distemper virus (CDV) is thought to be transmitted among individuals by direct contact and to present viral dynamics associated with a density-dependent multi-host carnivore community. In contrast, Canine Parvovirus (CPV) is mostly transmitted by indirect contact and does not depend only on the density, but also on the social behaviour of infected as well as susceptible hosts. The objective of this study was to assess how introduced American mink (*Neovison vison*) can act as a bridge-host between domestic dog (*Canis familiaris*) and Southern river otter (*Lontra provocax*) in different dog and mink population density scenarios. Our data show that otters are seropositive to both CDV and PV, as well as a molecular identity to Parvovirus in dogs and minks. Furthermore, a strong positive correlation between dog population density and observed seroprevalence of CDV in dogs, minks, and otters was recorded. For Parvovirus, the observed seroprevalence in mink and otters was not correlated to a higher dog population density, but instead a relationship between dog and mink population densities and social behaviour. Our results suggest that introduced American mink and domestic dogs are reservoirs of CDV and PV, both being diseases of major importance for the conservation of native endangered carnivores in Patagonia.

Key words: American mink, domestic dog, otters, Canine Parvovirus, Canine Distemper virus.

INTRODUCTION

Among the domestic hosts of infectious diseases, free-roaming dog populations are of interest in Chile because they are large and known to affect wildlife (González-Acuña *et al.*, 2003; Medina-Vogel, 2010; Acosta-Jamett *et al.*, 2011; Silva-Rodríguez & Sieving, 2012; Sepúlveda *et al.*, 2014). Furthermore, the intense migration dynamics of dog populations in Chile can modify the spread of an infectious disease in a way that is difficult to understand (Villatoro *et al.*, 2016). In south-central Chile, the average number of dogs ranges between 0.54 to 0.95 with a maximum of 1.28 dogs per rural household, with 1.6 to 2.4 males per 1 female and up to 60% of the local rural population may come from urban areas, as far as 1000 km away (Villatoro *et al.*, 2016). Silva-Rodríguez & Sieving (2012) found that households were the best predictor for dog occupancy during their study in rural sites in the south of Chile. Among domestic dog infectious diseases, there are two important viral diseases transmitted from them to wildlife species of conservation concern, Canine Parvovirus (CPV) and Canine Distemper Virus (CDV) (Frölich *et al.*, 2000; Acosta-Jamett *et al.*, 2011; Acosta-Jamett *et al.*, 2014; Millán *et al.*, 2015). Canine Parvovirus (CPV) is a DNA virus of the *Parvoviridae* family that is quite resistant to environmental conditions and can survive up to six months at room temperature (Parrish, 1990; Williams, 2001). It has been linked with mortality in mustelids in captivity (Gjeltema *et al.*, 2015), therefore, it might be able to threaten the viability of small carnivore isolated populations. On the other hand, Canine Distemper Virus (CDV) is a RNA virus, a member of the Morbillivirus genus of the Paramyxoviridae family; it is highly contagious among carnivores, it spreads rapidly in mustelids and induces a high mortality rate on unvaccinated mink (Hammer *et al.*, 2007). Another issue of wildlife conservation concern in Patagonia is the introduced American mink (*Neovison vison*). This semiaquatic mustelid registers stable populations since the 1970s, it is resistant to the presence of humans and its diet includes a substantial proportion of rodents (Medina, 1997; Medina *et al.*, 2013). Also, it cohabits with free-ranging domestic dogs associated with farming and housing near rivers as well as lakes shores and seashore. Minks are known to cause damage to hen houses and poultry, therefore, interspecies contact between American mink and domestic species is likely to occur (Philippa *et al.*, 2008; Sepúlveda *et al.*, 2014). American minks also share habitat with endangered Southern river otter (*Lontra provocax*) in freshwater and marine environments (Medina, 1997; Medina-Vogel *et al.*, 2013), suggesting that there is

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a potential function of mink as a bridge host of infectious diseases from domestic dogs to wild otter populations in Chile (Sepúlveda *et al.*, 2014). In North America, CDV has been reported in mink, otters, and domestic animals; North American river otters (*Lontra canadensis*) and Eurasian otters (*Lutra lutra*) have tested seropositive against CPV-2 and mink parvovirus (Kimber *et al.*, 2000); new antigenic types of CPV-2 have been isolated from stone martens (*Martes foina*) and other carnivores (Steinel *et al.*, 2001). Therefore, it is expected that similar situations of disease transmission of both CPV and CDV between domestic dog, alien American mink, and Southern river otter could happen in Southern Chile (Sepúlveda *et al.*, 2014).

During the last century, the population of Southern river otters (*Lontra provocax*) declined dramatically in its former territory (Medina, 1996) and as a result the species is currently listed as endangered by the International Union for the Conservation of Nature and Nature Resources (UICN, 2013). Although these otters were once widely distributed, the remaining population is currently spread out over two different areas: i) a small and fragmented northern population associated to freshwater systems (38ºS to 43ºS Latitude) and; ii) a patchy, but relatively extended southern population associated to the marine habitat of Chilean fiords and channels, as well as Argentinian and Chilean Tierra del Fuego and Cape Horn region (43°S to 58°S) (Medina, 1996). Several factors have been involved in the population decline of this mustelid in Chile, including excessive hunting and trapping in the past and a substantial loss of habitat during the last 70 years (Medina, 1996). However, there is almost no information on pathogen prevalence or disease in this species (Medina-Vogel, 2010; Sepúlveda *et al.*, 2014; Barros *et al.*, 2018) and increasing concerns about the potential importance of infectious disease spill-over from dog to otter, having the introduced American mink as a bridge host (Sepúlveda *et al.*, 2014).

The increasing human intervention of natural habitats and globalisation, resulting in the transport and introduction of alien species into other regions, enhance the emergence of new diseases in wildlife and make the re-emergence of old diseases not surprising (Daszak *et al.*, 2000; Medina-Vogel 2010). For instance, in California, USA, foxes (*Urocyon cinereargenteus*) and bobcat (*Lynx rufus*) live close to towns where important populations of stray dogs and cats have recorded a significantly high seropositive reaction to both CPV and calcivirus (Riley *et al.*, 2004). Many infectious diseases that originate from domestic dogs, cats and livestock have been reported in mustelids, such as CPV (Steinel *et al.*, 2001; Gjeltema *et al.*, 2015) and CDV (Frölich *et al.*, 2000; Williams, 2001; Philippa *et al.*, 2008). The transmission of CDV is thought to be through direct contact since the virus can survive only some hours at 25°C and up to 14 days at 5°C under test conditions (Shen & Gorham, 1980); also, the transmission is primarily by aerosol or by contact with oral, respiratory, ocular fluids and exudates containing the virus. Due to the relative fragility of the virus in the environment, a close association between infected and susceptible animals is necessary, for example, some carnivore behaviours such as sharing carcasses or latrines are a potential source of inter and intraspecies infection making the inter and intraspecies transmission of CDV quite plausible (Craft *et al.*, 2011, Sepúlveda *et al.*, 2014). In this sense, dense populations of susceptible individuals with special or characteristic behaviours are necessary to sustain CDV dynamics on a multi-host system of carnivores.

In contrast, CPV can survive for months under cool and moist conditions when protected from sunlight, hence the infection dose required for CPV may be very low. The transmission within dogs (Steinel *et al.*, 2001) and other wild carnivores occurs via contact with the virus shed in faeces (faecal-oral route), suggesting that indirect transmission rather than direct contact with infected animals may play a key role in the maintenance of this virus in a population, particularly among wild carnivores characterised by low contact rates. Transmission of CPV between domestic and wild carnivores may also occur through close contact or predation on smaller carnivores, and across long distances by fomites (Miranda & Thompson, 2016). Moreover, free-ranging carnivores at low densities, even solitary individuals, may be exposed at marking sites, latrines or other sites contaminated by faeces deposited by a virus shedder (Bakker & Parrish, 2001).

If CDV and CPV are being transmitted between domestic dogs, American minks, and otters in Chile, then these species should report a higher CDV seroprevalence in places with a higher population of dogs and CPV seroprevalence should follow a different pattern (Deem *et al.*, 2000; Almberg *et al.*, 2010). To validate this, molecular evidence of those transmissions should be found. The present study aimed to carry out a seroprevalence and molecular cross-sectional survey in populations of domestic dogs, minks and river otters in Southern Chile.

MATERIAL AND METHODS

STUDY AREA

This study was carried out in Southern Chile, 39º S and 45º S latitude (figure 1). Eleven sites were chosen based on independence of the Southern river otter home range size, dispersion pattern and distribution (figure 1) (Medina, 1996; Sepúlveda *et al.*, 2014).

All sites were located within a region characterised by a temperate-humid-cool climate with 2,000 mm to 3,000 mm of rain per year and an average humidity of around 90%. Rivers, lakes and marine coastal vegetation in this area is characterised by a type of forest known as Valdivian rainforest and Norpatagonic Valdivian rainforest. The average annual temperature in this region is below 10°C (Veblen & Schlegel, 1982; Toledo & Zapater, 1989).

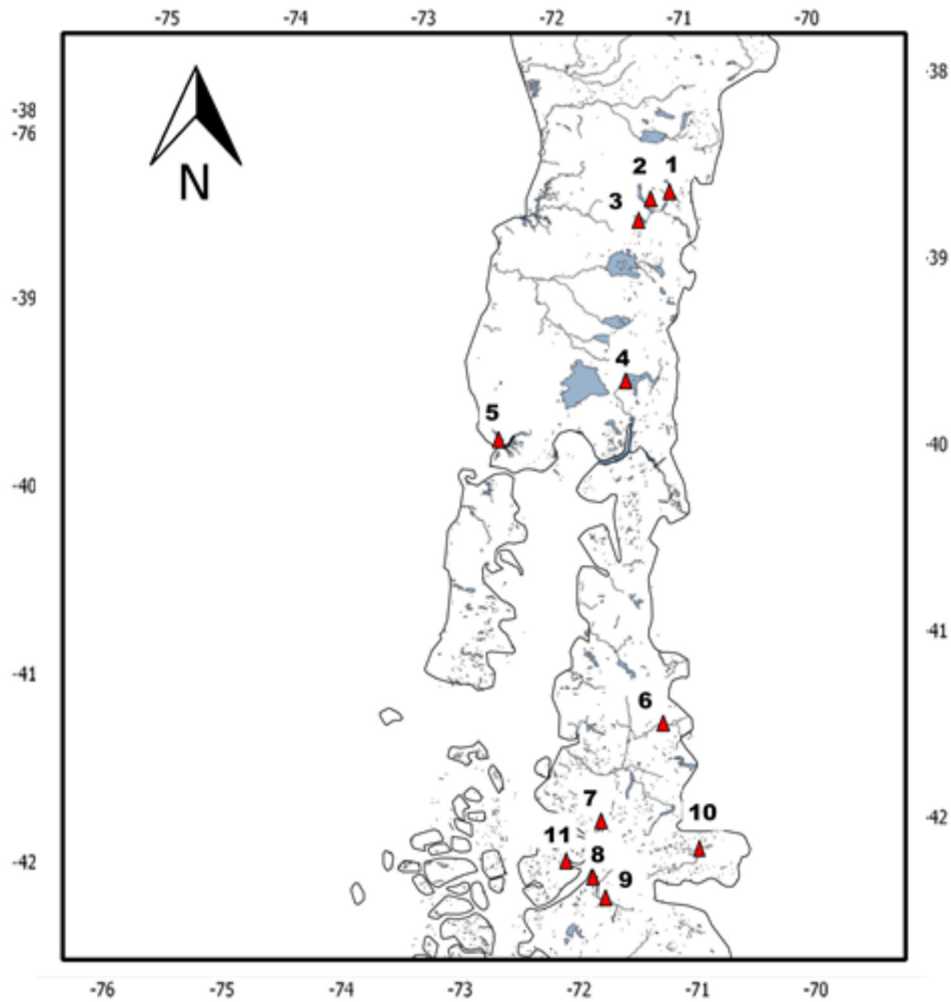


Figure 1. Geographic location of study sites: 1= Neltume Lake; 2= Liquiñe River; 3= Panguipulli Lake; 4= Todos Los Santos Lake; 5= Maullin River; 6= Palena River; 7= Cisnes seashore; 8= Cisnes Alto River; 9= Cisnes River; 10= Queulat Fiord; 11= Magdalena Island.

DOG AND AMERICAN MINK POPULATION DENSITY ESTIMATION

The sampling was defined in terms of the capture success (total individuals captured/number of traps) of American mink. We established a buffer zone of 4 km around each American mink captured in each sampling site, then these buffer zones merged to produce a wider area creating eleven sampling zones with an extension that depends on how separated and distant the minks were trapped. These sampling zones were divided into 1 km^2 cells which were then categorised as either having or not having human presence. Google Earth imagery was used to identify the presence of households. Cells with one or more households were designated as having a human presence and cells without households were designated as not having a human presence. To estimate the domestic dog population density, we counted the number of roofs per cell to have an approximate number of households per zone. In each of the zones we performed an on-ground survey of homeowners to estimate the number of dogs per house; the population density of domestic dogs was obtained as the ratio between the estimated number of dogs and the area (km^2) associated to the zones, following Acosta-Jamett *et al.* (2011) and Silva-Rodríguez & Sieving (2012). Finally, the estimated density of dogs per cell was divided into three categories of a similar sample size to denote study zones of low (≤4.0 dogs per km^2), medium (4.1 - 8.0 dogs per km^2), or high (> 8.1 dogs per km^2) dog population density. The population density of mink (minks/km) was estimated at each study zone using the following data: a) the number of trapped mink during the first 12 days of trapping (Harrington *et al.*, 2008; Medina-Vogel *et al.*, 2015); b) extrapolating the data of the length of home ranges of five male minks living in Cisnes River, providing an average of 2,213 m long (ranging between 1,422 m to 3,834 m), a female mink living in Magdalena Island,which had a lineal home range of 1,769 m, and another male living in Magdalena Island, whose home range was 2,069 m (Medina-Vogel *et al.*, 2013, 2015); and c) Minks intrasexual territoriality (Powell, 2000; Zuberogoitia

et al., 2010), assuming a proportion of males:female given by 1:1.2/km. Hence, the density of mink population in each study zone was obtained using the formula (TM/D) x 2.2, where TM=trapped minks and D=Total distance covered by the trapping transect (see Medina-Vogel *et al.*, 2015; for a detailed explanation).

ANIMAL SAMPLING

To capture American minks, wire cage traps with a double entrance (81 cm long, 21 cm high, and 23.5 cm wide) were used along with fresh or canned fish as bait. The trapping period lasted from January 2009 to February 2013. Traps were deployed along main river stretches, as well as along lakeshores and seashores; traps were set regularly spaced (around 500 m), considering the existence of mink field signs such as scats and footprints during a period between 10 to 20 days. Once captured, American minks were introduced into a mesh to perform a mechanical immobilisation to later inoculate the anaesthesia in the semimembranosus - semitendinosus muscle applying one single combined injection of ketamine-dexmedetomidine in a dose of 10-0.025 mg/kg IM, and blood samples (3-5 mL) were collected with cranial vena cava and intracadriae venipuncture using tubes with EDTA. These animals were then euthanised with thiopental by intracardiac injection (Biosano S.A., Santiago, Chile) (about 2 mL per individual) for post mortem examination. Likewise, Southern river otters were trapped using soft-catch leg-hold traps (Blundell *et al.*, 1999; Sepúlveda *et al.*, 2007) and they were handled using previously developed protocols (Soto-Azat *et al.*, 2006). They were anaesthetised with one single combine injection of ketamine-dexmedetomidine in a dose of 5-0.025 mg/kg IM and blood samples were taken from the jugular vein. Afterwards, otters were released on the same capture site. Both American mink and otter traps were checked once and twice a day, respectively. Simultaneously, near the place where minks and otters were captured, we randomly selected houses and asked dog owners for their informed consent to take a blood sample from their domestic dog (*Canis familiaris*). Dogs were manually restrained and 5 mL of blood were taken from their brachiocephalic vein, the blood samples were then processed as described for mink and otters. To obtain serum, the blood without anticoagulant was centrifuged for 10 minutes at 1,200 x g. All samples were stored in liquid nitrogen during fieldwork. In addition, a short interview with the owners was conducted to know if their dogs were permanently confined or otherwise allowed to roam freely outside their places and they were also asked about the dog vaccination records. The identification and age of the dogs were provided by the owners. All animal trapping and handling were carried out following the ethical protocols of the Bioethics Committee of Universidad Andrés Bello and the National Commission for Scientific and Technology (CONICYT) (Fondecyt 1100139 - Letter from Bioetical Comittee, there was no protocol number before 2013; Fondecyt 1171417 - Bioethics Approval Nº 007/2017). Otter trapping was done under permit number 1228 delivered by The Undersecretariat for Fisheries and Aquaculture of Chile (Subsecretaria de Pesca, Chile). As additional information on sampling, minks were removed from each study site after being trapped and sampled because of their invasive alien species status. On the other hand, after being trapped the otters were released in the same study site and were not trapped again in the same place for the following year. Finally, dogs were sampled one each year for each study site. As a result, the year was not considered as a variable, thus pseudo-replications were avoided.

LABORATORY ANALYSIS

Serum samples were tested for CPV and CDV antibody titers. Seropositivity to PV was analysed using a haemagglutination inhibition test, and titer ≥ 1:350 (see table 1) was considered positive. On the other hand, seropositivity to CDV was analysed using a seroneutralisation test and titer ≥ 1:16 (table 1) was considered positive. The detection of genomic DNA from CPV and CDV was performed through PCR from blood samples.

For the molecular analysis, DNA was extracted with the QIAamp DNA Mini Kit (Qiagen, Germany). PCR was used to screen CPV as described by Touihri *et al.*, (2009). To detect CPV, a region of 583bp of capsid protein gene was amplified by PCR with CPV primers (tables 2 and 3).

To determine the variant of parvovirus present in the positive samples, a PCR was performed to amplify and sequence 1,195bp of VP2 gene of CPV using CPV primers (tables 2 and 3).

To detect CDV, a region of 419bp of nucleocapsid protein gene (N) of CDV was amplified by RT-PCR with CDV primers (tables 2 and 3).

All PCR products were visualised using electrophoresis on 1% agarose gels with GelRed Nucleic Acid Stain (Biotium). The analysis was carried out in the Molecular Biology laboratory of the School of Veterinary Science, Universidad Andrés Bello and the Faculty of Agronomy and Forestry, Pontificia Universidad Católica de Chile. The PCR products from the VP2 gene of CPV were purified and sequenced bi-directionally at Macrogen Inc., Seoul, South Korea. Sequences were aligned and polymorphic sites were confirmed by eye according to the chromatogram using Sequencher 5.1 (Gene Codes Corporation, Ann Arbor, MI, USA). Sequences were compared with the GenBank database to confirm the presence of CPV and to compare the similarity of the amplified fragments for the different animal species.

STATISTICAL ANALYSIS

Observed seroprevalence was defined as the proportion of positive individuals among the totality of those sampled

Table 1. Observed seroprevalences (%) for PV (1:16) and CDV (1: 350) and estimated population density for dogs (km^2) and mink (trapping transect) in each study sites.

Study site	Population Density		Sample size			Seroprevalence PV			Seroprevalence CDV		
	Dog	Mink	Dog	Mink	Otter	Dog	Mink	Otter	Dog	Mink	Otter
Cisnes seashore	20.0	4.0	15	6	2	80	0	0	80	0	0
Palena River	9.1	2.0	0	1	0		100			0	
Neltume Lake	8.7	3.0	12	6	1	83	18	0	17	17	100
Panguipulli Lake	6.9	6.0	13	7	3	69	14	0	69	33	33
Maullin River	6.3	6.0	7	6	0	57	0		57	67	
Liquiñe River	5.1	3.9	4	1	0	50	0		50	0	
Cisnes River	1.3	7.5	10	1	0	30	0		30	0	
Todos Los Santos Lake	1.1	6.0	7	4	3	43	25	0	43	25	0
Cisnes Alto River	0.8	8.4	5	4	0	40	50		40	50	
Queulat Fiord	0.5	6.5	4	4		25	75		25	0	
Magdalena Island	0.0	13.0	1	19	3	100	37	33	100	20	0

Table 2. Observed seroprevalences (%) comparing both positives agents (PV and CDV) with the total positive of one of them, in each species.

Species	Dog density	Positives CDV	Positives PV	Both positives
Dogs	Low	3 (50%)	5 (100%)	2 (33%)
	Medium	7 (33%)	11 (52%)	4 (19%)
	Hight	37 (73%)	38 (79%)	29 (57%)
Minks	Low	5 (22%)	9 (39%)	3 (13%)
	Medium	1 (11%)	4 (44%)	0
	Hight	5 (19%)	6 (22%)	1 (4%)
Otters	Low	0	1 (33%)	0
	Medium	0	0	0
	Hight	2 (33%)	0	0

Table 3. Primers used for detection and sequencing of Parvovirus and Distemper.

	Name	bp	Sequence	Sample size	Function
Parvovirus	CPV-F	583	CAGGAAGATATCCAGAAGGA	20	Detection
Parvovirus	CPV-R	583	GGTGCTAGTTGATATGTAATAAACA	25	Detection
Parvovirus	VP2-561-F	1195	GAGCATTGGGCTTACCA	17	Sequencing
Parvovirus	VP2-1755-R	1195	TTAATATAATTTTCTAGGTGCTAGTTGAGA	30	Sequencing
Distemper	N-F	419	GTTAGCTAGTTTCATCCT	18	Detection
Distemper	N-R	419	GGTCCTCTGTTGTCTTGG	18	Detection

within each species (Philippa *et al.*, 2008). Exposure status (presence/absence) was recorded as binary outcomes (1/0) (Courchamp *et al.*, 2000). The differences in observed seroprevalence between more than two variables [species host, sex, age, study sites and both mink and dog population densities (Low, Medium, High)] were assessed applying Generalized Linear Models (GLM) by SYSTAT, where the tested variables were considered as the predictors and the exposure status obtained as the frequency of positive (1) or negative (0) seroprevalence (binary) was considered as the dependent variable. Subsequently, a non-parametric Mann-Whitney *U* test was used to assess differences between two variables. The Pearson correlation matrix was used to assess the correlation between two tested variables. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

The questionnaire for dog owners provided an approximation of how closely domestic animals interact

with wildlife. For each question, the percentages were calculated from the totality of the sample. A 64% of the dogs were male (usually adults), 87% were neutered, and 73% were allowed to roam free during part of the day. It was found that free or enclosed management of dogs has no significant effect on vaccination (P=0.18). Out of the 238 dog population surveyed, 38 (16%) were vaccinated against CDV and CPV at some point in their life.

Out of those individuals that were found seropositive to CPV, three were positive to CPV by PCR: one dog from Neltume Lake and two American minks from Cisnes Alto River, and Puerto Cisnes. As previously described, to characterise the virus sequencing and amplification of 1,195bp fragments were performed, however, after editing and cleaning the sequence for the analysis, these fragments were shortened to 438bp. Although this length cannot differentiate between CPV, Feline Panleukopenia, and mink Enteritis virus these short sequences showed that the two mink samples correspond to CPV, showing a 100% identity with CPV sequences at Genbank (Acc. Number: gb|HQ413321.1|). Interestingly, the dog sample showed 99% identity with CPV, 99% identity with Feline Panleukopenia, and 99% identity with Mink Enteritis virus (Acc. Number: gb|KP881687.1|). An alignment between these sequences showed 100% identity between minks and 99% identity between mink and dog samples. No positive sample to CDV was recorded. Since the number of positive samples to PCR was small, a statistical differentiation between infecting viruses with dogs and minks was not possible by molecular analysis, therefore, we will use Parvovirus (PV) to designate an exposure to CPVs for which the specific identification was unknown.

An average of 5.5 dogs/km^2 and 6.0 minks/km^2 population density per mink trapping transect were estimated in our study areas (table 1). Population size trends were different between dogs and minks: indeed, mink populations were smaller in areas where dog populations were bigger (Pearson´s coefficient equal to -0.85). None of the mustelids and dogs sampled showed any clinical signs neither of CPV nor CDV disease. Dogs had the highest seroprevalence for both diseases, followed by minks, and finally otters ($F_{2\text{-}291}$:34.7; P<0.01) (figures 2 and 3). CPVs recorded a higher prevalence than CDV, with a difference close to significant (Mann-Whitney U test 9.6; df: 1; P=0.06). Out of the total, 35 (47% of) dogs were seropositive to both PV and CDV, and 4 (7% of) minks were seropositive to both PV and CDV (table 2). Observed seroprevalence of PVs in dogs and minks showed no difference between gender or age, but a significant difference for PV seroprevalence in dogs regarding their population density was recorded ($F_{2\text{-}71}$:3.9; P=0.03), dogs had a higher observed seroprevalence in those zones with a higher population density. However, no relationship between dog population density and PVs seroprevalence in mink was inferred (figures 2 and 3, table 1).

Regarding CDV, seroprevalence in dogs and minks showed no difference between gender or age, but dogs had a significantly higher seroprevalence ($F_{2\text{-}75}$:6.3; P<0.01) in those areas with higher dog population density (figures 2 and 3), namely in those areas with smaller mink population density ($F_{2\text{-}75}$:3.3; P=0.04).

Otters did not show significant differences in PV or CDV seroprevalence concerning the sampling area, gender or age. Observed seroprevalence of PV in otters had a positive tendency towards those areas with higher mink population density (Pearson correlation Matrix= 0.43). In contrast, CDV seroprevalence observed in otters had a higher tendency to occur in those areas with higher dog population density (Pearson correlation Matrix= 0.43) (figures 2 and 3).

When grouping the data of the studied species, both diseases showed a higher observed seroprevalence in those sampling areas with larger dog population density ($F_{2\text{-}146}$:6.5; P<0.01), and in lower mink population density ($F_{2\text{-}146}$:3.7; P=0.03) (table 1). Although there was no significant difference, both diseases registered higher observed seroprevalence (%) in males than in females: dog (69/63), mink (27/16), and otter (17/11).

DISCUSSION

Domestic and introduced alien animals may act as amplifiers of infectious diseases and as a source of a pathogen for diseases that could otherwise not be maintained by native species with already low-density wild populations (Grenfall & Dobson, 1995; Woodroffe, 1999; Medina-Vogel, 2010). Pathogen spillover from domestic animals can occur when they are near wild ones (Lembo *et al.*, 2008; Sepúlveda *et al.*, 2014). Indirect or direct contact must exist for spillover to occur in diseases such as PV and CDV, also, population abundance of the domestic reservoir seems to be a very important issue. Several studies have found significant positive relationships between urbanisation, proximity to farms, presence of domestic dogs, and CPV and/or CDV seropositive foxes, wolves, and mustelids (Frölich *et al.*, 2000; Acosta-Jamett *et al.*, 2011; Acosta-Jamett *et al.*, 2014; Millán *et al.* 2015). Therefore, it is plausible that the host population density as well as how the species interact with each other affect the pattern of transmission of infectious diseases like CDV and CPV. For instance, Millán *et al.*, (2015) did not record any wolves positive to CDV, but 76% of the wolves that they studied presented evidence of exposure to CPV. Long term studies in the USA have shown that CPV is already enzootic in wolf populations (Mech *et al.*, 2008; Almberg *et al.*, 2009) where it can be maintained in the absence of reintroductions, but not CDV. This is consistent with the fact that CDV is an acute, highly immunising pathogen that requires high densities, and a large population of hosts for long term persistence; although CDV might also persist among terrestrial carnivores with small, patchily

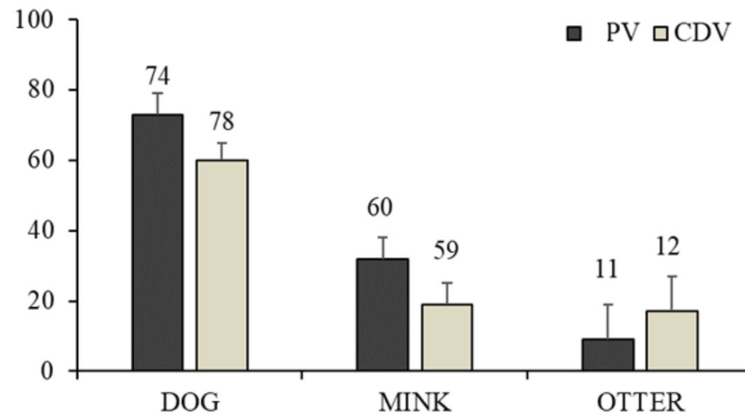
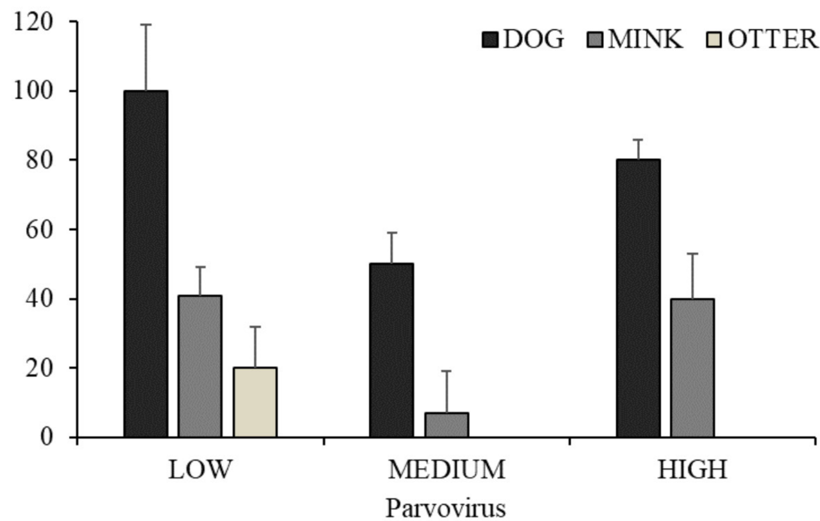


Figure 2. Observed seroprevalence (%) in domestic dogs, American mink, and Southern river otter in Southern Chile. Numbers indicate sample size; error bars indicate standard error.



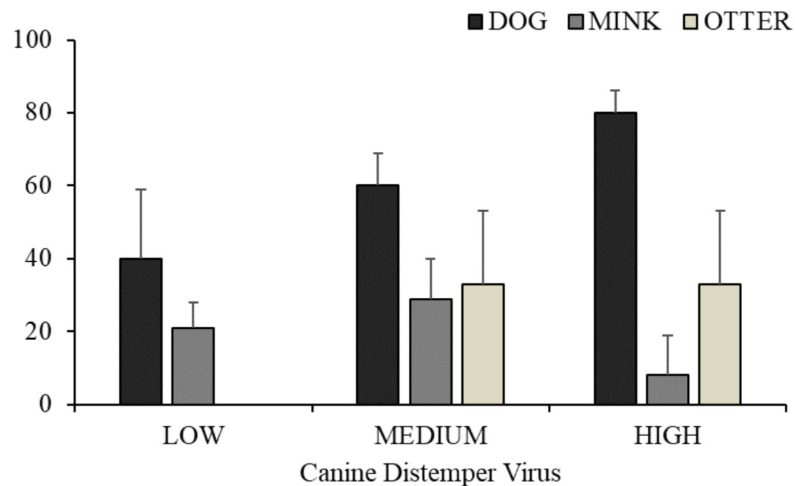


Figure 3. Observed seroprevalence (%) for PV and CDV in domestic dogs, American mink and Southern River otter in Southern Chile, according to estimated dog population size. Error bars indicate standard error.

distributed groups (Almberg *et al.*, 2010). Moreover, CDV is a pathogen with a short infection cycle that requires either large scales of hosts or multi-host transmission for its persistence, and it seems that wild carnivore species with naturally induced small populations cannot maintain CDV by themselves (Cleaveland *et al.*, 2000). However, the presence of a second competent host species can substantially increase the probability of long-term CDV persistence in a region (Almberg *et al.*, 2010). There are only a few studies concerning the spillover of CDV and CPV from domestic dogs to wild carnivores, where domestic as well as wild carnivore population densities have been estimated and their dynamics elucidated. Empirical studies seeking to identify disease population thresholds in wildlife find recurring obstacles, like small sample sizes and confounding factors (Lloyd-Smith *et al.*, 2005; Mech *et al.*, 2008, Cleaveland *et al.*, 2007; Almberg *et al.*, 2010). For instance, otters are susceptible to CDV and such is the case of American river otter (*Lontra canadensis*) (Kimber *et al.*, 2000) and captive Asian clawless otter (*Aonyx cinereus*) (Geisel, 1979; Madsen *et al.*, 1999; Mos *et al.*, 2003; De Bosschere *et al.*, 2005), however, only vague accounts of clinical canine parvovirus (CPV-2 variant) in otter species have been reported in the literature (Famini *et al.*, 2013; Gjeltema *et al.*, 2015; Miranda & Thompson, 2016). Another problem is the failure to find wild otters and minks with clinical signs of either CDV or CPV, this is probably because sick individuals remain in their dens during the disease and some die there (Barker & Parrish, 2001; Williams, 2001). This suggests that our results must be considered with caution due to the small sample size of the otters, the number of animals sampled per sampling zone, and the limitation of cross-sectional studies (Gilbert *et al.*, 2013). Moreover, we failed in finding molecular evidence for CDV, since infectious periods of this disease are short (Deem *et al.*, 2000); nevertheless, our sample size was comparable to that of Sobrino *et al.* (2008) and Kimber *et al.* (2000), but smaller than that of Delahay & Frölich (2000), none of which found antibodies against CDV.

The results we obtained regarding the observed seroprevalence for CDV were similar to those found by Sepúlveda *et al.* (2014) using 1:16 titer cut-off for mink (21.7% them, 17% us) and dogs (41.6% them, 60% us), but higher than those reported by Kimber *et al.* (2000) for river otter (*Lontra canadensis*) in North America because they recorded 4.7% (3 of 64) positive for CDV (1:8-1:768), and 7 of 64 (10.9%) otters positive for CPV-2 (range of titers 1:20-1:640). However, higher seroprevalences were found when compared to those obtained for American mink and other mustelids in France by Philippa *et al.* (2008) who recorded 9% of 127 European mink (*Mustela lutreola*), 20% of 210 polecats (*Mustela putorius*), 5% of 112 American mink, 33% of 21 stone marten (*Martes foina*), and 5% of 20 pine marten (*Martes martes*) in regions with almost no presence of free-ranging domestic dogs (Doherty *et al.*, 2017), although they considered positive a 1:10 titer. In our case, the observed seroprevalence in American mink was 33% for CDV with a titer 1:350, and 16% for CPV with a titer 1:16. Besides, these results are useful to begin the understanding of the ecology of CDV and CPV in a carnivore community where domestic dogs and alien American mink might be playing an important role (Cleaveland *et al.*, 2000; Gilbert *et al.*, 2013; Sepúlveda *et al.*, 2014); our cross-sectional assessment provides reliable information about exposure to viral infectious diseases of wild American minks and Southern river otters, adding support to the hypothesis of Sepúlveda *et al.* (2014) about behavioural aspects of transmission, with invasive species as host bridges from domestic to native species. Moreover, for the first time, CPV serological evidence in Southern river otters was documented in this study, as well as CPV serological and molecular evidence in wild American mink, and CDV serological evidence in otter from South America. Statistical differences in the observed seroprevalence of CDV and CPV in Southern river otter were not found, probably because of the small sample size. Nevertheless, we recorded a tendency of increased CDV seroprevalence in otter in those study areas with higher dog estimated population density, and similarly for CPV seroprevalence in otter in those study areas with higher mink estimated population densities (tables 1 and 2; figures 2 and 3).

Theoretical models suggest that whenever strong spatial segregation leads to distinct sub-grouping within a population, as it is the case for territorial species, interspecies transmission may be the dominant transmission pathway and the presence of an alternative host is required for pathogen establishment (Holt *et al.*, 2003; Keeling, 2005). Also, the influence of social hierarchy on disease dynamics becomes more important at low disease prevalence (Davidson *et al.*, 2008) and this seems to be the case for minks and otters, which have territorial as well as social hierarchical behaviour (Powell, 2000). Sepúlveda *et al.* (2014) found significant interactions between introduced American mink and both otter and dogs, either directly (harassment) or indirectly (latrines co-use). The indirect interactions between mink and dogs in latrines were not separated by more than two days (Sepúlveda *et al.*, 2014), an interval in which a pathogen, such as CDV and CPV, can remain viable in the environment (Shen & Gorham, 1980; Parrish, 1990; Williams, 2001). Similar observations were reported by Medina-Vogel *et al.* (2013) with a variable space overlap as well as latrines co-use between Southern river otter and mink, and aggressive encounters between both species in a dog free habitat. These facts led Sepúlveda *et al.* (2014) to theorise the feasibility of transmission of infectious diseases like CDV from dogs to River otter with mink acting as a *bridge host*, allowing our results to support this hypothesis. Moreover, the interspecies interactions mentioned above suggest a directional transmission from dog to mink, and from mink to otter.

With regard to interactions between domestic dogs and domestic cats with wild carnivores, transmissions were reported in a recent study in Madagascar, due to an important overlap in habitat use and specific sites (Rasambainarivo *et al.*, 2017). Frölich *et al.* (2000) found a significant difference in the number of seropositive foxes between urban, suburban and rural areas, indicating that free-living foxes can become infected with CDV by contact with domestic dogs. These authors conclude that dogs are contaminating the habitat of wild carnivores and, therefore, dog density would influence the seroprevalence of CDV antibodies in wild carnivores. Our results support the hypothesis that CDV and CPV are maintained in wild carnivore species due to the permanent presence of domestic dogs, we did not find molecular evidence of CDV in otters and mink but our results suggest that the exposure of mink to PVs could be the result of CPV infections.

In our study, dog-to-mink transmission seems to occur when minks visit farms attracted by rats, mice and poultry, and then can be infected by dogs (Philippa *et al.*, 2008; Sepúlveda *et al.*, 2014). The other possibility seems to be when dogs visit mink latrines (Sepúlveda *et al.*, 2014). Therefore, dog-to-mink transmission seems to take place as a result of the dynamics of scent communication since dogs use their faeces as territorial marks, and minks become in contact with those marks. These transmission rates appear to depend on domestic dog population size and density (table 1) and on the possibility that minks can get in contact with an infected spot. Instead, CPV and CDV transmission between mink and Southern river otters may occur mainly by direct interaction or when they are in close contact within a commonly used spraint site (Sepúlveda *et al.*, 2014; Medina-Vogel *et al.*, 2013). Transmission rates do appear to depend on the frequency of these contacts and on mink population densities, but not on Southern river otter population densities which are significantly lower: southern river otters in freshwater habitats in Chile record densities below 0.5 individuals per km of river (Sepúlveda *et al.*, 2007). Male mustelids have larger home ranges than females, leading to increased exposure to infections. Additionally, male increased stress during the breeding season may immunosuppress and increase their susceptibility to diseases (Powell, 2000; Cross *et al.*, 2009). Although we did not record gender statistical differences between observed seroprevalence in all three-study species, we found that seroprevalence was higher in males than in females. Male mink larger home range, strongest territorial behaviour, and larger displacement patterns could have increased the probability of becoming in contact with male dogs as well as male otters, which is supported by our results showing that this pattern is stronger for CDV than for PV.

As previously mentioned, our analysis supports the hypothesis of Sepúlveda *et al.* (2014) of American mink acting as a bridge host between domestic dogs and wild Southern river otter in Patagonia for infectious diseases such as PV and CDV, which is a matter of important conservational concern. These results raise a concern about the conservation of Patagonian otters because, from the perspective of the pathogen, one of the susceptible populations namely southern river otter and marine otter (*Lontra felina*) is made up of a small group of hosts (Sepúlveda *et al.*, 2007; Medina-Vogel *et al.*, 2007).

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REFERENCES

Acosta-Jamett G., Chalmers, W. S., Cunningham, A.A., Cleveland, S., Handel, I. G., & Bronsvoort, B. M. deC. (2011). Urban domestic dogs populations as a source of canine distemper virus for wild carnivores in the Coquimbo region of Chile. *Veterinay Microbiology 152*(3-4), 247-257. https://doi.org/10.1016/j.vetmic.2011.05.008

Acosta-Jamett, G., Cunningham, A. A., Bronsvoort, B. M., & Cleveland, S. (2014). Serosurvey of canine distemper virus and canine parvovirus in wild canids and domestic dogs at the rural interface in the Coquimbo Region, Chile. *European Journal of Wildlife Research 61*, 329-332. https://doi.org/10.1007/s10344-014-0886-0

Almberg, E. S., Mech, L. D., Smith, D. W., Sheldon, J. W., & Crabtree, R. L. (2009). A serological survey of infectious disease in Yellowstone National Park's canid community. *PLoS One* 4:e7042. https://doi.org/10.1371/journal.pone.0007042

Almberg, E. S., Cross, P. C., & Smith, D. W. (2010). Persistance of canine distemper virus in the Greater Yellowstone Ecosystem's carnivore community. *Ecological Aplications 20*(7), 2058-2074. https://doi.org/10.1890/09-1225.1

Barker, I. K., & Parrish, C. R. (2001). Parvovirus Infections. In: Williams, E.S. & Barker, I.K. 3th edition. *Infectious Diseases of Wild Mammals, 3rd ed. Iowa State University* Press, pp 131-146.

Barros, M., Cabezón, O., Dubey, J. P., Almería, S., Ribas, M. P., Escobar, LE, Ramos, B, & Medina-Vogel, G. (2018). *Toxoplasma gondii* infection in wild mustelids and cats across an urban-rural gradient. *PlosOne 13*(6), e0199085. https://doi.org/10.1371/journal.pone.0199085

Blundell G., Kern J., Bowyer T., & Duffy L. (1999). Capturing river otters: a comparison of Hancock and leg-hold traps. *Wildlife Society Bulletin 27*(1), 184-192.

Cleaveland, S., Appel, M. G. J., Chalmers, W. S. K., Chillingworth, C., Kaare, M., & Dye, C. (2000). Serological and demographic evidence for domestic dogs as a source of canine distemper virus infection for Serengeti wildlife. *Veterinary Microbiology*, *72*(3-4), 217-227. https://doi.org/10.1016/s0378-1135(99)00207-2

Cleaveland, S., Mlengeya, T., Kaare, M., Haydon, D., Lembo, T., Laurenson, M. K., & Paker, C. (2007). The conservation relevance of epidemiological research into carnivore viral diseases in the serengeti. *Conservation Biology 21*(3), 612-622. https://doi.org/10.1111/j.1523-1739.2007.00701.x

Courchamp, F., Say, L., & Pontier, D. (2000). Detection, identification, and correction of bias in an epidemiological study. *Journal of Wildlife Diseases 36*(1), 71-78. https://doi.org/10.7589/0090-3558-36.1.71

Craft, M. E., Volz, E., Packer, C., & Meyers, L. A. (2011). Disease transmission in territorial populations: the small-world network of Serengeti lions. *Journal of the Royal Society Interface 8*(59), 776-786. https://doi.org/10.1098/rsif.2010.0511

Cross, P. C., Drewe, J., Patrek, V., Pearce, G., Samuel, M. D., & Delahay, R. J. (2009). Wildlife population structure and parasite transmission: implications for disease management. In *Management of disease in wild mammals*. Delahay R., Smith G. & Hutchings M. R. (eds). Springer, Tokyo, Pp 9-29.

Daszak, P., Cunningham, A. A., & Hyatt, A. D. (2000). Emerging infectious diseases of wildlife - threats to biodiversity and human health. *Science*, *287*(5452), 443-449. https://doi.org/10.1126/science.287.5452.443

Davidson, R. S., Marion, G., & Hutchings, M. R. (2008). Effects of host social hierarchy on disease persistence. *Journal of Theoretical Biology*, *253*, 424-433. https://doi.org/10.1016/j.jtbi.2008.03.021

De Bosschere, H., Roles S., Lemmens, N., & Vanopdenbosch, E. (2005). Canine distemper virus in Asian clawless otter (*Aonyx cinereus*) littermates in captivity. *Vlaams Divergences Tijdschr* 74, 299.

Deem, S.L., Spelman, L. H., Yates, R. A., & Montali, R. J. (2000). Canine Distemper in Terrestrial Carnivores: a Review. *Journal of Zoo and Wildlife Medicine*, *31*(4), 441-451. https://doi.org/10.1638/1042-7260(2000)031[0441:CDITCA]2.0.CO;2

Delahay, R., & Frölich, K. (2000). Absence of antibodies against canine distemper virus in free-ranging populations of the Eurasian badger in Great Britain. *Journal of Wildlife Diseases 36*(3), 576-579. https://doi.org/10.7589/0090-3558-36.3.576

Doherty, T. S., Dickman, C. R., Glen, A. S., Newsome, T. M., Nimmo, D. G., Ritchie, E. G., Vanak, A. T., & Wirsing, A. J. (2017). The global impact of domestic dogs on threatened vertebrates. *Biological Conservation 210*, 56-59. https://doi.org/10.1016/j.biocon.2017.04.007

Famini, D., Gabriel, M. W., & Anderson, M. L. (2013). Case report of natural exposure, and one mortality of North American river otters (*Lontra canadensis*) to parvovirus in a rehabilitation center. *Proceedings of the Wildlife Society Western Section 2013 Annual Meeting, USA,* 16-17.

Frölich, K., Czupalla, O., Haas, L., Henschke, J., Dedek, J., & Fickel, J. (2000). Epizootiological investigation of canine distemper visrus in free-ranging carnivores from Germany. *Veterinary Microbiology 74*(4), 283-292. https://doi.org/10.1016/s0378-1135(00)00192-9

Geisel, O. (1979). Distemper in otters (*Lutra lutra*). *Berliner Und Munchener Tierarztliche Wochenschrift 92*(15), 304.

Gilbert, A. T., Fooks, A. R., Hayman, D. T. S, Horton, D. L., Müller, T., Plowright, R, Pel, A. J., Bowen, R, Wood, J. L. N., Mills, J., Cunningham, A. A., Rupprecht, C. E. (2013). Deciphering serology to understand the ecology of infectious diseases in wildlife. *EcoHealth 10*(3), 298-313. https://doi.org/10.1007/s10393-013-0856-0

Gjeltema, J., Murphy, H., & Rivera, S. (2015). Clinical canine parvovirus type 2C infection in a group of Asian small-clawed otters (*Aonyx cinerea*). *Journal of Zoo and Wildlife Medicine 46*(1), 120-123. https://doi.org/10.1638/2014-0090R1.1

González-Acuña, D., Ortega-Vásquez, R., Rivera-Ramírez, P., & Cable-lo-Cabalin, J. (2003). Verdacht auf Staupe beim Graufuchs (*Pseudalopex griseus*) im mittleren Chile (Fallbericht). *Zeitschrift fur Jagdwissenschaft 49*, 1-4. https://doi.org/10.1007/BF02189641

Grenfell, B. T., & Dobson, A. P. (1995). *Ecology of Infectious Diseases in Natural Populations*, Cambridge University Press.

Hammer, A. S., Dietz, H. H., & Hamilton-Dutoit, S. (2007). Immunohistochemical detection of 3 viral infections in paraffin-embedded tissue from mink (*Mustela vison*): a tissue-microarray-based study. *The Canadian Journal of Veterinary Research 71*(1), 8-13.

Harrington, L. A., Harrington, A. L., & MacDonald, D. W. (2008). Estimating the relative abundance of American mink *Mustela vison* on lowland rivers: evaluation and comparison of two techniques. *European Journal of Wildlife Research 54*(1), 79-87. https://doi.org/10.1007/s10344-007-0114-2

Holt, R. D., Dobson, A. P, Begon, M, Bowers, R. G, & Schauber, E. M. (2003). Parasite establishment in host communities. *Ecology Letters 6*(9), 837-842. https://doi.org/10.1046/j.1461-0248.2003.00501.x

Keeling, M. (2005). The implications of network structure for epidemic dynamics. *Theoretical Population Biology 67*, 1-8. https://doi.org/10.1016/j.tpb.2004.08.002

Kimber, K. R, Kollias, G. V, & Dubovi, E. J. (2000). Serologic survey of selected viral agents in recently captured wild North American river otters (*Lontra canadensis). Journal of Zoo and Wildlife Medicine 31*(2), 168-75. https://doi.org/10.1638/1042-7260(2000)031[0168:SSOSVA]2.0.CO;2

Lembo, T., Hampson, K., Haydon, D. T., Craf, M., Dobson, A., Dushoff, J., Ernest, E., Hoare, R., Kaare, M., Mlengeya, T., Mentzel, C., & Cleaveland, S. (2008). Exploring reservoir dynamics: a case study of rabies in the Serengeti ecosystem. *Journal of Applied Ecology 45*(4), 1246-1257. https://doi.org/10.1111/j.1365-2664.2008.01468.x

Lloyd-Smith, J. O., Cross, P. C., Briggs, C. J., Daugherty, M., Getz, W. M., Latto, J., Sanchez, M.S., Smith, A.B., & Swei, A. (2005). Should we expect population threshold for wildlife diseases? *Trends in Ecology and Evolution 20*(9), 511-519.

Madsen, A., Dietz, H., Henriksen, P., & Clausen, B. (1999). Survey of Danish free living otters *Lutra lutra* - a consecutive collection and necroscopy of dead bodies. *IUCN Otter Specialist Group Bulletin 16*(2), 65-76.

Mech, L. D., Goyal, S. M., Paul, W. J., & Newton, W. E. (2008). Demographic effects of canine parvovirus on a free-ranging wolf population over 30 years. *Journal of Wildlife Diseases 44*(4), 824-836. https://doi.org/10.7589/0090-3558-44.4.824

Medina G. (1996). Conservation status of *Lutra provocax* in Chile. *Pacific Conservation Biology 2*(4), 414-419. https://doi.org/10.1071/PC960414

Medina G. (1997). A comparison of the diet and distribution of southern river otter (*Lutra provocax*) and mink (*Mustela vison*) in Southern Chile. *Journal of Zoology 242*(2), 291-297. https://doi.org/10.1111/j.1469-7998.1997.tb05802.x

Medina-Vogel, G., Boher, F., Flores, G., Santibañez, A., & Soto-Azat, C. (2007). Spacing behavior of Marine otters (*Lontra felina*) in relation to land refuges and fishery wastes in Central Chile. *Journal of Mammalogy 88*(2), 487-494. https://doi.org/10.1644/06-MAMM-A081R1.1

Medina-Vogel, G. (2010). Ecología de enfermedades infecciosas emergentes y conservación de especies silvestres. *Archivos de Medicina Veterinaria 42*(1), 11-24. http://dx.doi.org/10.4067/S0301-732X2010000100003

Medina-Vogel, G., Barros, M., Organ, J. F., & Bonesi, L. (2013). Coexistence between the southern river otter and the alien invasive North American mink in marine habitats of southern Chile. *Journal of Zoology 290* (1), 27-34.

Medina-Vogel, G., Barros, M., Monsalve, R., & Pons, D.J. (2015). Assessment of the efficiency in trapping North American mink (*Neovison vison*) for population control in Patagonia. *Revista Chilena de Historia Natural* 88, 9. http://dx.doi.org/10.1186/S40693-015-0040-8

Millán, J., López-Bao, J. V., García, E. J., Oleaga, A., Llaneza, L., Palacios, V., de la Torre, A., Rodríguez, A., Dubovi, E.J., & Esperón, F. (2015). Patterns of exposure of Iberian wolves (*Canis lupus*) to canine viruses in human-dominated landscapes. *Ecohealth 13*(1), 123-134. https://doi.org/10.1007/s10393-015-1074-8

Miranda, C., & Thompson, G. (2016). Canine parvovirus: the worldwide occurrence of antigenic variants. *Journal of General Virology 97*(9), 2043-2057. https://doi.org/10.1099/jgv.0.000540

Mos, L., Ross, P., McIntosh, D., & Raverty, S. (2003). Canine distemper virus in river otters in British Columbia as an emergent risk for coastal pinnipeds. *Veterinary Record Open 152*, 237-239. https://doi.org/10.1136/vr.152.8.237

Parrish, C. R. (1990). Emergence, natural history, and variation of canine, mink, and feline parvoviruses. *Advances in Virus Research 38*, 403-450. https://doi.org/10.1016/s0065-3527(08)60867-2

Philippa, J., Fournier-Chambrillon, C., Fournier, P., Schaftenaar, W., van de Bildt, M., van Herbeijnen, R., Kuiken, T., Liabeuf, M., Ditcharry, S., Joubert, L., Bégnier, M., & Osterhaus A. (2008). Serological survey for selected viral pathogens in free-ranging endangered European

mink (*Mustela lutreola*) and other mustelids from south-western France. *Journal of Wildlife Diseases 44*(4), 791-801. https://doi.org/10.7589/0090-3558-44.4.791

Powell, R. A. (2000). Animal Home Range and Territories and Home Range Estimators. *In* L. Boitani, & T. K. Fuller (Eds,). *Research Techniques in Animal Ecology* (pp. 65-110). Columbia University Press.

Rasambainarivo, F., Farris, Z.J., Andrianalizah, H., & Parker, P. G. (2017). Interactions between carnivores in Madagascar and the risk of disease transmission. *EcoHealth 14*(4), 691-703. https://doi.org/10.1007/s10393-017-1280-7

Riley, S. P. D, Foley, J., & Chomel, B. (2004). Exposure to feline and canine pathogens in bobcats and gray foxes in urban and rural zones of a national park in California. *Journal of Wildlife Diseases 40*(1), 11-22. https://doi.org/10.7589/0090-3558-40.1.11

Sepúlveda, M. A, Bartheld, J. L, Monsalve, R., Gomez, V., & Medina-Vogel, G. (2007). Habitat use and spatial behaviour of the endangered Southern river otter (*Lontra provocax*) in riparian habitats of Chile: Conservation implications. *Biological Conservation 140*(3-4), 329-339. https://doi.org/10.1016/j.biocon.2007.08.026

Sepúlveda, M. A, Singer, R., Silva-Rodríguez, E., Stowhas, P., & Pelican, K. (2014). Domestic dogs in rural communities around protected areas: conservation problem or conflict solution? *PLoS ONE 9*(1), e86152. https://doi.org/10.1371/journal.pone.0086152

Sepúlveda, M. A, Singer, R. S, Silva-Rodríguez, E. A, Eguren, A., Stowhas, P., & Pelican, K. (2014). Invasive American mink: linking pathogen risk between domestic and endangered carnivores. *Ecohealth 11*(3), 409-419. https://doi.org/10.1007/s10393-014-0917-z

Shen, D., & Gorham, J. (1980). Survival of pathogenic distemper virus at 5C and 25C. *Veterinary Medicine Small Animal Clinician 75*(1), 69-70.

Silva-Rodríguez, E. A., & Sieving, K. E. (2012). Domestic dogs shape the landscape-scale distribution of a threatened forest ungulate. *Biological Conservation 150*(1), 103-110. https://doi.org/10.1016/j.biocon.2012.03.008

Sobrino, R., Arnal, M. C., Luco, D. F., & Gortázar, C. (2008). Prevalence of antibodies against canine distemper virus and canine parvovirus among foxes and wolves from Spain. *Veterinary Microbiology 126*(1-3), 251-256. https://doi.org/10.1016/j.vetmic.2007.06.014

Soto-Azat, C., Boher, F., Flores, G., Mora, E., Santibañez, A., & Medina-Vogel, G. (2006). Reversible anesthesia in wild marine otters (*Lontra felina*) using ketamine and medetomidine. *Journal of Zoo and Wildlife Medicine 37*(4), 535-538. https://doi.org/10.1638/05-067.1

Steinel, A., Parrish, C. R., Bloom, P. M. E, & Truyen, U. (2001). Parvovirus infections in wild carnivores. *Journal of Wildlife Diseases 37*(3), 594-607. https://doi.org/10.7589/0090-3558-37.3.594

Toledo, O. X., & Zapater, A. E. (1989). *Geografía General y Regional de Chile*. Editorial Universitaria. Santiago de Chile.

Touihri, L, Bouzid, I, Daoud, R, Desario, C, El Goulli, A. F., Decaro, N., Ghorbel, A., Buonavoglia Bahloul, C. (2009). Molecular characterization of canine parvovirus-2 variants circulating in Tunisia. *Virus Genes 38*(2), 249-258. https://doi.org/10.1007/s11262-008-0314-1

Veblen, T. T., & Schlegel, F. (1982). Reseña ecológica de los bosques del sur de Chile. *Bosque* (*Valdivia*), *4*(2), 73-115. https://doi.org/10.4206/bosque.1982.v4n2-03

Villatoro, F., Sepúlveda, M. A., Stowhas, P., & Silva-Rodríguez, E. A. (2016). Urban dogs in rural areas: Human-mediated movement defines dog populations in southern Chile. *Preventive Veterinary Medicine 135*, 59-66. https://doi.org/10.1016/j.prevetmed.2016.11.004

Williams, E. S. (2001). Canine Distemper. In E. S. Williams & I. K. Barker (Eds.), *Infectious Diseases of Wild Mammals* (3rd ed., pp. 50-59). Iowa State University Press.

Woodroffe, R. (1999). Managing disease threats to wild mammals. *Animal Conservation 2*(3), 185-193. https://doi.org/10.1111/j.1469-1795.1999.tb00064.x

Zuberogoitia, I., González-Oreja, J.A., Zabala, J., & Rodríguez-Refojos, C. (2010). Assessing the control/erradication of an invasive species, the American mink, based on field data; how much would cost? *Biodiversity and Conservation 19*(5), 1455-1469.

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SHORT COMMUNICATION

Detection of pathogenic leptospira as a cause of abortion in cattle-observations on diagnosis

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ABSTRACT. Leptospirosis is a zoonotic infectious disease caused by members of the genus *Leptospira,* which affects domestic and wild animals. Cases of abortion in cattle have been associated with this infection, but these are often not adequately confirmed. To determine the best diagnostic strategy for leptospirosis-associated cases of abortion, we evaluated some of the techniques used in the veterinary laboratory and found that the key issues are sample type and timing. In a retrospective anatomical and histopathological analysis, we studied 42 aborted foetuses with lesions consistent with leptospirosis to check for the presence of pathogenic leptospira by qPCR, as well as ascertaining the serologic status of the cows. In addition, in a prospective analysis, cows that had aborted foetuses were analysed within 2 days of the event by MAT and qPCR using blood and urine samples. Analysis of the foetuses indicated that only 14.3% of the selected cases (6 of 42) gave a positive qPCR result. Regarding cows that had recently aborted foetuses, 4 out of 11 sampled showed a positive qPCR, while MAT tests showed only negative results. The evidence provided in this study indicates that the time that has elapsed since a clinical event has occurred and the type of clinical sample taken are key elements in the successful confirmation of pathogenic leptospira as the cause of abortion.

Key words: Leptospirosis, pathogenic leptospira, abortion, dairy cattle, diagnostic.

INTRODUCTION

Leptospirosis is probably one of the most widespread and prevalent zoonotic diseases worldwide (Hartskeerl *et al.*, 2011). This infectious disease is caused by a group of spirochetes of the genus *Leptospira,* called pathogenic leptospira.

Pathogenic leptospira infection in cattle, which is associated with reproductive failure, is considered a major cause of economic loss (Bolin & Alt, 2001). In dairy cattle, abortion and stillbirth are the most serious clinical events caused by pathogenic leptospira infection (Ellis, 2015), followed by "Milk Drop Syndrome" (Alonso-Andicoberry *et al.*, 2001; Bolin, 2003). The negative economic impact can also be attributed to the cost of treatment, increases in culling and low pregnancy rates (Dhaliwal *et al.*, 1996). Gädicke and Monti (2013), have estimated that the economic losses in Chile could be as high as US$143 per lactation when a case of abortion occurs. Leptospirosis is often difficult to diagnose, and frequent misdiagnosis probably makes it the most neglected infectious disease in cattle (Martins & Lilenbaum, 2017). This is particularly significant when the diagnosis of a clinical case must be confirmed. Identification of the aetiology is essential to establish proper control and mitigation measures in the herd. Furthermore, an early and accurate diagnosis is of paramount importance to establish appropriate antibiotic treatment when pathogenic leptospira infection is suspected (Adler & De la Peña Moctezuma, 2010).

This brings to light a frequent and widespread diagnostic problem, namely confirmation of the aetiology of cattle abortion due to pathogenic leptospira. In southern Chile, most veterinary practitioners take a serum sample to detect antibodies in an aborted cow and the result obtained is interpreted as confirmation of pathogenic leptospira (Elder *et al.*, 1985). Although less frequent, the Pathology Department of our Faculty has also received requests for histopathological analysis as a diagnostic method through the identification of lesions, mainly in the foetus, consistent with infection from/by pathogenic leptospira in cases of abortion.

In the present study, we aimed to ascertain the type of diagnostic tool, as well as the clinical specimen and sampling time that should be used to accurately establish the aetiology of a case of abortion in cattle once pathogenic leptospira infection is suspected.

MATERIAL AND METHODS

To accomplish the objective of the present study, we organised the methodology in two independent but complementary observational surveys (I and II):

DESIGN SURVEY I

To assess pathogenic leptospira detection in an abortion case, a retrospective study was carried out. Primarily, this included information on all cases of aborted cattle recorded in the Veterinary Anatomical Pathology laboratory at

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the Institute of Animal Pathology, Faculty of Veterinary Sciences, Universidad Austral de Chile, between 2010 and 2019. As a second inclusion criterion, the samples of aborted foetuses that underwent further analysis were those displaying gross lesions consistent with pathogenic leptospira infection, such as jaundice, foci of necrosis in the liver, as well as signs of histopathological lesions, such as accumulations of mononuclear cells in the liver and/or kidney, areas of necrosis in the liver, vacuolization in the liver and/or kidney. In addition, the result of the microscopic agglutination test (MAT) of the cow was also taken into account.

For each selected sample, 10 to 15 sections of 5 µm thick tissue were taken from the paraffin-embedded tissue samples using a precision rotation microtome (Jung®), intended for DNA extraction. The paraffin sections were stored in 1.7 mL Eppendorf tubes. After this, the deparaffinization procedure was carried out according to Miller *et al.* (1997). Deparaffinized tissue samples were subjected to a DNA extraction-purification protocol using the High Pure PCR Template Preparation Kit (Roche, Indianapolis, IN, USA), following the manufacturer's instructions. The DNA templates obtained from the above protocol were analysed in a qPCR system (Roche LightCycler 2.0) using a TaqMan probe and targeting the *LipL32* gene which is specific only to pathogenic leptospira species (Stoddard *et al.*, 2009).

DESIGN SURVEY II

To show evidence of active infection by pathogenic leptospira in live cattle that have recently aborted foetuses, a field cross-sectional survey was performed.

Between January and December 2020, cows that had aborted within the previous 48 hours were selected and sampled for this study. The sampling was carried out from five dairy cattle herds located in three different districts of the Los Ríos region, Chile. To assess the infection status of the animals studied, urine samples (5-20 mL) were taken through direct stimulation of the vulvar area. The urine was collected in sterile 50 mL Falcon tubes. Also, to detect the pathogen in the whole blood, individual blood samples (5 to 10 mL) were taken by venipuncture of the coccygeal vein of each animal, using vacutainer tubes with anticoagulant and, in parallel, without coagulant for the detection of antibodies in the blood serum, using individual needles for each animal in both cases. Both types of samples were kept at room temperature until they were transferred to the Laboratory of Infectious Diseases, Institute of Preventive Veterinary Medicine, Universidad Austral de Chile. The sampling was carried out in strict accordance with the Universidad Austral de Chile's Guide for the Use of Animals for Research. (www.uach.cl/direccion/investigacion/uso_animales.htm).

Urine samples were pretreated using an immunomagnetic separation (IMS) protocol coupled to real time PCR (qPCR), according to a published protocol (Tomckowiack *et al.*, 2020). A 25 mL aliquot of each urine sample was centrifuged at 4,000 g for 15 min and the pellet was resuspended in 1 mL of phosphate buffered saline (PBS) [137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4 (pH 7)] and then transferred to a 1.5 mL microcentrifuge tube and recentrifuged at 11,000 g for 5 min. Finally, the supernatant was discarded, the pellet was resuspended in 1 mL of PBS and a 100 µL aliquot was submitted to be used in the IMS protocol (Tomckowiack *et al.*, 2020), before proceeding with DNA extraction by High Pure DNA Template Preparation Kit protocol (Roche, USA).

From a whole blood sample, an aliquot of 200 µL of blood was taken (with EDTA) from Vacutainer tubes (Becton Dickinson, USA), from which DNA was extracted using the High Pure DNA Template Preparation Kit (Roche, USA). This was performed as described in survey I.

Pathogenic leptospira cell numbers (genome equivalents) detected by qPCR were estimated according to a published protocol used in Tomckowiack *et al.* (2020), using the molecular weight of the genome of *Leptospira interrogans* serovar Hardjo type prajitno strain Hardjoprajitno (GenBank accession number EU357983.1) to establish a standard curve for the estimation of leptospira numbers by qPCR, according to a published algorithm (Dzieciol *et al.*, 2010).

Sera were tested for the presence of antibodies against six reference leptospira serovars, according to the published protocol (Salgado *et al.*, 2014), using the microagglutination test (MAT).

For survey I, the association between cow MAT and foetal tissue PCR results was evaluated by the McNemar test. For survey II, the proportion of positive results obtained by the diagnostic tests used for each of the clinical samples were compared. To do this, the ratio test was performed, using the Z statistic. The R software (R Core Team, year 2016®) was used, considering a significance level of 5%.

RESULTS AND DISCUSSION

To propose an adequate solution to the problem of pathogenic leptospira infection in cattle, we must first have a thorough understanding of the biology of this infection. Infection by pathogenic leptospira begins with bacteremia and the infection can then migrate to organs such as the liver and kidney, which is followed by leptospira urine shedding and, weeks later, antibody titers (Adler & De la Peña Moctezuma, 2010; Adler, 2014).

Because pathological analysis is also used as a diagnostic method, through the identification of lesions consistent with infection by pathogenic leptospira in cases of abortion, it seemed pertinent to us to retrospectively analyse a significant number of possible cases of abortions due to pathogenic leptospires. A total of 247 aborted bovine foetuses met the inclusion criteria in the retrospective study. Macroscopic and/or microscopic findings associated with pathogenic leptospira infection, as well as MAT results of the (mother) cow, led us to select a total of 42 aborted

foetuses. Overall, information from 7 (16.7%) of them showed only macroscopic lesions (enlarged liver, small areas of necrosis, jaundice). In addition, 10 (23.8%) displayed only microscopic lesions (inflammatory mononuclear (cell) infiltration), cloudy swelling) and 13 (30.9%) showed both types of lesions consistent with pathogenic leptospira infection. Finally, in 12 (28.6%) cases the sera showed MAT positive results without lesions. Although the lesions identified in these foetuses correspond with what has been considered suggestive for pathogenic leptospira infection, in most cases these lesions can be seen in other pathologies (Sebastian *et al.*, 2005; Smyth *et al.*, 1999). In this regard, Schlafer and Foster (2016) reported other findings, such as lesions in the placenta and lungs, which were not observed in the cases we studied because the majority of foetuses displayed a state of autolysis that made it difficult to identify these lesions (Smyth *et al.*, 1999). Therefore, these anatomical and histological findings should only be interpreted as presumptive information that requires a second confirmatory tool.

Therefore, in order to confirm those presumptive cases, the PCR technique on fixed tissue was used. Only 6 (14.3%) out of the 42 cases showed PCR positive results (table 1). Of these 6 cases, 1 sample case showed both microscopic and macroscopic lesions, while 2 showed only macroscopic lesions, and finally, 3 foetal sample cases did not show any visible lesions but exhibited high MAT antibody titers. However, molecular identification by PCR in paraffin-embedded samples may be underestimated due to a decrease in PCR efficiency. The study by Einerson *et al.* (2005) showed a loss of detection of up to 50% in their samples, attributed to the effect of the fixing reagents on the efficiency of the PCR.

Since the most commonly used diagnostic technique for leptospira infection is MAT (Thiermann, 1984), it seemed reasonable to monitor the serological status of the aborted cow. Thirty-one of the 42 aborted fetuses monitored (73%) produced positive serology results for one or more leptospira serovars. Hardjo was the predominant serovar, with 24 cases (77.4%), followed by Ballum, with 9 cases (29%), whilst the Canicola and Pomona serovars were identified in 5 cases (16.1%), and Autumnalis showed up in just 1 case (3.2%). The antibody titers ranged between 1:100 to 1:3200 (data not shown). There was a high percentage of selected foetuses with lesions consistent with pathogenic leptospira infection, although unconfirmed by PCR, with a positive MAT result for the mother. Any valid interpretation drawn from this finding must take into account the fact that, in southern Chile, there is a high pathogenic leptospira seroprevalence in dairy herds (Salgado *et al.*, 2014). Besides, MAT is useful for a herd-level diagnosis and may not be reliable for individual diagnoses (Otaka *et al.*, 2012), since most cows that show seroreactivity with low titers show no direct evidence of pathogenic leptospira shedding (Hamond *et al.*, 2014). So, samples with positive MAT from the mother indicate exposure, and not necessarily active infection, as a cause of abortion, thereby explaining the lack of a significant relationship ($P<0.05$) between MAT and PCR.

A key aspect that would allow us to determine the cause of abortion is the status of active infection by pathogenic leptospira in the mother through direct detection of the pathogen at the genomic level. In this way, there would be a confirmatory relationship between infection by this pathogen and a case of abortion. In survey II, the sampling of cows that had recently aborted was successful due to the valuable collaboration of veterinary practitioners. We found only 11 cows that had aborted foetuses and were sampled within 48 hours during the study period. None of the sera in the cows that had aborted showed positive MAT results. Of the 11 cows, 4 cows showed positive results which suggest an active infection status due to pathogenic leptospira. In the case of urine samples, only 1 out of 11 (9.1%) samples showed a positive result with a low concentration of 8.77 leptospira per mL. However, a higher proportion of positive results was observed when

Table 1. Aborted foetuses with a history associated with leptospirosis identified in the records of the Laboratory of Veterinary Anatomic Pathology with positive PCR (n=6).

ID animal	Ab titers	Serovar	Macroscopic lesion	Microscopic lesion	qPCR
781-11	1:400	Hardjo	Pale red mucous membranes and musculature	Liver and kidney: degenerative conditions	(+)
101-13	1:400 1:100	Hardjo Pomona	–	–	(+)
188-13	1:1600	Canicola	–	–	(+)
272-13	1:800 1:200	Ballum Hardjo	–	–	(+)
363-13	1:400	Hardjo	–	Liver: necrotic foci	(+)
395-14	1:400 1:100	Hardjo Ballum	–	Kidney: degenerative conditions	(+)

Table 2. Results of the analysis of the 11 cows for pathogenic leptospira infection.

Nº	ID animal	qPCR (Whole Blood) pathogenic leptospira per mL	IMS-qPCR (Urine) pathogenic leptospira per mL	MAT
1	3806	–	–	–
2	6487	$4.86 \cdot 10^2$	–	–
3	8731	–	$8.77 \cdot 10^0$	–
4	8732	–	–	–
5	8327	–	–	–
6	8238	$2.11 \cdot 10^4$	–	–
7	8042	-	-	-
8	8726	$6.14 \cdot 10^3$	-	-
9	8523	-	-	-
10	6693	-	-	-
11	9980	-	-	-

blood samples were used ($P<0.05$). Three out of 11 blood (27.3%) samples were positive, ranging in concentration from $4.86 \cdot 10^2$ to $2.11 \cdot 10^4$ pathogenic leptospira per mL (table 2). The reported finding regarding the detection of pathogenic leptospira in whole blood is consistent with published experimental investigations (Zuerner *et al.*, 2012) that studied the infection in golden hamsters by injecting this pathogen intraperitoneally and could be detected in blood vessels around 48 hours post inoculation, giving a graphic indication of the biology of this infection. The biology of infection indicates that leptospiremia is observed up to the first week after exposure, which is followed by pathogen migration to the target organs (Adler & De la Peña Moctezuma, 2010). As pathogenic leptospira decrease in concentration in the blood, the antibodies start to rise, reaching a detectable level between 7-14 days (Adler, 2014).

It seems that blood samples do not have a negative effect on polymerase efficiency, unlike urine samples which is likely to be due to PCR inhibitors (Rosenstraus *et al.*, 1998). The presence of PCR inhibitors in urine samples makes the analysis more difficult and expensive where DNA extraction protocols should consider inhibitor removal. To solve this problem, immunomagnetic separation (IMS) has been described as a technique to provide inhibitor-free PCR samples and improve their analytical sensitivity (Olsvik *et al.*, 1994; Taylor *et al.*, 1997). Recently, Tomckowiack *et al.* (2020) developed an immunomagnetic separation (IMS) protocol as pretreatment for qPCR that offered a cost-effective tool for urine analysis and solved the false negative problems of low bacterial loads in the specimen, allowing 30% higher detection of positive results than a conventional system. In the present study, the low bacterial load detected by the IMS-qPCR system in the positive urine sample (8.44 bacteria/mL) suggests that the animal has an initial kidney infection post bacteremia, where it would possibly have been classified as negative in a conventional molecular detection system without the pre-step of immunoseparation.

Most of the abortions caused by leptospira occur in the last trimester of pregnancy and are associated with a chronic infection in the individual aborting animal (Adler *et al.*, 2014; BonDurant, 2007). The present study reported the absence of antibody titers in MAT and the presence of pathogenic leptospira in blood, which could indicate a status of early or acute infection with initial bacteremia, and in this infectious scenario, an abortion is not an unexpected result in a pregnant cow.

This study showed that anatomical and histopathological information must be considered as a presumptive and non-confirmatory tool for pathogenic leptospira as a cause of abortions in cattle. Also, the detection of pathogen DNA in blood may provide new evidence of abortions in the early stages of this infection, suggesting that the evaluation of antibodies alone is not an accurate diagnostic strategy for the cause of abortion in cattle. Besides, it has been suggested that a chronic event and MAT values are either static flailing or are not detectable (Ellis *et al.*, 1982). The evidence provided in this study indicates that the time elapsed since a clinical event has occurred and the type of clinical sample taken are key elements in the successful confirmation of pathogenic leptospira as the cause of abortion.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

REFERENCES

Adler, B., & De la Peña Moctezuma, A. (2010). Leptospira and leptospirosis. *Veterinary Microbiology*, *140*, 287-296.

Adler, B. (2014). *Leptospira and leptospirosis*. Ed. (Vol. 387). Springer.

Alonso-Andicoberry, C., García-Peña, F. J., & Ortega-Mora, L. M. (2001). Epidemiología, diagnóstico y control de la leptospirosis bovina (Revisión). *Investigación agraria. Producción y sanidad animales*, *16*(2), 205-225.

Bolin, C. A. & Alt, D. P. (2001). Use of a monovalent leptospiral vaccine to prevent renal colonization and urinary shedding in cattle exposed to *Leptospira* borgpetersenii serovar hardjo. *American Journal of Veterinary Research*, *62*(7), 995-1000. https://doi.org/10.2460/ajvr.2001.62.995

Bolin, C. A. (2003). Diagnosis and control of bovine leptospirosis. In Proceedings of the 6th Western Dairy Management Conference, 12-14.

BonDurant, R. H. (2007). Selected diseases and conditions associated with bovine conceptus loss in the first trimester. *Theriogenology*, *68*(3), 461-473. https://doi.org/10.1016/j.theriogenology.2007.04.022

Dhaliwal, G. S., Murray, R. D., Dobson, H., Montgomery, J. & Ellis, W. A. (1996). Reduced conception rates in dairy cattle associated with serological evidence of *Leptospira* interrogans serovar hardjo infection. *Veterinary Record*, *139*(5), 110-114. https://doi.org/10.1136/vr.139.5.110

Dzieciol, M., Volgger, P., Khol, J., Baumgartner, W., Wagner, M. & Hein, I. (2010). A novel real-time PCR assay for specific detection and quantification of *Mycobacterium avium* subsp. paratuberculosis in milk with the inherent possibility of differentiation between viable and dead cells. *BMC Research Notes*, *3*, 1-8.

Einerson, R. R., Kurtin, P. J., Dayharsh, G. A., Kimlinger, T. K., & Remstein, E. D. (2005). FISH is superior to PCR in detecting t (14; 18) (q32; q21)–IgH/bcl-2 in follicular lymphoma using paraffin-embedded tissue samples. *American Journal of Clinical Pathology*, *124*(3), 421-429.

Elder, J. K., Pepper, P. M., Hill, M. W. M. & Ward, W. H. (1985). The significance of leptospiral titers associated with bovine abortion. *Australian Veterinary Journal*, *62*(8), 258-262. https://doi.org/10.1111/j.1751-0813.1985.tb14246.x

Ellis, W. A., O'brien, J. J., Neill, S. D., Ferguson, H. W., & Hanna, J. (1982). Bovine leptospirosis: microbiological and serological findings in aborted fetuses. *Veterinary Record, 110*, 147-150.

Ellis, W. A. (2015). Animal leptospirosis. In B. Adler (Ed.), *Leptospira and Leptospirosis* (pp. 99-137). Springer.

Gädicke, P. & Monti, G. (2013). Factors related to the level of occurrence of bovine abortion in Chilean dairy herds. *Preventive Veterinary Medicine, 110*(2), 183-189. https://doi.org/10.1016/j.prevetmed.2012.11.022

Hamond, C., Martins, G., Loureiro, A. P., Pestana, C., Lawson-Ferreira, R., Medeiros, M. A. & Lilenbaum, W. (2014). Urinary PCR as an increasingly useful tool for an accurate diagnosis of leptospirosis in livestock. *Veterinary Research Communications, 38*(1), 81-85. https://doi.org/10.1007/s11259-013-9582-x

Hartskeerl, R. A., Collares-Pereira, M. & Ellis, W. A. (2011). Emergence, control and re-emerging leptospirosis: dynamics of infection in the changing world. *Clinical Microbiology and Infection, 17*(4), 494-501. https://doi.org/10.1111/j.1469-0691.2011.03474.x

Martins, G. & Lilenbaum, W. (2017). Control of bovine leptospirosis: Aspects for consideration in a tropical environment. *Research in Veterinary Science, 112*, 156-160. https://doi.org/10.1016/j.rvsc.2017.03.021

Miller, J., Jenny, A., Rhyan, J., Saari, D., & Suarez, D. (1997). Detection of *Mycobacterium bovis* in formalin-fixed, paraffin-embedded tissues of cattle and elk by PCR amplification of an IS6110 sequence specific for *Mycobacterium tuberculosis* complex organisms. *Journal of Veterinary Diagnostic Investigation, 9*(3), 244-249. https://doi.org/10.1177/104063879700900304

Olsvik, O., Popovic, T., Skjerve, E., Cudjoe, K. S., Hornes, E., Ugelstad, J., & Uhlen, M. (1994). Magnetic separation techniques in diagnostic microbiology. *Clinical Microbiology Reviews, 7*(1), 43-54. https://doi.org/10.1128/CMR.7.1.43

Otaka, D. Y., Martins, G., Hamond, C., Penna, B., Medeiros, M. A. & Lilenbaum, W. (2012). Serology and PCR for bovine leptospirosis: herd and individual approaches. *Veterinary Record, 170*(13), 338-338. https://doi.org/10.1136/vr.100490

Rosenstraus, M., Wang, Z., Chang, S. Y., DeBonville, D. & Spadoro, J. P. (1998). An internal control for routine diagnostic PCR: design, properties, and effect on clinical performance. *Journal of Clinical Microbiology, 36*(1), 191-197. https://doi.org/10.1128/JCM.36.1.191-197.1998

Salgado, M., Otto, B., Sandoval, E., Reinhardt, G. & Boqvist, S. (2014). A cross sectional observational study to estimate herd level risk factors for *Leptospira* spp. serovars in small holder dairy cattle farms in southern Chile. *BMC Veterinary Research, 10*, 126. https://doi.org/10.1186/1746-6148-10-126

Schlafer, D. H., & Foster, R. A. (2016). Female genital system. *Jubb, Kennedy & Palmer's Pathology of Domestic Animals: Volume 3*, 358. https://doi.org/10.1016/B978-0-7020-5319-1.00015-3

Sebastian, M., Giles, R., Roberts, J., Poonacha, K., Harrison, L., Donahue, J., & Benirschke, K. (2005). Funisitis associated with leptospiral abortion in an equine placenta. *Veterinary Pathology, 42*(5), 659-662. https://doi.org/10.1354/vp.42-5-659

Smyth, A., Fitzpatrick, D. A., & Ellis, W. A. (1999). Stillbirth/perinatal weak calf syndrome: a study of calves infected with Leptospira. *Veterinary Record, 145*, 539-542.

Stoddard, R. A., Gee, J. E., Wilkins, P. P., McCaustland, K. & Hoffmaster, A. R. (2009). Detection of pathogenic *Leptospira* spp. through TaqMan polymerase chain reaction targeting the LipL32 gene. *Diagnostic Microbiology and Infectious Disease, 64*(3), 247-255. https://doi.org/10.1016/j.diagmicrobio.2009.03.014

Taylor, M. J., Ellis, W. A., Montgomery, J. M., Yan, K. T., McDowell, S. W. J. & Mackie, D. P. (1997). Magnetic immuno capture PCR assay (MIPA): detection of Leptospira borgpetersenii serovar hardjo. *Veterinary Microbiology, 56*(1-2), 135-145. https://doi.org/10.1016/S0378-1135(96)01351-X

Thiermann, A.B. (1984). Leptospirosis: current developments and trends. *Journal of the American Veterinary Medical Association*, *184*(6), 722.

Tomckowiack, C., Henriquez, C., Ramirez-Reveco, A., Muñoz, P., Collado, B., Herzberg, D., Folch Hugo & Salgado, M. (2020). Analytical evaluation of an immunomagnetic separation PCR assay to detect pathogenic Leptospira in cattle urine samples obtained under field conditions. *Journal of Veterinary Diagnostic Investigation 33*(1), 52-58. https://doi.org/10.1177/1040638720966299

Zuerner, R. L., Alt, D. P. & Palmer, M. V. (2012). Development of chronic and acute golden Syrian hamster infection models with *Leptospira* borgpetersenii serovar Hardjo. *Veterinary Pathology, 49*(2), 403-411. https://doi.org/10.1177/0300985811409252

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SHORT COMMUNICATION

Evaluation of the efficacy of essential oils of *Lavandula angustifolia* and *Eucalyptus globulus* for the control of *Varroa destructor* in *Apis mellifera*: A randomised field study

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ABSTRACT. *Varroa destructor* is the most harmful and widespread parasite that spreads disease in bees. *Eucalyptus spp* essential oils (EOs) have proved effective against *V. destructor*. Additionally, *Lavender spp* EOs treatment has caused mite mortality rates of 95% to 97% for the same parasite. In this study, 20 mL of each oil or the placebo were distributed on two sheets of papier-mâché located on the frames of the brood chamber inside each hive. The miticidal effects of *Lavandula angustifolia* and *Eucalyptus globulus* EOs were analysed. Parasitic load and mite fall were evaluated under field conditions. The mean infestation rate obtained from each of the three treatment groups at the beginning of the study was less than 3.6%. Then, the infestation rate increased gradually in each group until day 36. The infestation rate in the group treated with *L. angustifolia* was lower than in the control group by over two per cent and never exceeded 10%; the differences between the control group and the *L. angustifolia* group were statistically significant ($P<0.05$). In conclusion, *L. angustifolia* EO provided effective parasite control starting at the second treatment dose. However, *E. globulus* EO did not show a consistent parasite control. Further studies should consider the evaluation of EOs for the control of *V. destructor* in different weather conditions and other treatment delivery systems.

Key words: bees, parasite, mite, varroosis, ecological control.

INTRODUCTION

As pollinators, bees participate in the global economy and their contribution has been valued at between 235 and 285 billion US$ per year (Lautenbach *et al.*, 2012). Currently, a general weakening of honeybee populations, represented by colony losses, has been reported raising public concern and, in turn, the costs of managing bee colonies and pollination services have increased (Calderone, 2012).

The decline in pollinators has been associated with multiple factors such as natural disasters, environmental pollution, and a variety of pathologies (Potts *et al.*, 2010). According to Neira *et al.* (2004) varroosis is the most serious parasitic disease in bees. A large number of products have been tested to control this disease, and their repeated and improper use has resulted in the production of contaminated honey. There is a current trend towards the use of natural products, creating a constantly increasing demand for them. For this reason, varroosis diagnostic and treatment methods have been the subject of studies in several countries to improve its control (Gonzalez-Acuña *et al.*, 2005).

Current methods used for *V. destructor* control are the application of acaricides such as fluvalinate and coumaphos. Both were initially effective, but their recurrent use has led to the development of resistance in the mites (Milani, 1999).

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Chemicals applied by beekeepers against varroosis are a source of bee product contamination. There are maximum residue limits for authorised chemical substances. The contamination of bee products by acaricides can be minimised through careful use of chemotherapeutic products, however, the use of unauthorised products to control varroosis could become a major problem (Karazafiris *et al.*, 2011).

Cruzat & Baasch (2010) established that the control of varroosis has been managed mainly with artificially synthesised chemical products such as fluvalinate, flumethrin, amitraz, bromopropylate, and cymiazole. Nevertheless, these products can have dangerous consequences due to the accumulation of their residues in honey, wax, and propolis. In addition, their improper and repeated use can lead to significant resistance against these products in *V. destructor*.

EOs generally represent a less-expensive and safer alternative for both humans and bees (Calderone, 2012). Also, they are classified as food supplements that are safe for human consumption (Quarles, 1996). EOs can alter the behaviour, growth, and development as well as the ecdysis, mating and oviposition of insects (Khater, 2012). Additionally, the insecticidal activities of the components of lavender EO have been related to acetylcholinesterase inhibition, and eucalyptus EO has been shown to exhibit octopaminergic agonist activity (Rattan, 2010).

In Chile, a limited range of veterinary products has been authorised for apicultural use by the Agricultural and Livestock Service (SAG, 2019) and registered under the names Bayvarol®, Verostop® and Apilife Var®. The active molecule of the first two products is flumethrin, while the third product contains thymol, levomenthol, eucalyptus oil and camphor.

On the other hand, the *Chilean National System for the Certification of Organic Products* created under Decree No. 36 of 2006 of the Ministry of Agriculture allows, according to its Technical Standard Annex A List 5, the treatment of pests and diseases that affect beekeeping with natural treatments such as phytotherapy, aromatherapy, etheric EOs (camphor, eucalyptol, menthol, thymol), sulfur, oxalic acid, lactic acid, acetic acid, and formic acid (SAG, 2019).

Neira *et al.* (2004) established that applying lavender and laurel EOs under laboratory conditions resulted in approximately 90 to 100% mite fall. In addition, the mite mortality rates reached average values of nearly 40%.

Several studies on the use of EOs against *V. destructor* have been conducted under controlled laboratory conditions and have demonstrated miticidal effects (Imdorf *et al.*, 1999). Eucalyptus EO, which is rich in 1-8-cineol, has proved effective against *V. destructor* (Ghasemi *et al.*, 2011). Additionally, thyme, sage, rosemary, marjoram, dillsun and lavender EOs at concentrations of 1% and 2% (w/w) resulted in mite mortality rates ranging between 95% and 97%, respectively, and peppermint at 2% (w/w) killed more than 97% of *V. destructor* (Ariana *et al.*, 2002).

The effectiveness of different synthetic (amitraz, Apivar®) and natural (formulated from Api Life Var®, thymol oil and thymol alcohol) products authorised for the control of *V. destructor* were evaluated in a field study. All these treatments reduced the infestations of *V. destructor*, although they did not eliminate the parasite. However, the effectiveness of the treatment depended on the apiary to which it was applied. The variability in effectiveness detected among different apiaries represents a challenge for the identification of the significant factors that influence miticide effectiveness (Gracia *et al.*, 2017).

To our knowledge, no studies have currently investigated the residual effects of EOs or any long-term application protocols for them in production apiaries, specifically with regard to the dose, duration, residual effect, or application time and frequency. EOs selection was based on preliminary trials with unpublished data which evaluated the effects of *Syzygium aromaticum, Citrus sinensis*, *Lavandula angustifolia* and *Eucalyptus globulus* EOS on total mite fall, observing the best results with *Lavandula angustifolia* and *Eucalyptus globulus.* The study aimed to evaluate the effects of *Lavandula angustifolia* and *Eucalyptus globulus* EOs for varroosis control in *A. mellifera* in a 48-day, double-blind field trial.

MATERIAL AND METHODS

LOCATION

The research was conducted in the experimental apiary at the School of Agricultural and Veterinary Sciences, Universidad Viña del Mar, Chile, under regular production management practices during the months of March and April 2018. The climate conditions were Mediterranean temperate, with median temperatures of 19°C and 16°C in March and April, respectively (33°04’06”S, 71°33’27”W).

ESSENTIAL OIL APPLICATION

Twenty-four Langstroth-type hives naturally infested with *V. destructor* were randomly allocated to three groups of 8 beehives. The treatments applied were a) 5% *E. globulus* EO (group E) (1.8-cineole 60.2%; α-pinene 15.26%) and b) 5% *L. angustifolia* EO (group L) (linalool 36.53%; linalyl acetate 32.8%), both diluted in vegetable glycerine, and c) glycerine as the placebo (control group, C). Both essential oils are of commercial origin (Ac Es Eucalipto Org 5 ml; Ac Es Lavanda Org 5 ml, Sociedad Comercial Katmandú SpA, Chile).

During the treatments, 20 mL of each oil or the placebo were distributed on two sheets of papier-mâché located on the frames of the brood chamber inside each hive. Four applications were performed, on days 1, 11, 22 and 33 of the experimental period, to cover two consecutive reproductive periods of the bees and the parasite. The treatments were applied with a double-blind treatment design.

COLONY INFESTATION RATE

The colony infestation rate was evaluated in a sample of 200 bees from the brood frames of each colony. The frames were placed in a container with water and non-foaming detergent and were covered and stirred for two minutes. After settling for 10 minutes, the contents of each container were poured through a double screen. The first screen retained the bees and the second collected the *V. destructor* individuals. The bees and *V. destructor* individuals were then counted. Finally, the infestation rate was calculated according to the following formula (De Jong *et al.*, 1982):

$$\textit{Infestation rate (\%)} \\ = (\textit{V. destructor number}) / (\textit{bees number per sample}) \times 100$$

To measure the effects of the treatments and compare them with the effect of the placebo, the basal parasitic load was determined by the double-sieve sampling protocol at the beginning of the study (day 1, before the first treatment) and every 9 days thereafter (days 9, 18, 27, 36 and 45).

TOTAL MITE FALL

In each hive, from days 2-45 of the experiment, a daily mite fall count was performed as a complementary method for determining the parasitic load of the hive. A piece of white cardboard was placed on the bottom board of the hive and covered with glycerine to trap fallen *V. destructor* individuals. The cardboard was removed and replaced daily.

The infestation rate and total number of mite fall were tabulated and analysed for significant differences between treatments with *Friedman's* and *Dunn's* tests ($P<0.05$).

RESULTS AND DISCUSSION

The mean infestation rates obtained from each group at the beginning of the study were lower than 3.6%. Then, a gradual increase in infestation rate was observed in each of the three groups until day 36 (Group C 11.1%; Group L 9.4%; Group E 9.8%). At this point, the infestation rates decreased, reaching similar levels to those on day 18, especially in the groups treated with EOs.

Group L presented significantly lower parasitic loads than group C ($P<0.05$), as evidenced by the infestation rates during the 45 days of the study. Furthermore, the lower total drop of mites observed in group L responds to the lower infestation rate in the same group compared to groups L and C. Nonetheless, group E showed non-significant differences in the parasitic loads compared to group C (figures 1 and 2).

According to Gracia *et al.*, (2017) parasitic infestations are almost impossible to eradicate, therefore, only partial control of parasites is possible. Our results are consistent with those of these authors and demonstrate effective, easy and safe mite control to infestation rates lower than 8% with lavender EO. These effects were modulated by the local environmental, genetic and production conditions, which determine the efficiency of parasitic control (Bounous & Boga, 2005). Thus, EOs can control varroosis and are not considered contaminants by the technical standards of the National System of Certification of Organic Products of Chile (SAG, 2019).

The analysis of the parasitic infestation of the bee colonies showed that until the second dose (day 11), the infestation rates in the three groups did not show linear behaviour. However, after the second treatment, the infestation rates in group L was consistently lower than in group C (figure 1).

In this study, *L. angustifolia* EO was an efficient treatment because it stopped the sustained growth of the infestation rate in the treated hives. In contrast, in the control group, the infestation rate increased continuously. These results are consistent with those of Jean-Prost & Conte, 2007), who established that sustained infestation rate growth occurs because the multiplication of the parasite is associated with bee reproduction and the absence of antiparasitic treatments. In addition, the effects of *L. angustifolia* EO on the infestation rates and total mite fall could be due to the application in late summer and early autumn because the bee reproduction begins to decrease during this period (Avitabile, 1978). The decrease in bee reproduction rate leads to a decline in the adult mite population, resulting in a low infestation rate at the beginning of spring, which represents an improvement in the sanitary condition of the hive.

In total, during the 45 days of the study, 1,132 adult mites fell in group L, 1,802 adult mites fell in group E, and 2,019 adult mites fell in group C. There were statistically significant differences in mite fall between groups C and L as well as between groups L and E ($P<0.05$) (figure 2).

Daily measurements of adult mite fall were performed during the study as a complementary method of determining the degree of infestation in each group. However, this approach was not intended to measure the effect of each

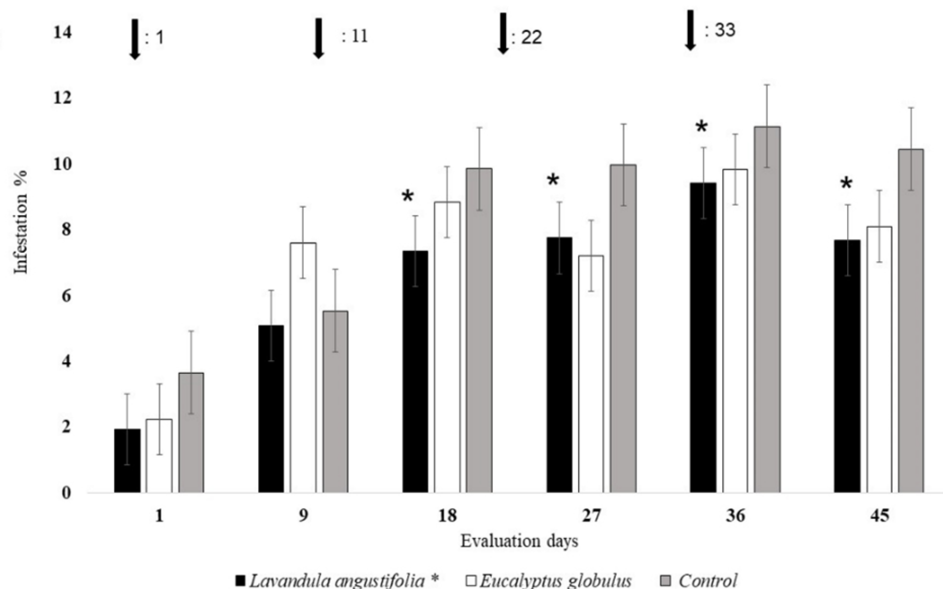

Figure 1. Mean infestation rate and standard error for each treatment group at different sampling times. Arrows indicate the treatment application times.

* indicates statistically significant differences between L and C according to Friedman's and Dunn's tests ($P<0.05$).

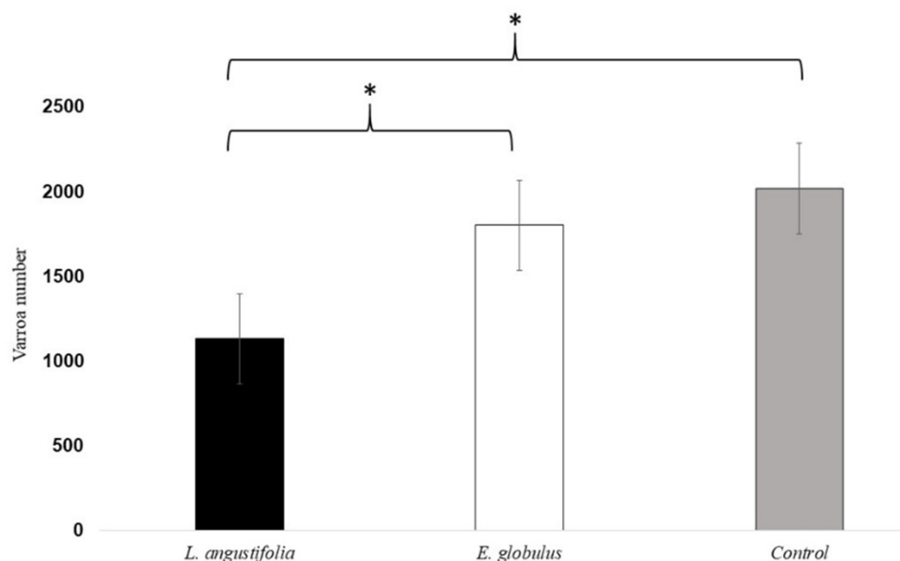

Figure 2. Total mite fall and standard error for each treatment group.
* indicates statistically significant differences according to Friedman's and Dunn's tests (*P*<0.05).

treatment since mite detachment can be caused by different factors, such as the size of the colonies, the typical handling of the hives, the grooming capacity of the families, the internal temperature of the hives, and the environmental temperature, as well as by the treatments applied (Spivak & Reuter, 1998).

Some limitations of our study included active behavioural defences of the hive that could affect the infestation rates individually, favourable weather conditions for mite reproduction such as temperature, humidity, or the availability of pollen and nectar, and the wrong entry of the bees in other hives that can affect the infestation rates. In addition, further studies should consider the evaluation of EOs for the control of *V. Destructor* in different weather conditions and other treatment delivery systems.

In conclusion, *L. angustifolia* EO provided effective parasite control. The infestation rates in the *L. angustifolia* EO treatment group were consistently lower than those in the control group starting at the second treatment dose due to the reproductive cycles of both species *Varroa destructor and Apis mellifera*. However, *E. globulus* EO did not show a consistent parasite control.

COMPETING INTERESTS STATEMENT

The authors declare that there is no conflict of interests regarding the publication of this article.

AUTHOR CONTRIBUTIONS

M.A., J.L.M. and A.C. designed research; M.A., J.L.M., G.B., C.S. and Y.O. performed research; M.A., J.L.M., G.B., C.S. and Y.O. contributed to acquisition of data; M.A., J.L.M., and H.M. analysed data; M.A., J.L.M., and H.M. wrote the paper.

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REFERENCES

Ariana, A., Ebadi, R., & Tahmasebi, G. (2002). Laboratory evaluation of some plant essences to control *Varroa destructor* (Acari: Varroidae). *Experimental & Applied Acarology*, *27*(4), 319. https://doi.org/10.1023/A:1023342118549

Avitabile, A. (1978). Brood rearing in honeybee colonies from late autumn to early spring. *Journal of Apicultural Research*, *17*(2), 69-73.

Bounous, C., & Boga, V. (2005). Fundamentos para el control de varroa y loque americana. *Montevideo, Uruguay*, *7*.

Calderone, N. W. (2012). Insect pollinated crops, insect pollinators and US agriculture: Trend analysis of aggregate data for the period 1992-2009. *PLOS ONE*, *7*(5), e37235. https://doi.org/10.1371/journal.pone.0037235

SAG. (2019). *Certificación de Productos Orgánicos*. https://www.sag.gob.cl/ambitos-de-accion/certificacion-de-productos-organicos. Retrieved 10 September 2021.

Cruzat, R., & Baasch, V. (2010). *Resultados y lecciones en productos en base a aceites esenciales microencapsulados para el control del ácaro varroa: Proyecto de innovación en Región del Maule: Pecuario/Apicultura*. Ministerio de Agricultura.

De Jong, D., De Andrea Roma, D., & Gonçalves, L. S. (1982). A comparative analysis of shaking solutions for the detection of varroa jacobsoni on adult honeybees (1). *Apidologie*, *13*(3), 297-306. https://doi.org/10.1051/apido:19820308

Ghasemi, V., Moharramipour, S., & Tahmasbi, G. (2011). Biological activity of some plant essential oils against *Varroa destructor* (Acari: Varroidae), an ectoparasitic mite of *Apis mellifera* (Hymenoptera: Apidae). *Experimental and Applied Acarology*, *55*(2), 147-154. https://doi.org/10.1007/s10493-011-9457-1

Gonzalez-Acuña, D., Abarca, D., Suarez, J., Salas, L. & Aguayo, O. (2005). Comparación de la eficacia del ácido fórmico y del fluvalinato, como métodos de control de Varroa destructor (Acari: Varroidae) en colmenas de Apis mellifera (Hymenoptera: Apidae), en Ñuble, centro sur de Chile. *Revista de la Sociedad Entomológica de Argentina, 64*(3), 35-42.

Gracia, M. J., Moreno, C., Ferrer, M., Sanz, A., Peribáñez, M. Á., & Estrada, R. (2017). Field efficacy of acaricides against *Varroa destructor*. *Plos one*, *12*(2), e0171633. https://doi.org/10.1371/journal.pone.0171633

Imdorf, A., Bogdanov, S., Ibáñez Ochoa, R., & Calderone, NW. (1999). Use of essential oils for the control of *Varroa jacobsoni* Oud in honey bee colonies. *Apidologie*, *30*(2-3), 209-228. https://doi.org/10.1051/apido:19990210

Jean-Prost, P., & Conte, Y. L. (2007). *Apicultura: Conocimiento de la abeja. Manejo de la colmena.* 4ª edición. Ediciones Mundiprensa.

Karazafiris, E., Tananaki, C., Thrasyvoulou, A., & Menkissoglu-Spiroudi, U. (2011). Pesticide residues in bee products. In E. Karazafiris, C. Tananaki, A. Thrasyvoulou & U Menkissoglu-Spiroudi, (Eds.), *Pesticides in the Modern World - Risks and Benefits* (pp. 89-126). IntechOpen Book Series.. https://doi.org/10.5772/19409

Khater, H. F. (2012). Ecosmart biorational insecticides: Alternative insect control strategies. In F. Perveen, (ed.), *Insecticides - Advances in Integrated Pest Management* (pp. 17-60). IntechOpen Book Series. https://doi.org/10.5772/27852

Lautenbach, S., Seppelt, R., Liebscher, J., & Dormann, C. F. (2012). Spatial and temporal trends of global pollination benefit. *Plos ONE*, *7*(4), e35954. https://doi.org/10.1371/journal.pone.0035954

Milani, N. (1999). The resistance of *Varroa jacobsoni* Oud. to acaricides. *Apidologie*, *30*(2-3), 229-234. https://doi.org/10.1051/apido:19990211

Neira, M., Heinsohn, P., Carrillo, R., Báez, A., & Fuentealba, J. (2004). Efecto de aceites esenciales de lavanda y laurel sobre el ácaro *Varroa destructor* Anderson & Truemann (Acari:Varroidae). *Agricultura Técnica*, *64*(3), 238-244. https://doi.org/10.4067/S0365-28072004000300003

Potts, S. G., Biesmeijer, J. C., Kremen, C., Neumann, P., Schweiger, O., & Kunin, W. E. (2010). Global pollinator declines: Trends, impacts and drivers. *Trends in Ecology & Evolution*, *25*(6), 345-353. https://doi.org/10.1016/j.tree.2010.01.007

Quarles, W. (1996). EPA exempts least-toxic pesticides. *IPM Practitioner : The Newsletter of Integrated Pest Management*. https://agris.fao.org/agris-search/search.do?Recordid=US201302908927

Rattan, R. S. (2010). *Mechanism of action of insecticidal secondary metabolites of plant origin*. https://pubag.nal.usda.gov/catalog/789666

Spivak, M., & Reuter, G. S. (1998). Performance of hygienic honey bee colonies in a commercial apiary. *Apidologie*, *29*(3), 291-302. https://doi.org/10.1051/apido:19980308

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CASE REPORT

Aneurysm of the pulmonary artery in a sheep with pulmonary adenocarcinoma

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ABSTRACT. Aneurysm of the pulmonary artery is a rare condition in animals, and to our knowledge it has never been reported in association with pulmonary neoplasia. This report describes a case of an adult female sheep of the "Churra Galega Bragançana" breed with an aneurysm of the pulmonary artery associated with lung cancer (ovine pulmonary adenocarcinoma).

Key words: aneurysm, pulmonary adenocarcinoma, sheep.

An aneurysm is a localised dilation or blood-filled sac of a thinner and weakened portion of a blood vessel. Aneurysms usually develop at the point where the blood vessel branches form, as it is structurally the most vulnerable part (Curtis *et al.*, 2019). They may occur in any blood vessel, but arteries seem to be more predisposed to the development of this lesion (Markovitz *et al.*, 1989). A true aneurysm comprises the three layers of the wall of a blood vessel: intima, media and adventitia whereas a pseudoaneurysm contains no layer of the wall of the vessel. They can result in thrombus development and subsequent embolization (Kim & Han, 2015). Aneurysms are potentially fatal if they rupture, with death occurring within minutes (Curtis *et al.*, 2019).

Aneurysms of the pulmonary artery are rarely seen in humans or animals. Most cases are idiopathic, but some causes include copper deficiency in pigs, parasites in cetaceans, syphilis, mycotic infection, chronic pulmonary hypertension, bacterial endocarditis, neoplasia, trauma, vasculitis and Marfan's syndrome in humans (Sadek *et al.*, 2008, Lafita *et al.*, 2007, Martineau *et al.*, 1986).

Aneurysms of the pulmonary artery associated with pulmonary neoplasia have been rarely reported in humans and, to the author's knowledge, have not yet been reported in veterinary medicine.

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An adult (3 years) female sheep of the "Churra Galega Bragançana" breed (autochthonous Portuguese breed) presented progressive weight loss (from 60 kg to 40 kg in 3 months), body condition of 1 (in a 1 to 5 scale), sporadic soft cough, dyspnea and change in the breathing pattern, wheelbarrow test positive, and flow of mucous and frothy fluid (50 mL) from both nostrils and crackle to auscultation. A thoracic radiography and electrocardiogram (ECG) were performed. Radiography revealed a nodular pattern with small and diffuse nodules in the pulmonary parenchyma and a larger lesion in the cause-dorsal lung field. The electrocardiographic trace showed an irregular narrow-QRS rhythm (QRS, 0.045s; normal, <0.06s) and a heart rate of approximately 105 bpm. The QRS had deep negative deflection (amplitude, 0.6 mV; normal, <0.3 mV). All QRS were preceded by a P wave (positive deflection in lead II; duration <0.04 s; amplitude <0.13 mV) and a consistent PR interval (<0.14s). The animal died within a few days of being examined.

At *post mortem* examination, the lungs failed to collapse and were enlarged and heavy with multifocal to coalescing subpleural pearly-white nodules, located in diaphragmatic pulmonary lobes (figure 1A). The nodules were dry and firm at cross section. Consolidation of cranial regions and alveolar emphysema areas were noted (figure 1B). The pulmonary artery had a focal dilatation at base of ~ 2.5 cm, interpreted as aneurysm, apparently with reduced wall thickness (figure 1C). Eccentric right ventricular hypertrophy was also observed. The mediastinal and bronchial lymph nodes had grey areas interpreted as metastatic neoplastic tissue. No additional gross lesions were detected in this animal.

Tissue samples were collected and placed in 10% buffered neutral formaldehyde for histological examination and stained with hematoxylin-eosin (H&E) and Masson´s trichrome. Histologically, within the grossly affected portion of the pulmonary artery, all tunica layers were present. However, in comparison with the adjacent and grossly non-affected region of the pulmonary artery, the aneurysm wall was thinner (1.135mm vs. 2.424mm), and the tunica media had thinning, disorganisation,

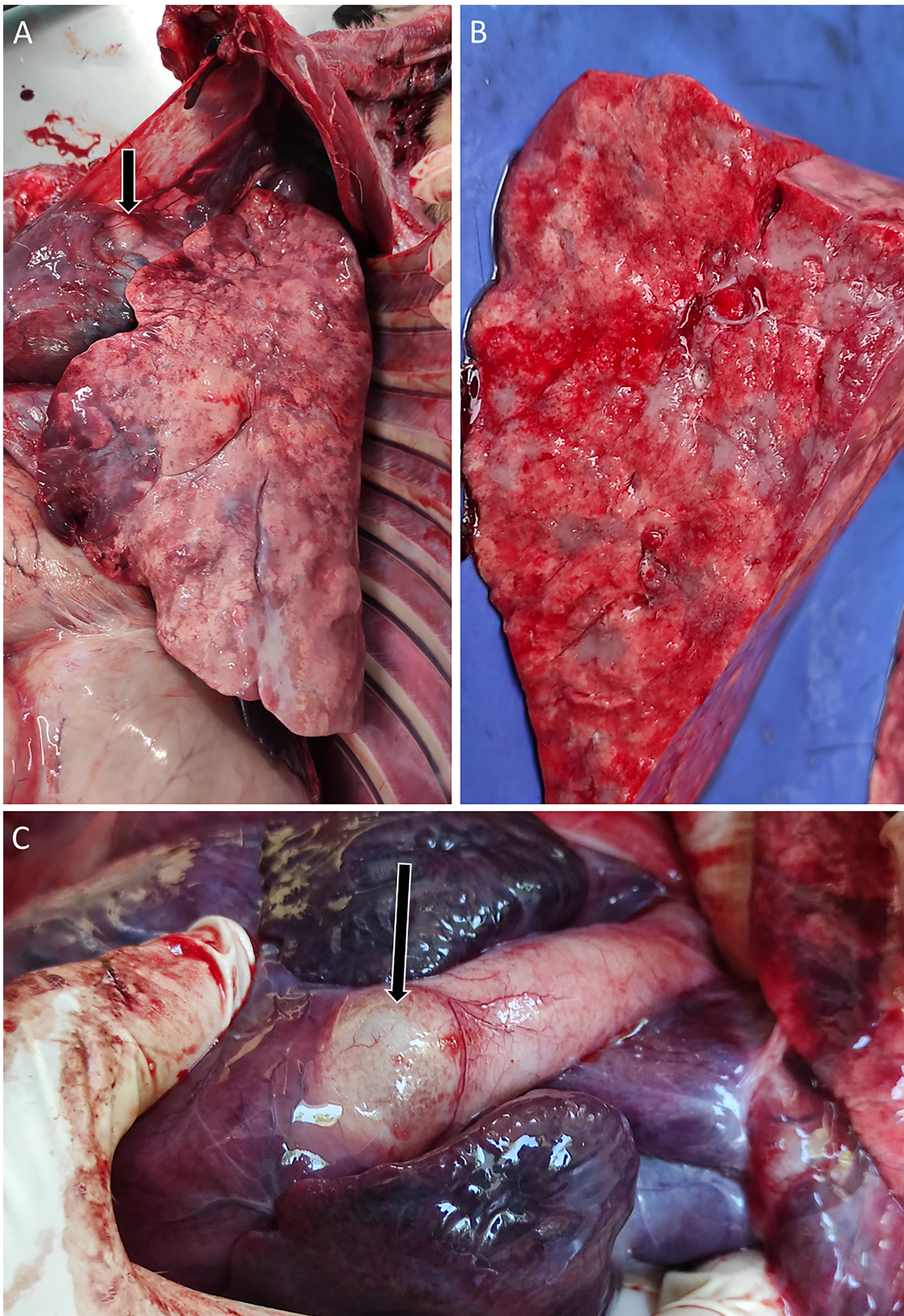

Figure 1. (A) Lungs of an adult female "Churra Galega Bragançana" sheep, are non-collapsed with multifocal to coalescing subpleural grey nodules, located in diaphragmatic pulmonary lobes. Aneurysm of the pulmonary artery is visible (arrow). (B) Macroscopic appearance of the lung at cut surface, whitish areas are observed corresponding to adenocarcinoma. (C) Pulmonary artery of an adult female "Churra Galega Bragançana" sheep with, focal dilatation of ~ 2.5 cm (aneurysm), close to pulmonary valve (arrow).

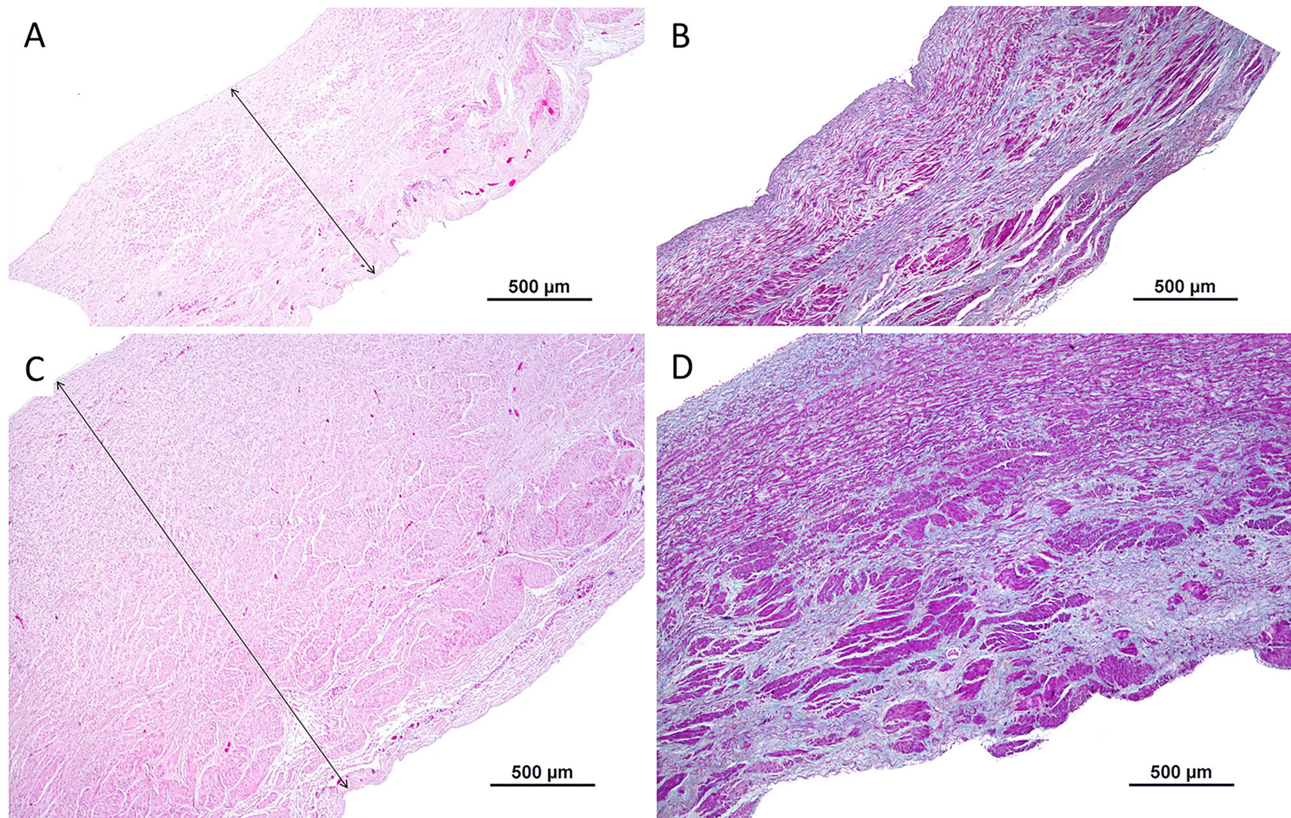

Figure 2. Pulmonary artery of an adult female "Churra Galega Bragançana" sheep. The wall of the artery is thin (aneurysm) (1.135mm); the tunica media exhibits thinning, disorganisation, fragmentation and loss of elastic fibres, and disorganisation and loss of smooth muscle cells: H&E (A) and Masson´s Trichrome stain (B). Pulmonary artery at the level of non-affected area (2.424mm): H&E (C) and Masson´s Trichrome stain (D).

fragmentation and loss of elastic fibres, and disorganisation and loss of smooth muscle (figures 2 A, B, C and D). Hypertrophy of cardiac myocytes of the right ventricle was also observed. The alveoli were lined with a single layer of cuboidal or columnar tumoural epithelial cells, within a moderate to abundant fibrous stroma. Tumour cells often form papillomatous projections into the alveoli. Anisokaryosis and anisocytosis were low to moderate with less than 2 mitoses per 10 high power field (400x). A large number of foamy macrophages were present in adjacent alveoli. Cranioventral areas showed suppurative bronchopneumonia with neutrophils in alveolar spaces and the bronchioloalveolar junction. Mediastinal lymph node metastasis was present.

The morphologic diagnosis was aneurysm of the pulmonary artery and pulmonary adenocarcinoma, with bronchopneumonia.

Aneurysm of the pulmonary artery is very uncommon in animals. To date, the bibliography only refers a few reports of true aneurysm in cattle (Breeze *et al.*, 1976) and cetaceans (Martineau *et al.*, 1986).

Aneurysm of the pulmonary artery could be congenital or acquired. In the present case, no prior clinical signs were observed. We speculate that the aneurysm, in this case, could have been acquired secondary to the associated neoplastic changes in the lung. Ovine pulmonary adenocarcinoma, also known as pulmonary adenomatosis or "jaagsiekte"(Griffiths *et al.*, 2010) is a contagious neoplasm of sheep and exceptionally goats, caused by a Betaretrovirus of the family Retroviridae (Lafita *et al.*, 2007, Youssef *et al.*, 2015). Although the typical form mainly affects the cranioventral lobes, the atypical form is characterised by tumour nodules in the diaphragmatic lobes, or diffuse, often associated with fibrosis as in the present case (Lafita *et al.*, 2007).

In human medicine, there are a few described cases of artery aneurysms or pseudoaneurysms of the pulmonary artery associated with pulmonary carcinoma (Kim & Han, 2015). This association may be caused by the erosion of the pulmonary artery or tumour expansion, which was not observed in this case (Kim & Han, 2015, Wiles *et al.*, 2021). The hyperplasia of the tunica media observed in the pulmonary vessels, the associated fibrosis and hypertrophy of the right ventricle suggest that the association of the aneurysm with pulmonary carcinoma is due to pulmonary hypertension and the consequent increase in pulmonary vascular resistance. The resultant increase in the post load of the right ventricle leads to its adaptation with dilatation of

the ventricular cavity and increase in myocardial thickness with myocyte hypertrophy (Koneru *et al.*, 2018, Wiles *et al.*, 2021) that predisposes and may be the cause of the aneurysm of the pulmonary artery.

Electrocardiogram findings may suggest a right ventricular enlargement (hypertrophy), according to the values described by Ahmed and Sanyal (2008). Nevertheless, there is no consensus in the literature on reference values for ECG interpretation in sheep and/or possible breed variability (Ahmed & Sanyal, 2008, Chalmeh *et al.*, 2015). It should be noted that the Churra Galega Bragançana breed, an indigenous breed from the north of Portugal, has a large ability to adapt to adverse environmental conditions, including very cold and hot temperatures.

Aneurysms are very difficult to diagnose in animals and often lead to sudden death. Although rare, they should be considered as a serious complication in animals with lung tumours.

To our knowledge, this is the first report of an aneurysm of the pulmonary artery in sheep and the first description of pulmonary neoplasia (ovine pulmonary adenocarcinoma) in animals associated with aneurysm of the pulmonary artery.

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REFERENCES

Ahmed, J., & Sanyal, S. (2008). Eletrocardiographic studies in Garol sheep and Black Bengal goats. *Research Journal of Cardiology, 1*, 1-8.

Breeze, R., Pirie, H., Selman, I., & Wiseman, A. (1976). Pulmonary arterial thrombo-embolism and pulmonary arterial mycotic aneurysms in cattle with vena caval thrombosis: a condition resembling the Hughes-Stovin syndrome. *Journal of Pathology, 119*(4), 229-237.

Chalmeh, A., Saadat, A., Zarei, M., & Badkoubeh, M. (2015). Electrocardiographic indices of clinically healthy Chios sheep. *Veterinary Research Communications, 5*, 99-102.

Curtis, B., Nofs, S., Ahearne, M., List, M., & Kiupel, M. (2019). Ruptured hepatic artery aneurysm in a domestic yak. *Journal of Veterinary Diagnostic Investigation, 31*(1), 74-77.

Griffiths, D., Martineau, H., & Cousens, C. (2010). Pathology and pathogenesis of ovine pulmonary adenocarcinoma. *Journal of Comparative Pathology, 142*(4), 260-283. https://doi.org/10.1016/j.jcpa.2009.12.013

Kim, J., & Han, S. (2015). A pulmonary artery pseudoaneurysm caused by concurrent chemoradiation therapy for lung cancer. *Pakistan Veterinary Journal*, *31*(1), 1-3. https://doi.org/10.12669/pjms.311.6001

Koneru, H., Biswas Roy, S., Islam, M., Abdelrazek, H., Bandyopadhyay, D., Madan, N., Patil, P. D., Panchabhai, T. S. (2018). Pulmonary artery pseudoaneurysm: A rare cause of fatal massive hemoptysis. *Case Reports in Pulmonology,* 8251967, https://doi.org/10.1155/2018/8251967

Lafita, V., Borge, M. A., & Demos, T. C. (2007). Pulmonary artery pseudoaneurysm: Etiology, presentation, diagnosis, and treatment. *Seminars in Interventional Radiology 24*(1), 119-123, https://doi.org/10.1055/s-2007-971202

Markovitz, L. J., Savage, E. B., Ratcliffe, M. B., Bavaria, J. E., Kreiner G., Iozzo R. V., Hargrove, W. C. (1989). Large animal model of left ventricular aneurysm. *Annals of Thoracic Surgery, 48*(6), 838-845. https://doi.org/10.1016/0003-4975(89)90682-6

Martineau, D., Lagacé, A., Béland, P., & Desjardins, C. (1986). Rupture of a dissecting aneurysm of the pulmonary trunk in a beluga whale (*Delphinapterus leucas*). *Journal of Wildlife Disseases*, *22*(2), 289-294.

Sadek, M., Hynecek R. L., Poblete, H., Levin, E., Shin, H-J, Lei, X., Faries P. L. (2008). Copper deficiency induces a connective tissue disorder state in a porcine native abdominal aortic aneurysm model. *The FASEB Journal, 22*(S1), 902.7. https://doi.org/10.1096/fasebj.22.1_supplement.902.7

Wiles, B., Comito, M., Labropoulos N., Santore, L. A., Bilfinger, T. (2021). High prevalence of abdominal aortic aneurysms in patients with lung cancer. *Journal of Vascular Surgery, 73*(3), 850-855. https://doi.org/DOI:10.1016/j.jvs.2020.05.069

Youssef, G., Wallace, W. A. H., Dagleish, M. P., Cousens, C., & Griffiths, D. J. 2015. Ovine pulmonary adenocarcinoma: A large animal model for human lung cancer. *ILAR Journal*, *56*(1) 99-115. https://doi.org/10.1093/ilar/ilv014

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REFEREES AND MANUSCRIPT REVIEW

Referees of *AJVS* will aid the Editorial Committee to determine whether the manuscript fulfills publication requirements. All articles submitted for publication will be assessed by two independent referees. The referees will be selected by the Editorial Committee, and may (but not necessarily) include those suggested by the author. In case of a disagreement between the referee's reports, a third referee will aid the Editorial Committee to reach a decision. Referees are obliged to keep all information from the articles confidential, including unpublished information. Authors should state any potential conflicts of interest at the time of submission of the manuscript. Such information will not alter established editorial and review policies but will assist the editorial staff in avoiding any potential conflicts that could give the appearance of a biased review. The final decision regarding acceptance of the manuscript will be taken when the Editorial Committee accepts the manuscript following correction according to the referees' comments and their own. Accepted articles must pay a publication fee prior to publication via Web-Pay.

TYPE OF ARTICLES

Review article

Reviews provide expert summaries of current knowledge in a particular field of veterinary science and do not necessarily have a set format. Articles should provide comprehensive depth and a balanced perspective, avoiding merely summarize the literature. A review should focus on fundamental concepts or issues, current research gaps, potential development of the field, controversies. The Editorial Committee asks for an expert to prepare a review on specific topics, which will also be refereed and edited. Articles are peer-reviewed and must not exceed 15,000 words in length and may contain no more than 4 figures/tables.

Original article

Report new and unpublished advances in veterinary science based on original research. Research Articles may also encompass confirming studies and disconfirming results which allow hypothesis elimination, reformulation and/or report on the non-reproducibility of previously published results. The format must include Abstract, Introduction, Materials and Methods, Results, Discussion, Declarations, and References. The articles have a maximum word count (including Introduction, Material and Methods, Results, and Discussion) of 12,000 words, and may contain no more than 10 figures/tables.

Short communication

Short Communications briefly inform of an advance, experimental result, new methodology, with the following format: abstract, introduction, material and methods, results and discussion (combined). Short communications also encourage submission of negative results and may report on the non-reproducibility of previously published results. Short communications articles are peer-reviewed, have maximum length of the manuscript

(including Abstract, Introduction, Materials and methods, Results and Discussion, Declarations and References) is 4,000 words and may contain no more than 2 figure and/or tables. Supplementary material may be included.

Case report

Case report: is a brief note that describes preliminary findings and contributes significantly to the understanding of the Veterinary Science. The maximum length is 1,300 words which includes the main body of the text and cites. An abstract of 50 words is required, plus 15 references and two tables or figures, or one of each. Acknowledgements can be included (when pertinent). Subtitles must not be used to divide the main body of the text.

Letters to the Editor

A letter judged suitable for publication will be printed in a "Letters to the Editor" section of AJVS. The purpose of this section is to provide a forum for scientific exchange relating to articles published in AJVS. To be acceptable for publication, a letter must adhere to the following guidelines.

- Only a letter that addresses matters of science and relates to information published in *AJVS* during the last year will be considered. In general, a letter should not exceed 500 words and should contain no more than 5 citations.
- A letter should provide supporting evidence based on published data for the points made or must develop logical scientific hypotheses. A letter based on conjecture or unsubstantiated claims will not be published. No new data may be presented in a letter.
- The Editorial Committee will evaluate each letter and determine whether a letter is appropriate for publication. If a letter is considered appropriate, the author(s) of original *AJVS* article(s) will be invited to write a letter of response. Normally both letters will be published together.
- All letters will be subject to acceptance and editing by the Editorial committee and editing by a technical editor.

JOURNAL STYLE AND LAYOUT

General presentation: Manuscripts must be written using 12 point Times New Roman font with one and a half-line spacing, on one side only of letter paper (21.5 x 27.9 cm) using 2 cm margins on all sides. Pages must be numbered consecutively in the top right corner, and lines must be numbered on the left, starting with number one, on all pages. Headings must be in upper case, left-justified on a separate line with no full stop following, e.g. MATERIAL AND METHODS. Only the first letter of sub-headings is capitalised. Primary sub-headings (e.g. Experimental design) should be left-justified; secondary sub-headings are left-justified and italicised. Do not use underlining and do not number sub-headings or itemised lists. In the text, numbers must be written in numerals. When a sentence begins with a number or when necessary for clarity, this should be written in words. A decimal point must be preceded by a number (e.g. 0.5 not .5). All measurements must be reported in SI units (www.nist.gov/pml/pubs/sp811/) unless it is normal practice in a discipline to use derivatives (e.g. the Curie international unit). Dates must be formatted as 07 September, 2021 in the text, but they may be abbreviated in tables and figures. Use the 24-hour clock for times of day (e. g. 13:00 h). Chemical nomenclature must be expressed using the Biochemical Society Standards (Biochem J 209, 1-27, 1983), generic names (in lower caps) must be used for medications. If brands and sources of medications need to be included, this should be included as a foot-note. Enzymes must be identified at first mention, in accordance with the Enzyme Commission of the International Union of Biochemistry. Latin terminology and abbreviations commonly used in scientific literature, such as *in vitro*, *in vivo*, *ad libitum* must be italicised. Scientific names of animal species should be mentioned once in the text, complete and in brackets, subsequently only the common or abbreviated (when possible) name should be used. Probability values must be presented as $P<0.05$ or $P<0.01$. Standard deviation, standard error of the mean and confidence intervals are abbreviated as follows: SD, SEM and CI, respectively.

Title

Title must be short, specific and informative. The title is centred in bold without using trade names or abbreviations. Only the first letter is capitalised. Scientific names of animal species must be mentioned in the title, in brackets, only in the case of non-domestic species.

Author's names and addresses

Author's names are written underneath the title, separated by a space. Use full name and separate authors by commas, as in the example: Christopher A. Westwood, Edward G. Bramley, Ian J. Lean. Superscript letters should be used after each author's name to identify affiliation as follows: Laboratory, Institute, Department, Organization, City, and Country. The corresponding author is indicated using the superscript letter followed by an asterisk, with email addresses indicated in the footnote.

Footnotes

These are used to indicate a web page address (URL) and to define abbreviations used in table titles, commercial brands, the name and address of companies. They must be indicated with numbers.

Abstract

The second page must contain an abstract of no more than 300 words that render the general significance and conceptual advance of the work clearly accessible to a broad readership. The abstract should describe the objectives of the study or research, the material and methods used, the principal results, and the most important conclusions. Non-standard abbreviations must not be used.

Key words

All article types require a minimum of 5 and a maximum of 8 keywords. They should be indicated below the Abstract. The use of key words containing more than two words (a phrase) must be avoided.

Introduction

The subheading "Introduction" is written on the next page following the Abstract. In the following line, the context of the study is briefly presented with no subheadings. The hypothesis and objectives of the study must be clearly and concisely presented.

Material and Methods

This section may be divided by subheadings and should contain sufficient detail so that when read in conjunction with

cited references, allow others to repeat the procedures. When the first reference in the text is made to medications or chemicals, the generic name, dose and route of administration should be indicated. For specialised equipment, the brand, model and manufacturer's name must be indicated. Studies involving animals or humans must mention the appropriate Bioethical Committee Certification.

Results

This section may be divided by subheadings and should contain a concise and logical description of the results obtained without discussion or reference to other work. The results can be supported by tables and/or figures that present the pertinent data. Data presented in tables and figures should not be repeated in the text. In the case of Original research articles only, this section and the Discussion are separated.

Discussion

This section may be divided by subheadings and should cover the key findings of the study, evaluate and interpret the results and relate these to other relevant results. The results should not be repeated, and new results must not be presented in this section. Care should be taken to ensure that the discussion is developed in a logical and concise manner, discussing the potential shortcomings and limitations on their interpretations. Conclusions that are not directly supported by the data of the study or other unpublished studies should not be presented.

DECLARATIONS

Competing Interests Statement

All financial and non-financial competing interests must be declared in this section. If authors do not have any competing interests, please state "The authors declare that they have no competing interests" in this section.

Ethics Statement

Studies involving animals must include a statement on ethics approval and for experimental studies involving animals, authors must also include a statement on informed consent from the client or owner. Any questionnaire associated to human studies, must also include appropriate permissions. For further details authors must see *Ethical oversight* at www.ajvs.cl/index.php/ajvs/editorialPolicies.

Author Contributions

The individual contributions of authors to the manuscript should be specified in this section. Please use initials to refer to each author's contribution in this section.

Funding

All sources of funding for the research reported should be declared. The role of the funding body in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript should be declared.

Acknowledgements

This section should be brief, including people or institutions that have made a direct contribution, provided necessary material or have provided the facilities for the study's development.

References

AJVS follows APA style described in the 7th edition APA Publication Manual. The following two templates can be used with the most common reference managers: Mendely and EndNote.

Examples:

Journal article:

Bumunang, E. W., McAllister, T. A., Zaheer, R., Polo, R. O., Stanford, K., King, R., Niu, Y. D., & Ateba, C. N. (2019). Characterization of non-O157 *Escherichia coli* from cattle faecal samples in the North-West Province of South Africa. *Microorganisms, 7*(8), 272. https://doi.org/ HYPERLINK "https://dx.doi.org/10.3390%2Fmicroorganisms7080272"10.3390/microorganisms7080272

Book:

Mayr, E. (1988). *Toward a new philosophy of biology: Observations of an evolutionist*. Harvard University Press.

Chapter in an edited book:

Gyles, J. M., & Fairbrother, F .C. L. (2010). Escherichia coli. In C. L. Gyles, J.F. Prescott, J. G. Songer, & C. O. Thoen, (Eds.), Pathogenesis of Bacterial Infections in Animals (pp. 276-308). Wiley-Blackwell.

Congresses and Proceedings:

Gallardo, R. A., Da Silva, A. P., Mendoza-Reilley A., Alvarado I., & Giroux, C. (2-5 August 2019). *False layer syndrome caused by IBV, genetic characterization and pathobiology insights*. American Association of Avian Pathologists Annual Meeting, Washington DC, USA.

Gómez, M., Rojas, M., Mieres, M., Moroni, M. & Muñoz, P. (2011). Clinical, clinicopathological and pathological findings in 7 domestic cats with paraparesis/plegia produced by nematodes in southern Chile. *Proceedings 24th Symposium ESVN-ECVN, Germany*.

Webpage:

Rattan, R. S. (2010). *Mechanism of action of insecticidal secondary metabolites of plant origin*. https://pubag.nal.usda.gov/catalog/789666

In-text citations:

Parenthetical citations: (Jackson, 2019; Smith *et al.*, 2020; Sapolsky & Jones, 2020)

Narrative citations: Jackson (2019), Smith *et al.* (2020), Sapolsky and Jones (2020)

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Tables

Tables should be presented at the end of the manuscript, after the "References" section. Please provide one table per page. The table caption should appear above the table and should be brief and self-explanatory and should be understandable without reference to the text. Tables must be numbered consecutively with Arabic numbers in the order in which they are referred to in the text. Each column must have a short or abbreviated heading. Use

horizontal lines for column headings only. Further information such as abbreviations, superscripts, and units should appear at the foot of the table. Significant differences between values must be indicated as superscripts using letters in lower case starting with letter a. Use up to 3 decimals. Indicate a zero value as 0.

Figures

Figures should be presented at the end of the manuscript, after the Tables, with their respective titles at the bottom and numbered consecutively using Arabic numerals in the order they are referred to in the text, e.g. Figure 1, not Fig. 1. Figures should also be submitted on separate files (one per file). Figures must be vertically oriented and be accompanied by a short descriptive caption that contains an explanation for all markers, lines and symbols used but no abbreviations. If the figure contains sections, these should be labelled as a, b, c, etc. in the top right corner and must be described in the caption. The authorship of non-original figures must be acknowledged, and when appropriate, authorization to reproduce these figures must be provided.

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