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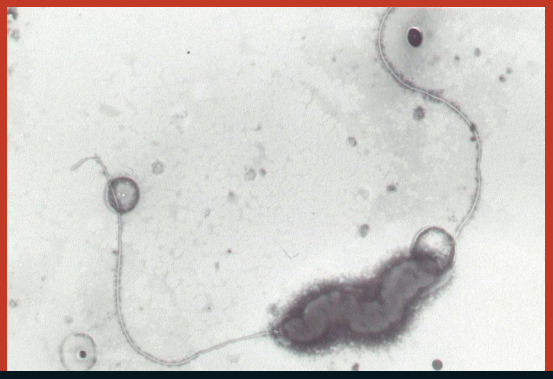

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REVIEW ARTICLE

ORIGINAL ARTICLES

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REVIEW ARTICLE

Adhesion mechanisms of *Actinobacillus pleuropneumoniae* to the porcine respiratory system and biofilm formation

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ABSTRACT. *Actinobacillus pleuropneumoniae* is a Gram-negative bacterium and the causative agent of porcine pleuropneumonia, a highly contagious disease of pigs characterised by fibrinohaemorrhagic necrotising pneumonia. Although it has been well controlled in some developed countries, outbreaks can occur in pigs of all ages in contact with asymptomatic carriers, leading to significant economic losses to the swine industry due to the high morbidity and mortality rates. Adhesion is a critical step in the colonisation of the swine respiratory tract and the pathogenesis of the porcine pleuropneumonia; however, a literature review of this process is not available to date. Therefore, this review aims to provide information regarding the molecules that have been described in the adhesion of *A. pleuropneumoniae* to cells and tissues of the porcine respiratory tract. Since adhesion is the first step in biofilm formation, we included a section to describe the genes involved in this process; some of these genes could participate directly or indirectly in the adhesion of *A. pleuropneumoniae* to the porcine respiratory system. Although the role of biofilms in porcine pleuropneumonia is still not clear, these molecules could be considered in the future as candidates for vaccine development.

Keywords: *Actinobacillus pleuropneumoniae*, porcine pleuropneumonia, adhesion mechanisms, biofilm formation.

INTRODUCTION

Porcine pleuropneumonia is a highly contagious disease in pigs of worldwide distribution. *Actinobacillus pleuropneumoniae* (AP) is a small encapsulated, gram-negative rod and the etiological agent of this disease, which is characterised by fibrinohaemorrhagic and necrotising pneumonia that often follows a fatal course during acute presentations (Chiers *et al.*, 2010). AP can be found in the nostrils, tonsils and lungs of infected pigs, and it can also be found in asymptomatic carriers previously infected or with a subclinical infection (Sidibé *et al.*, 1993; Chiers *et al.*, 2001). It is also known that pigs with chronic infection have deficient feed conversion and weight gain (Sassu *et al.*, 2018). In addition, research on naturally and experimentally infected animals suggests that the natural course of infection starts with the presence of AP in the upper respiratory tract, progressing all the way from the nasal cavities to the lungs; here the bacterium induces lesions and the production of neutralising antibodies. Also, tonsils seem to act as a reservoir of AP (Chiers *et al.*, 2001). Interestingly, it has been shown that there is no detection of neutralising antibodies in the serum of pigs that were positive for the presence of AP in the nasal cavity and/or tonsils, but negative for the presence of infected lung lesions, indicating a subclinical infection of pigs carrying the bacterium (Chiers *et al.*, 2002). Based on the requirement of nicotinamide adenine dinucleotide (NAD), two biotypes of AP have been described. In addition, there are 19 serotypes of AP based on differences in the antigenic properties of the capsular polysaccharides (Stringer *et al.*, 2021). It is known that there is a predominant serotype in herds endemically infected, however, more than one serotype has been isolated in some herds (Sidibé *et al.*, 1993). In addition, some of the described virulence factors are AP involved in adhesion, nutrient acquisition, induction of lung lesions, evasion of the immune system and persistence in the host (Chiers *et al.*, 2010). Furthermore, the severity of the disease is not only influenced by the bacterium, but also due to intrinsic factors such as the nutritional status and the immune system of the host and extrinsic factors related to environmental stress (Chiers *et al.*, 2010). There are some variations in the virulence among serotypes, and this could be in part attributed to the production of different combinations of Apx exotoxins, which differ among them in their cytotoxic and hemolytic activities (Hernández-Cuellar *et al.*, 2021). In this regard, ApxI expressed in serotypes 1, 5a, 5b, 9, 10, 11, 14, and 16 is highly haemolytic and cytotoxic, ApxII expressed in all serotypes but 10 and 14 are slightly haemolytic and moderately cytotoxic, and ApxIII expressed in serotypes 2, 3, 4, 6, 8, and 15 is non-haemolytic but highly cytotoxic (Sassu *et al.*, 2018). Also, it has been recently described that AP can internalise not only to phagocytic cells but

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to non-phagocytic endothelial cells (Plasencia-Muñoz *et al.*, 2021). However, the first step for the bacteria to colonise the porcine respiratory system is the adhesion to epithelial cells or extracellular matrix components. There is limited information available in the literature related to the adhesion mechanisms of AP even though this event represents the initial step in the establishment of the infection (Jacques & Paradis 1991). Therefore, the next section aims to present the most relevant findings regarding the adhesion molecules in AP described to date.

ADHESION MECHANISMS OF AP TO THE PORCINE RESPIRATORY TRACT

BINDING TO EXTRACELLULAR MATRIX COMPONENTS

Adhesion to porcine respiratory tract mucus was initially evaluated for 17 AP isolates. It was observed that ~70% of the isolates showed affinity to the mucus and this feature was independent of the serotype. It was also found that the presence of a capsule or a high capsular thickness decreased the adherence to the mucus (Bélanger *et al.*, 1992). In a similar study, AP serotype 1 was able to bind *in vitro* to swine-lung collagen in a Ca^{2+}-dependent manner. By using an overlay assay, it was shown that an unknown 60 kDa outer-membrane protein was able to bind to collagen and fibrinogen, but not to fibronectin and laminin (Enriquez-Verdugo *et al.*, 2004). Hammer-Barrera *et al.* (2004) showed that adhesion by AP serotype 1 was higher to swine buccal epithelial cells (BEC) in comparison with their cell counterparts of human or rat origin. Treatment with proteolytic enzymes and periodate highly decreased the adherence to swine BEC, suggesting the participation of cell-surface glycoproteins in the adhesion of AP to these cells. Interaction with fibrinogen or fibronectin resulted in reduced adherence to swine BEC, suggesting also the adhesion of bacteria to these extracellular matrix components (Hamer-Barrera *et al.*, 2004). We suggest that these extracellular matrix surfaces may be helpful for AP, an extracellular bacterium, to attach and progress from the upper to the lower porcine respiratory tract.

ROLE OF LPS AND CAPSULE IN THE ADHESION

Using porcine tracheal rings with ciliated epithelial cells maintained in culture, it was found that isolated AP serotypes 1, 2, 5, and 7 had variation in the adhesion capacity among serotypes or even within isolates of the same serotype. To analyse the role of the capsule on the adhesion, two capsulated isolates and their unencapsulated variants were tested and no differences were found in the adhesion index, suggesting that capsule was not involved in this process. There were differences in the lipopolysaccharide pattern of the bacterial isolates when a whole-cell lysate was subjected to a treatment with proteinase K. Based on this, isolates with a smooth-type lipopolysaccharide (75% of isolates with serotype 2 and 7) adhered in large number to porcine tracheal rings while isolates with a semi rough-type lipopolysaccharide (serotypes 1 and 5) adhered poorly (Bélanger *et al.*, 1990). It is worth mentioning that lipopolysaccharide varies in structure among bacteria but possessed three different regions attached covalently, the lipid A, an oligosaccharide core, and the O-antigen. Lipopolysaccharide with O-antigen is referred to as smooth type, while rough-type lipopolysaccharide does not contain O-antigen (Steimle *et al.*, 2016). Interestingly, purified lipopolysaccharides from homologous AP reference strains inhibited the bacterial adhesion to the porcine tracheal rings. Therefore, lipopolysaccharides were proposed for the first time as molecules important for the adhesion of AP to ciliated epithelial cells of the trachea (Bélanger *et al.*, 1990).

Similar research related to the adhesion capacity of lipopolysaccharides in AP showed by flow cytometry and electron microscopy that these molecules were well exposed at the surface of the encapsulated AP analysed. In addition, immunostaining showed that the lipopolysaccharide extracted from AP serotype 1 and 2 adhered to lung vascular endothelium and tracheal epithelium when incubated with porcine lung or tracheal frozen sections, respectively. To know which part of the lipopolysaccharides had the adhesion capacity, an extract of lipopolysaccharides from AP was obtained and hydrolysed. Through an adhesion-inhibition assay, it was found that the polysaccharide moiety was responsible for the adhesion of AP, while the lipid A was dispensable in this process (Paradis *et al.*, 1994). In addition, it was shown in an experiment trying to simulate the adhesion to cell membranes that AP serotype 1, 5b, and 7 were able to bind to phosphatidylethanolamine (PE) but not to other phospholipids. It was suggested that the lipopolysaccharide O-antigen was responsible for the binding to PE (Jeannotte *et al.*, 2003).

Contrary to the findings showing that lipopolysaccharides play an important role in the adhesion of AP to tracheal and lung epithelial cells, a different workgroup analysed the adhesion of several strains of AP to primary cultures of porcine lung epithelial cells (LEC). It was found that adhesion of AP was faster and up to 30-fold more efficient for LEC than for swine kidney cells. However, adhesion to LEC did not change for a transposon mutant with a modification in the lipid A moiety of the lipopolysaccharide or even resulted in a three-fold more adhesion for a mutant lacking O antigen compared to the parent strains. Furthermore, lipopolysaccharides purified from AP serotype 1, 3, 7, and 8 did not alter the adhesion of AP serotype 8 to LEC (Boekema *et al.*, 2003). These results clearly show that the mechanisms of adhesion for AP could be different depending on the surface of the porcine respiratory system.

In another study, 23 AP isolates were evaluated on their ability to adhere *in vitro* to porcine tracheal epithelial cells or frozen lung sections. Different to the frozen lung sections, adherence to the tracheal epithelial cells was very

poor and, in both cases, there was no correlation of the adherence with the serotype of the AP isolates. However, contrary to the aforementioned study, two unencapsulated variants adhered in greater numbers to the lung sections compared to the capsulated parent strains (Jacques *et al.*, 1991). Similarly, using a transposon mutagenesis system to generate an AP serotype 1 capsule-deficient mutant, it was found that the mutant strain showed more adhesion to porcine tracheal frozen sections than the parent strain. However, it was less virulent in pigs and it did not induce mortality. It was described that the product of mutation was the protein CpxC involved in polysaccharide transport across the cytoplasmic membrane during the biosynthesis of capsular polysaccharides. It was also concluded that the capsule was not important for adherence and may even mask an outer membrane protein important for adhesion (Rioux *et al.*, 2000).

OUTER MEMBRANE PROTEINS INVOLVED IN ADHESION

Adhesion of AP serotypes 2, 5a, 9, and 10 to alveolar epithelial cells showed that optimal adherence was obtained in NAD-restricted medium for strains 5a, 9, and 10. Interestingly, under this condition, it was expressed an outer membrane protein of 55 kDa and the presence of fimbriae was observed by electron microscopy. However, the sequence of the N-terminal of this outer membrane protein did not correspond to any known protein. Bacterial adhesion was significantly reduced when treated with proteolytic enzymes. This finding suggested that besides lipopolysaccharides, proteins are also important for the adhesion of AP. Furthermore, treatment of AP with a combination of pronase and sodium metaperiodate produced a higher inhibition of the adherence to alveolar epithelial cells compared to reagents being used separately. Therefore, glycoproteins could also be involved in the adhesion of AP to these cells (Overbeke *et al.*, 2002).

It was found that 170 genes were differentially expressed in AP attached to St. Jude porcine lung cell line (SJPL) compared with detached bacteria in the medium (planktonic). Two genes called *TadB* and *rcpA*, potentially involved in adhesion and biofilm formation were upregulated. Also, a gene (APL_0443) with high homology to the Hsf autotransporter adhesin of *Haemophilus influenzae* was upregulated (Auger *et al.*, 2009). This Hsf-like autotransporter called Apa1 (AP antigenic protein) was previously found to be expressed by AP in necrotic porcine lung tissue (Baltes *et al.*, 2004). Sequence analysis of the C-terminal region of Apa1 showed a translocator domain and six conserved HsfBD1-like or HsfBD2-like binding domains among different strains of AP. Adhesion to SJPL cell monolayers was tested by confocal microscopy through a GST fusion protein methodology in which GST was bound to the six ApaBD (HsfBD-like) domains. GST-ApaBD3 showed strong fluorescence while the other five domains had only basal fluorescence. It was confirmed the adhesion ability of ApaBD3 to epithelial cells through an adherence inhibition assay with a recombinant *E. coli*-ApaBD3 that expresses the domain on the surface (Xiao *et al.*, 2012). It was shown later an extra N-terminal domain (residues 124-612) of the trimeric autotransporter Apa1 called Adh that was required for adhesion, autoaggregation, and biofilm formation (Wang *et al.*, 2015).

In another study, the outer membrane protein Lip40 was described to mediate adherence of AP to SJPL cells using a mutant strain, Δ*lip40*. Interestingly, the mutant strain had also a reduced ability to invade the lungs of infected mice. Also, in an infection assay with pigs, the mutant strain produced fewer clinical signs (dyspnea, lethargy, and fever) and lung invasion than the wild-type or complemented strain (Liu *et al.*, 2018). These findings suggest a critical role of the adhesion process in the virulence of this bacterium.

GENES INVOLVED IN FIMBRIAE FORMATION

The presence of fimbriae in AP has been previously described (Utrera *et al.*, 1991, Dom *et al.*, 1994, Overbeke *et al.*, 2002) with the identification of ApfA, a 17 kDa type 4 fimbrial subunit protein (Zhang *et al.*, 2000). An operon (*apfABCD*) consisting of four genes involved in type 4 fimbrial biogenesis was also proposed (Stevenson *et al.*, 2003, Boekema *et al.*, 2004). ApfA was found to be highly conserved among the different serotypes of AP. Also, it was suggested as an adhesin since its expression was greatly upregulated upon contact of AP with the SJPL cell line. Adhesion to SJPL cell line and porcine iliac artery endothelial cell line (PIEC cells) decreased significantly for AP $4074^{\Delta apfA}$, a mutant strain deficient in ApfA. Furthermore, recombinant ApfA blocked the adhesion of AP to those cell lines. Interestingly, it was shown that ApfA mediates colonisation of AP to the lungs of infected mice, as the mutant strain AP $4074^{\Delta apfA}$ had reduced bacterial loads in lungs compared with mice infected with the wt AP strain 4074. Also, using a purified recombinant ApfA protein, it was found an elevated humoral immune response and protection against AP in an infection model in mice, proposing this fimbrial subunit as a promising vaccine candidate (Zhou *et al.*, 2013).

Two component systems (TCS) play important roles in adaptation to changes in the environment. Through genomic analysis, it has been described that AP have five pairs of TCS: ArcA/ArcB, CpxR/CpxA, NarP/NarQ, PhoB/PhoR, and QseB/QseC. It was analysed through a microarray the changes in the gene expression profile between a QseB/QseC deficient AP strain and the corresponding parent strain AP 4074. The expression of 44 genes was shown to be different, with 27 of them being up-regulated and 17 down-regulated. The expression levels of some of these genes, such as *PilM* were validated using qRT-PCR. Also, with an electrophoretic mobility shift assay (EMSA), it was shown that a phosphorylated recombinant QseB (rQseB-P) was able to bind to the promoter sequence of

PilM. An AP deficient in the expression of *PilM* showed a significant decrease in the adherence to SJPL cell line and was less virulent in pigs (Liu *et al.*, 2015). It was later found that the *apfABCD* and *PilMNOPQ* gene clusters were operons conserved in all the AP serovars and their products (*apfA*, *apfB*, *apfC*, *apfD*, *pilM*, *pilN*, *pilO*, *pilP*, and *pilQ*) are required for Tfp (a type IV pili) biogenesis, biofilm formation, and adhesion to SJPL cells (Liu *et al.*, 2018).

The presence of the *flp* operon consisting of 14 genes (*flp1-flp2-tadV-rcpCAB-tad-ZABCDEFG*) was described in AP. In reference strains with serotypes 1, 4, 5, 7, 12, and 13, the complete operon was identified. However, the *flp* promoter was absent in serotypes 2, 3, 6, 9, and 11, and for serotypes 10 and 15, the *flp1* gene was truncated resulting in the absence of pilus as observed by transmission electron microscopy. Adherence to SJPL cells resulted to be higher for piliated strains (Li *et al.*, 2012). Later, it was shown that the genes *flp1* and *tadD* were essential for Flp pilus biosynthesis using AP mutants in which biofilm formation and adherence to SJPL and porcine iliac artery endothelial (PIEC) cell lines was reduced. Also, those mutants lacking *flp1* and *tadD* resulted in deficient colonisation with reduced bacterial loads in the lungs of infected mice and pigs (Li *et al.*, 2019).

GLYCOSYLATION SYSTEMS

It was reported in AP the crystal structure of HMW1C, a glycosyltransferase of the GT41 family that was previously described in *Haemophilus influenzae*. HMW1C creates N-glycosidic linkages on HMW1, an adhesin that mediates adherence to respiratory epithelial cells (Kawai *et al.*, 2011). A recent study described the role of the cytoplasmic *N*-linked glycosylation system of AP (NGT) in the adhesion to A549 cells, human adenocarcinoma lung epithelial cells. A putative NGT locus consisting of *rimO* (methylthiotransferase) was proposed and the glycosyltransferases *ngt* and *agt*. Using AP strain HS143 to generate mutants deficient in *agt* and *ngt*, it was shown that the adhesion to cells was almost abrogated for the mutant strains HS143Δagt and HS143Δngt (Cuccui *et al.*, 2017). Table 1 summarises all the molecules that have been described in the adhesion of AP to surfaces related to the porcine respiratory system.

BIOFILM FORMATION

Biofilms are defined as communities of microbes embedded in an extracellular matrix, conferring them protection against environmental stress, host defence, and antibiotics (Hathroubi *et al.*, 2018). Most of the experimental studies in biofilm formation have been on abiotic surfaces such as polystyrene microplates. Although the ability to form biofilms has been associated with the virulence of AP, it is still not clear how this process contributes *in vivo* to the pathogenesis of the porcine pleuropneumoniae (Hathroubi *et al.*, 2018). In this respect, the presence of AP aggregates in the lungs of pigs naturally infected has been reported (Tremblay *et al.*, 2017). In addition, it was described the ability of AP to form biofilms on a biotic surface, using a monolayer culture of SJPL cells in which the bacterium formed biofilms at later times (~24h) in comparison with the highest biofilm formation in microplates at 4h. This biofilm formation was associated with an increase in the adhesion number of bacteria to the cells, and PNAG (a polymer of N-acetyl-D-glucosamine residues in beta (1,6) linkage) was shown to be an important component necessary for biofilm formation (Tremblay *et al.*, 2013). In addition, it was shown that medium replenishment was important to increase the biofilm biomass and delay bacterial dispersion. Using a drip flow system with constant nutrient supplementation, it was found that AP forms larger and more stable biofilms. In case of biofilm formation in microplates under static conditions, genes involved in energy metabolism were downregulated while genes involved in transport were upregulated in biofilm cells compared with planktonic cells, suggesting the need for an active nutrient supplementation of AP in biofilms. Also, it seems to be that the dispersion of AP in biofilms after 4h is driven by stress-related genes while at a growing phase, the bacterium expressed genes involved in transport and energy metabolism. For bacteria in biofilms coming from the drip flow system, genes involved in protein synthesis were upregulated in comparison with effluent bacteria (Tremblay *et al.*, 2013). In a different approach looking for genes involved in biofilm formation by AP, it was found 16 genes from a transposon library with around 1200 mutants. The genes associated with an increase in the biofilm formation were of unknown function while those associated with a deficient biofilm formation encoded proteins involved in transport, protein and nucleic acid synthesis (Grasteau *et al.*, 2011). Interestingly, it was found that sub-minimum inhibitory concentrations of penicillin G, an antibiotic used to control AP outbreaks, induced biofilm formation on polystyrene microplates in 9 out of 13 AP field isolates. These biofilms contained more PNAG, extracellular DNA and proteins compared with the control biofilms. Also, the expression of *pgaA* and genes of the envelope-stress two-component system CpxRA were up-regulated in AP under the presence of sub-minimum inhibitory concentrations of penicillin G, suggesting that the stress induced by the antibiotic on the cell wall of AP is associated with increased production of PNAG and the biofilm formation (Hathroubi *et al.*, 2015).

On the other hand, we have described in this review the adherence of AP to cells and tissues of the porcine respiratory system; however, adhesion is also the first step in biofilm formation. In this regard, fimbriae assembly in AP through the operons apfABCD, pilMNOPQ, and flp were required for cell adhesion, biofilm formation, and to confer virulence *in vivo* (Liu *et al.*, 2018; Li *et al.*, 2019).

Table 1. Adhesion mechanisms of *Actinobacillus pleuropneumoniae* to the porcine respiratory system.

Surface of adhesion	Type of experiment	Mechanism of Adhesion	Reference
Porcine respiratory tract mucus	*In vitro*	Presence of capsule or a high capsular thickness decreased the adherence	Bélanger *et al.* 1992
Swine-lung collagen (Type I, III, IV, and V) and fibrinogen	*In vitro*	Ca^{2+}-dependent/ A 60 kDa Outer-membrane protein	Enriquez-Verdugo *et al*. 2004
Swine buccal epithelial cells (BEC), fibronectin, and fibrinogen	*In vitro*	Cell-surface glycoproteins as treatment with proteolytic enzymes and periodate highly decreased the adherence to swine BEC	Hamer-Barrera *et al*. 2004
Porcine tracheal rings	*In vitro*	LPS (smooth type, O Antigen)	Bélanger *et al*. 1990
Lung vascular endothelium and tracheal epithelium	*In vitro*	LPS (Polysaccharide moiety)	Paradis *et al*. 1994
Phosphatidylethanolamine	*In vitro*	LPS (O antigen)	Jeannotte *et al*. 2003
Porcine lung epithelial cells	*In vitro*	LPS-independent	Boekema *et al*. 2003
Frozen lung sections	*In vitro*	Noncapsulated strains showed higher adhesion	Jacques *et al*. 1991
Porcine tracheal frozen sections	*In vitro/In vivo*	A capsule-deficient mutant showed higher adhesion	Rioux *et al*. 2000
Alveolar epithelial cells	*In vitro*	Fimbriae and a 55 kDa outer-membrane protein expressed in NAD-restricted medium	Overbeke *et al*. 2002
Not tested	*Microarray/ Transcriptomic profile*	*TadB*, *rcpA and Apa1* genes upregulated	Auger *et al*. 2009
St. Jude porcine lung cell line	*In vitro*	Apa1	Xiao *et al*. 2012, Wang *et al*. 2015
St. Jude porcine lung cell line	*In vitro/In vivo*	Lip40	Liu *et al*. 2018
Epithelium of alveolar cells and cilia of the terminal bronchioli epithelia	*In vitro*	Not tested but presence of fimbriae in AP was described	Dom *et al*. 1994
Not tested	*In silico/sequence analysis*	Fimbriae, Identification of ApfA protein by purification and amino acid sequence analysis	Zhang *et al*. 2000
PK-15 cells and St. Jude porcine lung cell line	*In vitro*	Operon *apfABCD* and *PilMNOPQ,* important for adhesion and biofilm formation	Liu *et al*. 2018
Not tested	*Cloning and In silico/sequence analysis*	ApfA and Operon (*apfABCD*) consisting of four genes involved in type 4 fimbrial biogenesis	Boekema *et al*. 2004, Stevenson *et al*. 2003
St. Jude porcine lung cell line and porcine iliac artery endothelial cell line	*In vitro/In vivo*	ApfA	Zhou *et al.* 2013
St. Jude porcine lung cell line	*In vitro/In vivo*	PilM	Liu *et al*. 2015
St. Jude porcine lung cell line	*In vitro*	Fimbriae, flp Operon consisting of 14 genes (flp1-flp2-tadV-rcpCAB-tadZABCDEFG)	Li *et al*. 2012, Li *et al*. 2019
Not tested	*In silico/sequence analysis and crystal structure*	ApHMW1C, a glycosyltransferase	
A549 cells, human adenocarcinoma lung epithelial cells	*In vitro* and *In silico/* sequence analysis	*Agt* and *Ngt*, glycosyltransferases	Cuccui *et al*. 2017

Also, O-antigen, a key component of lipopolysaccharides was not only important for cell adhesion but also to form biofilms (Hathroubi *et al.*, 2015). Finally, the trimeric autotransporter adhesin Apa1 participated in cell adhesion and biofilm formation (Wang *et al.*, 2015). Table 2 summarises more genes involved in biofilm formation for AP and their biological role; however, for most of those genes, it is not known whether they could be involved directly or indirectly in the process of adhesion of AP to cells and tissues of the porcine respiratory system. It is worth mentioning that many of the genes described in table 2 belong to stress-responding genes whose products may be important in the adaptation of AP to different environmental changes, for example, the two component systems (TCS) proteins CpxA, CpxR, and ArcA (Li *et al.*, 2018, Buettner et al 2008), the quorum sensing LuxS/

Table 2. Genes involved in biofilm formation by *Actinobacillus pleuropneumoniae*.

Gene	Function	Consequence of the mutation in AP	Reference
apf and *pil* operon genes	Formation of fimbriae (Type IV pilus, Tfp)	Deficient biofilm formation	Liu *et al*. 2018
flp1 and *tadD* (flp operon genes)	Formation of fimbriae (Type IVb pilus)	Deficient biofilm formation and attenuated virulence in mice and pigs.	Li *et al*. 2019
ArcA	Two-component systems (Metabolic adaptation to anaerobiocity)	Deficient biofilm formation. Attenuated virulence in the acute infection of pigs.	Buettner *et al*. 2008
CpxA/CpxR	Two-component systems	Deficient biofilm formation. Decreased expression of *RpoE* and *pgaC*. Attenuated virulence in mice.	Li *et al*. 2018
LuxS	Quorum sensing through AI-2 signaling molecule	Increased biofilm formation. Attenuated virulen in a mouse model/ Biofilm formation genes *pgaABC* were upregulated in early exponential phase. Some genes involved in adhesion were repressed at late exponential phase such as *apfABC* genes.	Li *et al*. 2008
hfq gene	RNA chaperone and posttranscriptional regulator, Hfq	Deficient biofilm formation, decreased level of *pgaC* transcript and PNAG content. Attenuated virulence in pigs.	Subashchandrabose *et al*. 2013
relA	relA-depedent (p)ppGpp-mediated stringent response, activated in nutritional starvation	Increased biofilm formation	Li *et al*. 2015
r*seA (mclA)* and *hns*	HN-S is a gene regulator and RseA is a negative regulator of the extracytoplasmic stress response sigma factor RpoE/σE	Increased biofilm formation. Attenuated virulence in mice/ Regulate the expression of the pgaABC operon.	Bossé *et al*. 2010, Dalai *et al*. 2009
TolC	An outer membrane channel, component of multidrug efflux pumps and type I secretion systems	Deficient initial adherence and biofilm formation. Decreased *pgaA* and *cpxR* expression. Decreased PNAG content in biofilm.	Li *et al*. 2016
TolC1, a TolC-like protein	An outer membrane channel, component of multidrug efflux pumps and type I secretion systems	Deficient biofilm formation and increased drug sensitivity	Li *et al*. 2016
VacJ	Outer membrane lipoprotein	Deficient biofilm formation	Xie *et al*. 2016b
Apa1 (Adh domain)	Trimeric Autotransporter serine protein	Deficient biofilm formation and adhesion. Attenuated virulence in piglets.	Wang *et al*. 2015
Apa1/Apa2	Trimeric Autotransporter serine protein	Deficient biofilm formation and adhesion to RAW246.7 macrophages. Attenuated virulence in mice.	Xiao *et al*. 2012
Aasp	An autotransporter serine protease	Deficient in biofilm formation	Tegetmeyer *et al.* 2009
Lon A	ATP-dependent protease (degradation of abnormal proteins in bacteria/stress tolerance)	Deficient biofilm formation. Attenuated virulence in mice.	Xie *et al*. 2016
ClpP	ClpP, the catalytic core of the Clp proteolytic complex. Stress tolerance.	Deficient in biofilm formation	Xie *et al*. 2013
pgaABC operon	PNAG or PGA (Polymer of N-acetyl-D-glucosamine residues in beta (1,6) linkage). Intercellular adhesion and attachment of cells to abiotic surfaces	Deficient biofilm formation	Izano *et al*. 2007

AI-2 system (Li *et al.*, 2008), the stress tolerance proteases LonA and ClpP (Xie *et al.*, 2013, Xie *et al.*, 2016a), the multidrug efflux channel protein TolC (Lie *et al.*, 2016), the RNA chaperone and posttranscriptional regulator Hfq (Subashchandrabose *et al* 2013), rseA, a regulator of sigma E (Bossé *et al.*, 2010), and RelA, an enzyme that participates in the (p)ppGpp-mediated stringent response (Li *et al.*, 2015). PNAG was shown to be an important component of the AP biofilm matrix, and AP strains able to form biofilms failed in this process when treated with

dispersin B, a PNAG-hydrolysing enzyme (Izano *et al.*, 2007). Dispersin B is an enzyme that induces the release of adherent cells from mature biofilms through catalysing the hydrolysis of linear polymers of N-acetyl-D-glucosamines (Kaplan *et al.*, 2004). Interestingly, the *pgaABC* operon genes encoding for PNAG were regulated by the TCS CpxA/CpxR (Li *et al* 2018), TolC (Li *et al.*, 2016), Hfq (Subashchandrabose *et al.*, 2013), LuxS (Li *et al.*, 2008), rseA, and the histone-like nucleoid structuring protein H-NS (Bossé *et al.*, 2010). Although the mutation of most of these genes in AP resulted in a deficient *in vitro* biofilm formation and an *in vivo* attenuated virulence, for *LuxS* and H-NS, however, it was an increased biofilm formation with attenuated virulence (Bossé *et al.*, 2010, Li *et al.*, 2008). These differences challenge the notion of the association of the *in vitro* biofilm formation on abiotic surfaces with the pathogenicity in an infection model. It is worth mentioning that some of these differences may be due to the animal model used, the experimental infection route, and the role of the genes in the pathogenicity independently of the contribution to form biofilms. Figure 1 describes the molecules involved in biofilm formation and/or adhesion to the porcine respiratory system, as well as the regulation of the *pgaABCD* operon.

CONCLUDING REMARKS

In this review, we have described the adhesion features of AP to the porcine extracellular matrix components such as mucus, collagen, fibronectin, and fibrinogen. These surfaces may be the initial contact of AP (an extracellular bacterium) to progress throughout the porcine respiratory system till reaching the lungs. Lipopolysaccharides were also initially proposed as molecules involved in adhesion to cells of the upper porcine respiratory tract. However, as we mentioned before, these molecules were dispensable for adhesion in a different cell model. We suggest that this difference could be explained not only because of a different model but the former protocols of lipopolysaccharides extraction that were contaminated with proteins and other cellular products. We consider that new studies with purified lipopolysaccharides or mutant strains deficient in the synthesis of these molecules will be helpful to analyse their exact role in the adherence of AP. Interestingly, unencapsulated AP strains and capsule-deficient mutants showed higher adhesion capacity to lung and tracheal epithelial tissue, thus, the capsule is not only dispensable in cell adhesion, but it seems that it masks other molecules important for adhesion such as

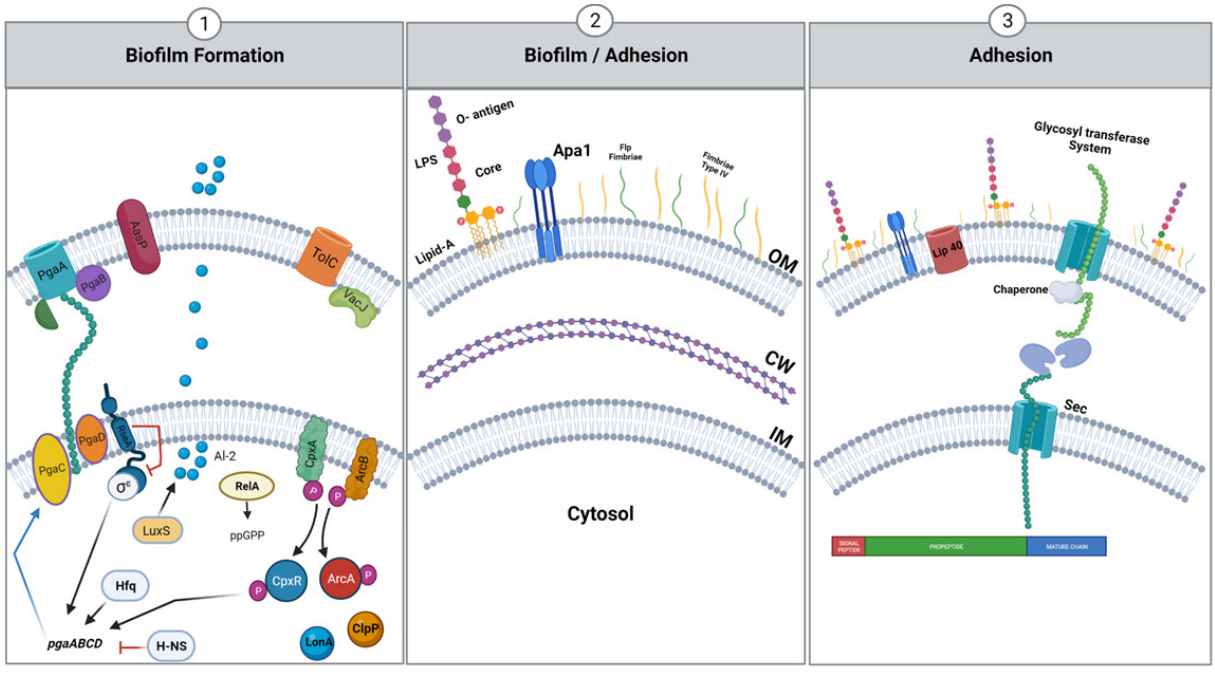

Figure 1. Molecules involved in biofilm formation and/or adhesion to the porcine respiratory system by Actinobacillus pleuropneumoniae. PNAG (Polymer of N-acetyl-D-glucosamine residues in beta (1,6) linkage) is a key component of biofilms. pgaABCD operon is necessary for PNAG synthesis and its expression is regulated by the chaperone Hfq, the global gene regulator H-NS, the two-component system (TCS) CpxA/CpxR, and RseA (the repressor of RpoE/σE). Other molecules involved in biofilm formation are the proteases ClpP, LonA, and Aasp, the outer membrane proteins VacJ and TolC, the relA hydrolase, the TCS ArcA, and the LuxS/Al-2 quorum-sensing system. The lipoprotein Lip40 and the glycosyltransferase system NGT participate in the adhesion to porcine respiratory cells. It is suggested that HMW1C, another glycosyltransferase may be involved in this process. Flp and type IV fimbriae, the trimeric autotransporter Apa1, and the lipopolysaccharide (o-antigen) were shown to participate in biofilm formation and adhesion to the porcine respiratory epithelial cells. OM, outer membrane; IM, inner membrane; CW, cell wall. This figure was created by BioRender software (https://www.biorender.com).

outer membrane proteins. However, how these molecules are exposed in AP with an intact capsule is not clear. In this case, the adhesion of AP must be through the type IV pilus and Flp pilus. It is worth mentioning that most of the studies were performed *in vitro*. Furthermore, most of the research related to the adhesion of AP employed the SJPL cell line which was mistakenly classified as being of simian instead of pig origin, as previously thought. Therefore, a more appropriate cell line model must be considered in the future. On the other hand, biofilm formation, a sessile mode of growth, has been associated with the virulence of bacteria. Although it is still not clear the role of biofilms during infection by AP, it was shown that AP can grow as aggregates on porcine respiratory tissue and biotic surfaces such as cell monolayers. We have presented information related to the genes involved in biofilm formation by AP at different stages, under different conditions, and comparing with the planktonic bacteria. For those genes, many of them with unknown functions, it is not known whether they are involved in adhesion to the porcine respiratory tract or in a different biological process affecting biofilm formation. Finally, most of the commercially available vaccines for porcine pleuropneumonia are based on the use of whole-cell bacterins (first-generation vaccines), an attenuated form of a specific AP serovar or a combination of serovars. One of the limitations of these vaccines is a partial protection against heterologous serovars and the lack of important virulence factors produced in live bacteria such as the Apx toxins. Although lipopolysaccharides of AP are known to participate in the adhesion to the porcine respiratory system, the potential as antigens to generate vaccines was not as expected due to the high heterogeneity of LPS among serotypes and the same was true for capsular polysaccharides. Because of this, it seems to be that the most promising antigens to generate vaccines in AP are outer membrane proteins and lipoproteins with conserved sequences among serotypes. In this regard, this review may be helpful to find conserved molecules with antigenic properties involved directly or indirectly in the adhesion of AP to the porcine respiratory tract or in biofilm formation to develop new vaccines that may confer protection against porcine pleuropneumonia.

CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest.

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ORIGINAL ARTICLE

Characterisation of dairy female calf management practices in southern Chile

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ABSTRACT. The objective of this study was to characterise husbandry and technical-productive practices at the calf rearing stage in dairy farms in Los Lagos Region, southern Chile. A face-to-face survey was applied to 22 dairy farms in Los Lagos Region in 2017. All farms performed artificial calf rearing under either of two systems: total barn confinement (48%) or a mixed system that considers the first stage with confinement and the second stage in open-air paddocks (52%). More than half (52%) of the farms supplied fresh colostrum to the calf from its dam and the rest of the farms used bottle or oesophageal tube. Only 30% of the farms evaluated colostrum quality using colostrometer (densimeter) or refractometer. After the colostrum supply, milk replacers, waste milk, or a mixture of both were used for calf feeding. Most of the farms (66.7%) did not have automated milk-feeding systems and used bottles (88.9%) and buckets (11.1%) instead. On average, calves were handled by 1.5 caretakers (SD: 0.63) of which 63.4% (SD: 40.2) were men. The average age for caretakers was 43.9 years (SD: 12.7), with 23.8% being less than 35 years old. Overall, results from this study can be used to identify key managements that could improve calves' rearing productive traits.

Keywords: calves, dairy, health, nutrition, production.

INTRODUCTION

Currently, the demands of high production dairy farms have increased the incidence of reproductive, locomotion and metabolic-health problems (Riberio *et al.*, 2017; Carvalho *et al.*, 2019). These problems can lead to poor welfare (Calderón-Amor & Gallo, 2020), a decrease in milk production, economic revenues, all of which could be prevented by improving the rearing of replacement heifers (Probo *et al.*, 2018; Machado *et al.*, 2020). Heifer rearing influences the productivity and profitability of dairy herds (Mohd *et al.*, 2015), which represents between 15 to 20% of the total costs in dairy production systems (Heinrichs *et al.*, 1993).

When rearing replacement heifers, it is pivotal to consider production management such as practices during parturition (for both, the dam and the offspring), supply of colostrum, post-weaning feeding, grouping, health control, and housing infrastructure that guarantees a well-being environment (Murray *et al.*, 2015; Bach *et al.*, 2006; Raboisson *et al.*, 2014; Staněk *et al.*, 2014; Diao *et al.*, 2017). Despite the economic importance of rearing replacement heifers, aspects related to feeding and general management are usually overlooked (Dobos *et al.*, 2001). According to Abuelo *et al.* (2019), in Australian dairy farms, this disinterest is reflected in high pre-weaning morbidity and mortality rates, reaching values close to 35% and 7%, respectively.

In Chile, milk production reached 2,275 million litres in 2020, of which 85% is produced in the southern part of the country (La Araucanía, Los Ríos, and Los Lagos regions with 6.7%, 31.4% and 46.3% of the total milk produced, respectively), which highlights the importance of dairy farms from this area (ODEPA, 2021). Here, dairy production, as indicated by Toro-Mujica *et al.* (2020) is performed under extensive and semi-extensive production systems, which are based on natural grasslands, improved natural grasslands, sown grasslands, and supplementary crops. In these systems, rearing replacement calves is commonly done in two stages. The first stage is carried out in roofed sheds in which individual and/or collective pens are kept. The second stage, called mixed, includes a mixture of an open-air patio and collective pens with a shed. Notwithstanding the above, given the natural variability of dairy production systems in southern Chile, Toro-Mujica *et al.* (2020) suggested that it would be possible to find heterogeneity in productive management performed during the calf rearing stage. Eventually, knowing these productive managements could lead to identifying improvements to reduce mortality rates, morbidity, and increase growth parameters and rumen development (Silper *et al.*, 2014). Furthermore, it has been observed that growth rate during the calf rearing period affects mammary gland development due to a reduced peripubertal allometric mammary growth phase and altered responses to mammogenic stimuli (Geiger *et al.*, 2016). Subsequent lactations are also compromised as a result of trying to get calves to their pubertal body

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weight earlier due to epigenetic programming (Soberon *et al.*, 2012; Margerison *et al.*, 2013). Until now, there is scarce scientific data on calf management practices in Chile. Thus, the objective of this study was to characterise female dairy calf husbandry and technical-productive practices at the calf rearing stage in dairy farms in Los Lagos Region, southern Chile.

MATERIAL AND METHODS

The study was conducted according to the guidelines of the Committee of Ethics in Research in Social Sciences and Humanities of Pontificia Universidad Católica de Chile (protocol code 151216004 approved in 2016).

DESCRIPTION OF THE STUDIED REGION

The study was performed in Los Lagos Region, latitude 39°17'14" to 40°40'51" and longitude 71°35'33" to 73°43'29". This region has an area of 48,584 km^2 distributed in four provinces (Chiloé, Llanquihue, Osorno and Palena). Around 87% of the Chilean bovine production for both milk and beef is concentrated in the provinces of Llanquihue and Osorno (INE, 2007). Annual rainfall ranges from 865 to 1071 mm, with monthly average temperatures ranging from 6.6 to 16.2 ° C (DMC, 2020). In most of the provinces, the prevailing climate is temperate (Cfb) (figure 1).

FARM SELECTION AND DATA COLLECTION

A survey was designed to characterise productive management practices commonly used at the rearing stage in dairy farms in Los Lagos Region, as it concentrates the greater milk production from the country. The survey was applied to 22 dairy farms that belong to the Chilean Federation of Dairy Producers from Los Lagos Region in February 2017. This trade association had approximately 500 active members in Los Lagos Region. We chose farms that were representative of the range of a milk production greater than 1,500,000 L/year. In Chile, these types of farms produce 90% of the total milk production (Consorcio Lechero, 2019; ODEPA, 2021).

Before applying surveys, selected farms were randomly contacted by phone for collaboration, and only 22 farmers volunteered to participate in the study. A similar recruitment method was previously reported by Vasseur *et al.* (2010) and Santman-Berends *et al.* (2014). In-person surveys were conducted with farm managers using a printed hard copy. The questionnaire had 54 questions, which allowed the

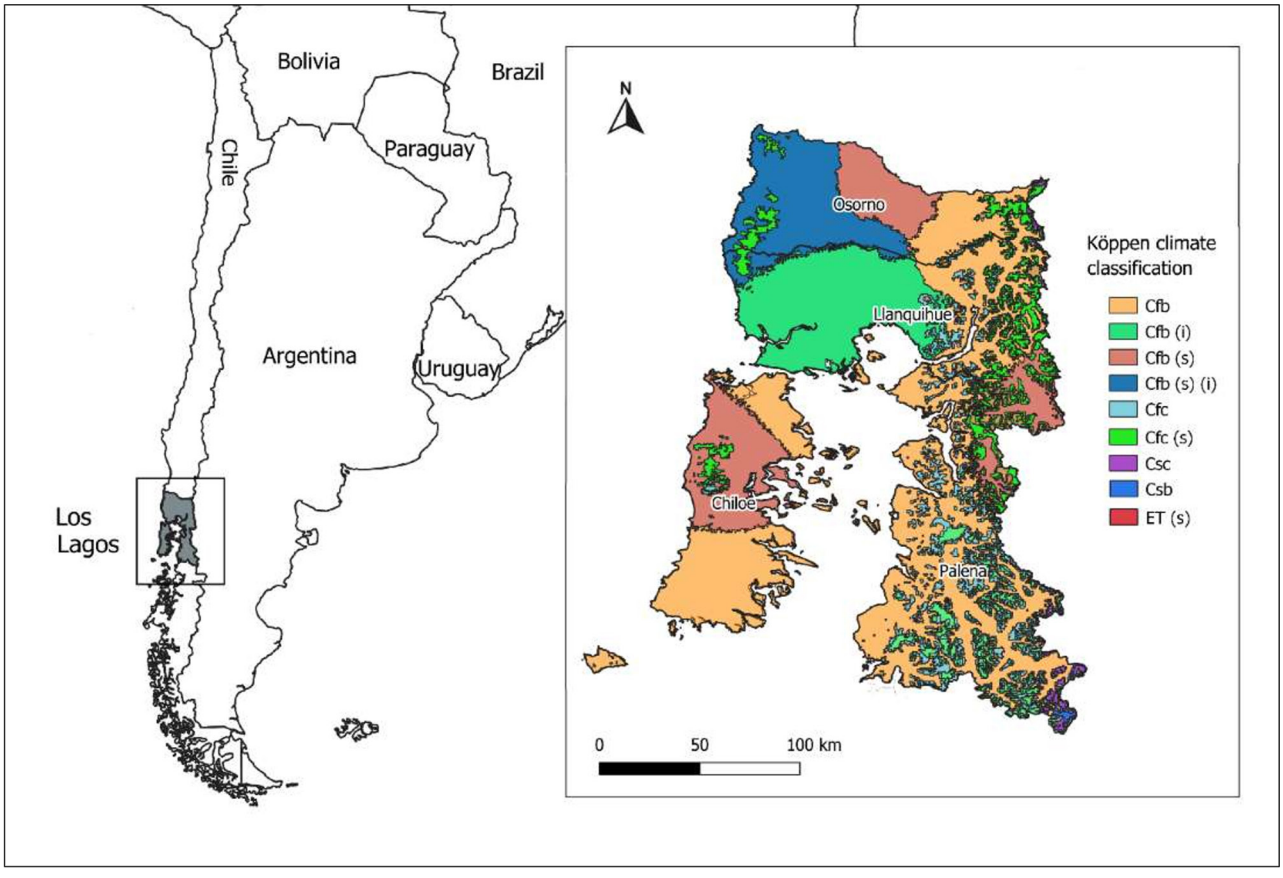

Figure 1. The study zone at Los Lagos Region in southern Chile.

identification of 95 variables. The full survey can be found as supplementary material. Before statistical analysis, the data were reviewed and then, for continuous variables, the outliers were identified using box plot graphics for their subsequent removal from the data set. In the categorical variables, the classification levels used in the survey were kept.

SURVEY DEVELOPMENT

Two professionals (E.V.-B.-P. and P.T.-M.) with PhD in animal production and an agronomist (R.G.) developed the questions for the survey. The survey included general farm data (i.e., production system, size, and location) and specific questions related to the rearing stage, which included information on housing space, feeding, mortality, weaning and health parameters. The survey was divided into three sections: I. Farm data that included 13 general questions regarding farm characteristics (size, production system, and location); II. Calf management that included 33 questions and III. Staff data that included 8 questions. Before using the final survey, a pilot survey was performed on 2 dairy producers from Los Lagos Region to evaluate clarity, the accuracy of response options, use of technical language and overall flow.

STATISTICAL ANALYSIS

The information collected in each survey was coded and stored in an Excel database (Microsoft Excel, Microsoft Corp., Redmond, WA). Firstly, data were used to describe the farms through quantitative descriptive statistics (mean, variances, minimums, and maximums) and qualitatively (percentage frequency). Secondly, paired relationships between calves' mortality and all other variables were searched through regression analysis (quantitative variables), contingency tables, and X^2 test (qualitative variables). The statistical software SPSS 11.5 was used for all statistical analyses.

RESULTS

PRODUCTION SYSTEMS CHARACTERISATION

The average surface of the farms was 448 ha. Around 48% of this area was used for dairy farming, 44% for pasture production and 11% for crop production (table 1). Most (80%) of the surveyed farms had grazing production systems, while the remaining 20% use mixed production systems (grazing animals in spring-summer and confined in winter with feeding based on conserved forages and concentrates). All farms performed milking twice daily and cows had an average lactation length of 307 days.

On average, surveyed farms had 689 females (calves, heifers, and cows); however, wide variability was observed between farms, ranging from 90 to more than 3000 females. Of the total number of cows, on average, 74% were lactating and had a calving interval of 382 days. More than half (59%) of the surveyed farms performed artificial insemination, while 27% combined artificial insemination and herd bull. The most common breed was Holstein (67%), however, 57% of the surveyed farms had more than two breeds.

CALF RAISING HUSBANDRY PRACTICES

Housing. All farms performed artificial calf rearing under either of 2 systems: total barn confinement (47.6%) and mixed system that considers the first stage with confinement and the second stage in open-air paddocks (52.4%). In more than half of the surveyed farms (61.9%), calves management was carried out in groups of 5 to 50 animals ($\bar{x} \pm SD$: 17.5 ± 14.3). This management was performed from day 1 (at birth) in 26% of the farms, while 11% of the farms delayed it until one month of age.

In all surveyed farms, the bedding material used at calf barns was wood chips. Only 25% of the surveyed farms had calf barns with temperature control. Regarding calf barn capacity, this was related to the number of cows in the herd (X^2 20.98, df. 9, p = 0.013), with a capacity between 101 to 200 animals with a space per animal of 1.5 to 2 m^2. The farm stocking rate was 0.92 cows/ha (standard deviation: 0.37) and the animal stocking rate for dairy production was 1.93 cows/ha (standard deviation: 0.59).

Health. Most farms (85.7%) do vaccination and deworming programs. More than 80% of the farms reported mortality rates below 10%, and there was no relation between calf birth weight and calf mortality rate (X^2 6.22 df.12; P=0.904) (figure 2). With regard to the type of health problems observed at the calf barn, the surveyed caretakers declared that 100% were of respiratory or digestive origin (table 2). In this study, there were not significant relationships between the presence of respiratory and digestive problems and mortality rates (P=0.62).

Calf feeding practices. The results for newborn calf feeding management (table 3) showed that 52.4% of the farms supplied fresh colostrum to the calf from its dam. In the case that fresh colostrum from the dam was not being used, it was obtained from another recently calved cow during the milking routine (78.5%) or from frozen colostrum (21.4%). In both cases, the colostrum was supplied either through a bottle (68.8%) or by using an oesophageal tube (31.4%).

In more than half (52.4%) of the surveyed farms, the evaluation of passive immunity transfer was carried out instead of evaluating colostrum quality, using refractometers or through blood tests. After colostrum supply, milk replacers, waste milk, or a mixture of both was used for calf feeding. Milk replacer was used in 90.5% of the farms, 71.4% of the farms used waste milk, and a mixture

Table 1. Descriptive statistics from 22 surveyed dairy farms from Los Lagos Region.

Quantitative variables	Mean	SE	Minimum	Maximum
Total surface (ha)	448.5	77.0	50	1020
Surface for dairy (total %)	48.8	4.9	18.5	79.2
Grazing surface (%)	44.1	4.2	12.0	70
Cropping surface (%)	11.3	2.4	1.3	42.3
Total number of females	688.5	182.7	90	3152
% of lactating cows	74.1	3.4	31.1	95.0
Calving interval (days)	381.7	7.4	330	424
Lactation length (days)	307.7	13.4	180	380
Qualitative variables	**Category**		**Frequency**	
Production system	Grazing		80.0	
	Partial total mixed ration		20.0	
Milking per day	Two		100	
Breeding type	Artificial insemination (AI)		59.1	
	AI and herd bull		27.3	
	Herd bull		13.6	
Breed[1]	Holstein		66.7	
	Jersey		28.6	
	Red Friesian		33.3	
	Black Friesian		14.3	
	Other		23.8	
Number of breeds used	1		42.9	
	2		47.6	
	3		9.5	
Synchronised calving	Yes		71.4	
	No		28.6	
Calving distribution	All-year-round		19.0	
	Seasonal[2]		81.0	

[1]Percentage of farms with the breed.
[2]Concentration of calving in spring.

of milk replacer and waste milk was used in 33.3% of them. Most of the farms used more than one feed (milk substitute + waste milk or milk replacer + mixture), and only 27% of the farms exclusively used milk replacer. Most of the farms (66.7%) did not have automated milk-feeding systems, and bottle feed (88.9%) and buckets (11.1%) were used. Milk feeding was provided in most of the surveyed farms twice a day (71.4%), however, due to automation, there were farms where a higher number of feedings was provided (*ad libitum*). Weaning time ranged from 1 to 4 months, and then feeding with starter concentrate and forage began from day 1 or at least from week 1, with one or two rations or *ad libitum*. Most farms (95.2%) used forage feeding based mostly on corn silage (78.9%). In most farms (66.7%), forage feeding was used for 2 months. Lastly, no relationship was found between calving time and feeding method. As observed in table 4, the seasonal breeding systems tended to present lower mortalities than non-seasonal breeding systems.

Personnel in charge of the calves. On average, calves were handled by 1.5 caretakers (standard deviation: 0.63) of which 63.4% (standard deviation: 40.2) were men. The number of people in charge of the calves was positively related (P=0.003) to the number of cows on the farm. The average age for caretakers was 43.9 years (standard deviation: 12.7), with 23.8% being less than 35 years old. The educational level of 45% of the personnel responsible for the calves is basic education, while 35% had secondary education (table 5).

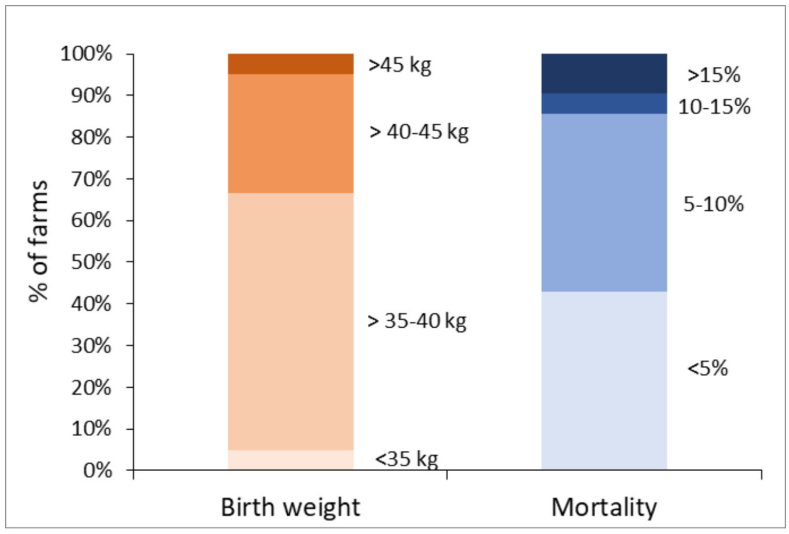

Figure 2. Distribution of calf's birth weights and mortality in 22 surveyed dairy farms from Los Lagos Region.

Weaning criteria parameters. To transfer calves from the calf barn to the next productive stage/housing (after weaning), a combination of weight and age was the most used criterion (52.4% of the surveyed farms) (table 2). Most surveyed farms (71.4%) produce female calves as replacements. Calf weight at weaning ranged between 71 to 120 kg in most farms (76%).

DISCUSSION

PRODUCTION SYSTEMS CHARACTERISATION

As reflected by this survey, grazing systems are predominant in the humid temperate region of southern Chile (Keim *et al.*, 2015). Synchronised calving was a common practice, with the higher (around 70%) concentration of calving taking place during the springtime as a strategy used to reduce feeding costs and calves' survival rate, which is typical of grazing systems in humid temperate regions to match calving with a pasture growth that equals nutritional demands (Roche *et al.*, 2017). In 71% of the surveyed farms, male calves were reared and sold for beef production. Male dairy calves represent 45% of the total animals used for beef production in Chile and Los Lagos region, they account for more than 2/3 of beef animals (INE, 2015).

CALF RAISING HUSBANDRY PRACTICES

Housing. At the surveyed farms, calf barn capacity was lower than the minimum of 3.3 m^2 per calf recommended by Nordlund and Halbach (2019) and is considered highly determinant for air quality, having a major impact on the quality and moisture of the bedded surface in which the calves lie. Stocking rates are similar to those reported by Toro-Mujica *et al.* (2020) for dairy production systems in southern Chile. In addition, according to Svensson and Liberg (2006), group-housed calves should be maintained in pens of less than 10 calves, however, optimal health and growth performance can be achieved in groups of 20 to 25, as larger groups of calves are associated with an increased risk of respiratory disease (Nordlund & Halbach, 2019). In future studies, it will be of great importance to identify the criteria used for the cleaning and disinfection of pens, as well as the considerations for changing the bedding material, since this has an impact on the health of the animals.

Health. In this study, the lack of relationship between the presence of respiratory and digestive problems and mortality rates would be a consequence of the different recovery capacity of calves between farms, since the most common causes of pre-weaning mortality in dairy calves are neonatal diarrhoea and bovine respiratory disease (Pempek *et al.*, 2017). As reported, the absence of other health problems could be a consequence of vaccination and deworming schedules, a common practice observed in more than 85% of the surveyed farms.

More than 80% of the farms reported mortality rates below 10%. Mortality rates with ranges between 0 to 11% have been reported by different authors (Santman-Berends *et al.*, 2014; Cuttance *et al.*, 2017; Tautenhahn *et al.*, 2020). In this study, mortality was not related to calving time and feeding method. Reiten *et al.* (2018) reported that this would be linked to the incidence and survival rate of season-dependent diseases such as diarrhoea and respiratory diseases. Regarding feeding methods, lower mortalities were observed when automated feeding methods were used. For the use of automatic feeding, it is necessary to handle the calves in groups. Relationships between grouping and calf health are controversial, for example, Svensson *et al.* (2006) associated group management with a higher risk of

Table 2. Characteristics of calf management in 22 surveyed dairy farms from Los Lagos Region.

Variable	Category	Percentage
Female calves' productive purpose	For sale	19.0
	Replacement	71.4
	Other	9.5
Weight at birth (kg)	< 35	4.8
	35-40	61.9
	40-45	28.6
	> 45	4.8
Calves weaning criteria	Weight	38.1
	Age	4.8
	Weight and age	52.4
	Need for space for new calves	4.8
Weaning weight (kg)	51-70	9.5
	71-90	38.1
	91-120	38.1
	More than 121	14.3
Rearing place	Pen	47.6
	Mixed (outdoor + pen)	52.4
Animal capacity (n° head)	Less than 100	23.8
	101 a 200	52.4
	201-300	9.5
	More than 300	14.3
Controlled temperature	Yes	25
	No	75
Space availability (m^2/animal)	Less than 1 m^2 /animal	0.0
	1-1.5 m^2/animal	15.8
	1.5-2 m^2/animal	57.9
	More than 2 m^2/animal	26.3
Diseases	Respiratory	42.9
	Digestive	33.3
	Respiratory and digestive	23.8
Vaccination and deworming program	Yes	85.7
	No	14.3
Mortality (%)	Less than 5	42.9
	5-10	42.9
	10-15	4.8
	15-20	4.8
	20-25	4.8

enteric and respiratory diseases, whereas Hänninen *et al.* (2003) and Babu *et al.* (2009) found that calves housed in groups had lower incidences of diarrhoea and were less likely to have respiratory disease compared with individually housed calves. Medrano-Galarza *et al.* (2017) reported that group size is one of the main contributors to health issues rather than group housing *per se*. However, in the present study, this was not observed. In this regard, Svensson and Liberg (2006) stated that group size is one of several risk factors associated with calf health and therefore many other variables can affect calf health.

In this study, there are few data on the clinical methodology and follow-up of the causes of declared mortality in calves. It must be considered that it is essential to prepare medical records, with data on mortality and morbidity, for the analysis of epidemiological behaviour to make long-term clinical management effective (Vasseur *et al.* 2010). Future studies should investigate further whether there are comprehensive preventive medicine protocols, with staff training on the factors that affect animal health (facilities, biosecurity, cleaning, clinical examinations) and clinical monitoring (laboratory diagnoses and treatments).

Table 3. Calf feeding management in 22 surveyed dairy farms from Los Lagos Region.

Management	Options	%
Colostrum origin	From the dam	52.4
	From stored colostrum	47.6
Colostrum type	Fresh	78.8
	Frozen	21.4
Colostrum supply	Bottle feed	68.8
	Oesophageal tubing	31.4
Colostrum quantity and frequency	4 L/d, in 2 feedings	33.3
	6 L/d, in 2 feedings	66.7
Colostrum check quality*	Yes	30
	No	70
Evaluation of passive transfer of immunity	Yes	52.4
	No	47.6
Milk feeding		
Type of milk	Milk replacer	90.5
	Waste milk	71.4
	Milk replacer and waste milk	38.1
Use of automatic milk feeding (Yes)		33.3
Type of feeding	Bottle feed	88.8
	Buckets	11.1
Lactation length	1 to 2 months	14.3
	2 to 3 months	52.4
	3 to 4 months	33.3
Milk quantity	2-4 L/d	15.0
	4-6 L/d	75.0
	6-8 L/d	10.0
Time of supply	Morning	4.8
	Afternoon	4.8
	Morning and afternoon	71.4
	Other time during the day	19.0
Concentrate feed		
Use of concentrate feed	Yes	100
	No	0
Feeding frequency	Ad libitum	71.4
	One daily ration	9.5
	Two daily rations	19.0
Quantity	Less than 1 kg	25
	1-2 kg	75
Forage feed		
Use of forage feeding	Yes	95.2
	No	4.8
Type of forage	Alfalfa hay	5.3
	Corn silage	78.9
	Meadow silage	10.5
	Other	5.3
Use of forage feeding (months)	1	4.8
	2	66.7
	3	23.8
	4	4.8

*Use of either refractometry or blood tests.

Table 4. Relationship between mortality and breeding time and mortality and type of feeding in 22 surveyed dairy farms from Los Lagos Region.

Mortality	Calving		Feeding	
	Non-seasonal	Seasonal	Manual	Automatic
Less than 5%	22.2%	77.8%	0%*	100%*
Between 5-10%	0%	100%	66.7%	33.3%
More than 10%	66.7%*	33.3%*	33.3%*	66.7%*

*Values that differ from expected values $P<0.05$.

Table 5. Personnel in charge of the calves in 22 surveyed dairy farms from Los Lagos Region.

Variable	Category	Percentage
Educational level	Basic	45
	High school	35
	Technical	15
	University	5
Training at farm	Yes	81
	No	19
Training frequency	Less than a month	17.6
	Monthly	17.6
	Every 6 months	52.9
	Every 12 months	11.8
Personnel who perform handling and / or procedures in calves	Farm staff	52.4
	Veterinarian	28.6
	Both	19.0
Age (years)	18-25	9.5
	26-35	14.3
	36-45	38.1
	46-55	9.5
	More than 55	28.6
Work satisfaction	Yes	85
	No	15

Calf feeding practices. More than half of the surveyed farms used fresh colostrum for the calf from its dam, however, this contrast with Stanek *et al.* (2014), where all farmers supplied colostrum artificially. The latter is a recommended procedure as it ensures the recommended volume of colostrum supply (Moran, 2002).

The use of tubing for colostrum supply was observed in 23% of the farms, a value that exceeds the reported 8% of dairy farms in Canada (Medrano-Galarza *et al.*, 2017) and 5% in the Czech Republic (Staněk *et al.*, 2014). Large quantities of colostrum supplied through oesophageal tubing have been associated with reduced apparent efficiency of IgG absorption (AEA) and slightly lower serum IgG concentration compared with colostrum administered by nipple bottle (Lee *et al.*, 1983). Colostrum administered by oesophageal tubing enters the rumen before moving into the abomasum and intestine (Lateur-Rowet *et al.*, 1983). Thereafter, it takes 2 to 4 h for the colostrum to leave the rumen. This interval may be the reason for lower AEA, because the intestine may mature during this time, thereby reducing the number of actively absorbing cells in the intestine.

In this study, most surveyed farms used colostrum from recently calved cows followed by those using frozen colostrum mostly supplied using bottles. As Costa *et al.* (2017) reported, the colostrum storage method, either by refrigeration, freezing or at room temperature (after fermentation or chemical treatment) for its subsequent use is a frequent practice that does not affect the nutritional composition or its immunoglobulin content. Irrespective of the type of supply, the amount of colostrum delivered was between 4 to 6 litres divided into two feedings. This

amount of colostrum was less than that indicated by Renaud *et al.* (2020) in farms in Ontario, where around 9.6 litres of colostrum were supplied within the first 24 hours of life. The amount of colostrum, along with its cleanliness, quality, and speed of administration, are some of the factors that Godden *et al.* (2019) and Pempek *et al.* (2017) associated with the improvement of serum IgG levels and with the survival rate and health of calves. However, in the present study, a relationship between the amount of colostrum supplied and calf mortality (X^2 1.97, df 4, p = 0.741) was not observed.

Assessing colostrum quality before feeding is recommended as an important productive practice (Godden *et al.*, 2019). Regarding colostrum quality, only 30% of the farms evaluated this parameter, using a colostrometer (densimeter), or refractometer. In a Canadian survey on calf management practices, only 23% of the farms evaluated colostrum quality (Medrano-Galarza *et al.*, 2017), while Barry *et al.* (2019) reported 12.8% in Irish dairy farms. In contrast, a Czech survey reported that 44.1% of farmers measured colostrum quality (Staněk *et al.*, 2014). As noted by Turini *et al.* (2020) the supply of high-quality colostrum ensures the delivery of immunity reducing pre-weaning morbidity and mortality. In this regard, values greater than ≥10 g/L of IgG using radial immunodiffusion (Weaver *et al.*, 2000) or ≥5.2 g/dL of total serum protein (Buczinski *et al.*, 2018) are considered adequate.

The percentage of surveyed farms with automated milk feeders was higher than that described by Medrano-Galarza *et al.* (2017) in Canadian farms, who reported their use in 16% of the surveyed farms and Stanek *et al.* (2014) in Czech dairy farms (2.2%). Among the advantages mentioned by Stanek *et al.* (2014) for the use of automated milk feeders, the most important were to provide greater amounts of milk, facilitate greater number of feedings, and greater social interaction. However, the health of the animals can be compromised if this feeding system is not handled properly (Svensson *et al.*, 2006).

Personnel in charge of the calves. The number of persons working with calves was related to the number of lactating cows (P=0.005) and the size of the rearing place (P<0.001). However, Sischo *et al.* (2019) reported that in smaller dairy farms, it is usually common for a caretaker to be responsible for more than one task and, therefore, there would be an increase in the number of animals handled with the consequent increase in hours dedicated to the activity by the same worker.

On average, personnel in charge of the calves had more than 36 years of age (table 5), which differs from the reports of Sischo *et al.* (2019) in North American dairy farms, where 40% of the calf managers were under 30 years old, however, it should be noted that most the staff corresponded to immigrants or their descendants.

The educational level of the personnel in charge of the calves was consistent with the average years of schooling in the region which reached 10.1 years in 2017 (CASEN, 2018). In this regard, only 5% of the personnel had university education, which contrasts with the reports of Sischo *et al.* (2019) who mentioned percentages of 37, 11 and 24% for calf managers, calf feeders and treaters, respectively. It is important to note that the latter study was done in North America where the caretakers of calves and technical staff can be composed of qualified personnel coming from Latin American countries, which contrasts with the conditions of the present study.

Although the specialisation in animal production of the personnel in charge of the calves is scarce, this has changed at the local level through training courses, thus 82.4% of the farm's staff received at least one training per year. Within the training topics related to calving management, as indicated by Schuenemann *et al.* (2013), those related to newborn-calf care practices (e.g., time and amount of colostrum administered) were fundamental for calves' performance. In Chilean farms, it could be suggested to employ calf-specific personnel that may lead to increasing the specialisations of skills in the staff and the ability of veterinary practices to target training courses. In the United Kingdom (Mahendran *et al.*, 2022) the development and implementation of standard operating procedures in conjunction with veterinary practices is becoming more common and even a requirement for some dairy contracts and this could be applied in Chile in the near future.

Weaning criteria parameters. In this study, the criterion for weaning partly agrees with studies from Europe. In the United Kingdom, farmers use age as the main weaning criteria, with an age ranging from 6 to 12 weeks (Mahendran *et al.*, 2022). In Czech dairy herds, Stanek *et al.* (2014) reported that calving in group pens predominated (67% of farms) and the main weaning criterion was age (61.7%) followed by intake of starter and concentrated feeds. It is noteworthy mentioning that none of the farmers used dry feed intake as a weaning criterion, which is a recommended practice as it is related to rumen development (Moran, 2002). In Switzerland, weaning calves with concentrate-dependent feeding regime has been shown to be an effective strategy to allow a faster physiological development without a negative impact on rumen development, weight gain, or health status (Roth *et al.*, 2009). In line with this, Benetton *et al.* (2019) suggested that weaning based on individual concentrate intakes can lower overall milk consumption and maintain similar postweaning weights compared with calves weaned at a fixed age.

IMPLICATIONS AND LIMITATIONS OF THE STUDY

It is worth mentioning that this survey was performed on a limited number of dairy farms (n = 22) from Los Lagos Region, that according to farm surface, the number of animals and milk production per year represent large scale dairy farms (Consorcio Lechero, 2019). Large-scale dairy farms

represent 10% of total dairy farmers in Chile (Consorcio Lechero, 2019). These farmers are characterised by using more technology and recording more data compared with smallholder dairy farms (Chang'a *et al.*, 2010). Thus, it may be expected that rearing conditions in most of the dairy farms in the country, would be poorest compared with the selected group that we are reporting in this study. Further studies will need to consider increasing sample size, including different types of production systems and farm size levels. In addition, future surveys should include an analysis of medical records from each farm.

Dairy farmers would benefit from the findings in this study since it has been demonstrated that benchmarking motivates them to improve dairy calf management (Summer *et al.*, 2018). The survey showed some management practices that need to be improved and were not completely achieved or performed by a large proportion of the respondents. According to Mee (2008), key features of successful newborn dairy calf management are ensuring heifers and cows are moved in time to calve to suitable maternity housing, with discreet calving supervision and appropriate timing of any necessary calving assistance, immediate parturient evaluation of at-risk newborn calves and prompt movement of the newborn calf to hygienic calf housing. Furthermore, colostrum management is the single most important management factor in determining calf health and survival (Godden *et al.*, 2019), and it is recommended that calves should receive colostrum three times during the first 24h at a dose of 5-6% BW, which was not the selected criteria used by farmers, who let calves to get fresh colostrum from their dams or received 4 - 6 kg artificially divided into two feedings. This may be one of the reasons for the 20% of respondents with mortality rates greater than 10%, as AEA may be not successfully achieved. The results of the study suggest that other practices or managements should be further improved, for example: space per calf (>3 m^2/calf), weaning criteria (based on starter intake, rather than age or weight), and lack of colostrum quality measurements, among others.

In this study, twenty-two dairy farms from southern Chile were characterised by grazing systems where cows are milked twice a day and the use of artificial insemination. In the case of newborn calves, colostrum was supplied directly from the dam or through other approaches such as oesophageal tubing. Evaluation of colostrum quality and passive transfer of immunity, as well as automated feeding, are not predominant in this region but appear to be an upward trend.

Due to the limited sample size, it was not possible to identify that the calving system and feeding method were related to calf mortality. The impossibility of identifying significant relationships between variables arises from the heterogeneity of the dairies, both in dimensional terms and type of production systems as well as in relation to calf management. In this way, and considering the multiple variables that conditioned calf mortality, more research is required, with a greater number of farms, which will provide a suitable data set for multivariate analysis. It is very important to note that 22 dairy farms were surveyed and therefore our data is not a reflection of the Chilean dairy production.

Overall, results from this study can be used to identify key managements that could improve calves' rearing productive traits but caution must be paid as our data does not represent Chilean dairy production systems. For example, farmers should reduce barn-stocking rate, supervise pens disinfection and cleanliness, avoid humidity of bedding, measure colostrum quality, supply colostrum artificially, improve management of automatic feeding systems and implement a clinical protocol to monitor calves' health.

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ORIGINAL ARTICLE

Virulence and antimicrobial resistance of *Escherichia coli* isolated from chicken meat, beef, and raw milk

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ABSTRACT. Two hundred and thirty-five strains of *E. coli* were recovered from beef, chicken meat, and raw milk sold in butcher shops and markets in the town of Jijel, East Algeria. A PCR investigation revealed the predominance of bacterial strains with extraintestinal pathogenic *E. coli* (ExPEC) genes (19.91%). Enteroaggregative (EAEC) and enteroinvasive (EIEC) bacteria were also detected. Strains from phylogenetic groups A and B1 were the most common; they accounted for 62.35% and 21.17% in chicken meat, 67.53% and 16.88% in raw milk and 64.38% and 25.97% in beef, respectively, followed by the strains from phylogenetic groups B2 and D. Moreover, *E. coli* from phylogenetic group D was more abundant in chicken than in beef or raw milk samples ($P<0.05$). Antibiogram results revealed the presence of three major antibiotic-susceptibility groups and more than a hundred antibiotypes. Between 50% and 70% of strains were resistant to antibiotics of the first group (ampicillin, amoxicillin, trimethoprim, sulfonamide and tetracycline), 20% to 50% to antibiotics of the second group (amoxicillin plus clavulanic acid, kanamycin, streptomycin, ciprofloxacin, flumequine and neomycin) and less than 20% to those of the third group (cephalothin, gentamicin and colistin). Additionally, the resistance to flumequine, trimethoprim, sulfamethoxazole-trimethoprim and tetracycline was more frequent in chicken meat strains than in those from beef and milk. It is important to point out that the strains carrying more than two virulence factors belonged to the B2 or D phylogenetic groups and had weaker resistance to antibiotics. The strains from the A and B1 groups had fewer virulence factors and showed high resistance to antibiotics.

Keywords: Chicken meat, beef, raw milk, *Escherichia coli*, virulence factor, phylogeny, antibiotic resistance.

INTRODUCTION

Foodborne diseases and microbial food safety are becoming global public health concerns. Most foodborne diseases are generally caused by the consumption of contaminated beverages or food products like raw milk, beef and chicken meat. A variety of pathogens are involved in this type of infections, such as pathogenic *E. coli* strains of zoonotic origin (Rivera-Betancourt *et al.*, 2004). Furthermore, pathogenic *E. coli* strains were detected in beef-processing plants as reported in several studies (Johnson *et al.*, 2005; Holko *et al.*, 2006). *E. coli* are natural inhabitants of the digestive tract of humans and animals. However, some strains can be pathogenic for humans and animals (Kaper *et al.*, 2004; Holko *et al.*, 2006). Pathogenic *E. coli* can be categorised as intestinal pathogenic *E. coli* or extraintestinal pathogenic *E. coli* (ExPEC) (Russo & Johnson, 2000). Among the intestinal pathogenic *E. coli*, enterohemorrhagic *E. coli* (EHEC) are responsible for severe clinical symptoms, such as haemorrhagic colitis and the potential lethal haemolytic uremic syndrome (Karmali *et al.*, 2010). EHEC strains are zoonotic pathogens because domestic ruminants, mainly cattle, sheep, and goats have been considered as major natural reservoirs for EHEC (Ferens & Hovde, 2011). Pathogenic *E. coli* strains with common genetic characteristics have been found in humans and animals (Clermont *et al.*, 2011). These pathogenic strains have been divided into numerous categories or pathotypes on the basis of their distinct virulence properties and the clinical symptoms of the hosts. The intestinal strains include enterotoxigenic (ETEC), enteropathogenic (EPEC), enteroaggregative (EAEC), enteroinvasive (EIEC), and enterohaemorrhagic *E. coli* (EHEC). Extraintestinal infections (sepsis, urinary tract infections and neonatal meningitis strains) are caused by ExPEC (extraintestinal pathogenic *E. coli*) (Rodriguez-Siek *et al.*, 2005). Generally, virulence genes are used as targets to determine the pathogenic potential of any given *E. coli* isolate (Holko *et al.*, 2006; Cheng *et al.*, 2020; Kim *et al.,* 2022). Moreover, the virulence factors and virulence genes are similar in strains of the same pathotype. It has been reported that human and animal pathogenic *E. coli* strains can also be assigned to one of the main phylogenetic groups, A, B1, B2 and D (Clermont *et al.*, 2000) and share common genetic backgrounds (Clermont *et al.*, 2011). Whether animals are a source for human pathogenic *E. coli* or not is still a matter of debate. Nevertheless, *E. coli* strains with virulence genes have been detected in food products of avian and cattle origin.

Antimicrobial resistance (AMR) is recognised as a global problem in human and veterinary medicine. The indiscriminate use of antimicrobials in both medicines, as well as their use as growth-promoting factors in husbandry, has caused an increase in antibiotic residues in the environment and they have also been found in food products and pathogenic samples of human and animal origin. The accumulation of these residues promotes selective pressure, enhancing the selection of resistant

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bacteria, since several genes coding for antibiotic resistance are located on mobile genetic elements (Cheng *et al.*, 2020). Therefore, animal food of animal origin can constitute an ideal environment for the emergence of new pathogenic and resistant bacterial strains by the acquisition of different virulence and resistance determinants (Kim *et al.*, 2022). The analysis of bacteria present in dairy food products can thus provide information on the bacterial gene reservoir that may be useful for predicting risk for human populations. Finally, to estimate the extent of the antimicrobial resistance (AMR) problem and follow its evolution, surveillance programs have been established in many countries worldwide.

The main objectives of the present study were (i) to make a collection of *E. coli* strains isolated from three types of food products (chopped chicken, beef and raw milk) sold in butcher and traditional milk shops in Jijel (Eastern Algeria), (ii) to screen virulence genes and determine the phylogenetic group for each strain, (iii) to evaluate the resistance of the isolated strains to commonly used antimicrobial agents in human and veterinary medicine in Algeria.

MATERIAL AND METHODS

SAMPLE COLLECTION

Three hundred samples of chicken meat, beef and raw milk were collected randomly (at least one per month) from 10 butcher shops localised in the wilaya of Jijel, a region located in eastern Algeria. The samples consisted of 1 kg of bovine meat, 1 liter of milk and a whole broiler. All the samples were purchased from butcher and milk sales outlets located in the Jijel region. All animals came from jijlians herds and the sampling period was from September 2017 to June 2019. Collected samples were sent to the laboratory in sterile bags on ice and were processed on the same day at the Microbiological Laboratory of the Microbiology and Biochemistry Department (Mohamed Seddik Benyahia University, Jijel, Algeria).

ISOLATION AND PRIMARY IDENTIFICATION

E. coli were isolated from the samples as previously described by Zhao *et al.* (2001). Briefly, 25g portions of chicken meat or beef and 25ml samples of raw milk were aseptically taken from collected samples. After homogenisation in 225 ml of sterile buffered Peptone Water (Institut Pasteur, Algiers, Algeria (IPA)), 1 ml of the suspension of each sample was plated onto approximately 15 ml of Violet Red Bile Glucose Agar (VRBG, IPA) and incubated at 37 °C for 24 hours. Primary identification procedures involved subculturing the presumptive *E. coli* colonies in Brilliant Green Bile Broth (BGBB, IPA) containing an inverted Durham tube and in Tryptone Water without indole (IPA). The samples were incubated at 37 °C for 24 h. After the incubation, the produced gas, visible as a bubble in the inverted Durham tube, indicated a positive result. Kovacs (IPA) reagent (0.2-0.3 ml) was added to Tryptone Water to detect indole production; the appearance of a red colour on the upper layer indicated a positive reaction. The presumptive *E. coli* was recovered and confirmed by Gram staining and biochemical tests by using API 20E test (BioMérieux, Marcy l'Etoile, France) (Badri *et al.*, 2009). It has been shown that an arbitrarily selected *E. coli* colony has an 86% probability of representing the quantitatively predominant clone in the sample (Lidin-Janson *et al.*, 1978). Two hundred thirty-five *E. coli* strains (85 from chicken meat, 73 from beef, and 77 from raw milk) were collected, identified and grown separately with agitation at 37 °C in Luria Bertoni broth (LB). They were then stored at -80 °C in LB broth containing 30% glycerol in 96-well microtiter plates for further analysis.

SCREENING FOR POTENTIALLY PATHOGENIC *E. coli*

A total of 235 *E. coli* strains was screened for the presence of three virulence genes associated with EHEC (*eae, stx1 and stx2*), ten genes associated with ExPEC (*f17A, cnf, papEF*, *afa/draBC*, *fyua*, *clbN*, *hlyf*, *kpsMT(K1)*, *hlyA*, and *sfa/focDE*), one with EIEC (*ipah*) and three virulence genes associated with EAEC (*AAprobe, aap* and *aggr*). DNA was extracted from overnight cultures using NaOH and subjected to multiplex and uniplex PCR (Diallo *et al.*, 2013).

A triplex PCR was performed to detect *eae, stx*$_1$ and *stx*$_2$ genes as described previously (Paton & Paton 1998). EHEC O157:H7 RIMD 050992 (Sakaï) (Hayashi *et al.*, 2001) was used as a positive control. For the ExPEC, triplex PCRs were performed to detect *sfa/focDE* (Le Bouguenec *et al.*, 1992), *kpsMT* K1 (Johnson & Stell, 2000), and *hlyA* (Johnson & Stell, 2000) and *papEF* (Yamamoto *et al.*, 1995) *afa/draBC*, and *fyua* (Johnson & Stell, 2000). A duplex PCR was conducted to detect *clbN* (Johnson & Stell, 2000) and *hlyF* (Moulin-Schouleur *et al.*, 2007). A uniplex PCR was performed for the *f17A* gene (Bertin *et al.*, 1996) and another to detect the *cnf* gene (Yamamoto *et al.,* 1995). For EAEC, a triplex PCR was used to identify *AAprobe*, *aap*, and *aggr,* as described by Cerna *et al.* (2003). Finally, for EIEC, a uniplex PCR was performed to detect *ipah* (Aranda *et al.*, 2007). The following control strains were used: J96 for *sfa/focDE*, *hlyA*, *papEF*, and *fuyA* (Johnson *et al.*, 1997), SP15 for *kpsMT K1*, and *hlyF* (Johnson *et al.*, 2000), A30 for *afa/draBC* (Johnson and Stell 2000), IHE3034 for *clbN* (Korhonen *et al.*, 1985), 25KH9 for *f17A* (Girardeau *et al.*, 1988), S5 for *cnf* (Smith, 1974), O42 for *AAprobe*, *aap*, and *aggr* and EDL1284 for *ipah* (Aranda *et al.*, 2007) (table 1).

PHYLOGENETIC GROUP CLASSIFICATION

Phylogenetic grouping of *E. coli* strains was conducted using multiplex PCR with the *chuA* and *yjaA* genes and

Table 1. PCR conditions and the different multiplex and uniplex carried out for the detection of virulence genes of isolated *E. coli* strains.

	GENES CIBLES	SEQUENCES (3'-5')		Strains (+)	Target (bp)	Denaturation	Hybridation	Elongation	cycles	And	REFERENCES
ExPEC	*f17A*	P1(322) P2(323)	*GCAGAAAATTCAATTTATCCTTGG* *CTGATAAGCGATGGTGTAATTAAC*	*25KH9*	537	95/2 then 95/45	56/45	72/1,5	35	72/10	Bertin *et al.*, 1996
	cnf	cnfxbiss308 cnfxbisas309	*CAA TGG CAA CAA AAA TAC CTT* *GAA CGA CGT TCT TCA TAA GTA TC*	*28C*	1147	95/2 then 95/45	56/45	72/1,5	35	72/10	Yamamoto *et al.*, 1995
	papEF	pap3 pap4	*GCA ACA GCA ACG CTG GTT GCA TCA T* *AGA GAG AGC CAC TCT TAT ACG GAC A*	*J96*	336	94/4 then 94/1	63/0,5	72/2	30	72/7	Yamamoto *et al.*, 1995
	afa/draBC	Afa f Afa r	*GGC AGA GGG CCG GCA ACA GGC* *CCC GTA ACG CGC CAG CAT CTC*	*A30*	559						J.R. Johnson., 2000
	fyuA	fyua/f fyua/r	*TGA TTA ACC CCG CGA CGG GAA* *CGC AGT AGG CAC GAT GTT GTA*	*J96*	880						J.R. Johnson., 2000
	clbN	69	*GTT TTG CTC GCC AGA TAG TCA TTC* *CAG TTC GGG TAT GTG TGG AAG G*	*IHE3034*	733	95/2 then 95/45	56/45	72/1,5	35	72/10	J.R. Johnson., 2008
	hlyF	70	*TCG TTT AGG GTG CTT ACC TTC AAC* *TTT GGC GGT TTA GGC ATT CC*	*SP15*	444						Moulin-Schouler, 2007
	kpsMT (K1)	k1.f kapsII r	*TAG CAA ACG TTC TAT TGG TGC* *CAT CCA GAC GAT AAG CAT GAG CA*	*SP15*	153	94/4 then 94/1	63/0,5	72/2	30	72/7	J.R. Johnson., 2000
	hlyA	hly1 hly2	*AAC AAG GAT AAG CAC TGT TCT GGC T* *ACC ATA TAA GCG GTC ATT CCC GTC A*	*J96*	1177						J.R. Johnson., 2000
	sfa/focDE	sfaD(595) sfaE(596)	*CTC CGG AGA ACT GGG TGC ATC TTA C* *CGG AGG AGT AAT TAC AAA CCT GGC A*	*J96*	410						Yamamoto *et al.*, 1995
EHEC	*eae*	eaeB52 eaeB53	*GAC CCG GCA CAA GCA TAA GC* *CCA CCT GCA GCA ACA AGA GG*	Sakai O157H7	384 Kb	Need special program (In every cycle conditions must be changed automatically)					Paton & Paton (1998)
	stx1	slt-I B54 slt-I B55	*ATA AAT CGC CAT TCG TTG ACT AC* *AGA ACG CCC ACT GAG ATC ATC*		180 Kb						
	stx2	slt-II B56 slt-II B57	*GGC ACT GTC TGA AAC TGC TCC* *TGC CCA GTT ATC TGA CAT TCT G*		255 Kb						
EAEC	*AAprobe*	*EAEC1* *EAEC2*	*CTG GCG AAA GAC TGT ATC AT* *CAA TGT ATA GAA ATC CGC TGT T*	*O42*	629	94/4 then 94/1	63/0,5	72/2	30	72/7	Cerna *et al.*, 2003
	aap	*Aap1* *Aap2*	*CTT GGG TAT CAG CCT GAA TG* *AAC CCA TTC GGT TAG AGC AC*		310						
	aggR	*AggR1* *AggR2*	*CTA ATT GTA CAA TCG ATG TA* *AGA GTC CAT CTC TTT GAT AAG*		457						
EIEC	*ipah*	ipaH1 ipaH2	*GTT CCT TGA CCG CCT TTC CGA TAG CGT C* *GCC GGT CAG CCA CCC TCT GAG AGT AC*	*EIEC 85b*	600	94/4 then 94/1	63/0,5	72/2	30	72/7	Cerna *et al.*, 2003

the DNA fragment *TSPE4.C2*, according to the method described by Clermont *et al.* (2000). Representative *E. coli* reference collection strains were used as template control.

PCR was performed in 0.5 ml Eppendorf tubes on a Techne Progene (UK) thermal cycler with a reaction of 50 µl volume. The DNA template (5 µl containing100-200 pg of DNA) was added to 45 µl reaction mixture containing 0.1 mM each dATP, dCTP, dGTP, and dTTP (Invitrogen); 5 µl buffer solution 10X (Biolabs, New England); PCR primers (Tbl1), 1.5 U of Taq DNA polymerase (Biolabs, New England) were added to the reaction. The amplification products were analysed by gel electrophoresis on a 2.0% agarose gel (ROTI1 Garose, Roth GmbH, Germany), stained with ethidium bromide, and photographed at UV exposure. PCR Conditions, primers and programs were summarised in table 1.

ANTIBIOTIC SUSCEPTIBILITY TESTING

All *E. coli* strains were subjected to antimicrobial susceptibility testing. The tests were performed using the disk diffusion method according to the CLSI standards (CLSI, 2009) on Mueller-Hinton agar (Bio-Rad Laboratories). *E. coli* ATCC 25922 was used as the control strain. The 16 antibiotic disks (Bio-Rad Laboratories) used in this study were Ampicillin (Amp) 10 µg, (Amoxicillin + alvulanic acid (Amx+AC) 20+10 µg), Amoxicillin (Amx) 25 µg, Cephalothin (CF) 30 µg, Gentamicin (Gn) 10 µg, Kanamycin (K) 30 µg, Streptomycin (S) 10 µg, Colistin (CT) 50µg, Neomycin (N) 30 µg, Ciprofloxacin (Cip) 5 µg, Flumequin (UB30) 30 µg, Trimethoprim (TMP) 5 µg, Sulfamethoxazole-Trimethoprim (Sxt) 23.75 µg + 1.25 µg, Sulphonamid (SSS) 300 µg, Tetracycline (Tet) 75 µg, Chloramphenicol (C) 30 µg. Susceptibility breakpoints for all the antimicrobials followed the recommendations of CLSI (CLSI, 2009). Strains were classified as multiresistant when they exhibited resistance to three or more classes of antimicrobial agents (Schwarz *et al.*, 2010). It should be noted that identification tests, PCR tests and antibiotic sensitivity tests were carried out at Inserm Umr1043, Inra Usc1360, University of Toulouse, INP, ENVT, France.

STATISTICAL ANALYSIS

The different phylogenetic groups and antimicrobial resistance data were compared using the chi-square test (R. software version 2.14.1 (2011-12-22) for Windows) and ANOVA (Statistica software 8.1) test was used to compare de prevalence of antibiorestance versus prevalence of virulence factors. A P value of < 0.05 was considered statistically significant.

RESULTS

E. coli STRAINS COLLECTION

Three hundred samples were purchased and analysed (100 samples of each food product) and two hundred thirty-five (235) *E. coli* strains were collected (85 strains from chicken meat, 73 from beef, and 77 from raw milk).

CHARACTERISATION OF *E. coli* STRAINS FROM FOOD PRODUCTS

Prevalence of pathogenic E. coli. All the two hundred thirty-five recovered strains were submitted to PCR to detect 17 virulence genes. Ninety-seven potentially virulent strains were identified (41.27% of investigated strains). The comparisons between the samples of different origins showed that 47 (55.25%) of chicken meat samples, 25 (34.24%) of beef samples and 29 (37.66%) of milk samples contained the bacteria with virulence genes.

The prevalence of virulence genes ranged from 0% for *eae*, *stx1*, *sfa/focDE*, and *kpsMT* (*K1*) to 19.91% (46 strains) for *hlyf*. Globally, the adhesin-coding genes, *papEF* 5 (2.16%), *afa/draBC* 5 (2.16%), and *f17* 3 (1.20%) were the most prevalent, followed by *ipaH* (two strains) and *aggr* (two strains). The *fyuA* gene, found in 20 strains (8.65%), was more common than *clbN* (seven strains, 3.03%) and *cnf* (three strains, 1.2%), *hlyA* (one strain), and stx2 (one strain) (table 2). The *hlyf* and *fyua* genes were detected more frequently in the strains isolated from chicken meat than beef and raw milk samples ($P<0.05$). The *eae, stx1, sfa/focDE, kpsMT (K1), and afa/draBC* were not amplified in any isolated strains. Potentially virulent *E. coli* strains tended to show few antibiotic-resistance patterns. However, our study clearly showed that chicken meat, beef, and raw milk act as reservoirs for *E. coli* strains carrying virulence factors associated with resistance to many antibiotics. The distribution of the various targeted sequences revealed 30 virulence gene patterns (labelled with EC followed by arabic number, from one (EC1) to thirty (EC30). Our results reveal that certain strains belonging to phylogenetic groups A, B1, B2, or D and carrying at least 1 virulence gene are at the same time resistant to at least 5 antibiotics, such is the case respectively of phylogenetic group A with 31 strains distributed over 3 patterns (EC1, EC2, EC5), phylogenetic group B1 with 11 strains distributed over 3 patterns (EC1, EC13, EC28), phylogenetic group D with 5 strains distributed over 4 patterns (EC11, EC12, EC13, EC17) and finally the B2 phylogenetic group with 11 strains spread over 3 patterns (EC15, EC19, EC21).

Phylogenetic group. It was shown that the phylogenetic group A was the most common; 67.53% (52/77) in raw milk, 64.38% (47/73) in beef and 62.35% (53/85) in chicken meat, followed by group B1 in beef (25.97%, 20/73), in chicken meat (21.17%, 18/85) and in raw milk (16.88%,

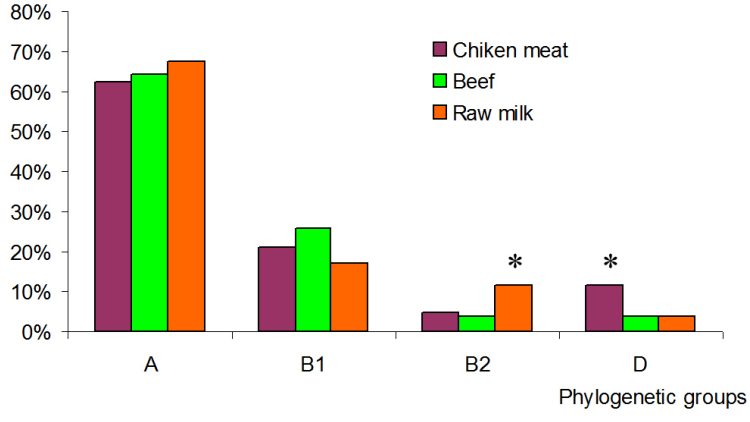
Table 2. Prevalence of virulence factors among 235 *E. coli* strains isolated from chicken meat, beef, and raw milk samples.

		Chicken meat n = 85	Beef n = 73	Raw milk n = 77	Total no. of positive strains n = 235	Stat.
EHEC	*stx1*	–	–	–	–	ns
	stx2	–	1 (1.36%)	–	1 (0.4%)	
	eae	–	–	–	–	
ExPEC	*f17*	–	1 (1.36%)	1 (1.29%)	2 (0.8%)	ns
	cnf	–	2 (2%)	1 (1.29%)	3 (1.2%)	
	hlyF	24(28.23%)	9 (12.32%)	13 (16.88%)	46 (19.91%)	
	papEF	4 (4.7%)	1 (1.36%)	–	5 (2.12%)	
	afa/draBC	3 (3.52%)	2 (2%)	–	5 (2.12%)	
	sfa/focDE	–	–	–	–	
	hlyA	1 (1.17%)	–	2 (2.59%)	3 (1.2%)	
	kpsMT (K1)	–	–	–	–	
	fyua	10 (11.76%)	5 (6.84%)	5 (6.49%)	20 (8.51%)	
	clbN	2 (2.35%)	3 (4.10%)	4 (5.19%)	7 (2.97%)	
EIEC	*ipah*	–	–	1 (1,29%)	1 (0,4%)	ns
EAEC	*aggr*	1 (1.17%)	1 (1.36%)	–	2 (0.8%)	ns
	aap	1 (1.17%)	–	1 (1.29%)	2 (0.8%)	

-: Absent= (0%), ns: No Significant (P>0.05).

13/77). The phylogenetic group B2 was significantly more frequent in raw milk 11.68% (9/77) than chicken meat 4.7% (4/85) and beef 3.98% (3/73) samples (P<0.05). The phylogenetic group D was more common in chicken meat (11.76%, 10/85) than in beef (3.89%, 3/73) and in raw milk (3.89%, 3/77) (P<0.05) (figure 1). Moreover, the comparison of the percentages of strains carrying Vfs and belonging to phylogenetic group B2 (68.75%) showed that they are higher than those recorded in strains belonging to phylogenetic groups D (37.5%) A (30.26%) and B1 (27.45) respectively (table 3).

Antibacterial susceptibility. Figure 2 shows the results of the susceptibility of the isolated 235 *E. coli* strains to 16 antibiotics. The highest prevalence of resistance was recorded for tetracycline with 64.25% (151/235 strains), followed by amoxicillin with 54.04% (127/235 strains), ampicillin with 53.61% (126/235 strains), sulfonamide with 43.82% (103/235 strains), trimethoprim with 37.87% (89/235 strains), trimethoprim-sulfamethoxazole with 37.44% (88/235 strains), streptomycin with 31.06% (73/235 strains) oxacillin plus clavulanic acid with 19.14% (45/235 strains), ciprofloxacin with 17.87% (42/235 strains),



*= Significant difference (P<0.05)
- (Chicken meat, Beef), (Chicken meat, Raw milk) Significant difference (P<0.05)
- (Raw milk, Beef), (Raw milk, Chicken meat) Significant difference (P<0.05)

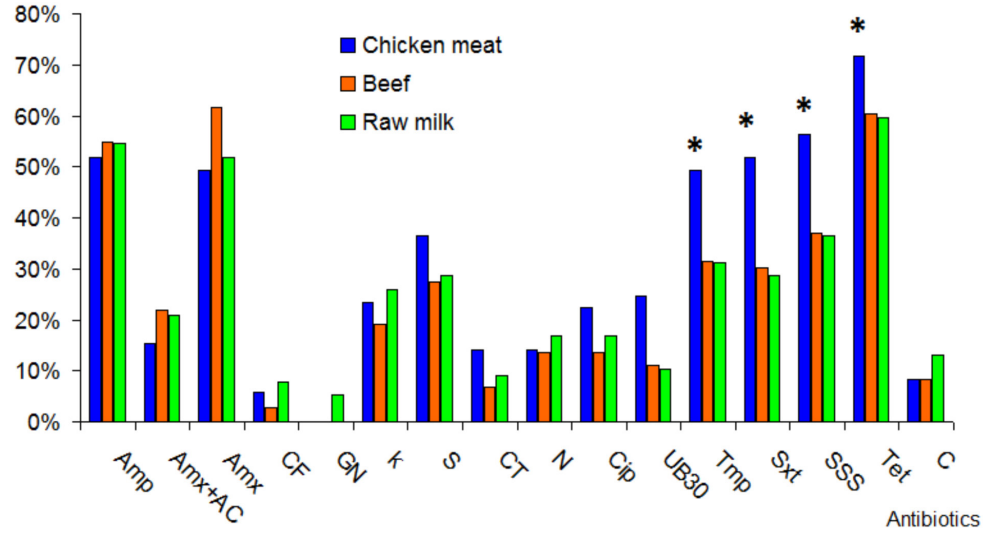
Figure 1. Phylogenetic group classification of *E. coli* strains isolated from chicken meat (n = 85), beef (n = 73), and raw milk (n = 77).

Table 3. Virulence, phylogeny, and antibiotic-resistance patterns identified among 235 *E. coli* strains isolated from chicken meat, beef, and raw milk samples.

	Patterns	Virulence gene carriage	Phylogeny group				ATB
			A n=(152)	B1 n=(51)	B2 n=(16)	D n=(16)	
Chicken meat	*EC1*	*hlyf*	14 (9%)	5 (9.8%)		3 (18.75%)	Amp., Amx., Amx+AC., GN., K., N., Cip., UB30., SSS., Tmp., Sxt Tet., C.
	EC2	*ipah*	1 (0.6%)				Amp,. Amx., K., C., Sxt., Tmp., Cip., UB30., SSS., Tet
	EC3	*hlyA*	1 (0.6%)				–
	EC4	*fyua*	3 (1.9%)				S., Tet., SSS.
	EC5	*papEF*	1 (0.6%)				Amp. k., S., Tet., SSS.
	EC6	*afa/draBC*	3 (1.9%)				
	EC7	*aggr*	1 (0.6%)				–
	EC8	*hlyF-fyuA*	2 (1.3%)				Amp., Amx., K., N., S., Cip., Sxt., SSS., Tet.
	EC9	*clbN- fyuA*			2 (1.25%)		S., Tet., SSS.
	EC10	*papEF- fyua*	2 (1.3%)				Amx+AC., Amx.
	EC11	*hlyf- aap-*				1 (6.25%)	S, Tmp, Sxt, UB30, SSS
	EC12	*papEF- fyua*				1 (6.25%)	Amp., Amx+AC., C., Tet., Tmp., Sxt., Cip., SSS., Amx.
Beef	*EC13*	*hlyF*	4(2.6%)	2 (3.9%)		1 (6.25%)	Amp., Amx., S., Tet., Tmp., SXT., SSS.
	EC14	*afa/draBC*	2 (1.3%)				Amx+AC., Aux., S., Tet., Tmp., SXT., SSS.
	EC15	*f17*			1 (6.25%)		Amp., Amx., Amx+AC., SSS., Tmp., Sxt., Tet., Aux.
	EC16	*aggr*	1 (0.6%)				–
	EC17	*fyua*				2	Amp., Amx., Tet., S., SSS., Tmp., Sxt.
	EC18	*Stx2*	1 (0.6%)				Amp., Amx., K., N., S., Cip., Sxt., SSS., Tet.
	EC19	*clbN- Cnf- fyua*			1 (6.25%)		Amp., K., N., S., Tet., C., SSS., Amx.
	EC20	*clbN- papEF - fyua*			1 (6.25%)		Tet.
	EC21	*clbN- cnf- fyua- ipah*			1 (6.25%)		Amp, K, N, S, Tet, SSS, Amx
Raw milk	*EC22*	*hlyA*	2 (1.3%)				–
	EC23	*f17*			1 (6.25%)		Amp., Amx., Amx+AC., SSS., Tmp., Sxt., Tet., Aux.
	EC24	*hlyF*	7	5 (9.8%)		1 (6.25%)	Amp., Amx., Amx+AC., GN., N., Cip., UB30., SSS., Tmp., Sxt., Tet., C.
	EC25	*fyua*	1 (0.6%)	1 (1.9%)			Amx+AC, Amx. Tet
	EC26	*clbN- hlyF*			1 (6.25%)		–
	EC27	*clbN- fyuA*			1 (6.25%)		–
	EC28	*aap*		1 (1.9%)			Amp., Amx+AC., K., N., S., Tet., C., Tmp,. CT., SSS., Amx.
	EC29	*clbN- cnf- fyua-*			1 (6.25%)		–
	EC30	*clbN- f17- fyua-*			1 (6.25%)		S., SSS.
Tot			46 (30.26%)	14 (27.45%)	11 (68.75%)	6 (37.5%)	

-: Sensitive to all tested antibiotics.

flumequine with 15.74% (37/235 strains), neomycin with 14.89 (35/235 strains) and colistin with 10.21% (24/235 strains). Resistance to cephalothin, trimethoprim, trimethoprim-sulfamethoxazole and sulfonamide was significantly higher for strains from chicken meat than beef and raw milk samples (P<0.05) (figure 2). In addition, 62.12% (146/235) of strains were resistant to at least one antibiotic. The prevalence of multiresistant strains ranged from 1 isolate resistant to 13 antibiotics (representing 0.42%) to 23 strains resistant to 3 antibiotics (9.78%). It can also be noted that thirteen antibiotypes were observed (table 4). All antibiotypes were present in chicken meat samples. Eighteen strains (7.65%) were resistant only to tetracycline. Nine strains (3.82%) were resistant to three antibiotics (tetracycline, ampicillin and amoxicillin) and four to 7 antibiotics (1.87%). Moreover, some strains showed simultaneous resistance to fluoroquinolone, gentamycin, streptomycin, and chloramphenicol.



(Amp: Ampicillin), (Amx+AC: Amoxicillin plus Clavulanic acid), (Amx: Amoxicillin), (CF: Cephalothin), (Gn: Gentamicin), (K: Kanamycin), (S: Streptomycin), (CT: Colistin), (N: Neomycin), (Cip: Ciprofloxacin) , (UB30: flumequine), (TMP: Trimethoprim), (Sxt: Sulfamethoxazole-Trimethoprim), (SSS: Sulfonamide), (Tet: Tetracycline), (C: Chloramphenicol).
* = (Chicken meat, beef) (P<0.05)
- (Chicken meat, raw milk) Significant difference (P<0.05)

Figure 2. The antibiotic susceptibility of the 235 *E. coli* strains isolated from chicken meat, beef, and raw milk.

Table 4. The most frequent antibiotic resistance patterns in the 235 *E. coli* strains isolated from food products (chicken meat, beef, and raw milk)

Resistance patterns	Chicken meat n = 85	Beef n = 73	Raw milk n = 77	Total n = 235
	b(%)[a]	b(%)[a]	b(%)[a]	b(%)[a]
Tet.	7(8.23)	6(8.21)	5(6;49)	18(7.76)
Amp., Amx+AC.	1(1.17)	4(5.47)	2(2.59)	7(2.97)
Tet., Amp., Amx.	1(1.17)	6(8.21)	2(2.59)	9(3.82)
Tet., Tmp., SXT., SSS.	2(2.35)	–	2(2.59)	4(1.70)
Amp., Amx., Tmp., Sxt., SSS.	3(3.52)	–	–	3(1.27)
Am., Amx+AC., SSS., Tm., Sxt., Tet..	2(2.35)	–	–	2(0.85)
Amp., Amx., Tet., S., SSS., Tmp., Sxt.	1(1.17)	2(2.73)	1(1.29)	4(1.70)
Amp., Amx., Cip., UB30., SSS., Sxt., K., Tet.	1(1.17)			1(0.42)
Amp., Tmp., Sxt., Tet., SSS., Amx., UB30., Cip., C.	1(1.17)	1(1.36)	1(1.29)	3(1.29)
Amp,. Amx., K., C., Sxt., Tmp., Cip., UB30., SSS., Tet.	1(1.17)		1(1.29)	2(0.85)
Amp., Amx., Amx+AC., Cip., Tmp., Sxt., SSS., K., S., T., C.	2(2.35)	–	–	2(0.85)
Amp., Amx., K., N., S., Cip., UB30., Tmp., Sxt., SSS., Tet., C.	1(1.17)	–	–	1(0.42)
Amp., Amx., Amx+AC., GN., K., N., Cip., UB30., SSS., Tmp., Sxt., Tet., C.	1(1.17)	–	–	1(0.42)

a: Number of the most frequent antibiotic-resistance patterns.
b: N° antibiotic-resistance pattern.

DISCUSSION

The consumption of animal products such as meat and milk is the origin of many health problems, as revealed by several disease outbreaks and investigations of food products sold in different countries. Therefore, determining the microbial content of raw meat and milk products is an important concern for the meat and milk industries. Although many researchers have attempted to identify the foodborne pathogens in meat and raw milk, relatively few reports are available on the prevalence of commensal *E. coli* as a faecal contamination indicator (Schlundt, 2002; Aslam *et al.*, 2003).

The main objective of the present study was to demonstrate that commensal *E. coli* might be a source of contamination of food products of animal origin. To our knowledge, this study is the first to report an in-depth analysis of *E. coli* strains isolated from chicken meat, beef, and raw milk sold in Jijel's area, Eastern Algeria. We performed systematic phylogenetic grouping, screening of virulence genes and the evaluation of resistance to different antimicrobial agents. Two hundred and thirty-five *E. coli* strains isolated from these food products were classified into four phylogenetic groups (A, B1, B2, and D) using the triplex PCR method of Clermont *et al.* (2000). Most *E. coli* strains are belonged to the A and B1 phylogenetic groups. Other authors have reported similar results (Unno *et al.*, 2009; Lagerstrom & Hadly, 2021; Lozica *et al.*, 2021). However, in contrast to the results obtained by Soufi *et al.* (2009), we found in the present study that the number of strains classified in the phylogenetic group D was higher in chicken meat than in beef and raw milk. The large number of A and B1 strains recorded in the present study could be explained by the fact that the samples were obtained from healthy animals, and they were probably of faecal origin. These groups are generally associated with commensal strains, whereas in most cases, enteropathogenic strains are assigned to group D and uropathogenic strains are assigned to group B2 (Lozica *et al.*, 2021). We should mention here that enterohemorrhagic *E. coli* O157H7 belongs to the phylogenic group A.

The PCR screening showed that the ExPEC bacteria of group B2 (in all sample types) account for 5.53% of the total. The ExPEC have been associated with both human and animal diseases (Manges & Johnson, 2012). It has been reported that foods of animal origin, such as the samples analysed here, are important vehicles for these bacteria and could be implicated in infections of humans and dairy animals (Joanne *et al.*, 2011; Amer *et al.*, 2020). It is important to note that the prevalence of ExPEC recorded in this study was much higher than that recorded in Spain by Quinto and Cepeda (1997) (0.4%) and in Ontario (Canada) by Steele *et al.* (1997) (0.87%). However, our results were slightly lower than that reported by Johnson *et al.* (2005) (46%) in poultry samples from 10 retail markets in the Minneapolis-Saint Paul area (USA) between 2001 and 2003. Moreover, in our study, the prevalence of *hlyF* in chicken meat was higher than in beef and raw milk as reported by James *et al.* (2009).

Here, only one isolate of *E. coli* from beef encoded Shiga-toxin (*stx2*) gene. This result is in agreement with the report of Abdullah *et al.* (2010). It should also be noted that Garcia-Aljaro *et al.* (2009) reported that Stx2 which is of phage origin can be the cause of carriage and dissemination of antibiotic resistance genes such as chloramphenicol, tetracycline, sulfamethoxazole, streptomycin, trimethoprim, and trimethoprim-sulfamethoxazole.

Usually, the *E. coli* strains belonging to B2 and D phylogroups carry more virulence-associated genes and have lower antimicrobial resistance rates than the so-called nonpathogenic commensal strains (phylogenetic group A and B1) (Cocchi *et al.*, 2007; Cortes *et al.*, 2010). The present study found the virulence-associated genes in the strains belonging to phylogenetic groups B1, B2, D and one strain in the A group. Most of our strains carrying two or more virulence-associated genes belonged to group D or B2, as previously observed by Cortes *et al.* (2010). Moreover, it should be noted that our results reveal the presence of strains carrying gene virulence can also be multiresistant and belonging to the phylogroups recognised as regrouping the most pathogenic strains like B1, B2 and D. Such is the case of the patterns cited in the results section (table 3). This observation should alert us to the probable carrying of antibiotic resistance genes which may be associated with virulence genes.

Antibiotic resistance is also a global problem that encourages researchers to survey the evolution of antibiotic resistance of different microbial pathogens. It is important to preserve the efficacy of the antibiotics used in human and veterinary medicine and prevent the dissemination of antimicrobial resistance genes in the environment. The appearance of resistance profiles is usually caused by the extensive use of antibiotics, leading to the spread of resistance genes among the bacteria (Smith *et al.*, 2007; Gyles, 2008). The association between the use of antibiotics in animal feeds and the emergence of resistant bacteria has been known for a long time (Levy *et al.*, 1976; Aarestrup *et al.*, 1998; Lathers, 2001; Barbieri *et al.*, 2017; Messaili *et al.*, 2019). Therefore, examining resistant clones in foods of animal origin is important to determine the impact of on-farm use of antimicrobials and the possible food-borne transmission of the resistant clones to humans and is an idea that should be considered in our future work (Lerminiaux *et al.*, 2019; Thomrongsuwannakij *et al.*, 2022).

Our results reveal the presence, in food products, of strains carrying virulence genes and resistant to the various antibiotics prescribed in human and animal medicine and even some of them are prohibited from use in Algeria either due to their toxicity or are reserved for the treatment of certain serious bacterial infections such as cholera disease, e.g. Chloramphenicol. The obtained results agree with previously published studies on chicken meat (Amara

et al., 1995; Hammoudi & Aggad, 2008; Aggad *et al.*, 2010; Messaili *et al.*, 2019) and beef samples (Schwaiger *et al.*, 2012).

Our data also revealed the presence of three major antibiotic-resistance groups. In the first group, between 50% and 70% of strains were resistant to Amp, Amx, Tmp, SSS and Tet. In the second one, 20% to 50% of resistant strains were observed (Amx+AC, K, S, Cip, UB30, and N) and the third group included less than 20% of resistant strains (CF, GN, and CT). Furthermore, antibiotic resistance to UB30, Tmp, Sxt and Tet was higher in chicken-derived strains than that observed in beef and milk strains. Similar results have been observed in Saudi Arabia as reported by Abdullah et al (2010). We should also note the appearance of multiresistance as most strains (63.7%) were resistant to at least 2 antibiotics.

In Algeria, numerous antibiotics are often administrated concomitantly for prophylaxis or to fight infections. The high incidence of antibiotic resistance and multiresistance of *E. coli* is possibly caused by the extensive and indiscriminate use of antibiotics and the likely dissemination of resistance genes in the poultry industry and bovine herds in Algeria. Such practices, especially without prior antibiotic sensitivity testing of bacterial strains, may lead to the development of a pool of antibiotic-resistant genes and the selection of increasing numbers of resistant *E. coli* gene clones as reported by Chique *et al.* (2019). Antibiotic resistance is frequently encoded by conjugative plasmids or transposons; thus, *E. coli* of avian origin could be a source of antibiotic resistance in other bacterial species, including human and other animal pathogens (Miles *et al.*, 2006). An increase in the pool of antibiotic-resistant bacteria could heavily impair the treatment of human and animal diseases.

Finally, in Algeria, beef, chicken meat, and raw milk represent the major sources of animal proteins for a large part of the population. The poor hygiene associated with the food processing plants and the sale of the products in butcher shops and other inadequately controlled outlets carries a high risk of microbial contamination. In addition, gene exchange may also contribute to the rising prevalence of pathogens in foods as reported by previous studies (Cohen & Karib, 2006; Garcia-Aljaro *et al.*, 2009; Murase *et al.*, 2016; Kim *et al.*, 2022). Pathogenic bacteria such as *E. coli* may be transferred to raw milk, which should be considered as a potential vehicle for transmitting bacterial pathogens (Mohamed-Zeinhom & Gihan, 2014). Since many people still drink uncooked milk, especially in rural areas, some effort should be put into informing the population about the health risks associated with the consumption of raw unpasteurized milk or poultry consumption (Manges *et al.*, 2007; Johar *et al.*, 2021).

We can conclude that the phylogenetic groups A and B1 were the most prevalent among the 235 *E. coli* strains isolated from three different food products (beef, chicken meat and raw milk), followed by groups B2 and D. The screening of the virulence genes for the isolated and identified strains allowed us to reveal the presence of pathogenic strains belonging to the different groups of pathovars. A high occurrence of the ExpEC was recorded with the predominance of the *Hlyf* gene, followed by the EPEC with the presence of EIECs and EAEC and a single strain affiliated with the EHEC pathovars. Antibiotic sensitivity tests revealed the presence of polyresistant strains to antibiotics in the different types of analysed food product samples. Also, our results show that several strains carrying virulence genes are resistant to at least two types of antibiotics and even to chloramphenicol, which is normally prohibited for use in veterinary medicine. Finally, the obtained results should be taken into account in future studies, focusing on the role of meat products and raw milk bacteria as vectors for the transmission of virulence genes and genes that are responsible for antibiotic resistance.

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ORIGINAL ARTICLE

Current attitudes towards the use of perioperative analgesics in small animals by Uruguayan veterinarians

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ABSTRACT. In recent decades, several articles have reported significant progress regarding pain treatment in veterinary medicine. This study aims to analyse the attitudes of veterinarians working in small animal practices in Uruguay towards the use of analgesics during the perioperative period. Veterinarians in charge of clinics performing surgeries were interviewed, including clinics located in the capital city, Montevideo (n=59) and the rest of the country (n=81), based on data obtained from the National Veterinary Census in 2010. Most interviews were conducted in person, and if not possible, they were carried out through video calls. According to gender, 54% of interviewees were women and 46% were men, with 95% of them practising in urban areas. The most used drugs were nonsteroidal anti-inflammatory drugs (NSAIDs), with dipyrone (89%) being the most popular. Amongst opioids, pure agonists presented minimal inclusion in analgesic treatment (13%), with tramadol (93%) being the most used opioid. Drugs belonging to other groups were less mentioned. Thirty-eight per cent of the respondents considered that their training in pain management was inadequate. Veterinarians categorised the intensity of pain caused by several surgical procedures as "severe", however, they used weak opioids such as tramadol or NSAIDs as a single therapy to treat most cases. The scarce use of analgesic drugs and techniques that provide profound analgesia (such as mu-opioid receptor agonists) by Uruguayan veterinarians reveals the need for open discussion, adjustment of attitudes and continuing education on pain management.

Keywords: Analgesics, surgery, animal welfare, pain management.

INTRODUCTION

Over the last few years, there has been a growing interest in evaluating and treating pain in animals which has been reflected in an increased number of publications on the topic. Although the scenario regarding the use of analgesics in the perioperative period seems to be improving, research shows that it is still suboptimal (Capner *et al.*, 1999; Hansen, 1993; Hugonnard *et al.*, 2004; Lorena *et al.*, 2014; Raekallio *et al.*, 2003; Williams *et al.*, 2005).

The first study that looked into the use of analgesics in veterinary medicine was carried out in the United States, and it reported that in a teaching hospital only 6% of the cats (*Felis catus*) received analgesia in the postoperative period, compared to 40% of the dogs (*Canis familiaris*) (Hansen and Hardie, 1993). In 1999, cats in the United Kingdom received less analgesia than dogs, and treatment of pain in veterinary medicine was suboptimal (Lascelles *et al.*, 1999). Oligoanalgesia in veterinary medicine has also been reported in South Africa, Finland, France, New Zealand, Canada, and more recently in Brazil (Dohoo & Dohoo, 1996; Hewson *et al.*, 2006; Joubert, 2001; Lorena *et al.*, 2014; Raekallio *et al.*, 2003; Williams *et al.*, 2005).

In France, the difficulty to recognise pain, lack of knowledge about the use of analgesics and fear of their side effects were the main reasons for providing insufficient analgesia (Hugonnard *et al.*, 2004). Sociodemographic factors such as gender, years of professional experience, accessibility to drugs and urbanisation of the region studied, influence veterinarians' attitudes toward pain and analgesia. Variants such as religion, environment, gender, age, income, career orientation, and whether they lived with companion or farm animals were considered. Those who showed more interest in animal welfare were students who had companion animals of their own, who lived in urban areas, the youngest ones or the ones who were at the beginning of their professional careers. Moreover, women proved to be more sensitive to animal welfare than men (Ostovic *et al.*, 2017).

This study aimed to determine the current attitudes of Uruguayan veterinarians regarding the use of analgesics in cats and dogs during the perioperative period.

MATERIAL AND METHODS

In this study, 142 veterinarians in charge of small animal clinics were interviewed in person or through video call. A random selection of veterinarians was used, applying stratification by state/department to avoid geographical bias. The stratification was based on the National Census of Veterinarians of Uruguay, conducted in 2010 by the University of the Republic (Universidad de la República, Udelar), Ministry of Livestock, Agriculture and Fisheries (Ministerio de Ganadería, Agricultura y Pesca, MGAP) and the Society of Veterinary Medicine of Uruguay (Sociedad de Medicina Veterinaria del Uruguay, SMVU), which shows

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that 59% of the veterinarians practise veterinary medicine in the capital city and the remaining 41% are distributed across the country (Gil & Piaggio, 2010).

Those clinics registered in the MGAP as authorised businesses under decree 160/997 were contacted since the census did not present data on how these professionals were distributed throughout the different states (commonly referred to as "departments" in Uruguay). In addition, veterinary clinics that met the other established criteria, including being registered with the National Zoonosis Commission and distributor "Laboratorios Sur", but were not involved with the MGAP, were also added in order to increase the sample.

The distribution of questionnaires was planned based on the geographic location of 303 small animal clinics that perform surgery according to the MGAP, pursuing a minimum of 61 questionnaires from Montevideo and 81 questionnaires from the remaining departments. The stratification in Montevideo was carried out through the zonal community centres.

The questionnaire used was based on previously published questionnaires (Capner *et al.*, 1999; Hunt *et al.*, 2015; Lascelles *et al.*, 1999; Lorena *et al.*, 2014; Williams *et al.*, 2005) and it collected the following information:

Part I: Demographic data including gender, year of graduation, geographic location, highest academic degree obtained, number of veterinarians and technicians working in the practice.

Part II: Drugs and procedures used regularly to provide pre and/or post-operative analgesia; the expected duration of analgesia after a single preoperative dose of opioid, nonsteroidal anti-inflammatory drug (NSAID), local anaesthetic, dissociative anaesthetic and gabapentinoids, techniques employed including the use of local anaesthetic blockade of peripheral nerves, epidural analgesia, homoeopathy or acupuncture. In this part, the veterinarian indicated the drug or the technique employed but the specification of the technique was not mandatory.

Part III: Perioperative drugs used and the factors affecting the veterinarian's decision (side effects, cost, information about toxicity and dose, requirement to keep records of use and analgesic efficacy). This part was composed of three tables: opioids, NSAIDs and corticosteroids. Open-ended questions were used to ask respondents about the potential side effects of the different drugs.

Part IV: Different attitudes towards the treatment of pain in different situations. The veterinarians answered eleven statements based on a scale from 1 (completely agree) to 10 (completely disagree).

Part V: Different procedures were listed in a table (laparotomy, fractures, mastectomy, ovariohysterectomy, dental procedures and orchiectomy) and the interviewees answered in which cases they administered analgesics and when (pre, intra and/or postoperatively, including prescriptions for continued treatment at home), indicating in those cases which drug(s), dose, frequency and route of administration were preferred.

Part VI: Severity of pain in the first 12 hours after specific surgical procedures in cats and dogs. The numerical choices ranged from 1 (painless) to 10 (worst pain possible).

Part VII: Information related to continuing education, if respondents thought that their knowledge in the area was appropriate, forms of updating their knowledge, and whether the veterinarian was the main responsible for monitoring the patients postoperatively.

STATISTICAL ANALYSIS

The statistical significance of the association between variables that were recorded as dichotomous (presence or absence of a postgraduate degree, residence in the capital city or other departments, prescription or nonprescription of a certain drug), was verified by Fisher's exact test, while the association of other categorical variables was contrasted using the Chi-squared test of independence. The significance of the difference between the number of veterinarians in Montevideo and the rest of the country was tested using a difference of proportions test based on a normal approximation. The association of location of professional practice, gender, specialisation, or species treated with degrees of importance or agreement/disagreement with a statement, was verified by classifying the range of scores into three categories: 1 to 3 (agreement), 4 to 6 (indifferent) and 7 to 10 (disagreement), and posteriorly applying Chi-squared tests of independence. A Fleiss' Kappa test was used to analyse the concordance between pain perceptions. Logistic regression was used to study the tendency to administer analgesics to cats and dogs given several input variables (gender, postgraduate studies, years since graduation, Montevideo vs. other departments, working with colleagues and pain perception). A P-value of <0.05 was considered to indicate statistical significance in all cases. All statistical analyses were performed in R 3.6.3.

RESULTS

A total of 142 veterinarians were surveyed throughout the country, 43% were female and 57% were male respondents (table 1). Most veterinarians worked exclusively in small animal practice (78.9%) and had graduated recently (58.5% after 2000). Amongst the respondents, 66.9% worked together with other colleagues as part of a team. Only 19% had a specialisation (short-term courses), residency or postgraduate degree (master's or PhD).

Table 1. Demographic data obtained from 142 Uruguayan veterinarians, interviewed about the use of analgesics in small animals.

	Montevideo	Other departments	Total
Location of practice	43%	57%	100%
Female	49%	60.7%	54%
Male	51%	39.3%	46%
Exclusive small animal practice	91.8%	69.1%	78.9%
Mixed small and large animal practice	8.2%	30.9%	21.1%
Graduated 1979-1990	47.6%	52.4%	15.6%
Graduated 1991-2000	45.7%	54.3%	25.9%
Graduated after 2000	57.0%	43.0%	58.5%
Has a postgraduate degree*	29.5% [a]	11.1% [b]	19%
Work alone	40.4%	59.6%	33.1%
Work with colleagues	60.0%	40.0%	66.9%

*(P=0.06).

In the second part of the interview, the veterinarians were asked about the use of opioids, NSAIDs and other drugs or techniques employed for pain management. A list of all the opioids and NSAIDs available in the country was provided, and it included an open option referred to as "others/specify". Concerning the use of opioids, tramadol (93%) was the most used in acute pain by Uruguayan veterinarians, while other opioids such as morphine and fentanyl were used less frequently (12.7% and 11.9% respectively) (table 2). Forty per cent of the veterinarians with a postgraduate degree used morphine and fentanyl, while veterinarians without any type of preparation (hereinafter non specialised) veterinarians rarely used them (P<0.01). In addition, 77.7% of the veterinarians who used these drugs worked in the metropolitan area (P<0.01). Opioids such as methadone, butorphanol, buprenorphine and meperidine were barely mentioned. There was no difference between genders regarding the use of opioids.

The most commonly used NSAIDs were dipyrone (88.7%) and carprofen (71.1%). Other NSAIDs used were meloxicam (57.7%), firocoxib (52.1%), ketoprofen (46.5%), flunixin meglumine (31.7%) and tolfenamic acid (10.5%) (table 2). All the respondents mentioned at least one NSAID. No difference between genders was observed in the use of NSAIDs.

The use of dissociative agents for pain management was given little consideration by veterinarians in their daily routine (8.5%). The most frequently mentioned local anaesthetic was lidocaine (87.1%), followed by bupivacaine (10.5%). Local anaesthetics were used in procedures such as peripheral infiltrative blocks (72%), perineural blocks (30.3%), intra-articular blocks (9.2%) and finally epidural anaesthesia (7.7%). The use of opioids through epidural administration was rarely mentioned. Only a few veterinarians (12%) mentioned considering gabapentinoids for the treatment of acute pain.

Table 2. Percentage of opioids and non-steroidal anti-inflammatory drugs (NSAIDs) used in the perioperative period by 142 Uruguayan veterinarians interviewed about the use of analgesics in small animals.

Drug Type	Drug	n	%
NSAIDs	Dipyrone	126	89
	Carprofen	101	71
	Meloxicam	82	58
	Firocoxib	74	52
	Ketoprofen	67	47
	Flumixin meglumine	45	32
	Tolfenamic acid	16	11
Opioids	Tramadol	132	93
	Morphine	19	13
	Fentanyl	17	12
	Butorphanol	7	5
	Methadone	1	1
	Buprenorphine	1	1
	Meperidine	0	

Among other therapies available for the participants to choose from, acupuncture was the most popular one (8.5%), while homoeopathy, fentanyl patches and physiotherapy were barely mentioned.

In the third part of the interview, the veterinarians were asked to classify as very important, important, not so important, not important or not applicable, different determining factors for the use of opioids and NSAIDs in the perioperative period (including side effects, cost, sedation, available information about the drug and its analgesic/anti-inflammatory effect).

Analgesic effect (96.2%) and information about efficacy, toxicity and dosage (90.9%) were the factors considered by most veterinarians when using opioids in dogs as well

as cats (95.4% and 89.9% respectively). Less than 10% of the veterinarians mentioned considering the side effects to be related to the use of opioids, associating them to central nervous system (sedation) and gastrointestinal effects, such as vomiting. When classifying the data by department, Montevideo considered the analgesic effect to be "very important", while in the rest of the country less relevance was given to this point ($P<0.05$). Analgesia was considered of "extreme importance" by veterinarians who used more potent opioids, while it was considered "very important" and "important" by those who did not use these drugs ($P<0.05$).

Anti-inflammatory (96.4%) and analgesic effects (95%) were considered important factors for the use of NSAIDs. Gastrointestinal effects such as gastritis, ulcers and vomiting were taken into consideration by most veterinarians when using NSAIDs.

The fourth part of the interview contained a list of statements regarding the recognition and treatment of pain on a scale from 0 to 10, being 0 completely agree, 5 indifferent, and 10 completely disagree. Only 13% of the respondents agreed with the statement "a certain degree of pain in the postoperative period is good because it keeps the animal inactive". Most veterinarians who disagreed with the previous statement worked in Montevideo (60%), whereas 40% worked in the rest of the country ($P=0.045$). For the statement "animals should receive the same consideration for pain relief as humans", a high number of professionals in the area agreed (89%), as well as with the phrase "preventing pain is better than relieving it" (89%). The majority of the respondents (82%) disagreed with the statement "analgesics' side effects exceed their benefits", in this case, those veterinarians with a postgraduate degree showed more emphasis in their disagreement (96%) than those without one (78%). Roughly half of the respondents (51%) disagreed with the phrase "recovery from anaesthesia can be prolonged by relieving pain", and those who agreed were more distributed throughout the country (64%) rather than located in the capital city (36%) ($P=0.022$, table 1).

The fifth part of the questionnaire consisted of a list of the surgical procedures above mentioned, and the interviewees had to answer in which cases they administered analgesics and when. Dipyrone and tramadol were the most used drugs in dogs while meloxicam and tramadol were the most used in cats. Tramadol (35%), dipyrone (25%) and meloxicam (9%) were the most used in dogs that underwent laparotomy, while in cats the most used drugs for this procedure were tramadol (35%), meloxicam (21%), dipyrone (17%) and tolfenamic acid (8%). Veterinarians provided analgesia in most cases for both dogs (94%) and cats (88%) ($P>0.05$). During the postoperative period, a low proportion of dogs (23%) and cats (22%) received additional doses of analgesics ($P>0.05$). During orthopaedic postoperative care, analgesics were more frequently prescribed to dogs (92%) than cats (76%) ($P<0.05$). When compared in which moment they used analgesia (pre, intra and/or postoperatively), there were no differences between the variables considered in the logistic regression (gender, postgraduate studies, years since graduation, Montevideo vs. other departments, working with colleagues and pain perception) (table 3). The variable year of graduation was not statistically significant but it presented borderline values, which suggests that it could be influencing the use of analgesia in the postoperative period: veterinarians who had graduated recently were more likely to prescribe analgesics ($0.1>P>0.05$). Veterinarians with postgraduate studies prescribed more analgesia in cats. This tendency was seen with cats only. Veterinarians working in metropolitan areas expressed higher use of analgesics in the postoperative period following ovariohysterectomies in dogs. Veterinarians working with other colleagues provided more analgesia in the postoperative period following fracture repairs in cats. The perception of pain was especially important at the time of prescribing analgesics, mainly in cats. The greater the perception of pain the respondent had, the more likely he/she was to administer analgesia.

Regarding pain intensity associated with each procedure (part VI), scores greater than 5 on the simple descriptive scale were considered as severe pain (Gerbershagen *et al.*, 2011). The procedures that veterinarians perceived as the most painful in dogs were fracture repairs (94%) and ear canal ablation (82%), followed by cruciate ligament repair (79%) and mastectomy (75%) (table 4). In cats, fracture repair (91%) and diaphragmatic hernia (81%) presented the highest pain scores, followed by ovariohysterectomy (63%) and dental procedures with several extractions (60%)

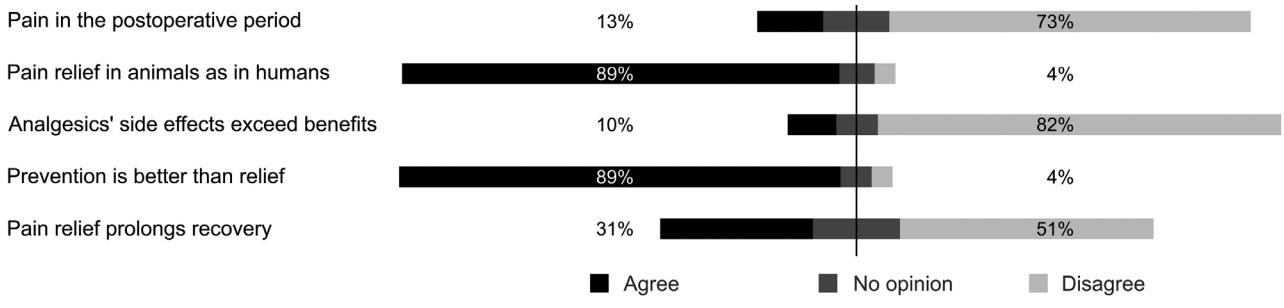

Figure 1. Attitudes towards statements related to the treatment of pain in small animals by 142 Uruguayan veterinarians interviewed.

Table 3. Results of the logistic regression for postoperative or prescription doses of analgesics in three procedures for cats and dogs by 142 Uruguayan veterinarians interviewed about the use of analgesics in small animals.

Species	DOGS					CATS					
Procedure	Fracture repair	Orchiectomy		Ovariohysterectomy		Fracture repair		Orchiectomy		Ovariohysterectomy	
Timeframe	Postoperative[a]	Postoperative	Prescription	Postoperative	Prescription	Postoperative	Prescription	Postoperative	Prescription	Postoperative	Prescription
Gender											
Female (ref.)											
Male	0.97 (0.21, 4.53)	0.65 (0.27, 1.51)	0.98 (0.40, 2.41)	1.09 (0.45, 2.73)	0.70 (0.22, 2.22)	0.97 (0.23, 4.12)	0.57 (0.12, 2.52)	1.16 (0.47, 2.92)	1.22 (0.51, 2.95)	1.28 (0.52, 3.21)	0.85 (0.35, 2.09)
Graduation year	0.08 (0.002, 0.17)*	0.01 (-0.03, 0.06)	0.04 (-0.001, 0.09)^	0.04 (-0.006, 0.09)^	0.05 (-0.01, 0.11)	0.001 (-0.07, 0.07)	0.05 (-0.03, 0.13)	-0.01 (-0.06, 0.05)	0.03 (-1.74, 0.08)	0.005 (-0.04, 0.05)	0.045 (-0.001, 0.09)^
Postgraduate degree											
No (ref.)											
Yes	3.35 (0.41, 73.02)	1.52 (0.53, 4.68)	1.93 (0.64, 6.68)	1.54 (0.46, 6.13)	3.39 (0.55, 66.0)	0.77 (0.14, 4.59)	2.31 (0.29, 48.3)	2.57 (0.80, 9.48)	3.30 (1.11, 10.9)*	3.48 (0.98, 16.8)	3.26 (0.93, 15.46)^
Location of practice											
Rest of the country (ref.)											
Montevideo	2.60 (0.49, 17.04)	2.19 (0.94, 5.24)^	1.31 (0.54, 3.23)	2.82 (1.16, 7.23)*	2.16 (0.68, 7.80)	0.76 (0.17, 3.24)	5.45 (1.08, 42.3)*	2.19 (0.90, 5.55)^	0.82 (0.34, 1.96)	2.11 (0.87, 5.30)	0.97 (0.39, 2.38)
Practice with colleagues											
No (ref.)											
Yes	1.51 (0.29, 7.34)	2.03 (0.85, 4.89)	1.14 (0.44, 2.84)	1.69 (0.69, 4.09)	1.15 (0.35, 3.59)	5.47 (1.28, 27.3)*	1.09 (0.21, 5.07)	1.77 (0.70, 4.52)	0.98 (0.39, 2.42)	1.18 (0.46, 2.98)	0.83 (0.31, 2.08)
Pain perception	0.03 (-7.04, 0.68)	0.13 (-0.1, 0.36)	0.23 (-0.001, 0.47)^	0.14 (-0.09, 0.38)	0.23 (-0.06, 0.53)	0.33 (-0.19, 0.89)	-0.07 (-0.93, 0.58)	0.24 (0.04, 0.45)*	0.19 (0.005, 0.41)*	0.30 (0.07, 0.54)*	0.14 (-0.09, 0.36)

Point estimates of odds ratios are presented with the 95% confidence interval (lower and upper limits) in parentheses.
Point estimates of the slopes are presented for graduation year (quantitative) and pain perception (treated as quantitative for this analysis), with the 95% confidence interval (lower and upper limits) in parentheses.
[a] Results for prescription were not reported as the vast majority of practitioners applied prescription rendering the regression useless.
^ Effect marginally significant ($0.1>P>0.05$); *effect significant ($0.05>P>0.01$).

Table 4. Distribution (percentage) of professionals according to pain perception for eight different procedures in dogs and six procedures in cats.

		Pain severity										
Procedures in dogs	Total responses	1	2	3	4	5	6	7	8	9	10	Median (range)
Fracture repair	140	0.0%	0.0%	0.0%	0.0%	1.4%	1.4%	3.6%	11.4%	12.1%	70.0%	10 (5-10)
Cruciate ligament repair	142	0.0%	0.7%	0.0%	0.0%	5.6%	6.3%	13.3%	26.6%	8.4%	39.2%	8 (2-10)
Exploratory laparotomy	141	0.0%	0.7%	1.4%	2.1%	13.5%	7.8%	14.9%	21.3%	9.2%	29.1%	8 (2-10)
Ovariohysterectomy	142	0.0%	0.0%	2.1%	0.7%	10.6%	4.9%	15.5%	25.4%	8.5%	32.4%	8 (3-10)
Castration	142	0.7%	0.0%	2.7%	2.1%	15.8%	8.9%	20.5%	22.6%	6.2%	20.5%	7 (1-10)
Dental procedure	141	0.0%	1.4%	2.1%	0.7%	15.6%	7.1%	11.3%	23.4%	8.5%	29.8%	8 (2-10)
Ear canal ablation	126	0.0%	0.0%	1.6%	0.8%	4.8%	1.6%	9.5%	12.7%	13.5%	55.6%	10 (3-10)
Mastectomy	140	0.0%	0.0%	0.0%	1.4%	4.3%	7.1%	12.1%	22.9%	13.6%	38.6%	9 (4-10)
Procedure in cats												
Fracture repair	140	0.0%	0.0%	0.0%	0.0%	2.9%	1.4%	4.3%	12.9%	10.7%	67.9%	10 (4-10)
Diaphragmatic hernia	130	0.0%	0.0%	0.0%	0.0%	6.2%	2.3%	10.8%	17.7%	10.8%	52.3%	10 (4-10)
Exploratory laparotomy	141	0.0%	0.7%	2.1%	2.1%	15.6%	5.7%	12.8%	24.8%	10.6%	25.5%	8 (2-10)
Ovariohysterectomy	141	0.0%	0.0%	2.1%	3.5%	9.2%	8.5%	13.5%	22.0%	9.2%	31.9%	8 (3-10)
Castration	141	1.4%	1.4%	4.3%	6.4%	13.5%	7.8%	21.3%	21.3%	2.8%	19.9%	7 (1-10)
Dental procedure	140	0.0%	1.4%	2.9%	1.4%	12.1%	6.4%	15.7%	17.1%	10.0%	32.9%	8 (2-10)

Table 5. Detailed Fleiss' kappa coefficients for the pain perception ratings attributed to the procedures listed in table 4.

Rating	Kappa	z	*P*
2	–0.004	–1.133	0.257
3	0.002	0.701	0.483
4	0.013	3.857	<0.001
5	0.018	5.443	<0.001
6	0.008	2.509	0.012
7	0.013	3.969	<0.001
8	0.006	1.952	0.051
9	0.002	0.636	0.525
10	0.090	27.850	<0.001

(table 5). Exploratory laparotomies in dogs were considered more painful by veterinarians working in metropolitan areas (65%) than veterinarians working in other regions of the country (53%) (U=1921.5, $P<0.05$), something similar occurred with cats (68% and 58% respectively) (U=2001, $P<0.05$). There was a low concordance (kappa=0.0347, 95% confidence interval 0.0315-0.0379) between veterinarians regarding the degree of pain attributed to different procedures and it was found to be significant ($P<0.001$) only due to the high number of interviews. When analysing by pain rating (table 5), it is evident that the greatest discrepancies are found near both ends of the pain scale, meaning that while there was agreement on certain procedures that were extremely painful, there was less agreement on the scores for procedures that are found to cause pain less than extreme.

Veterinarians considered that their training for recognition and treatment of pain was provided by clinical experience (89%) and conferences (68%), and a smaller proportion mentioned university education (38%). Moreover, national (69%) and regional events (70%) were considered the best ways of updating their knowledge on the topic.

Many veterinarians (62%) indicated that their knowledge to recognise and treating pain in small animals was adequate. The majority of the veterinarians (91%) reported being responsible for perioperative monitoring of the patients and pain assessment.

DISCUSSION

This study was based on a total of 142 interviews, all of which were completed satisfactorily and mostly in person. The sampling method used was stratified random sampling and was adopted to avoid bias, allowing to work with a representative sample of the population of small animal veterinarians in the whole country.

No significant differences were found among the professionals interviewed, in concordance with previous studies (Lorena *et al.*, 2014). It was observed that before 2004 most veterinarian graduates were men and from 2005 onwards the majority were women. In Uruguay, since 1990, men and women have entered veterinary school in equal proportion, which explains the results observed in this study (Gil & Piaggio, 2010). A similar event was reported in Canada, where the change occurred in 1994 (Dohoo & Dohoo, 1996; Hewson *et al.*, 2006). Similar to other studies, the majority of the respondents had graduated more than 10 years ago (66%).

Few Uruguayan veterinarians had a postgraduate degree or had specialised in a certain area (19%), similar to what was reported in New Zealand (Williams *et al.*, 2005). In Brazil and Italy, half of the respondents had postgraduate education (Catanzaro *et al.*, 2016; Lorena *et al.*, 2014). In the Uruguayan national veterinary census carried out in 2010, only 17% had a postgraduate degree (Gil & Piaggio, 2010). The reason could be that in Uruguay, to date, there is no specific anaesthesiology discipline in undergraduate or postgraduate programs. Brazil has implemented residency programs since 1983, including a College of Veterinary Anaesthesiology that awards the title of specialist in the area.

Tramadol was used by most of the respondents (93%) and to lesser extent morphine (12.7%) and fentanyl (11.9%). Research has proved that the use of tramadol in dogs is questionable (Donati *et al.*, 2021), recommending more effective opioids for the treatment of acute pain in this species. This is explained by the fact that dogs produce lower concentrations of the metabolite (O-desmetiltramadol) responsible for the analgesic effect than other species (Budsberg *et al.*, 2018; KuKanich, 2013; Ruel & Steagall, 2019). It has been described that morphine is more effective than tramadol for dogs undergoing soft tissue procedures such as ovariohysterectomy, ovariectomy and mastectomy (Kongara *et al.*, 2012, 2013; Teixeira *et al.*, 2013). Tramadol was also preferred by most Brazilian and Colombian veterinarians, although more effective opioids such as morphine were used more frequently (51% of the Brazilians and more than 45% of the Colombians) (Lorena *et al.*, 2014; Morales-Vallecilla *et al.*, 2019). Different results were obtained in other countries, for example in Canada the most popular opioids were butorphanol and meperidine (Hewson *et al.*, 2006), while in the United Kingdom buprenorphine was also used, apart from butorphanol (Capner *et al.*, 1999) and in South Africa meperidine was the most popular opioid (Joubert, 2001).

Veterinarians with postgraduate education proved to use opioids with greater analgesic efficacy (mainly in cats), such as morphine and fentanyl, this has also been proved in previous studies (Hunt *et al.*, 2015; Lorena *et al.*, 2014) and could explain why these opioids are not frequently chosen in Uruguay. The availability of opioid drugs in different countries could explain these differences as well (Lorena *et al.*, 2014).

We can conclude that more than 80% of the veterinarians in Uruguay do not use opioids effectively enough to manage surgical pain. This means that the majority of the surgeries that cause moderate to severe pain, such as fracture repairs, are performed without the use of an effective opioid, analgesic infusions with other drugs or peripheral nerve blocks. Guidelines for anaesthesia and pain management in small animals recommend including full mu-opioid receptor agonists in the analgesic plan for patients that will undergo surgery (Grubb *et al.*, 2020; K. Mathews *et al.*, 2015; Steagall MV *et al.*, 2022). The fact that in Uruguay only 13% of the veterinarians are using fentanyl or morphine has implications on animal welfare, considering that many animals go through procedures such as orchiectomy or ovariohysterectomy experiencing pain.

In Uruguay, the acquisition of opioids conducted by the Ministry of Public Health does not consider their use in veterinary medicine, therefore, there is restrictive access to this group of drugs in human pharmacy. There are no full mu-opioid receptor agonists approved for use in veterinary medicine, a factor that could also contribute to the infrequent prescription of these drugs by Uruguayan veterinarians.

In the United States there are regulations in place allowing the patients better access and care by approving these drugs beyond state borders and outside their own clinics (American Veterinary Medical Association [AVMA], n.d.). Similarly, in Brazil the rules dictating the access to opioids were reviewed and adjusted to changes in society and the market (National Agency Health Surveillance [ANVISA], 2017). A report about opioid distribution in Uruguay concluded that the risk of deviation of these drugs in facilities regulated by the Ministry of Public Health is low (Ministry of Public Health [MSP], 2019). This indicates that Uruguay has an effective regulation system of the market, to which veterinarians have almost no access.

Analgesic effect was the most notorious factor influencing veterinarians when choosing treatment with opioids. In contrast, in Brazil, the most common factors were adverse effects and costs. Brazilian and Canadian veterinarians showed more concern towards the excitatory effect in cats and respiratory depression in both cats and dogs (Dohoo & Dohoo, 1996; Lorena *et al.*, 2014), while Uruguayan veterinarians were more concerned about emesis and sedation.

In this study, all the respondents used NSAIDs, dipyrone was the most popular being used by 88.7% of the veterinarians, followed by carprofen (71.1%) and meloxicam (57.7%). Dipyrone was also chosen among most respondents in New Zealand (Williams *et al.*, 2005). Despite its weak anti-inflammatory effect (Monteiro and Steagall, 2019), its analgesic effect and low incidence of adverse effects have been reported and it is recommended to use it as a single medication or in multimodal therapies in dogs

(Silva *et al.*, 2021). In the United Kingdom, veterinarians used meloxicam and carprofen more frequently (Hunt *et al.*, 2015), while in France and Brazil, ketoprofen and meloxicam were the most commonly used drugs of this group (Hugonnard *et al.*, 2004; Lorena *et al.*, 2014). The frequent use of meloxicam in cats seen in this research could be due to several publications reporting its analgesic efficacy in patients undergoing abdominal surgery, and its safety in cats and dogs (Mathews *et al.*, 2001; Slingsby and Waterman-Pearson, 2002). Additionally, in countries such as Brazil and Uruguay, meloxicam for veterinary use can be found in the market in different pharmaceutical presentations including different routes of administration.

Nonsteroidal anti-inflammatories are well tolerated by most animals, with a small number of patients (less than 10%) having to discontinue the treatment due to adverse effects. Some of the side effects known to be caused by NSAIDs include emesis, diarrhoea, gastrointestinal ulcers, nephropathy and hepatopathy. In addition, there are pre-existing conditions and other factors that make the use of NSAIDs contraindicated for certain patients. Therefore, it is important to investigate and consider alternative therapies and promote their use in companion animals (KuKanich, 2013).

According to most respondents, the analgesic effect was one of the main factors considered for the use of anti-inflammatories, both in cats and dogs, similar to the results obtained in the United Kingdom (Hunt *et al.*, 2015). The potential adverse effects and the information available were the most influential factors for Brazilian veterinarians when choosing the treatment (Lorena *et al.*, 2014).

Lidocaine was used by most veterinarians (87.3%), to perform peripheral nerve blocks by infiltration, followed by perineural blocks, these results were similar to the results obtained in New Zealand (Williams *et al.*, 2005).

Analgesic and anaesthetic drugs are commonly used through the epidural route because it provides efficient analgesia and/or anaesthesia (Jones, 2001; Sarotti *et al.*, 2015; Valverde, 2008). However, in Uruguay, epidural drug administration was notoriously low (7.7%) according to this study. It should be noted that the use of epidural drug administration was more frequent in New Zealand based on a study carried on in 2003 (Williams *et al.*, 2005) than in Uruguay in 2019. In Brazil, 30.4% of the respondents employed epidural anaesthesia/analgesia with local anaesthetics and 22.2% with opioids, exceeding the numbers obtained in our territory (Lorena, *et al.*, 2014).

A minority of veterinarians (8.5%) considered dissociative anaesthetics as a component of pain management for acute pain, which contrasts with Brazil where 52% of the veterinarians used these and they were even more popular in the United Kingdom (97%) (Hunt *et al.*, 2015; Lorena *et al.*, 2014). Administered at subanesthetic doses, ketamine provides an antihyperalgesic effect due to its antagonism of NMDA receptors. After evaluating its analgesic efficacy, several studies reported its benefits, including a decrease in minimum alveolar concentration (MAC), decreased requirement for rescue analgesia, decrease in pain scores based on acute pain scales, decrease in hyperalgesia associated with surgical sites (Muir *et al.*, 2003; Pascoe *et al.*, 2007; Sarrau *et al.*, 2007; Slingsby and Waterman-Pearson, 2000; Solano *et al.*, 2006; Wagner *et al.*, 2002).

Gabapentinoids were not commonly used by Uruguayan veterinarians to treat acute pain (12%), unlike British veterinarians (68.8%) (Hunt *et al.*, 2015). The Global Pain Council of the World Small Animal Veterinarian Association (WSAVA) recommends the use of gabapentin for preventing or treating postoperative pain or when there is low availability of analgesic drugs (Mathews *et al.*, 2014). The analgesic efficacy of gabapentin has been described when used as an adjuvant for the treatment of perioperative pain in cats and dogs (Crociolli *et al.*, 2015; Schmierer *et al.*, 2020; Steagall *et al.*, 2018; Vettorato & Corletto, 2011). However, some studies question its efficacy in patients with acute pain (Wagner *et al.*, 2010).

Acupuncture was the most popular alternative therapy amongst the respondents (8.5%) while homoeopathy, fentanyl patches and physiotherapy were barely mentioned. In Brazil the most common ones were acupuncture (17%) and homoeopathy (13.8%) (Lorena *et al.*, 2014); Finnish veterinarians incorporated physiotherapy (37%), acupuncture (16%) and homoeopathy (3%) (Raekallio *et al.*, 2003). This difference between Uruguay and other countries could be due to the unavailability of undergraduate and postgraduate courses in such areas, therefore, there is a significant lack of knowledge about alternative therapies offered nowadays in veterinary medicine. Although there is a lack of evidence regarding the efficacy of acupuncture, pharmacopuncture, homoeopathy and physiotherapy which makes the subject quite controversial, these therapies may be effective techniques when associated with conventional drugs such as NSAIDs and opioids (Cassu *et al.*, 2011, 2012; Marques *et al.*, 2015; Nascimento *et al.*, 2019; Pacca *et al.*, 2015; Tomacheuski *et al.*, 2020; Zidan *et al.*, 2018).

Most respondents disagreed with the idea that a certain degree of pain in the postoperative period is good because it keeps the animal inactive, and veterinarians from Montevideo put the most emphasis on this idea (72%). Also, veterinarians of the metropolitan area use more full agonist opioids and considered exploratory laparotomies more painful in dogs and cats. It is significantly worrying that 41% of the respondents still think that pain can be beneficial for recovery. It has been stated that urban areas in greater contact with companion animals show more concern for animal welfare (Ostovic *et al.*, 2017), which could explain the findings in this study. Similar to the results obtained in Brazil, in Uruguay 86% of the veterinarians agreed that animals should receive the same consideration for pain relief as humans. Only 46% of the respondents understood that treating pain does not prolong postoperative recovery, which differs from the results obtained in the neighbouring country (Lorena,

2010). As seen in Brasil, in Uruguay the gender factor did not influence the prescription of analgesics, however, the year of graduation did (the more years that had passed since graduation, the lower the prescription of analgesics) (Lorena *et al.*, 2014).

The greater the perception of pain generated by a procedure, the more likely the administration of analgesia is, except in orthopaedic surgeries where this relationship is not so clear. Based on other studies, orthopaedic procedures have been considered to be the most painful surgeries (Gómez de Segura *et al.*, 2003; Hewson *et al.*, 2006; Lorena *et al.*, 2014). Regarding procedures that veterinarians considered equally painful for cats and dogs, the latter ones received more analgesia. This pattern was repeated in several studies, in which veterinarians provided more analgesia to dogs than cats, even though they perceived certain procedures as equally painful for both species (Capner *et al.*, 1999; Hunt *et al.*, 2015; Joubert, 2001; Lascelles *et al.*, 1999; Reimann *et al.*, 2017; Williams *et al.*, 2005).

The experience gained with practice was considered the best source to acquire knowledge about pain recognition and treatment by most professionals in Uruguay, Brazil, Canada, New Zealand and the United Kingdom (Hewson *et al.*, 2006; Lascelles *et al.*, 1999; Lorena *et al.*, 2014; Williams *et al.*, 2005). Seventy per cent of the respondents thought that the most appropriate way of acquiring new knowledge was through national and regional academic events. Most Uruguayan veterinarians believed that their knowledge of the subject is adequate, similarly to the results obtained in Canada and New Zealand (Hewson *et al.*, 2006; Williams *et al.*, 2005). In contrast, most Brazilian and British respondents classified their knowledge as "insufficient" (Lascelles *et al.*, 1999).

The undergraduate training in the area was considered to be inadequate by most respondents, something similar occurred in New Zealand (Williams *et al.*, 2005). In Canada, undergraduate education was also reported not to be a useful source of information (Dohoo & Dohoo, 1996). In Uruguay, the lack of undergraduate and postgraduate education on pain management could explain the results obtained.

Postoperative monitoring was the responsibility of most Uruguayan veterinarians (91%), differing from lower values obtained in the United Kingdom, and New Zealand (Capner *et al.*, 1999; Lascelles *et al.*, 1999; Williams *et al.*, 2005). In Uruguay, there are no anaesthesia courses for technicians and perhaps this explains the results obtained.

A possible limitation of this study may be related to the availability of veterinarians to answer the questionnaire since many of them were at their workplace at the time of the interview and this factor could have influenced the quality of the answers obtained.

The results obtained in this study reveal that Uruguayan veterinary professionals acknowledge that animals experience severe pain when undergoing diverse surgical procedures, however, they use weak opioids or NSAIDs alone to treat such pain. The high cost of the drugs, the low number of opioids approved for veterinary use and the lack of postgraduate education in the area seem to explain the differences obtained in this study compared to other countries.

Based on these concerning results, an important debate about animal welfare arises aiming at improving professional training at the University of the Republic. Also, a serious discussion with ministerial authorities about facilitating veterinary access to more potent opioids with affordable costs is needed. In conclusion, the low use of analgesic drugs and complementary techniques which provide profound analgesia by Uruguayan veterinarians reveals the need for open discussion, adjustment of attitudes and continuing education on pain relief in animals.

COMPETING INTERESTS STATEMENT

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

All authors have read and approved the current manuscript. JGB was involved in the execution of the study, the statistical analysis and the elaboration of the manuscript. GF contributed to the study design, sampling and data analysis. LR was involved in the execution of the study and elaboration of the manuscript. VM and ER participated in the execution of the project. SPL participated in the critical analysis of the project. NCO was responsible for the study design, execution and critical analysis of the project.

ETHICS STATEMENT

This study was approved by the Ethical Committee of the Faculty of Humanities at Universidad de la República (protocol approval number 121900-500704-21).

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ORIGINAL ARTICLE

Force-sensitive resistors to measure the distribution of weight in the pads of sound dogs in static standing position

Chun-ki Choi[a], Jinsu Kang[b], Namsoo Kim[b], Soon-wuk Jeong[a], Suyoung Heo[b*]

ABSTRACT. The purpose of this study was to measure how weight is distributed in the pads of each of the 4 limbs of dogs and evaluate the intra-investigator reproducibility and inter-investigator reliability of the measurement method. Eight dogs were examined 3 times a day by 3 investigators at 1 week intervals for 3 weeks to determine the weight distribution to each of the pads. The force-sensitive resistor was used for measurement and specific software (PetLAB2) was used to calculate the weight applied to each pad. The intra-investigator reproducibility showed moderate to good reliability (ICC range, 0.575-0.873) and the inter-investigator reliability was moderate (ICC range, 0.525-0.746). Based on this study, it can be observed whether the weight distributed to each pad approaches the normal value after treatment in patients with orthopaedic and neurologic diseases. It is expected that this experimental method will be one of the objective indicators to evaluate the degree of recovery in patients with orthopaedic and neurologic diseases.

Keywords: Force-sensitive resistor, pad, weight distribution, reliability, dog.

INTRODUCTION

The evaluation of limb use in dogs with orthopaedic diseases is a major aspect of examinations (Sharkey, 2013). An objective evaluation of limb use involves measuring weight bearing in a static or dynamic state using force plates or pressure-sensitive walkways (Lascelles *et al.*, 2006). Static weight distribution is used to assess the amount of weight each limb bears during standing, whereas dynamic weight distribution is assessed during walking/running, both of which are important assessments in veterinary patients with orthopaedic conditions (Hyytiäinen *et al.*, 2012). In veterinary medicine, these objective measures have been used to evaluate surgical interventions, determine analgesic efficacy, develop surgical models for pain, and monitor patients in rehabilitation programs effectively (Seibert *et al.*, 2012; Tomas *et al.*, 2014; Tomas *et al.*, 2015).

Various tools and methods are used in veterinary clinics to measure static and dynamic weight distribution, and measurement objectivity has been confirmed for the dynamic state evaluation using a pressure-sensitive walkway in healthy Labradors (Light *et al.*, 2010). However, variables such as head position and velocity may affect the kinematic and force plate data in the dynamic state (McLaughlin & Roush, 1994; McLaughlin, 2001).

Static weight distribution is defined as the percentage of body weight distributed to each limb during standing. In sound dogs, 30% of the weight is distributed to each forelimb and 20% of the weight is distributed to each hindlimb (Bosscher *et al.*, 2017). Patients with pain or instability associated with orthopaedic or neurologic disease may alter their weight distribution during standing. Depending on the severity of the disease, it can vary from subtle changes in weight distribution to complete non-weight bearing. In the process of recovery through surgery, the weight bearing of the affected limb is expected to be slightly loaded. Measurement of the static weight distribution has been used previously to assess response to treatment after total hip replacement surgery (Seibert *et al.*, 2012).

In human medicine, the human foot has been divided into regions corresponding to transversal and horizontal cuts to obtain a detailed assessment of weight distribution on the plantar surface (Hennig & Rosenbaum, 1991). The classification of these compartments is important for the measurements because of the complexity of the human foot, allowing for a detailed study of numerous foot disorders (Hessert *et al.*, 2005; Hughes *et al.*, 1987; Yavuz *et al.*, 2009; Zammit *et al.*, 2008). The foot pressure distribution can provide essential information and thus assist in medical diagnoses (Vigneshwaran & Murali 2020). Similarly, in veterinary medicine, many have reported the pressure distribution of the pads and peak vertical force and vertical impulse applied to each pad in a static and dynamic state (Besancon *et al.*, 2004; Souza *et al.*, 2013; Souza *et al.*, 2014) and, further, one study have been conducted by dividing the pads into four quadrants to measure vertical force distribution (Braun *et al.*, 2019).

Additionally, while a few studies have evaluated the vertical force applied to each pad, this is a dynamic state measurement; research on the pressure applied to each pad in a static state is scarce (Besancon *et al.*, 2004; Marghitu *et al.*, 2003; Souza *et al.*, 2013). Recently, body weight distribution in static state in small dogs without orthopaedic or neurosurgical disease has been studied but without measuring the value given to each pad (Linder *et al.*, 2021). In both human and veterinary medicine, many researchers have reported the pressure distribution of the

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pads and peak vertical force and vertical impulse applied to each pad in a static and dynamic state (Besancon *et al.*, 2004, Souza *et al.*, 2013, Souza *et al.*, 2014).

This study aimed to determine the distribution of weight in the pads of the four limbs during static standing in sound dogs and evaluate the intra-investigator reproducibility and inter-investigator reliability of force-sensitive resistor. We hypothesised that measurements collected using a force-sensitive resistor would be both reproducible and reliable.

MATERIAL AND METHODS

ANIMALS

After owner consent was obtained, 8 client-owned adult dogs (3 Malteses, 2 Pomeranians, 1 Beagle, and 2 mixed breeds) were included in this study. This study was approved by Jeonbuk National University's Institutional Animal Care and Use Committee (Number: JBNU 2021-0173). The body weights of the dogs used in the study ranged from 2.7 kg. to 11.3 kg. and the average body weight was 5.2 ± 2.8 kg. (mean ± standard deviation (SD)). All dogs underwent physical, orthopaedic, neurologic, and gait evaluations to rule out any lameness that could affect weight distribution.

EXPERIMENTAL DESIGN

Static weight distribution was recorded on a 30 cm × 32 cm piezoresistive-type force-sensitive resistor (Kitronyx, Seoul, South Korea) (figure 1a) equipped with a total of 2,304 nodes. The force-sensitive resistor was connected to a dedicated computer equipped with specific software (PetLAB2; Kitronyx, Seoul, South Korea) designed for data acquisition, storage, and graphic conversion. Two force-sensitive resistors were placed on a digital scale (Jiangyin Ditai Electronics CO, Jiangsu, China) (figure 1b) and calibrated.

After the dog's forelimbs and hindlimbs were placed on each sensor, the exact weight applied to each limb was recorded. Before the analysis, the sensors were calibrated according to the measured weight. During the measurement process, the investigator restricted the dog's movement by carefully wrapping the head. The investigator was placed directly in front of the dog to ensure that the head and neck were facing forward. The measurement time was 1 minute, and the moment when the weight bearing was applied to all pads was determined as the measurement moment. Measurements were taken only when the dog was standing still and looking straight ahead (figure 2a).

As a simplified schematic for measurement, the PetLAB2 software program was used and the hardware was comprised of a digital weighing scale, force-sensitive resistor, and data acquisition electronics (figure 2b). Once the area of each pad was manually set according to the shape of the pad represented by the graphic, the program calculated the pressure in the area and converted it into the weight of each pad (figure 3). The total body weight was set at 100% and the distributed weight of a total of 20 pads (forelimbs and hindlimbs) was calculated as a percentage (%). The sensor used in this study was manufactured using the principle of outputting an electrical signal generated in proportion to the digital signal to indicate how the weight (kg.) was distributed to each pad.

All procedures were conducted by three investigators (small-animal surgery residents) who were instructed on the measurement method through lectures and handouts prior to recording any measurements. On the first day, 8 dogs were measured three times by the three investigators to obtain 72 measurements, and the same measurement was repeated after 7 and 14 days to obtain a total of 216 measurements. For the accuracy of the measurements, animals were given a 5 min. rest between measurements. The experimental order of the investigators and dogs was randomly determined using randomization software (http://www.random.org/) three measurement days.

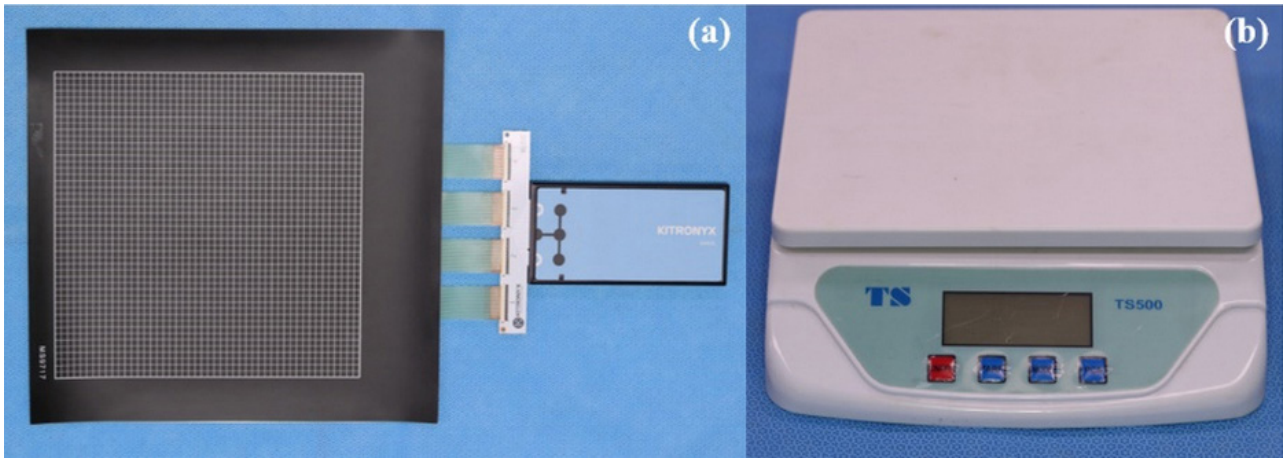

Figure 1. The force-sensitive resistor (MS9717) connected to data acquisition electronics (Baikal force controller) (a) and digital weighing scale (TS500) (b).

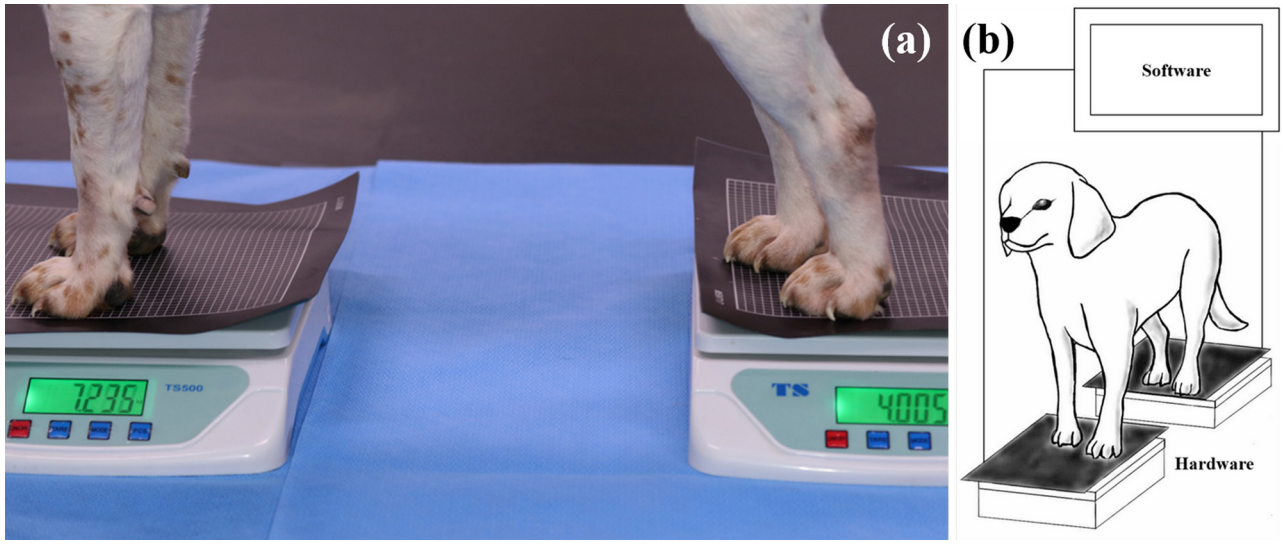

Figure 2. Placement of the dog and devices during measurement (a). As a simplified schematic for measurement, the software was created using the PetLAB2 program, and the hardware is composed of a digital weighing scale, force-sensitive resistor, and data acquisition electronics (b).

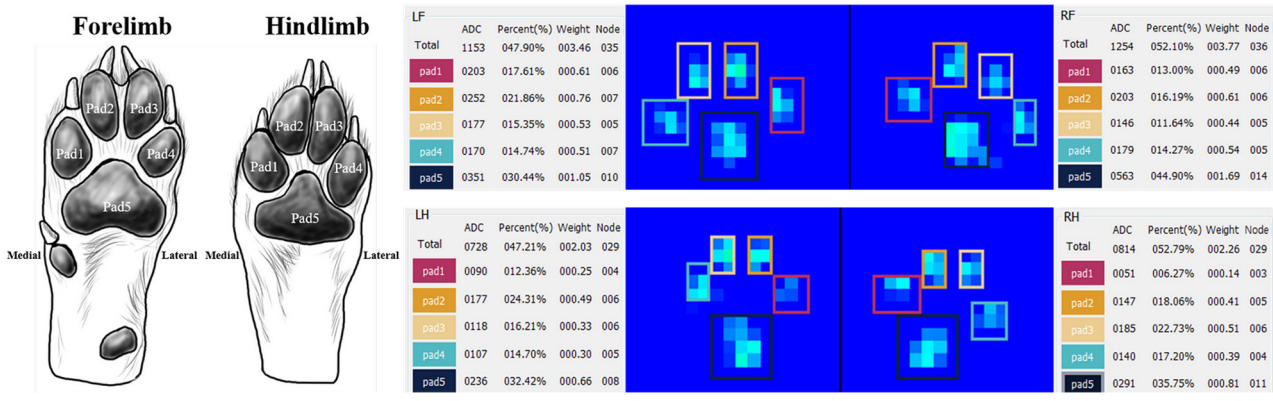

Figure 3. Pads of both forelimbs and hindlimbs were designated as Pad 1, 2, 3, and 4 in the lateral direction from the medial direction, and the metacarpal/metatarsal pad was designated as Pad 5. The picture on the right shows the manual setting of the area of each pad to measure the weight using the PetLAB2 program.

STATISTICAL ANALYSIS

SPSS version 23 (SPSS Inc, Chicago, IL, USA) was used to verify normality assumptions and to confirm the quality of the data obtained in the study. The normal distribution of percentage value for each pad was investigated using the Kolmogorov-Smirnov test. The data were presented as the mean ± SD.

The evaluation of intra-investigator reproducibility and inter-investigator reliability for the force-sensitive resistor was performed using the intraclass correlation coefficient (ICC). The interpretation of the ICC was evaluated using the criteria introduced by Portney and Watkins based on the 95% confidence interval of the ICC estimate. Specifically, values < 0.5 indicated poor reliability, those between 0.5 and 0.75 indicated moderate reliability, those between 0.75 and 0.9 indicated good reliability, and those > 0.90 indicated excellent reliability.

RESULTS

A total of 216 measurements were obtained and analysed. As previously described, the area for each pad was used to calculate each pad's weight out of the total weight distributed. These data were found to be normally distributed. The figure 4 shows the mean value of the weight applied to each pad as a percentage of the total weight. The data showed that weight-bearing was higher for the metacarpal pad than for the metatarsal pad. The tables 1 and 2 present the results of the reproducibility and

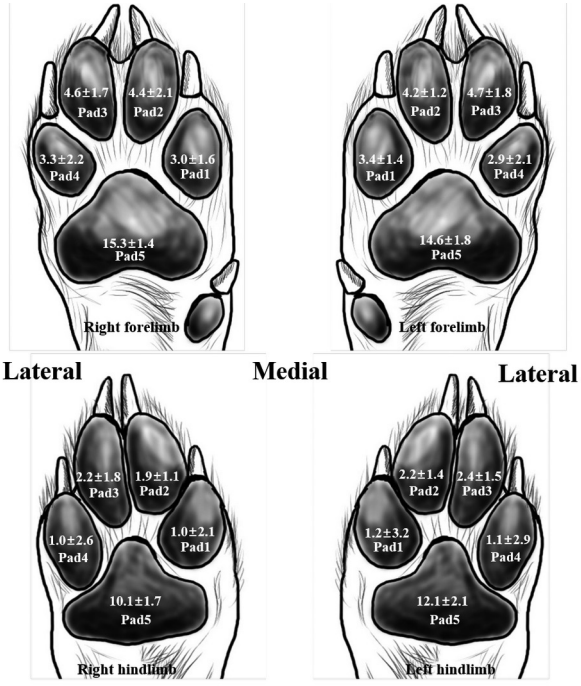

Figure 4. Value of the weight given to each pad expressed as percentages (mean ± SD) for the four limbs of each of the 8 dogs.

reliability evaluation respectively. The intra-investigator reproducibility showed moderate to good reliability (ICC range, 0.575-0.873), and the inter-investigator reliability was moderate (ICC range, 0.525-0.746).

DISCUSSION

To the best of our knowledge, no study has been conducted on the distribution of total body weight to each pad in standing sound dogs. Therefore, we investigated the normal weight value applied to each pad through a force-sensitive resistor in a static state.

When diagnosing lameness in dogs, dynamic state evaluation is more sensitive than subjective lameness scoring scales (Quinn *et al.*, 2007; Waxman *et al.*, 2008). However, it is difficult to perform dynamic measurements using a pressure-sensitive walkway in veterinary patients with severe neurological and orthopaedic diseases. These patients cannot ambulate normally due to severe pain, and the measurement value cannot be regarded as reliable. Even if the pain is not severe, in the case of severely nervous patients, it is difficult to perform dynamic measurements because the animal is not ambulating normally, and therefore, the test is not accurate. In addition, compared to kinetic

Table 1. Intra-investigator reproducibility (95% confidence intervals).

	Left forelimb (CI value)					Right forelimb (CI value)				
	Pad 1	Pad 2	Pad 3	Pad 4	Pad 5	Pad 1	Pad 2	Pad 3	Pad 4	Pad 5
INV 1	0.658	0.751	0.732	0.575	0.791	0.601	0.645	0.705	0.744	0.821
INV 2	0.651	0.742	0.717	0.719	0.836	0.741	0.733	0.732	0.736	0.823
INV 3	0.708	0.693	0.873	0.789	0.793	0.742	0.716	0.707	0.670	0.866
	Left hindlimb (CI value)					Right hindlimb (CI value)				
	Pad 1	Pad 2	Pad 3	Pad 4	Pad 5	Pad 1	Pad 2	Pad 3	Pad 4	Pad 5
INV 1	0.707	0.635	0.636	0.740	0.836	0.727	0.654	0.773	0.815	0.851
INV 2	0.624	0.854	0.779	0.717	0.707	0.743	0.757	0.753	0.733	0.727
INV 3	0.791	0.781	0.699	0.711	0.827	0.720	0.733	0.806	0.767	0.838

INV: investigator, Pad 5: metacarpal or metatarsal pad.

Table 2. Inter-investigator reliability (95% confidence intervals).

	Left forelimb (CI value)					Right forelimb (CI value)				
	Pad 1	Pad 2	Pad 3	Pad 4	Pad 5	Pad 1	Pad 2	Pad 3	Pad 4	Pad 5
1st	0.681	0.685	0.671	0.677	0.711	0.690	0.717	0.746	0.706	0.717
2nd	0.617	0.631	0.618	0.639	0.714	0.654	0.659	0.621	0.595	0.691
3rd	0.644	0.531	0.606	0.615	0.697	0.673	0.650	0.676	0.639	0.687
	Left hindlimb (CI value)					Right hindlimb (CI value)				
	Pad 1	Pad 2	Pad 3	Pad 4	Pad 5	Pad 1	Pad 2	Pad 3	Pad 4	Pad 5
1st	0.665	0.706	0.678	0.665	0.725	0.617	0.673	0.661	0.544	0.702
2nd	0.525	0.647	0.679	0.615	0.686	0.589	0.541	0.651	0.669	0.678
3rd	0.630	0.557	0.614	0.632	0.681	0.627	0.567	0.592	0.594	0.697

1st: the first day of measurement, 2nd: 7 days later, 3rd: 14 day later, Pad 5: metacarpal or metatarsal pad.

measurement in static state, the method of measuring the dynamic state requires more space and data acquisition skills and is more expensive (Bosscher *et al.*, 2017; Clough *et al.*, 2018; Cole & Millis 2017).

The comparison between the values measured in this study, set to a normal value, and the values obtained from dogs with orthopaedic and neurologic diseases, shows how the weight distribution on each pad has been altered. In these patients, if the weight applied to the pad is continuously measured in the process of recovery after surgical or/and rehabilitation treatment, it is expected that as it approaches the normal value, this may be a more objective indicator of the patient's recovery.

For the evaluation of the reliability of the equipment and programs used in this study, the intra-investigator reproducibility showed moderate to good reliability, while the inter-investigator reliability was moderate. This may be related to various factors, such as the dog's temper or the investigator's ability to soothe the dogs; however, to increase the objectivity of the values measured by the equipment, a method that can increase the reliability between investigators is necessary.

One limitation of this study was the necessity of adjusting both the forelimbs and hindlimbs of the dog to fit on the sensor. Therefore, when the limb moved away from the sensor, the investigator needed to handle the limb which may have altered the patient's neutral position. Even if the dog's body does not deviate significantly from the sensor, it is impossible to measure when the dog is holding the leg up or sitting down due to severe pain, and in this case, there is a limitation in that the accurate weight load cannot be measured.

In addition, the force-sensitive resistor used in this study could not be used to accurately measure the weight, so it was necessary to measure the exact weight by placing the resistor on a digital scale and placing the dog's forelimbs and hindlimbs on the resistor. In small dogs, the distance between the two pieces of equipment was narrow, so it was possible to measure it as if it was almost attached. However, for medium to large–sized dogs, there was more distance between the two pieces of equipment, which could have affected the dog's balance. Therefore, the authors and the research team are considering a method to accurately measure the weight with the force-sensitive resistor.

We hope the results of this study will help veterinarians evaluate the limbs and assess abnormal weight distribution of pads by comparing them with normal values measured in this study. In addition, it may be another indicator that can objectively be used to evaluate the patient's recovery through assessment of the gradual changes in the weight distributed to the pads compared to normal values. Further research is needed, but various advantages are expected compared to dynamic state measurements and we anticipate it will be a more useful test method if measurement elements are added through clinical application and discussion.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing interests.

ETHICS STATEMENT

This experiment was carried out at the Jeonbuk Animal Medical Center (JAMC), College of Veterinary Medicine, Iksan, Korea, and it was approved by the Committee of Animal Experiments of the Jeonbuk National University, Jeonju, Korea, for the use of animals in the experiment.

AUTHOR CONTRIBUTIONS

CC and JK conceived this study. SJ and SH designed the study. CC and JK conducted the experiment and collected important background information. CC and JK analysed the data. All authors were involved in data interpretation, write up and final approval of the manuscript.

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ORIGINAL ARTICLE

Report of *Oslerus rostratus* (Strongylida: Filaroididae) in cats from the Canary Islands, Spain

Katherine Garcia-Livia[a,b], Maria Valladares Salmerón[c], Sandra Pacheco[d], Basilio Valladares[a], Pilar Foronda[a,b*]

ABSTRACT. Metastrongylid species infecting wild and domestic cats worldwide are increasingly being reported. Between 2017 and 2019, a total of 202 faecal samples of domestic cats from the island of Tenerife (Canary Islands, Spain) were analysed by microscopy and molecular techniques. Morphological analyses showed that 8.91% (18/202) of the faecal samples presented first stage larvae (L1) of metastrongylid species. Total DNA was isolated and tested by PCR targeting a 508 bp fragment of the ITS-2 gene. The nucleotide sequences obtained showed high homology (100%) with the species *Oslerus rostratus*. This work contributes to the knowledge of the wide distribution of *O. rostratus* worldwide, being Tenerife (Canary Islands, Spain), close to the African continent, the new geographic location for this metastrongylid species. Further molecular studies involving new geographic areas from the island of Tenerife, as well as neighbouring islands, are needed to provide relevant insight and better understand the epidemiology of *O. rostratus* and other metastrongylid species in wild and domestic cats from the Canary Islands.

Keywords: Oslerus rostratus, metastrongylids, lungworm, domestic cat, Canary Islands, Spain.

INTRODUCTION

Metastrongylid species (Nematoda: Metastrongyloidea) have been frequently reported to infect felids worldwide. These parasites are recognised as important etiological agents in the pathology of the cardio-pulmonary system of felids and have gained the attention of the veterinary community (Traversa *et al.*, 2010; Brianti *et al.*, 2014). Infections by metastrongylid nematodes in felids can be asymptomatic or may show a variety of clinical signs and symptoms depending on the age and immune status of the host, the parasite species, and the parasitic burden. Besides, depending on the degree of infection, it can be fatal (Di Cesare *et al.*, 2011; Traversa & Di Cesare, 2013; Pennisi *et al.*, 2015).

The signs of infection by metastrongylids species are similar to those of other feline respiratory diseases such as feline asthma, and allergic bronchitis, among others (Foster & Martin, 2011). In addition, the first-stage larvae (L1) of the different metastrongylid species found in the faeces of infected hosts have similar morphometric characteristics (Traversa & Di Cesare, 2013), which makes their identification a challenge and, therefore, it should be considered by veterinarians in order to make a correct diagnosis, highlighting the usefulness of molecular tools, especially in epidemiological surveys on lungworm infections in both domestic and wild animals (Otranto *et al.*, 2013; Brianti *et al.*, 2014; Penagos-Tabares *et al.*, 2018).

The metastrongyloid *Aelurostrongylus abstrusus* (Strongylida: Filaroididae) is the most common and widespread nematode reported to infect the domestic cat, followed by the trichurid *Eucoleus aerophilus* (syn. *Capillaria aerophila*) (Anderson, 2000; Traversa *et al.*, 2010; Di Cesare *et al.*, 2015; Traversa and Di Cesare, 2016; Giannelli *et al.*, 2017). Besides, other metastrongylid nematode species have been cited to affect cats, namely *Angiostrongylus vasorum* (Traversa and Guglielmini, 2008), *Oslerus rostratus* (Brianti *et al.*, 2014), *Gurtlia paralysans* (Moroni *et al.*, 2012), and *Troglostrongylus* species (Brianti *et al.*, 2012), among others.

In Europe, these nematodes have mainly been studied in wild and domestic cats, *Felis silvestris* and *Felis catus*, respectively (Traversa & Di Cesare, 2016). In the Canary Islands (Spain), an archipelago composed of eight islands and islets situated close to the NW side of Africa, studies related to the distribution and prevalence of metastrongylid species in cats are scarce. An anatomopathological study on domestic dead cats, carried out in 1992, was the first to provide data about the presence of *A. abstrusus* in this archipelago (Valladares *et al.*, 1992). Also, an epidemiological survey carried out on the island of Gran Canaria revealed an overall prevalence of 10.4% of *A. abstrusus* in feral *F. catus* (Rodríguez-Ponce *et al.*, 2016). Recently, *G. paralysans* was first reported in the Canary Islands parasitising the eye of a domestic cat (Udiz-Rodríguez *et al.*, 2018). Considering data is currently scarce, the present study aimed to detect the presence, as well as the identity and prevalence, of the metastrongylid

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lungworm species that could be affecting domestic cats from Tenerife (Canary Islands, Spain).

MATERIAL AND METHODS

Between 2017 and 2019, a total of 202 faecal samples of domestic cats from 1 to 3 years old from Tenerife, Canary Islands (Spain), were sent for routinely parasitological analyses to the Finca España Laboratory, a private centre that performs a parasitic diagnosis service for veterinary clinics. The remaining sample, previously diagnosed, was stored frozen at -20ºC. Later, this collection was analysed for the search for metastrongylid larvae. Due to the current legislation (R.D. 53/2013) and considering the origin of these samples, previously analysed at a laboratory centre for routine analyses, no ethical approval was required.

After thawing, all faecal samples were concentrated by using a modification of Richie's formaldehyde-ether method, in which the formaldehyde-ether was replaced by ethyl acetate (Young *et al.*, 1979) and analysed to screen the samples for metastrongylid L1 larvae. It was not possible to find out any information about the previous provenance or travel histories of the animals and their outdoor activities. Larval body length, the position of the oral opening and tail morphology of the larvae found in the faecal samples were the main features considered in the morphological identification. Data obtained were compared with the descriptions reported elsewhere (Traversa and Di Cesare, 2013; 2016), commonly used for *A. abstrusus*, *Troglostrongylus brevior* and *O. rostratus* differentiation. Digital images and measurements in µm were taken using the optical microscope Leica DM2500 and the Leica LAS AF 4.12 software, respectively.

For the molecular study, genomic DNA was extracted using the faecal concentrate which was diluted in 250 µl of a solution containing 30 mM Tris-HCL (pH 8.0), 10 mM EDTA and 0.4% SDS. Then, 3 µl of proteinase K (20 mg ml $^{-1}$) was added to the samples and incubated at 56 ºC overnight. After having inactivated the proteinase K, DNA extraction continued following the instructions of the method used by Lopez *et al.* (2015). The quantity and quality of the extracted DNA were determined with the spectrophotometer Nanodrop ND-1000 (Thermo Fisher Scientific, Wilmington, DE, USA). DNA was stored at -20 ºC until further processing. A fragment of the internal transcribed spacer 2 (ITS-2) was amplified using the primers NC1 (5'-ACGTCTGGTTCAGGGTTGTT-3') and NC2 (5'-TTAGTTTCTTTTCCTCCGCT-3') as previously described by Gasser *et al.* (1993). Approximately 20-50 ng of genomic DNA were added to each PCR. PCR reactions were performed in a total volume of 25 µl, including 10× buffer Mg^{2+} free (Bioline, London), 2.5 µl of each dNTP (10 mM), 1 µl of each primer (12.5 ng/ml), 0.125 µl of Biotaq polymerase (5 U/ml) (Bioline, London), 0.75 mM $MgCl_2$, 2 µl of DNA template and water. The ITS-2 fragment was amplified using the following cycling conditions: 94 °C for 2 min (first polymerase activation and denaturation), followed by 35 cycles of 94 °C for 1 min (denaturation), 58 °C for 1 min (annealing), and 72 °C for 1 min (extension), with a final extra extension step at 72 °C for 5 min. All PCR products were resolved on 1.5% agarose gels. The amplicons were sequenced in both directions in SEGAI (Universidad de La Laguna sequencing services, Spain) and Macrogen Inc. (Madrid, Spain). The obtained sequences were edited with the MEGA X program (Kumar *et al.*, 2018) and subsequently aligned with the ClustalW program included in MEGA X. To elucidate any homologies or similarities previously published in GenBank, a BLAST search was carried out. The molecular identification, carried out in MEGA X, was achieved by phylogenetic analysis through the Neighbor-Joining distance method (Saitou & Nei, 1987) with at least 1000 bootstrap replications. *Toxocara cati* was used as the outgroup. Nucleotide sequence data reported in this publication is available in the GenBank database under the accession numbers: MW263070, MW263071 and MW263072.

RESULTS

First stage larvae (L1) of metastrongylid species were detected by microscopical analysis in 18 out of 202 (8.91%) faecal samples from domestic cats. A total of 50 first stage L1 larvae were measured, obtaining body length values ranging from 333 to 390 µm, with 361.1 as the mean value and 17 of standard deviation. When compared with the larval length reference of metastrongylid species, the measurements values obtained were within the interval range reported for *A. abstrusus* (360-415 µm), *T. brevior* (300-357 µm) and *O. rostratus* (335-412 µm). These measured larvae were characterised by a rounded head with a cylindrical buccal capsule and a central oral opening surrounded by a cuticular ring with dorsal and ventral prominences. The tail was slightly undulated, with a deep ventral and a shallow dorsal one ending with a minute spine, matching this morphology description with the reported for the species *O. rostratus* (figure 1).

Since using the morphological and morphometric diagnosis of the L1 larvae of metastrongylid species could cause misidentification, molecular analyses based on the ITS-2 gene were carried out. Eventually, only three out of 18 samples were successfully sequenced. The three nucleotide sequences obtained, isolate 017 (434 bp), isolate 976 (468 bp) and isolate 694 (431 bp), displayed 99-100% homology with *O. rostratus* sequence of *F. catus* from Sardinia, Italy (GenBank: KM009118) and with other *O. rostratus* sequence of *F. silvestris* from Hesse, Germany (GenBank: KP987220). Furthermore, phylogenetic analysis based on 454 bp of the alignment confirmed these results, since the sequences from domestic cats obtained in this study were grouped into the *O. rostratus* clade with high bootstrap value (100) (figure 2).

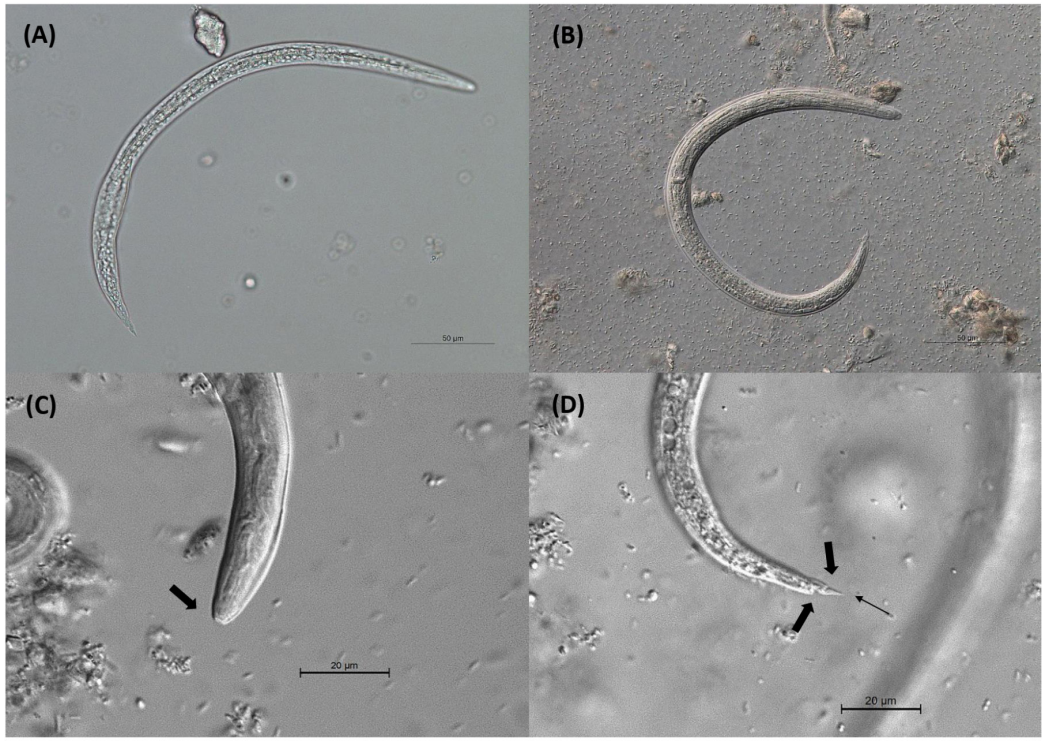

Figure 1. First-stage larvae (L1) of *Oslerus rostratus* (A, B) recovered from cat fecal samples; (C) magnification of the anterior end, showing a rounded head with a central oral opening surrounded by a cuticular ring with dorsal and ventral prominences (bold arrow); (D) magnification of the tail, showing a deeper notch on the ventral side and a shallower one on the dorsal side (bold arrows). At the proximal edge of the dorsal notch there is a minute cuticular spine (light arrow).

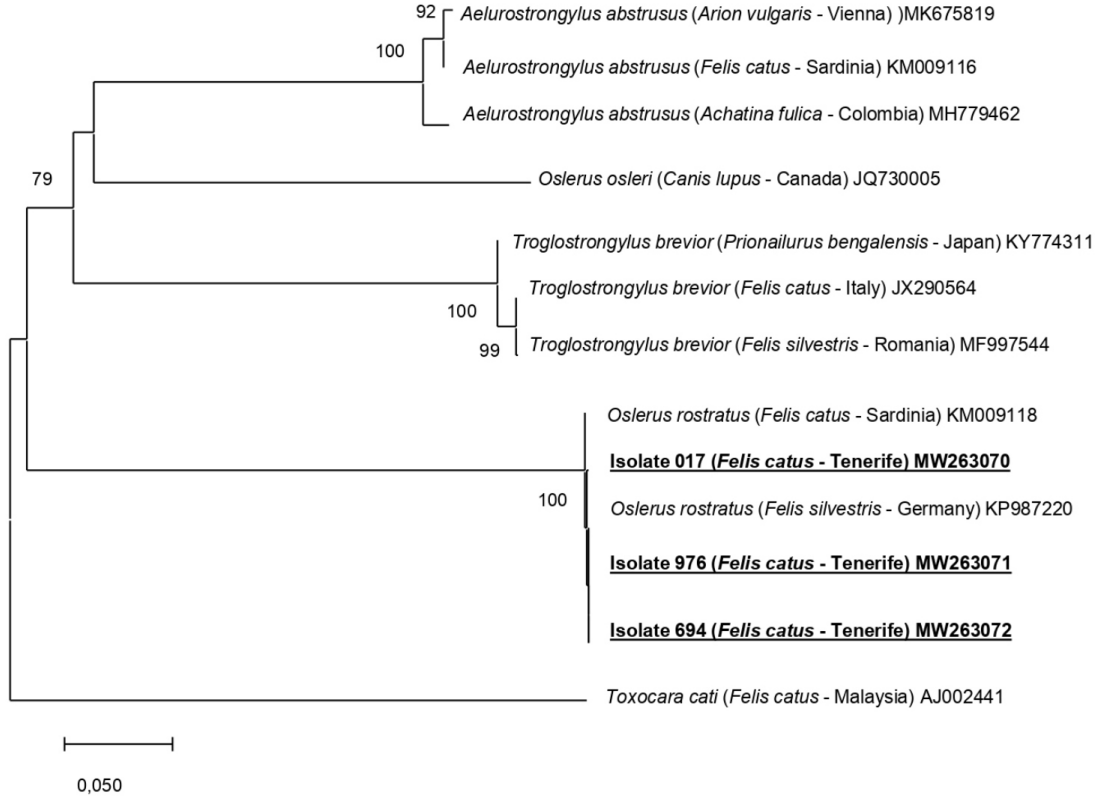

Figure 2. Phylogenetic analysis using the Neighbor-Joining method with p-distance and 1000 bootstrap replications based on a 454 bp fragment of the internal transcribed spacer 2 (ITS-2). New sequences obtained in this study are typed in bold, underlined text. *Toxocara cati* was used as the outgroup.

DISCUSSION

The results obtained in this study contribute to deepening the knowledge about feline lungworms distribution. *Oslerus rostratus*, after its first description as *Anafilaroides rostratus* in cats from Jerusalem (Gerichter, 1949), was reported in cats from Sri Lanka island (Seneviratna, 1958) and Hawaii island (Ash, 1962), as well as in bobcats from Virginia and Georgia (USA) (Klewer, 1958; Watson *et al.*, 1981). In Europe, this nematode has been reported in domestic cats from mainland Spain (Juste *et al.*, 1992), in feral cats from Majorca and domestic cats from Ibiza islands (Spain) (Millan & Casanova, 2009; Jefferies *et al.*, 2010), in a stray cat from Sicilia and a domestic cat from Sardinia islands (Italy) (Brianti *et al.*, 2014; Varcasia *et al.*, 2015) and domestic cats from Hungary (Kiszely *et al.*, 2019). Recently, *Oslerus sp.* has been found in South America, more specifically in Brazil and Chile, parasitising two felid species, the jaguarundi (*Puma yagouaroundi*) and the guignas (*Leopardus guigna*), respectively (Corrêa *et al.*, 2019; Acuña-Olea *et al.*, 2020). Therefore, our study provides new data on the distribution of *O. rostratus*, being the seventh report on islands.

With regard to the prevalence, in Europe, a study carried out by Giannelli *et al.* (2017) showed that 10.6% (n= 210/1990) of the sampled domestic cats were infected by lungworms. The prevalence of lungworm infection reported for Spain was 6.5% (n= 13/200), being similar to the prevalence obtained in our study, 8.91% (n= 18/202). In the same study, 3.8% (n= 8/210) of the lungworm infections in domestic cats were caused by the species *O. rostratus*. However, the higher prevalence was obtained for *O. rostratus* in other regions, such as Sri Lanka, Virginia (USA) and Majorca island (Spain), where 60%, 96% and 24% of prevalence were reported, respectively (Seneviratna, 1958; Klewer, 1958; Watson *et al.*, 1981; Millán & Casanova, 2009). Despite this, the reports of this nematode in domestic cats are usually regarded as singles cases (Gerichter, 1949; Juste *et al.*, 1992; Brianti *et al.*, 2014; Varcasia *et al.*, 2015).

According to the life cycle of *O. rostratus*, it is similar to other metastrongylid species, with a wide range of mollusc species acting as intermediate hosts (Seneviratna, 1959). Furthermore, species of lizards, frogs, birds, and small mammals can act as paratenic hosts, thus contributing to the dispersion and transmission of metastrongylids species among this fauna, including cats (Bowman, 2000, 2002). In the Canary Islands, there are numerous species of terrestrial gastropods, some of them endemic, as well as lizards, frogs, birds and small mammals (Izquierdo *et al.*, 2004). In this sense, some introduced metastrongylid species, such as *Angiostrongylus cantonensis*, have been successfully adapted to the Canaries habitat since it was previously found in rats (*Rattus rattus*) and terrestrial snails from Tenerife (Foronda *et al.*, 2010; Martin-Alonso *et al.*, 2015). In addition, an epidemiological study carried out in this archipelago reported the presence of A. *cantonensis*, *A. vasorum* and *A. abstrusus* in terrestrial native slug and snails species collected in the islands of Tenerife, Gran Canaria, El Hierro, Lanzarote, La Palma and Fuerteventura (Segeritz *et al.*, 2021). More studies would be necessary to confirm if the life cycle of *O. rostratus* is well established in the island of Tenerife and if the free ranging domestic cats could have made them available to infect potential intermediate and paratenic hosts.

On the other hand, veterinarians from the island of Tenerife have reported the presence of *A. abstrusus* larvae in the faeces of domestic cats (author's pers. obs.). However, the larvae diagnosis in those clinical cases has been made only by morphological techniques, so *O. rostratus* could be more prevalent in the Canary Islands than currently thought, being misdiagnosed as *A. abstrusus* due to the overlapping morphological features and individual variations of the metastrongylid L1 larvae (Traversa & Di Cesare, 2013). Furthermore, if both metastrongylid species *A. abstrusus* and *O. rostratus* have been detected in the Canary Islands, coinfections by these two metastrongylid species in a domestic cat could be occuring, as previously reported by other studies carried out in Spain, Sicily, and Hungary (Juste *et al.*, 1992; Brianti *et al.*, 2014; Kiszely *et al.*, 2019; Gianelli *et al.*, 2017). Even though molecular analyses from this study could not confirm the presence of coinfection, they should not be discarded.

The current climate change together with ecological factors, international trade, travel and migration, and animals transport are factors than can influence the establishment, maintenance, and transmission of metastrongylid species in previously unreported areas (Patz *et al.*, 2000; Traversa *et al.*, 2010; Brianti *et al.*, 2014; Otranto, 2015; Traversa & Di Cesare, 2016). An example is the Canarian Archipelago, where a total of two metastrongylid species have been reported infecting cats: *A. abstrusus* in feral cats from Gran Canaria (Rodríguez-Ponce *et al.*, 2016) and *G. paralysans* in domestic cat from Tenerife (Udiz-Rodríguez *et al.*, 2018). Therefore, the report of *O. rostratus* in our study constitutes the third report of a metastrongylid species parasitising cats in the Canary Islands, Spain.

This work contributes to the knowledge of the wide distribution of *O. rostratus,* previously reported in Europe, Asia and America, being Tenerife (Canary Islands, Spain), the new geographic location for this metastrongylid species. Further studies involving neighbouring islands of the Canary Islands will provide relevant insight to better understand the epidemiology of *O. rostratus* and other metastrongylid species in wild and domestic cats from the Canary Islands.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing interests.

ETHICS STATEMENT

Due to the current legislation (R.D. 53/2013) and considering the origin of these samples, previously analysed at a laboratory centre for routine analyses, no ethical approval was required.

AUTHOR CONTRIBUTIONS

KGL: methodology, data analysis and interpretation, writing original draft, review and editing. MVS and SP provided resources, review and editing of the manuscript. BV and PF conception and design of the study, resources, validation, review and editing the manuscript, visualisation, and supervision. All authors read and approved the final manuscript version to be submitted.

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Austral Journal of Veterinary Sciences (*AJVS*) publishes, in English, original scientific contributions such as original articles, reviews, short communications, and case reports which can include clinical observations, descriptions of methods or techniques, and advances in all aspects of veterinary science and animal welfare.

SUBMISSION

Manuscripts should be submitted via the online platform www.ajvs.cl and must include:

- An electronic version of the main text (MS Word format) that includes tables (preferably in MS Word format), charts (Excel format), figures and photographs (TIFF at a 300 dpi size, they must also be uploaded individually) in it; supplementary material must be added separately.
- A cover letter to the Editor signed by the corresponding author must include:
  1. An explanation of why your manuscript should be published in *AJVS*, including a general overview about the novelty and contribution to the field of the data presented.
  2. An explanation of any issues relating to Journal Policies. A declaration of any potential Competing interests (Please see www.ajvs.cl/index.php/ajvs/editorialPolicies for further details).
  3. Confirmation that all authors have read and approved the final version of the manuscript for submission (The statement must include the email address of all the authors).
  4. Confirmation that the content of the manuscript has not been published, or submitted for publication elsewhere.
  5. Specification of the type of manuscript that is being submitted.
  6. If you are submitting a manuscript to a particular special issue, please refer to its specific name in your covering letter.
  7. List of 3 potential reviewers.
  8. Mention any researchers who should NOT review your manuscript.
- Manuscripts should fulfill the editorial requirements in order to continue the review process.

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)

COMPLEMENTARY INSTRUCTIONS

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.

Changes to Authorship

Authors are expected to consider carefully the list and order of authors before submitting their manuscript and provide the definitive list of authors at the time of the original submission. Any addition, deletion or rearrangement of author names in the authorship list should be made only before the manuscript has been accepted and previous approvement by the journal Editor. Requests of authorship changes please submit a letter to the Editor contain gin the following information: (a) the reason for the change in author list and (b) written confirmation (e-mail, letter) from all authors that they agree with the addition, removal or rearrangement. In the case of addition or removal of authors, this includes confirmation from the author being added or removed. No further changes are possible after manuscript has been accepted.

Proofs

A proof will be sent to the corresponding author for proof-reading in PDF format, and must be returned within the specified time, otherwise the Editor reserves the right to carefully proof-read the article but without assuming responsibility for errors, to continue with the publication process. Alterations to the proof that do not correspond to minor errors will be charged to the authors. Neither the Editor nor the Publisher accept any responsibility for errors that had not been indicated by the authors.

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