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Ana Isabel Abad Fau

Caracterización de patógenos entéricos de diferentes orígenes desde una perspectiva One Health
Characterization of enteric pathogens from different origins from a One Health perspective

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CARACTERIZACIÓN DE PATÓGENOS ENTÉRICOS DE DIFERENTES ORÍGENES DESDE UNA PERSPECTIVA ONE HEALTH
CHARACTERIZATION OF ENTERIC PATHOGENS FROM DIFFERENT ORIGINS FROM A ONE HEALTH PERSPECTIVE

Autor

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Tesis Doctoral

Caracterización de patógenos entéricos de diferentes orígenes desde una perspectiva One Health

Characterization of enteric pathogens from different origins from a One Health perspective

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2024

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CERTIFICAN:

Que Dª Ana Isabel Abad Fau ha realizado, bajo nuestra dirección los trabajos correspondientes a su Tesis Doctoral titulada "Caracterización de patógenos entéricos de diferentes orígenes desde una perspectiva One Health", que se corresponde con el plan de investigación aprobado por la Comisión de Doctorado y cumple los requisitos exigidos por la legislación vigente para optar al Grado de Doctor por la Universidad de Zaragoza, por lo que autorizamos su presentación para que pueda ser juzgado por el tribunal correspondiente. Asimismo, certificamos que durante el desarrollo de la Tesis Doctoral se realizó una estancia superior a tres meses en el Leiden University Medical Center (Países Bajos), bajo la supervisión del Dr. Wiep Klaas Smits.

En Zaragoza, a 03 de diciembre de 2024

Fdo.: Rosa Mº Bolea Bailo

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Abreviaturas

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English		Español	
μg	Micrograme	μg	Microgramo
μL	Microlitre	μL	Microlitro
μm	Micrometre	μm	Micrómetro
AMR	Antimicrobial resistance	AMR	Resistencia a los antimicrobianos
APEC	Aviar pathogen *E. coli*	APEC	*E. coli* patógena aviar
BHI	Brain heart infusion	BHI	Infusión cerebro corazón
bp	Base pairs	pb	Pares de bases
CA-CDI	Community adquired *C. difficile* infection	CA-CDI	Infección por *C. difficile* adquirido en comunidad
CC	Clonal complex	CC	Complejo clonal
CD	Clindamycin	CD	Clindamicina
CDC	Centers for Disease Control and Prevention	CDC	Centro de Control y Prevención de Enfermedades
CDI	*C. difficile* infection	CDI	Infección por *C. difficile*
CDMN	*C. difficile* moxalactam norfloxacin selective supplement	CDMN	Suplemento selectivo para *C. difficile* con moxalactam y norfloxacina
CdtLoc	Locus CDT	CdtLoc	Locus CDT
CECT	The Spanish type culture collection	CECT	Colección Española de Cultivos Tipo
cgMLST	Core genome Multilocus sequence typing	cgMLST	Tipificación por secuencia multilocus del genoma central
cgST	Core genome Sequence Type	cgST	Tipo de secuencia del genoma central
cgWGS	Core geneme Wole genoma sequence	cgWGS	Secuenciación completa del genoma central
CI	Confidence interval	IC	Intervalo de confianza
CLO	Comercial agar CLO, bioMérieux	CLO	Agar comercial CLO, bioMérieux
CLSI	Clinical and Laboratory Standards Institute	CLSI	El Instituto de Normas Clínicas y de Laboratorio
DAEC	Difusely adherent *E. coli*	DAEC	*E. coli* difusamente adherente
ddNTPs	Dideoxynucleotide	ddNPTs	Didesoxinucleótidos

English		Español	
DNA	Deoxyribonucleic acid	ADN	Ácido desoxirribonucleico
dNTP	Deoxynucleotide triphosphate	dNTP	Desoxirribonucleótidos trifosfato
E	Erythromycin	E	Eritromicina
EAEC	Enteroaggregative *E. coli*	EAEC	*E.coli* enteroagregativa
ECDC	The European Centre for Disease Prevention and Control	ECDC	Centro Europeo para la Prevención y el Control de las Enfermedades
EHEC	Enterohemorrhagic *E. coli*	EHEC	*E. coli* enterohemorrágica
EIEC	Enteroinvasive *E. coli*	EIEC	*E. coli* enteroinvasiva
EPEC	Enteropathogen *E. coli*	EPEC	*E. coli* enteropatógena
ERE	Epizoonotic rabbit enteropathy	ERE	Enteropatía epizoótica del conejo
ESBL	Extended spectrum beta-lactamase	BLEE	Beta-lactamasas de espectro extendido
ETEC	Enterotoxigenic *E. coli*	ETEC	*E. coli* enterotoxigénica
E-test	Epsilometer test	E-test	Test de epsilometría
EUCAST	The European Committee on Antimicrobial Susceptibility Testing	EUCAST	Comité europeo del antibiograma
ExPEC	Extraintestinal patogenic *E. coli*	ExPEC	*E. coli* patógena extraintestinal
g	Grame	g	Gramo
g	Gravitational force	g	Fuerza centrífuga relativa
h	Hour	h	Hora
HA-CDI	*C. difficile* infección-hospital adquired	HA-CDI	Infección por *C. difficile* de adquisición hospitalaria
HP-CIA	Highest priority critically important antibiotics	HP-CIA	Antibióticos críticamente importantes de prioridad alta
HUS	Hemolytic uremic syndrome	SUH	Síndrome urémico hemolítico
ICE	Integrative conjugative element	ICE	Elemento conjugativo integrativo
IF	Impact factor	FI	Factor de impacto
IS	Inssertion sequence	IS	Secuencia de inserción
L	Litre	L	Litro
LB Broth	Lysogeny broth	Caldo LB	Caldo de lisogenia

Abreviaturas

English		Español	
LTs	*E. coli* thermolabile toxins	LTs	Toxinas termolábiles de *E. coli*
MCK	Macconkey agar	MCK	Agar Macconkey
MDR	Multidrug resistance	MDR	Resistencia a múltiples drogas
MGE	Mobile genetic element	EGM	Elemento genético móvil
MIC	Minimum inhibitory concentration	CMI	Concentración mínima inhibitoria
min	Minute	min	Minuto
MIR	Miniature inverted repeat	MIR	Secuencia de repetición invertida
mL	Mililitre	mL	Mililitro
MLVA	Multiple-locus variable number tandem repeat analysis	MLVA	Análisis multi-locus de número variable de repeticiones en tándem
mm	Millimetre	mm	Milímetro
MSLT	Multilocus sequence typing	MLST	Tipificación por secuencia multilocus
MTZ	Metronidazole	MTZ	Metronidazol
MXF	Moxifloxacin	MXF	Moxifloxacina
NCBI	National Center for Biotechnology Information	NCBI	Centro Nacional para la Información Biotecnológica
nd	Not done	nd	No hecho
ND	No date	SF	Sin fecha
NGS	Next generation sequencing	NGS	Secuenciación de próxima generación
° C	Celsius degrees	° C	Grados Celsius
OR	Odds ratio	OR	Razón de probabilidades
PAI	Pathogenicity islands	PAI	Islas de patogenicidad
PaLoc	Pathogenicity locus	PaLoc	Locus de patogenicidad
PCR	Polimerase chain reaction	PCR	Reacción en cadena de la polimerasa
PL	Plasmid	PL	Plásmido
REA	Restriction enzime analysis	REA	Análisis por encimas de restricción
rpm	Revolutions per minute	rpm	Revoluciones por minuto
RT	Ribotype	RT	Ribotipo
SNP	Single nucleotide polymorphism	SNP	Polimorfismos de nucleótido único

English		Español	
ST	Sequence type	ST	Tipo de secuencia
STEC	Shiga toxin-producing *E. coli*	STEC	*E. coli* productora de shiga-toxinas
STs	*E. coli* thermostable toxins	STs	Toxinas termoestables de *E. coli*
Stx	Shiga-toxins	Stx	Shiga-toxinas
TBE	Tris-borate-EDTA	TBE	Tris-borato-EDTA
TE	Tetracycline	TE	Tetraciclina
TSA	Tryptone ssoya Agar	TSA	Agar triptona soja
TSC	Tryptose sulfite cycloserine agar	TSC	Agar triptona sulfito cicloserina
UPEC	Uropathogenic *E. coli*	UPEC	*E. coli* uropatogénica
UPGMA	Unweighted Pair Group Method using Arithmetic Averages	UPGMA	Agrupamiento pareado no ponderado utilizando media aritmética
UTI	Urinary tract infection	ITU	Infección del Tracto urinario
UV	Ultra violet	UV	Ultravioleta
VA	Vancomycin	VA	Vancomicina
WGS	Whole genoma sequence	WGS	Secuenciación completa del genoma

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Resumen

Resumen

Los enteropatógenos son organismos capaces de causar enfermedades en el tracto digestivo. En muchos casos, estos patógenos pueden afectar a múltiples especies simultáneamente, incluyendo a los seres humanos. Estas enfermedades representan una amenaza constante, no solo por el impacto en la salud, sino también por las pérdidas económicas que generan, especialmente en el caso de los animales de producción.

Siguiendo esta línea, el objetivo de esta tesis doctoral es el estudio en profundidad de tres enteropatógenos desde una perspectiva One Health. Para ello, se han seleccionado tres bacterias distintas por su relevancia clínica y epidemiológica: *Escherichia coli*, *Clostridioides difficile* y *Clostridium perfringens*, abarcando diversas especies afectadas y su interacción con el medio ambiente.

En el caso de *E. coli*, un patógeno clásico que también puede formar parte de la flora intestinal habitual de animales y personas, se estudió su presencia tanto en perros con patología urinaria como en conejos. *E. coli* puede presentarse de varias formas, conocidas como patotipos. En el caso del primer estudio de esta tesis, realizado en perros, los más encontrados fueron aquellos caracterizados como ExPEC. El estudio genético y fenotípico de sensibilidad a los antibióticos de los aislados reveló un elevado porcentaje (71,15 %) de multirresistencia. Además, algunos de los genes de resistencia se encontraron asociados a elementos genéticos móviles, lo que es especialmente relevante dada la creciente preocupación por el aumento de la resistencia antibiótica en poblaciones bacterianas, que limita la eficacia terapéutica de los antimicrobianos.

En la especie cunícola, el estudio de *E. coli* se centró en sus factores de virulencia, específicamente en la intimina, el factor de virulencia que con mayor frecuencia se asocia a casos de colibacilosis en conejos. También se destacó la presencia de un brote asociado a *E. coli* lactosa negativo, aunque no se encontraron factores de virulencia asociados. Además, se investigó la presencia de *C. difficile* y *C. perfringens*, encontrándose una baja prevalencia de *C. difficile* en las poblaciones de conejos de carne y un mayor aislamiento de *C. perfringens* en el intestino delgado.

La resistencia ambiental de *C. difficile* y *C. perfringens* se exploró mediante el análisis de muestras de aguas residuales. Ambas bacterias son especialmente resistentes en el medio ambiente debido a su capacidad de formar esporas. Se encontró una cantidad elevada de ambas bacterias (24 % y 75 %, respectivamente). La presencia de enterotoxina en los aislados de *C. perfringens* puede suponer un riesgo de diseminación de esta toxina al medio ambiente, actuando como reservorio para cepas que afecten el tracto digestivo de animales

y personas, causando enfermedad. Los resultados del análisis de resistencia antibiótica revelaron resistencias heterogéneas entre los aislados de ambas especies, generalmente mostrando niveles bajos de resistencia.

En la especie porcina, la presencia de *C. difficile* se ha asociado con diarrea neonatal. Sin embargo, tras el estudio de 475 lechones, no se encontró una asociación estadísticamente significativa entre la presencia de *C. difficile* y la diarrea. Además, un estudio posterior sobre las relaciones genéticas entre los aislados reveló heterogeneidad dentro de las granjas, indicando que no solo hay una población clonal afectando a los animales, sino varias cepas transmitiéndose simultáneamente. Los lechones fueron los principales implicados en la transmisión dentro de la granja a lo largo del tiempo, con el periodo neonatal siendo el más relevante en la propagación del patógeno.

Finalmente, la caracterización genética de los aislados de *C. difficile* de varias especies reveló una tendencia a agruparse según la especie de origen, aunque las diferencias generales fueron pequeñas, con una similitud del ribotipo 126 superior a la esperada. La presencia de genes accesorios conservados indica una alta estabilidad dentro de este ribotipo.

En conclusión, esta tesis destaca varios aspectos en la lucha contra los enteropatógenos. En primer lugar, se evidenció el riesgo de transmisión zoonótica y las complejas relaciones entre bacterias de animales, humanos y el medio ambiente. Asimismo, se subrayó la importancia de la variabilidad en la respuesta del hospedador, lo que implica que diferentes especies pueden reaccionar de manera distinta a la misma bacteria. Además, se identificaron diferencias en la resistencia antimicrobiana entre las especies bacterianas estudiadas, lo que resalta la necesidad de enfoques individualizados para la vigilancia epidemiológica. Estos hallazgos refuerzan la relevancia del enfoque One Health para la prevención y control de zoonosis, ya que integrando la salud humana, animal y ambiental se pueden desarrollar estrategias más efectivas para combatir estas enfermedades.

Summary

Enteropathogens are organisms capable of causing disease in the digestive tract. In many cases, these pathogens can affect a myriad of species simultaneously, including human beings. These diseases are a constant threat, not only due to the direct impact on health, but also due to the economic losses they generate, especially in production animals.

Hence, the objective of this doctoral thesis is to carry out an in-depth study of three enteropathogens from a One Health perspective. To achieve this, three different bacteria have been selected because of their clinical and epidemiological relevance: *Escherichia coli*, *Clostridioides difficile* and *Clostridium perfringens,* encompassing diverse affected species and their interaction with the environment

In *E. coli*, a classic pathogen than can also be part of common flora in animals and humans, its presence in dogs with urinary pathology as well as rabbits was studied. *E. coli* can be presented in various forms, known as pathotypes. In the case of the first study of this thesis, based on dogs, the most common pathotype found was ExPEC. In the genetic and phenotypic study of sensibility to antimicrobials, a high percentage (71.15 %) of multidrug resistance was found. Besides, some resistance genes were associated to genetic mobile elements, which is of special interest due to the increasing preoccupation in the rise of antimicrobial resistance in bacterial populations, which limits the effectiveness of antimicrobials.

In rabbits, the study of *E. coli* was centered in its virulence factors, especially intimin, the most frequently virulence factor associated with rabbit's colibacillosis. An outbreak of lactose negative *E. coli* was also identified, although no virulence factor associated was found. In addition, the presence of *C. difficile* and *C. perfringens* was studied, finding a low presence of *C. difficile* in meat rabbit populations and an increased isolation of *C. perfringens* in small intestine.

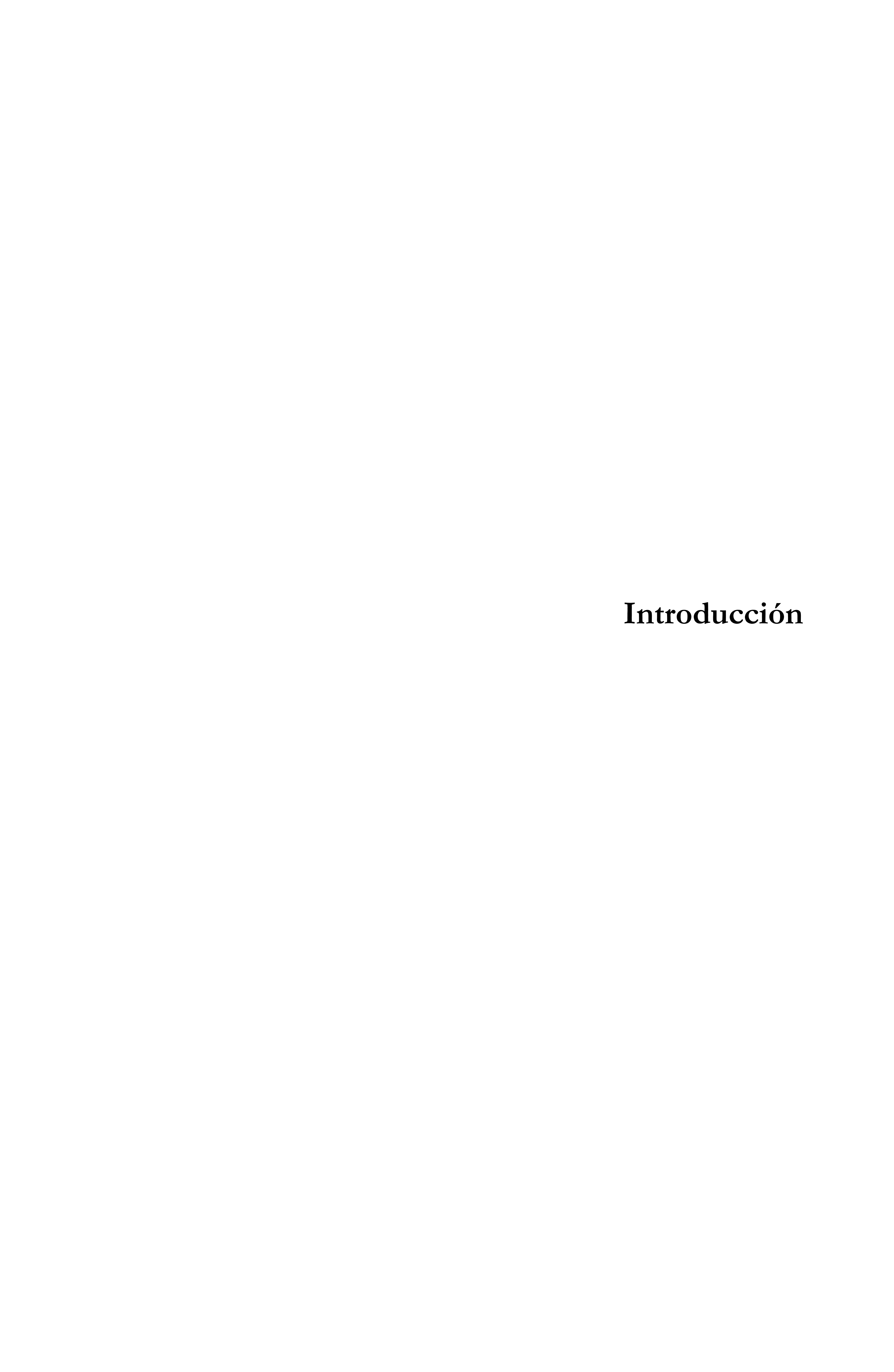
Ambiental resistance of *C. difficile* and *C. perfringens* was analyzed through the study of wastewater. Both bacteria are especially resistant in environmental conditions because of its capability to form spores, as shown by a high presence of both bacteria (24% y 75%, respectively). The presence of *C. perfringens* enterotoxin can be a risk factor in the dissemination of this toxin to the environment, acting as a reservoir to strains that affect the digestive system of animals and humans, causing disease. The results of the antimicrobial resistance analysis showed heterogeneous resistances in the isolates of both species, generally showing low levels of resistance.

Summary

In swine, the presence of *C. difficile* has been linked to neonatal diarrhea. However, after analyzing 475 piglets, no statistically significant association was found between the presence of *C. difficile* and diarrhea. Besides, a later study of the genetical relationship showed heterogenicity across farms, indicating that there is not only one clonal complex affecting animals, but various strains transmitting simultaneously. Piglets were the main factors of transmission in the farm through time, with the neonatal period being the most relevant in pathogen propagation.

Finally, genetic characterization of *C. difficile* isolates from several species displays a tendency to grouping according to their origin (specie), although differences were small, with a similarity across ribotype 126 higher than expected. The presence of conserved accessory genes implies a high stability within the ribotype.

In conclusion, this work highlights several aspects in the fight against enteropathogens. Firstly, the risk of zoonotic transmission was shown, as well as the complex relationship between animal, human and environmental bacteria. Likewise, the importance of variability in host response was stressed, which implies that different species can react in different ways to the same bacteria. In addition, difference in antimicrobial resistance between studied bacterial species was identified, which features the necessity of individualized approaches in epidemiological vigilance. This data enhances the relevance of the One Health approach in the prevention and control of zoonosis, integrating human, animal and environmental health to develop more effective strategies to fight these diseases.



Introducción

Introducción

Desde el inicio de la especie humana, personas, animales y patógenos han convivido juntos. Sin embargo, el término zoonosis— enfermedades compartidas por el ser humano y los animales— no fue introducido hasta el siglo XIX por el doctor Rudolf Virchow, aunque el concepto ya había sido explorado previamente (Singh et al., 2023). Desde entonces, la comprensión de la dinámica y epidemiologia de las infecciones no ha hecho más que crecer, hasta que en 2004 se proponen los principios de Manhattan con el objetivo de establecer una aplicación más completa en la gestión de y prevención de enfermedades (Cook et al., 2004). De esta conferencia, titulada "One World, One Health" surge la filosofía One Health, que ha experimentado un creciente interés desde entonces y ha sido recomendada como estrategia para el manejo de la salud, incluyendo planes en organizaciones tan importantes como la Unión Europea (por ejemplo, A European One Health Action Plan against Antimicrobial Resistance (AMR) o One Health European Joint Programme), el Centro de Control de Enfermedades de Estados Unidos (Global Health Security Agenda, Healthy Pets, Healthy People Program) o la organización mundial de la salud (CDC, 2022; Horizon Staff, 2023; WHO, 2023). Esta filosofía parte de la idea de que solamente existe una sola salud con diversos agentes implicados que se relacionan constantemente entre sí: personas, animales y medio ambiente. Por ello, las acciones que se tomen en uno de estos sectores tienen la capacidad de afectar, tanto positiva como negativamente, al resto.

En este contexto, los enteropatógenos son un tipo de organismos capaces de causar enfermedad en el tracto intestinal. Este tipo de agentes puede incluir virus, parásitos, hongos y, el objetivo de esta tesis doctoral, bacterias.

La presencia de enteropatógenos representa una preocupación en diversos sectores, ya que no solo afecta la salud, sino que también conlleva consecuencias en términos económicos, tanto en animales como en personas (Bartsch et al., 2016; Boolchandani et al., 2022; Hasan et al., 2021; Pinior et al., 2017; Zimmermann et al., 2019). Estos agentes patógenos tienen la capacidad de causar enfermedades gastrointestinales, desencadenando un amplio espectro de síntomas que van desde leves molestias hasta casos más graves de diarreas. Las pérdidas económicas derivadas de las diarreas causadas por enteropatógenos son multifacéticas. En el ámbito de la producción animal, se observa una disminución en la tasa de crecimiento, un aumento en la mortalidad y una reducción en la eficiencia de conversión alimentaria (Du et al, 2023).

Asimismo, los costes asociados con el tratamiento y la gestión de enfermedades diarreicas pueden ser abundantes, abarcando desde la adquisición de medicamentos hasta la implementación de prácticas de bioseguridad y medidas preventivas. En concreto, el aumento de resistencias antibióticas y su diseminación entre las distintas especies y el medio ambiente supone actualmente una de las principales amenazas contra la salud pública (European

Antimicrobial Resistance Collaborators, 2022). Este fenómeno se traduce en una disminución de la efectividad de los antibióticos disponibles, lo cual se asocia con un aumento en la mortalidad al restringir las opciones terapéuticas frente a enfermedades que, desde la instauración del uso generalizado de antibióticos, se consideraban controladas. Ante esta situación, es necesario abordar de manera integral la gestión de las resistencias antibióticas, destacando la necesidad de estrategias efectivas para preservar la eficacia de los tratamientos antimicrobianos y garantizar la salud pública a largo plazo.

En el espectro de las enfermedades gastrointestinales que afectan a los animales se destacan diversas causas, algunas de las cuales han adquirido un protagonismo debido a su incidencia y repercusiones. Entre las causas más comunes de estas patologías, destacan tres agentes patógenos que ejercen una influencia notable en la salud animal y humana a nivel mundial y de los que va a ser objeto esta tesis: *Escherichia coli* (*E. coli*), *Clostridioides difficile* (*C. difficile*) y *Clostridium perfringens* (*C. perfringens*).

En primer lugar, *Escherichia coli* (*E. coli*) se constituye como una de las principales amenazas, siendo reconocida como una causa de enfermedades gastrointestinales en la especie humana y en animales de distintas especies. En el informe más reciente de vigilancia de resistencias antibióticas en la Unión Europea, se observó que *E. coli* fue la bacteria más frecuentemente aislada, evidenciando casos frecuentes de multirresistencia (MDR) (ECDC, 2023). Además, también ocupó el cuarto lugar entre las zoonosis más comunes (EFSA & ECDC, 2023). La versatilidad y adaptabilidad de *E. coli* como bacteria patógena la convierten en un desafío constante para la sanidad, ya que su presencia y capacidad para inducir diarrea, en ocasiones severas, subrayan la importancia de comprender este patógeno a nivel mundial.

C. difficile es reconocido actualmente como un patógeno emergente. Aunque históricamente se ha considerado un agente nosocomial, en los últimos años este organismo ha sido aislado en suelo, alimentos y animales, abriendo las puertas a la valoración de otras vías de infección que no habían sido consideradas hasta ahora (Mitchell et al., 2022). Su elevada resistencia natural a antibióticos, unido a la aparición de nuevas cepas hipervirulentas y de resistencias a los tratamientos de elección, hacen de este agente una amenaza especialmente relevante en el panorama de la salud global.

Por último, *C. perfringens* es un patógeno habitual en casos de diarreas en animales, estableciendo una conexión estrecha con el entorno en el que se desarrollan. Su presencia ubicua en el medio ambiente y su capacidad para formar esporas contribuyen a su persistencia, convirtiéndola en una amenaza habitual para la salud animal y humana, especialmente asociada en el ser humano al consumo de alimentos (toxiinfección).

Así, estos tres patógenos representan un conjunto de desafíos a los que se enfrentan los sistemas de producción animal en todo el mundo. Comprender sus dinámicas, modos de transmisión y factores desencadenantes es esencial para desarrollar estrategias efectivas de prevención y control, no solo para preservar la salud animal, sino también para mitigar las pérdidas económicas asociadas a estas enfermedades gastrointestinales y proteger la salud pública.

Escherichia coli

E. coli es una bacteria Gram negativa, anaerobia facultativa, que forma parte de la microbiota intestinal de animales y humanos y que abarca una amplia diversidad de cepas con diferentes características y funciones, como la síntesis de vitaminas K y B, destacando como comensales habituales (Burkholder & McVeigh, 1942). Aunque comúnmente se asocia con infecciones gastrointestinales, es necesario reconocer que *E. coli* es un enteropatógeno versátil que puede afectar a otros sistemas y no siempre se manifiesta a través de diarrea o sintomatología digestiva.

En general, las cepas de *E. coli* patógenas se clasifican en patotipos, es decir, grupos que tienen en común características, factores de virulencia y sintomatología clínica (Kaper et al., 2004). A continuación, se exploran algunos de los distintos patotipos de *E. coli*:

1. *E. coli* enteropatógena (EPEC). Este patotipo es conocido por su capacidad para adherirse a las células intestinales, provocando la formación de lesiones en la mucosa, descritas histológicamente como "lesiones de adhesión y borrado". Fue el primer patotipo descrito y, aunque no siempre causa diarrea, puede desencadenar infecciones intestinales en animales jóvenes. En humanos es una de las causas más importantes de diarrea infantil en países en vías de desarrollo (Kaur & Dudeja, 2023).
2. *E. coli* enterotoxigénica (ETEC). Este patotipo produce toxinas que afectan la mucosa intestinal y estimulan la secreción de líquidos. Estas toxinas pueden ser de dos tipos: termolábiles (LTs) o termorresistentes (STs). Es una de las causas más comunes de diarrea en animales de producción, en muchas ocasiones siendo el único agente infeccioso aislado, aunque también puede causar diarrea en humanos (Dubreuil et al., 2016).
3. *E. coli* enterohemorrágica (EHEC). En general, la expresión clínica de este tipo de cepas es en forma de diarrea, tanto hemorrágica (colitis hemorrágica) como no hemorrágica, o produciendo el síndrome urémico-hemorrágico en la especie humana. Especialmente vinculada al ganado bovino, la enfermedad en seres humanos se suele asociar al consumo de carne de esta especie (infección alimentaria). La

patogenicidad de estas cepas está asociada a dos toxinas, las verotoxinas 1 y 2, también llamadas Shiga-toxinas, ya que su estructura y su actividad son semejantes a la toxina Shiga producida por *Shigella dysenteriae (Gray et al., 2014).*

4. *E. coli* enteroinvasiva (EIEC). Muy relacionadas con *Shigella spp.*, este patotipo tiene la capacidad de invadir células intestinales y provocar lesiones en la mucosa. Se asocia comúnmente con diarrea acuosa. A pesar de su carácter invasivo, las lesiones más profundas de la submucosa son poco frecuentes (Prats & Llovet, 1995).
5. *E. coli* enteroagregativa (EAEC). Este patotipo se caracteriza por su capacidad para adherirse en grandes cantidades a las células intestinales, principalmente en el colon, formando agregados (patrón de auto-agregación). En la especie humana suele estar asociado con diarreas persistentes. Aunque los mecanismos de acción siguen sin estar enteramente caracterizados, se pueden distinguir dos tipos: las cepas típicas que presentan el gen *AggR*, y las atípicas que no lo poseen (Kaper et al., 2004; Nataro & Kaper, 1998).
6. *E. coli* difusamente adherente (DAEC). Se caracteriza por su peculiar forma de adherencia a monocapas de células HEp-2, en forma de patrón difuso. Este patógeno ha sido identificado como una causa potencial de diarrea, y afecta a diversas funciones de las células intestinales. La mayor parte de estas cepas produce una adhesina fimbrial denominada F1845. La infección por DAEC podría presentar características proinflamatorias, lo que posiblemente se asocie con implicaciones en el desarrollo de enfermedades intestinales inflamatorias (Servin, 2005).
7. *E. coli* patógena extraintestinal (ExPEC). Aunque no está considerado como uno de los patotipos clásicos, esta clasificación hace referencia a aquellas cepas patógenas que causan infecciones extraintestinales. Estas cepas pueden causar cuadros clínicos muy variados, aunque habitualmente incluyen infecciones del tracto urinario, meningitis o bacteriemia (Russo & Johnson, 2000).

Con este contexto evolutivo y la elevada diversidad de las cepas de *E. coli*, la clasificación de los distintos patotipos está en constante revisión, como así denota la reciente propuesta de la inclusión del patotipo ExPEC o la inclusión del patotipo ECPA (*E. coli* patógena aviar), no descrita en esta introducción.

E. coli en conejo

La colibacilosis es una enfermedad que ha afectado en cunicultura, históricamente, a animales recién nacidos o a animales jóvenes (Zarzuelo Pastor, 1988). En condiciones normales, las cepas de *E. coli* cumplen funciones beneficiosas en el equilibrio de la microbiota intestinal. Sin embargo, ciertas cepas patógenas pueden causar problemas gastrointestinales, especialmente

cuando hay desequilibrios en la microbiota o factores de estrés ambiental. Es el primer agente asociado con enteritis-diarrea en gazapos lactantes, hasta los 28 días, aunque sigue siendo una causa importante durante toda la etapa productiva. La mayoría de las cepas que causan estos problemas se pueden clasificar como EPEC, aunque otros patotipos también son capaces de causar enfermedad (Rosell Pujol, 200). Tampoco son infrecuentes los casos de colibacilosis en los que existen otros procesos infecciosos simultáneamente. En general, los casos de colibacilosis en conejos suelen estar asociados al filogrupo B1, y varios estudios han encontrado resistencias antibióticas, incluyendo resistencia a colistina, antibiótico habitualmente efectivo frente a *E. coli* (Silva et al., 2023). La importancia de estas cepas emergentes resistentes a colistina radica en el uso de la colistina en la especie humana como antibiótico de último recurso, por lo que la diseminación de estas cepas resistentes supone un prolema para la salud pública (Danaei et al, 2023).

E. coli en perros

Las cepas patógenas de *E. coli* en perros representan un área de interés en la investigación veterinaria, ya que estas variantes pueden desencadenar diversas condiciones clínicas. Estos patógenos, en lugar de actuar como comensales, pueden dar lugar a infecciones gastrointestinales, urinarias y sistémicas en la especie canina.

En esta especie, aunque es habitual aislar *E. coli* pertenecientes a todos los patotipos, la forma más frecuente de infección es la forma uropatógena, que provoca infecciones del tracto urinario. Estas infecciones pueden presentarse con síntomas como micción frecuente, dolor al orinar y cambios en el comportamiento del animal. Tampoco son infrecuentes los casos de piometra o bacteriemia (EFSA Panel on Animal Health and Welfare et al., 2022).

Además, la presencia de cepas resistentes a antibióticos, cada vez más, presenta un riesgo para la salud pública y una limitación de las opciones terapéuticas que existen para tratar las enfermedades de estos animales. En la especie canina resultan de especial interés las resistencias BLEE (beta-lactamasas de espectro extendido), que en muchas ocasiones se encuentran también en cepas con resistencias a otros antibióticos (Marchetti et al., 2021). Además, al ser los perros animales de compañía frecuentemente asociados a los seres humanos, existe el riesgo de que estos animales hagan de reservorio de estas cepas multirresistentes, no solo por el intercambio que pueda existir en el domicilio de los dueños, si no al relacionarse los perros con el entorno urbano (por ejemplo, parques) (Damborg et al., 2023).

E. coli en la especie humana: zoonosis

Desde una perspectiva One Health, *E. coli* emerge como un patógeno de gran importancia. La resistencia antimicrobiana en cepas de *E. coli* comparte similitudes entre poblaciones humanas y animales, destacando la necesidad de abordar la resistencia desde una perspectiva que integre a todos los agentes de la cadena. La contaminación ambiental y alimentaria por cepas patógenas de *E. coli* subraya la interconexión entre la salud humana y animal, recalcando la necesidad de enfoques colaborativos en la gestión de la medicina animal.

De especial interés en la especie humana son los casos de toxiinfecciones, especialmente de aquellas cepas verotoxigénicas. El serotipo más frecuentemente implicado en estos brotes es el O157:H7, aunque también se han detectado otros serotipos en brotes de infecciones alimentarias, como el O26. La intoxicación alimentaria es, por tanto, la principal fuente de infección de los seres humanos por esta zoonosis (Ministerio de agricultura, pesca y alimentación, 2023).

Además de estos casos, que suelen estar asociados con la especie bovina y, en menor medida, con la porcina, existen otras especies que son capaces de afectar al ser humano por la trasmisión de esta zoonosis. En concreto, algunos estudios han puesto de manifiesto la posible trasmisión de *E. coli* uropatogénicas desde animales de compañía, como el perro, a la especie humana (Jacob & Lorber, 2015; Nam et al., 2013), con las consiguientes implicaciones que ello tendría para la salud pública y animal. En la especie humana, *E. coli* puede causar infecciones en el tracto urinario, con mayor frecuencia en mujeres debido a la anatomía de la uretra femenina. La prevalencia de infecciones urinarias por *E. coli* destaca su versatilidad en la afectación de diferentes sistemas del cuerpo humano (Flores-Mireles et al., 2015). En otras ocasiones, también se han aislado cepas de *E. coli* extraintestinales y multirresistentes asociadas a animales de compañía, lo que defiende el interés que el estudio de estos patógenos en animales puede tener de relevancia para la salud pública (Ahmed et al., 2015; Procter et al., 2014).

Clostridioides difficile

C. difficile es un patógeno con forma de bacilo, Gram positivo y anaerobio. Tiene capacidad de generar esporas, por lo que su resistencia a los medios comunes de desinfección es muy alta, además de tener capacidad de permanecer en superficies y fómites por varios meses (Deng et al., 2015). Normalmente, esta bacteria se considera flora habitual intestinal en el ser humano, aunque en bajas cantidades (Ferretti et al., 2023; Vedantam et al., 2012). Cuando se produce un cambio en la composición de la microflora (por ejemplo, tras el uso de antibióticos) es cuando se produce la colonización del intestino de forma oportunista por parte de *C. difficile*. De hecho, diversos estudios relacionan la

presencia de una microbiota sana con un efecto protector contra la germinación y colonización por *C. difficile* (Britton & Young, 2014; Pike & Theriot, 2021).

En la especie humana, el tratamiento con antibióticos, la edad avanzada y las estancias prolongadas en hospitales son los principales factores de riesgo, de ahí que tradicionalmente se haya considerado una enfermedad nosocomial (Czepiel et al., 2019). Sin embargo, con la aparición de cepas hipervirulentas, especialmente de los ribotipos 027 y 078 (Bauer et al., 2011), y, con los cambios de epidemiologia fundamentalmente asociados al 078 (personas más jóvenes y con frecuencia infecciones asociadas a la comunidad (Goorhuis et al., 2008), se pone de manifiesto la necesidad de profundizar en los métodos de trasmisión de este patógeno, además de la necesidad de explorar nuevas vías de infección prácticamente ignoradas hasta hoy.

La elevada resistencia antibiótica observada en *C. difficile* constituye una preocupación en el ámbito de la salud pública. Este patógeno, reconocido por su asociación con infecciones intestinales, ha desarrollado una notable capacidad para resistir múltiples clases de antibióticos. Tal resistencia, que incluye agentes considerados de primera elección en el tratamiento de infecciones bacterianas, ha planteado desafíos en el manejo clínico de las infecciones por *C. difficile*. De especial interés es la aparición de mecanismos de resistencia frente al metronidazol y a la vancomicina, dos de los tratamientos clásicos frente a *C. difficile* (siendo la vancomicina el tratamiento de elección, habiendo desbancado al metronidazol como primera opción terapéutica desde 2021 (Fitzpatrick et al., 2024; Jieun et al., 2022). La persistencia de cepas resistentes a antibióticos es un fenómeno intrínseco de este microorganismo, y su prevalencia subraya la importancia de gestionar estratégicamente la resistencia antimicrobiana en este contexto. La necesidad de medidas eficaces de control y prevención se vuelve aún más importante dado el potencial impacto de la resistencia antibiótica en la eficacia de las terapias y la gestión clínica de las infecciones asociadas a *C. difficile* (Peng et al., 2017).Cabe destacar que *C. difficile* no se limita a afectar exclusivamente a los seres humanos: se trata de un patógeno multiespecie. Este aspecto se traduce en estudios que evidencian la transmisión de cepas de *C. difficile* entre humanos y animales, particularmente cerdos (Moloney et al., 2021; Knight & Riley, 2019; Squire & Riley, 2013). La relación entre las cepas que afectan a estos animales y las que afectan a los humanos hace replantearse lo que conocemos sobre las rutas de transmisión y la influencia de factores ambientales en la propagación de esta bacteria. Por todo ello, se destaca la importancia de comprender la dinámica de este patógeno no solo en el ámbito clínico, sino también en el contexto de la salud pública y la interfaz humano-animal.

C. difficile en conejo

C. difficile es un patógeno que ha sido poco estudiado en la especie cunícola. Además, el hecho de que la distribución de la producción de carne de conejo sea tan limitada no hace más que reforzar este hecho (en Europa, el 83 % de la producción se encuentra entre España, Francia e Italia (European Commission, 2017), siendo España el cuarto productor de carne de conejo, a nivel mundial (FAO, SF)).

En uno de los mayores estudios epidemiológicos de *C. difficile* en conejos, realizado en Italia entre el 2007 y el 2013 (Drigo et al., 2015), encontraron que, aunque poco frecuente, este agente se encuentra circulando en granjas comerciales dedicadas a la producción de carne con fin al consumo humano. Además, las cepas aisladas en varias ocasiones presentaron resistencia a los antibióticos, incluyendo sensibilidad reducida al metronidazol.

La presentación clínica de las infecciones por *C. difficile* en conejos generalmente se manifiesta como diarrea, incluyendo inflamación y daño a la mucosa intestinal (Taha et al., 2019). En el conejo, los órganos más afectados son el yeyuno y el íleon, seguido por el ciego (Keel & Songer, 2006).

La investigación sobre *C. difficile* en conejos ha sido más limitada en comparación con estudios en otras especies, pero su presencia y capacidad patógena en estos animales acentúan la importancia de comprender su papel en la salud gastrointestinal de los conejos. Además, considerando el potencial zoonótico de *C. difficile*, la investigación en este ámbito adquiere una relevancia adicional, que justifica su inclusión en esta tesis doctoral.

C. difficile en cerdo

C. difficile también ha sido identificado en cerdos. La presencia de este agente se ha asociado con problemas gastrointestinales, incluyendo diarrea, aunque su papel en la salud porcina ha sido cuestionado recientemente, ya que es una bacteria que también se puede aislar en animales sanos (Uzal et al., 2023). La capacidad de formar esporas de *C. difficile* contribuye a su persistencia en el entorno, aumentando el riesgo de transmisión entre cerdos y a través del ambiente.

Se ha observado que ciertos ribotipos de *C. difficile* en cerdos son similares o idénticos a los encontrados en humanos, lo que sugiere la posibilidad de transmisión interespecie (Putsathit et al., 2019; Squire & Riley, 2013).

Clínicamente, *C. difficile* produce en cerdos una diarrea de moderada intensidad, especialmente en lechones lactantes hasta la semana de edad. Estas infecciones, a diferencia de otras especies, no parecen estar asociadas al consumo de antibióticos (Waters et al., 1998). Las lesiones afectan al ciego y/o colon, siendo frecuente el edema de mesocolon, con la mucosa ligeramente afectada o con

lesiones severas de necrosis multifocal a difusa. Microscópicamente, se observa una colitis o tiflocolitis erosiva, ulcerosa, necrotizante y neutrofílica, siendo frecuente encontrar lesiones "tipo volcán", que se corresponden con erosiones en la mucosa, a través de las cuales se produce exudación de fibrina y neutrófilos (Keel & Songer, 2006). El impacto económico de la diarrea neonatal en la producción porcina es de especial relevancia, ya que las infecciones pueden resultar tanto en la pérdida de animales por mortalidad como en un descenso de los índices de transformación del animal asociados a la infección.

C. difficile en la especie humana

C. difficile representa un desafío en la salud humana, especialmente con el aumento de las infecciones asociadas a la comunidad (CA-CDI), aunque menos prevalentes que su vertiente nosocomial (HA-CDI). En general, la infección por CA-CDI se ha vinculado con pacientes más jóvenes sin exposición previa a antibióticos ni asociación con el ámbito sanitario, y que presentan una mortalidad más baja que en su variante nosocomial (Kim & Zhu, 2017).

El cambio epidemiológico marcado por la emergencia de ribotipos como el 027 ha supuestos nuevos desafíoslas estrategias convencionales de prevención y control. La capacidad de *C. difficile* para generar nuevos ribotipos sugiere una evolución constante que requiere una respuesta clínica y de investigación rápida.

En la especie humana, una de las fuentes de trasmisión de *C. difficile* entre pacientes son las manos de los trabajadores, debido a la falta efectividad de los jabones en base de alcohol, que se usan frecuentemente en los hospitales para una higeniciación rápida de las manos del personal hospitalario, pero incapaz de eliminar las esporas de *C. difficile*, las cuales son resistentes al alcohol (Jabbar et al., 2010). También es muy frecuente la trasmisión por el ambiente, ya sea a través de fómites, como puede ser el instrumental quirúrgico, especialmente el asociado a colonoscopias, (Borji et al., 2022) como por trasmisión intrapacientes en el propio hospital (McHaney-Lindstrom et al., 2019).

El potencial zoonótico de *C. difficile* se manifiesta en la identificación de cepas similares en humanos y animales, especialmente en cerdos. La transmisión interespecie plantea interrogantes sobre la dinámica de este patógeno, destacando la importancia de la colaboración entre disciplinas (Hensgens et al., 2012; Indra et al., 2009).

Clostridium perfringens

Al igual que en el caso de *C. difficile*, *C. perfringens* es un bacilo anaerobio, Gram positivo, con capacidad de formar esporas. Por esta razón, es un agente que tiene una elevada presencia en el medio ambiente, y cuya versatilidad le permite tanto actuar como agente patógeno como permanecer largos periodosen el ambiente.

Ubicuo en la naturaleza, *C. perfringens* se encuentra comúnmente en el suelo, agua, y en el tracto gastrointestinal de animales y humanos (Brynestad & Granum, 2002). Aunque su presencia es frecuente en estos entornos, cabe destacar que no siempre desencadena enfermedad. Además, su capacidad patógena suele estar muy asociada a los factores de virulencia que posea esta bacteria. Por ello, *C. perfringens* es frecuentemente catalogado en toxinotipos, una clasificación basada en los tipos de toxinas que producen. Cada toxinotipo está asociado a distintas enfermedades y especies animales. Esta especificidad de especie es particularmente marcada, contribuyendo a su adaptación a diferentes hospedadores y ambientes (Uzal et al., 2018; Uzal et al., 2010). De manera general, el toxinotipo A es el más frecuentemente aislado, aunque en muchos casos no es capaz de causar enfermedad; en casos en que produce enfermedad suele asociarse a enterocolitis o a abomasitis en rumiantes. El tipo B y C están asociados a enteritis necrotizante en rumiantes, con el C afectando también a lechones, mientras que el tipo D causa enterotoxemia. El tipo E causa enteritis en vacuno, ovino y conejo, y el F causa gastroenteritis hemorrágica en perro y colitis en caballo. Uno de los tipos más importantes es el tipo F, cuya enterotoxina es responsable de su asociación con infecciones alimentarias en la especie humana. Por último, el tipo G se relaciona con enteritis necrótica en aves (Anju et al., 2021).

C. perfringens en conejo

Las enfermedades producidas por el género *Clostridium* han sido un problema en el conejo asociado a importantes pérdidas económicas, tanto por su mortalidad como por las pérdidas de ganancias productivas asociadas a la diarrea que provoca (Solans et al., 2019). Entre estos otros Clostridia se destacan cuatro: *C. spiroforme, C. perfringens, C. piliforme* y *C. cuniculi.* De estos, el más alejado de las características clásicas del género es *C. piliforme*, causante de la enfermedad de Tyzzer, puesto que es un bacilo Gram negativo intracelular. Las lesiones macroscópicas clásicas de *C. perfringens* afectan al intestino, hígado y corazón, con afección. En el digestivo es frecuente el edema intenso en el ciego, en el hígado necrosis miliar multifocal y, en el corazón, miocarditis y miocardosis. Clínicamente, puede producir diarrea acuosa de forma aguda en animales jóvenes, con una elevada mortalidad, mientras que en animales adultos es más frecuente que se produzca retraso en el crecimiento, con una mortalidad cercana solamente al 5 % (Mansfield & Fox, 2019; Rosell Pujol, 2000).

El género *Clostridium*, incluyendo a *C. perfringens*, está normalmente asociado a cuadros de enterotoxemia (Gelberg, 2017). A diferencia de otras especies, donde *C. perfringens* es la causa más habitual de enterotoxemia, en conejos es *C. spiroforme* el causante más implicado (Rosell Pujol, 2000). La toxina que producen estas bacterias está estrechamente relacionada con la toxina iota de *C. perfringens*. En general, la diarrea está asociada al consumo de antibióticos,

aunque también se han descritos casos de enterotoxemia espontánea en gazapos recién destetados. En ambos casos, la enterotoxemia, se manifiesta cuando las bacterias proliferan y producen toxinas.

Además, la descripción de *C. cuniculi* asociado a la enteropatía enzootica del conejo (Djukovic et al., 2018) complican aún más el panorama de las enfermedades digestivas del conejo. Esta enfermedad ha sido vinculada en otras ocasiones con el género *Clostridium* (Bäuerl et al., 2014), aunque sus causas siguen siendo desconocidas (Puón-Peláez et al., 2020). Además, las coinfecciones entre bacterias, especialmente junto a *E. coli*, son frecuentes (Solans et al., 2019).

C. perfringens en el medio ambiente

C. perfringens exhibe una presencia ubicua en el medio ambiente, siendo común en suelos, agua y materiales orgánicos (Uzal et al., 2014). Aunque, en general, su presencia no desencadena problemas de forma directa, la versatilidad de *C. perfringens* también implica la capacidad de volverse un riesgo potencial en determinadas circunstancias.

En situaciones donde la carga bacteriana es elevada o cuando se producen condiciones propicias para la proliferación bacteriana, *C. perfringens* puede convertirse en un peligro. La formación de esporas resistentes contribuye a su persistencia en el medio ambiente, y en ciertos contextos, la presencia de cepas toxigénicas puede desencadenar problemas de salud, tanto en animales como en humanos.

Particularmente, en cuerpos de agua utilizados para recreación o como fuentes de suministro de alimentos, la presencia elevada de *C. perfringens* puede indicar contaminación fecal y sugerir un riesgo potencial para la Salud Pública. La producción de enterotoxinas por algunas cepas de *C. perfringens* puede estar asociada con intoxicaciones alimentarias, lo que subraya la importancia de monitorear y gestionar la presencia de esta bacteria en entornos críticos (Vierheilig et al., 2013).

C. perfringens en la especie humana

C. perfringens, a pesar de ser parte común de la microbiota intestinal humana, puede desempeñar un papel dual en la salud de las personas. En condiciones normales, esta bacteria anaerobia no causa enfermedades significativas y coexiste de manera comensal en el tracto gastrointestinal, contribuyendo al equilibrio de la microbiota (Kiu & Hall, 2018).

Sin embargo, *C. perfringens* tiene la capacidad de producir diversas toxinas, y la presencia de cepas toxigénicas puede dar lugar a infecciones, especialmente asociadas con la ingestión de alimentos contaminados. La alta capacidad de *C. perfringens* para sobrevivir a elevadas temperaturas y su rápida multiplicación hace que la infección por este agente sea una causa importante de intoxicación alimentaria (Zea & Salazar, 2012; Kiu & Hall ,2018). En este caso, son las células vegetativas, que sobreviven a la presencia de los ácidos gástricos, las que generan toxinas en este ambiente. Los casos de diarrea están asociados especialmente a la enterotoxina, que se une al epitelio intestinal, produciendo el acortamiento de las vellosidades y descamación del epitelio, y, con ello, la diarrea (Shrestha et al., 2018).

Además, *C. perfringens* también es conocido por ser causante de la gangrena gaseosa. Las células vegetativas o esporas invaden tejidos lesionados, produciendo graves necrosis, que pueden llegar a ocasionar la muerte (Uzal et al., 2014; Yao & Annamaraju, 2023).

Cabe destacar que, a pesar de su potencial patógeno, la mayoría de las cepas de *C. perfringens* en el intestino humano no son toxigénicas y no causan enfermedad. La presencia de la bacteria en la microbiota intestinal es parte de la flora normal, y su impacto negativo generalmente está asociado con circunstancias específicas, como la contaminación de alimentos (Ravinder et al., 2015).

Objetivos y justificación

El objetivo general de esta tesis doctoral es la valoración de la presencia y de la importancia de ciertos enteropatógenos en distintas especies animales y en el medio ambiente, tales como *Escherichia coli, Clostridioides difficile* y *Clostridium perfringens*. Además, se pretende su caracterización, la cual estará principalmente enfocada al análisis de perfiles de resistencia antibiótica y de los principales factores de virulencia que presentes en cada especie bacteriana.

Para cumplir con el objetivo general, se han planteado los siguientes objetivos específicos:

1. Describir cepas de *E. coli* con capacidad uropatogénica en perros, su sensibilidad antimicrobiana y factores de virulencia más importantes. Además, caracterizar genéticamente las cepas con mayor resistencia fenotípica antimicrobiana.
2. Realizar un estudio de la presencia de *C. difficile* en conejos y valorar su asociación con diarrea o síntomas clínicos digestivos. También se valoran la aparición de otros patógenos frecuentes en problemas digestivos, concretamente *E. coli* y *C. perfringens.*
3. Estudiar la presencia de *C. perfringens* y *C. difficile* en aguas residuales de origen humano y nosocomial. Asimismo, caracterización de los aislados encontrados.
4. Analizar la aparición temporal de *C. difficile* en granjas de cerdos, caracterizar los aislados encontrados y evaluar los perfiles de resistencia antibiótica.
5. Realizar una revisión de los métodos más utilizados para caracterizar molecularmente aislados de *C. difficile*
6. Realizar un estudio de secuenciación completa del genoma (WGS) en varias cepas del ribotipo 126 de *C. difficile*, procedentes de distintas especies animales. Utilizar genómica comparativa para evaluar las asociaciones y diferencias de los aislados.

A lo largo del tiempo, los patógenos entéricos han representado una constante fuente de pérdida económica y de bienestar en diversas comunidades, tanto humanas como animales. Entre estos enteropatógenos clásicos se encuentra *E. coli*, reconocido por su capacidad para provocar enfermedades gastrointestinales y, en algunos casos, condiciones más graves. Sin embargo, la dinámica de las enfermedades infecciosas continúa evolucionando, y la emergencia de nuevos retos, como *C. difficile*, subraya la constante necesidad de gestionar estos problemas de manera continua y adaptativa. Estos patógenos, con sus características únicas y la capacidad de afectar tanto a humanos como a animales, imponen desafíos constantes para la salud pública, la medicina veterinaria y la gestión del medio ambiente. La comprensión de la interacción entre estos patógenos y su entorno, así como la vigilancia de las amenazas emergentes, son esenciales para mitigar los impactos negativos que estos agentes infecciosos pueden tener en la economía y el bienestar general.

Objetivos y justificación

Con la evolución de la filosofía One Health, se destaca la creciente importancia de reconocer que la salud es una entidad única, donde lo que afecta a los seres humanos repercute directamente en los animales y el medio ambiente, y viceversa, debido a la interconexión intrínseca entre estos componentes. La perspectiva One Health promueve una comprensión holística de los sistemas de salud, reconociendo que la salud de una especie no puede considerarse de manera aislada.

La presencia ubicua de los agentes estudiados, sumada a sus diversas formas de transmisión, justifica la necesidad de continuar la investigación en este ámbito. La complejidad de la interacción entre estos patógenos, el medio ambiente y las diferentes rutas de transmisión, algunas aún por descubrir, plantea desafíos que requieren un enfoque científico continuo. Además, en este contexto, no se debe ignorar la influencia de los portadores asintomáticos, cuyo papel en la transmisión de estas bacterias es esencial pero aún no completamente comprendido. El estudio de estos factores es fundamental para diseñar estrategias de prevención y control efectivas, así como para identificar posibles puntos críticos en la cadena de transmisión que podrían pasar desapercibidos con los enfoques tradicionales.

Además, las bacterias tienen capacidad de transferir genes horizontalmente, no limitándose solo a su propia especie, sino también estableciendo intercambios genéticos con otras bacterias de géneros considerablemente distintos. Este fenómeno es particularmente preocupante en el caso de la resistencia a los antibióticos, donde la transferencia horizontal de genes presenta graves consecuencias un plazo muy corto de tiempo. Este proceso puede llevarse a cabo a través de diversas modalidades, como la transmisión horizontal directa, la participación de plásmidos que actúan como vectores de genes de resistencia o la intervención de elementos genéticos móviles, entre otros mecanismos. La facilidad con la que se puede llevar a cabo la transferencia de genes relacionados con la resistencia antimicrobiana destaca la importancia de abordar este fenómeno tanto desde la investigación como de la práctica clínica, dado que puede contribuir significativamente a la rápida propagación y persistencia de la resistencia a los antibióticos en diversas poblaciones bacterianas.

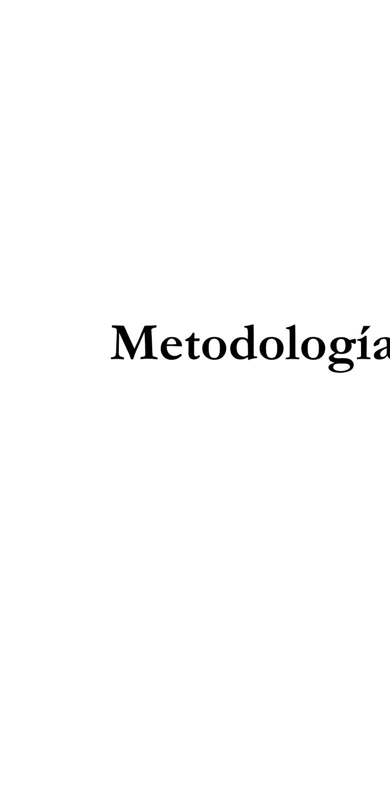
En el marco de esta tesis, se propone el estudio de tres agentes bacterianos específicamente seleccionados debido a sus características distintivas. *E. coli*, una bacteria ubicua, desempeña un papel dual como comensal y patógeno, siendo en ocasiones empleada como indicador de resistencia antimicrobiana por estas características (Castillo et al., 2022). La consideración de *E. coli* como una enfermedad re-emergente destaca la importancia de comprender su dinámica epidemiológica en constante evolución. Su presencia tanto en la microbiota normal como en casos patológicos subraya su versatilidad y la necesidad de abordar su papel en la resistencia a los antimicrobianos desde un enfoque integral que considere tanto la salud humana como animal.

En este sentido, desde su cambio de epidemiologia, *C. difficile* también ha pasado a considerarse una enfermedad emergente, tanto por el aumento en la virulencia de los casos debido a la aparición de cepas hipervirulentas, como por el incremento de infecciones asociadas a la comunidad (Kim & Zhu, 2017). Además, se plantea la consideración de *C. difficile* como una zoonosis emergente, explorando la posibilidad de contagio entre animales y personas. Esta perspectiva amplía el alcance de la investigación, destacando la importancia de evaluar la transmisión potencial entre distintas especies y su impacto en la salud general.

En el cierre de la selección de agentes bacterianos de esta tesis se seleccionó *C. perfringens*, una bacteria frecuentemente asociada a sintomatología digestiva, que suele recibir poca atención por no causar brotes con alta letalidad de forma frecuente, pero está siempre presente en diversos entornos. El estudio de esta bacteria no solo aporta beneficios directos, como su utilidad como indicador de contaminación fecal o la necesidad de investigarlo en casos de brotes alimentarios, sino que también destaca por su ubicuidad y su capacidad de resistir numerosos factores ambientales. La presencia habitual de *C. perfringens* en diversos contextos sugiere su relevancia como un indicador general en estudios de microbiología ambiental y clínica, remarcando la importancia de entender su papel en la salud pública y su potencial impacto en situaciones de contaminación y brotes infecciosos.

Por último, el aumento de la resistencia antibiótica constituye una preocupación constante en el panorama global actual. Esta preocupación ha impulsado cambios en las estrategias de gestión, marcadas por un aumento en la vigilancia epidemiológica y restricciones en el uso de antibióticos, especialmente en el ámbito de los animales de compañía y de producción. La aparición vinculada a estos animales de resistencias antimicrobianas frente a agentes comúnmente utilizados como primera opción en la medicina humana acentúa la necesidad de una evaluación constante de estas resistencias. Este aspecto adquiere importancia dado que, además de afectar a los animales donde se identifican los problemas, estas resistencias pueden transmitirse y afectar negativamente al ser humano, ya sea a través de animales de compañía como mascotas o mediante el consumo de carne procedente de animales de producción. La comprensión de esta problemática es esencial para implementar medidas eficaces que mitiguen los riesgos asociados con la resistencia antimicrobiana y preserven la eficacia de los tratamientos en medicina humana y veterinaria.

Metodología



En el complejo escenario de las enfermedades infecciosas, el diagnóstico constituye el inicio del proceso que conduce al tratamiento adecuado y, con ello, hacia la recuperación de la salud, tanto a nivel individual como poblacional.

Dentro del amplio abanico de las enfermedades infecciosas, las enfermedades entéricas suponen un área de especial preocupación, siendo frecuentemente causa de pérdidas económicas importantes en la industria de la producción animal, y una de las principales causas de pérdidas de la salud tanto en animales como en la especie humana. La patología digestiva de origen infeccioso presenta una morbilidad y mortalidad importantes a nivel mundial y el diagnóstico precoz no solo es crucial para el tratamiento individualizado, sino también para la implementación de medidas de salud pública que prevengan su propagación.

El diagnóstico de las enfermedades entéricas, con su diversidad de agentes etiológicos que incluyen bacterias, virus, parásitos y hongos, plantea desafíos únicos. La similitud de los síntomas entre diferentes patologías entéricas, como la diarrea, el dolor abdominal y la fiebre, requiere un enfoque diagnóstico profundo y diferencial. Es aquí donde la precisión de las metodologías de diagnóstico, desde el cultivo microbiológico hasta las avanzadas técnicas de biología molecular, cobra especial relevancia.

En este sentido, en esta tesis, se han utilizado tres enfoques distintos para el diagnóstico de los patógenos seleccionados: microbiológico, anatomopatológico y de biología molecular. A continuación, se pretende contextualizar cada uno de estos enfoques, así como explicar las técnicas utilizadas. El objetivo de esta sección es, por tanto, facilitar una visión global de las mismas para más tarde detallar los protocolos seguidos en cada trabajo en sus propios apartados de material y métodos.

Diagnóstico Microbiológico Tradicional

La medicina veterinaria tradicional siempre ha necesitado de otras disciplinas para poder llevar a cabo exploraciones diagnósticas complejas. En el campo de las enfermedades infecciosas, la microbiología siempre ha desempeñado un papel esencial, tanto para el diagnóstico como para los estudios epidemiológicos de las poblaciones.

Uno de los primeros pasos en el descubrimiento de los microorganismos fue el descubrimiento de los "animálculos", en 1676, por Anton van Leeuwenhoek, describiendo por primera vez lo que posteriormente serían protozoos, bacterias y levaduras, con los que sentó las primeras bases de la disciplina de la microbiología (Osorio Abarzúa, 2020). Sin embargo, no fue hasta varios siglos después, en la década de 1860, cuando Louis Pasteur demostraba la relación entre estos microorganismos y las enfermedades, la teoría germinal de las enfermedades (Institut Pasteur, 2016). Unos años después, en 1882, Robert

Metodología

Koch, a partir del aislamiento de *Bacillus anthracis,* desarrolló lo que hoy se conoce como los postulados de Koch (actualmente conocidos como postulados de Henle-Koch), que deben cumplirse para establecer una relación causal entre un microorganismo específico y una enfermedad (Day, 2013).

Prácticamente de forma simultánea, en 1892, Dmitri Ivanovsky filtró el jugo de hojas de tabaco enfermas y descubrió un organismo más pequeño que las bacterias y que podía causar enfermedad: los virus. Este hallazgo inició una nueva dimensión en la microbiología, introduciendo un nuevo tipo de agentes patógenos capaces de infectar todas las formas de vida, desde plantas y animales hasta humanos (Lecoq, 2001).

En este sentido, la microbiología, con su comprensión de los microorganismos y su papel en la salud y la enfermedad, sirve como un puente entre la medicina humana y veterinaria. Los mismos principios y metodologías utilizados para el diagnóstico y estudio de enfermedades infecciosas en humanos se aplican también en el ámbito veterinario, y muchas de las enfermedades con las que se sentaron las bases de la microbiología actual son, de hecho, zoonosis. Los microorganismos no reconocen fronteras entre especies, y muchas enfermedades infecciosas pueden afectar tanto a humanos como a animales.

Estos agentes microscópicos juegan roles protagonistas en el equilibrio de los ecosistemas y en la patogénesis de una amplia gama de enfermedades. El diagnóstico microbiológico, por tanto, se convierte en un pilar fundamental en la medicina clínica, permitiendo no solo la detección de estos organismos sino también la comprensión de su comportamiento, resistencia, y la manera en que interactúan con sus huéspedes. Por ello, en esta sección se van a explicar las distintas metodologías frecuentemente utilizadas en el campo de la microbiología, en concreto de la bacteriología, todas ellas utilizadas a lo largo de esta tesis en los distintos capítulos, profundizando en los fundamentos teóricos detrás de las mismas.

1. Aislamiento y uso de medios específicos

El primer paso necesario antes de la identificación bacteriana tradicional es el aislamiento de los microorganismos. En general, consiste en la separación de los microorganismos que existen en una muestra y su cultivo en medios que proporcionen condiciones óptimas para su crecimiento. Una de las condiciones más sencillas para diferenciar los microorganismos es según su requerimiento de oxígeno para su metabolismo: aerobio o anaerobio. Los anaerobios son organismos que pueden crecer y desarrollarse en ausencia total de oxígeno, mientras que los aerobios necesitan oxígeno para su crecimiento. Por otro lado, los anaerobios facultativos tienen la capacidad de vivir tanto en presencia como en ausencia de oxígeno, adaptándose a condiciones variables. En este sentido, las bacterias caracterizadas en esta tesis son de anaerobia para *C. difficile* y *C. perfringens* y anaerobia facultativa para *E. coli*.

La existencia de diferencias en el metabolismo bacteriano ha permitido el desarrollo de medios de cultivo específicos, diseñados para fomentar el crecimiento de ciertos microorganismos mientras se inhibe el de otros. Estos medios pueden ser manipulados en términos de composición nutricional, pH y presencia de agentes antimicrobianos, entre otras estrategias.

En relación con los medios utilizados en esta tesis, el agar sangre, agar nutritivo y el agar Triptona-Soja se clasifican como medios generales. Estos medios no requieren ningún tipo de suplementación especial y proporcionan un entorno de cultivo adecuado, versátil y no selectivo para una amplia variedad de bacterias.

En cuanto a los agares MacConkey, Agar Triptona Sulfito Cicloserina (TSC) y el agar CLO utilizados, se clasifican como medios específicos y diferenciales. Los medios específicos están diseñados para restringir el crecimiento de ciertos microorganismos mientras favorecen el crecimiento de otros. Además, en muchas ocasiones los medios diferenciales contienen colorantes o químicos que producen características fenotípicas o patrones de crecimiento distintivos, lo que permite la identificación de microorganismos basada en la interpretación de los resultados.

El mecanismo que utiliza el agar MacConkey para considerarse medio selectivo se basa en la incorporación de sales biliares y cristal violeta en su composición. Estos compuestos tienen la capacidad de inhibir el crecimiento de bacterias Gram positivas al dañar su membrana celular. Como resultado, las bacterias Gram negativas pueden crecer sin competencia en este medio. Además, el agar MacConkey contiene lactosa y un indicador de pH, generalmente rojo neutro. Cuando las bacterias fermentan la lactosa, producen ácido láctico como producto de desecho. Este ácido acidifica el medio, lo que provoca un cambio de color en el indicador de pH. Este cambio de color permite detectar la fermentación de lactosa y diferenciar entre bacterias lactosa-fermentadoras (que formarán colonias de color rosado o rojo) y bacterias no lactosa-fermentadoras (que formarán colonias incoloras o transparentes). En este medio, que se utilizó para la identificación de *E. coli*, las colonias deben verse de color rosa al ser, en general, una bacteria lactosa positiva. También se han descrito cepas de *E. coli* lactosa negativas (Mazumder et al., 2022), aunque durante el desarrollo de los diferentes trabajos únicamente se han descrito en el segundo estudio.

En el agar TSC, la presencia de cicloserina es esencial para inhibir crecimientos indeseados, lo que resulta en el desarrollo de colonias de menor tamaño. Esto facilita la observación de las colonias de *C. perfringens* al disminuir el ennegrecimiento difuso que a menudo puede dificultar la identificación precisa, ya que la identificación de esta bacteria se realiza en

base a su capacidad de producir sulfuro de hidrogeno y una coloración negra. Además, la incorporación de lecitina en el medio genera una zona de precipitación alrededor de las colonias que producen lecitinasa, lo que mejora la identificación de este microorganismo. El último medio utilizado, CLO agar, a través de una mezcla comercial de peptonas, taurocolato y ciertos antibióticos, favorece la germinación de las esporas mientas que dificulta el crecimiento de otros microorganismos, permitiendo la identificación de *C. difficile*. La composición del agar es similar a la utilizada en la fabricación del caldo selectivo de *C. difficile*, con la diferencia de que la base es un caldo de infusión cerebro-corazón (brain heart infusion, BHI) en lugar de agar. Esta modificación se realiza con el objetivo de proporcionar un medio líquido adecuado para el crecimiento óptimo de la bacteria.

De manera similar, la morfología de cada microorganismo es dependiente tanto de la especie como del medio en el que crezca, por lo que su estudio puede proporcionar información sobre la identidad de los agentes. Algunas de las características más frecuentemente estudiadas son la forma, el tamaño, color, textura u olor.

2. Tinción de Gram

La tinción de Gram es una técnica de tinción diferencial que permite distinguir entre bacterias Gram positivas y Gram negativas, según las diferencias en la composición de su pared celular. Esta tinción se basa en el uso de dos colorantes distintos, un fijador y una solución de lavado para eliminar el primer colorante (Tripathi & Sapra, 2024).

Aunque existen distintas variaciones del protocolo, en esta sección únicamente se explicará protocolo seguido durante esta tesis y sus fundamentos.

En primer lugar, las bacterias han de ser fijadas a la llama en un portaobjetos transparente, partiendo de una colonia pura para una clara identificación. El primer paso consiste en la tinción de las bacterias con cristal violeta durante 30 segundos. Este colorante básico se une a las moléculas de peptidoglicano en la pared celular. Posteriormente, se lava y se añade lugol durante otros 30 segundos. El yodo molecular entra en las células y forma con el colorante cristal violeta un complejo insoluble en agua, ayudando a fijarlo en las bacterias. A continuación, se lava con una solución de alcohol-acetona. Este paso es crucial porque elimina el colorante de las bacterias Gram negativas, que tienen una capa delgada de peptidoglicano en su pared celular y una membrana externa adicional, pero no pared celular. En las bacterias Gram positivas, que tienen una pared celular gruesa de peptidoglicano, pero carecen de membrana externa, el alcohol no penetra fácilmente y el colorante se mantiene. Finalmente, se aplica durante otros 30 segundos fuchina diluida, que tiñe las bacterias Gram negativas de color rosado, mientras que las bacterias Gram positivas permanecen de color violeta o azul oscuro.

A la observación al microscopio de los microorganismos seleccionados en esta tesis, los tres presentan morfología bacilar, siendo *E. coli* Gram negativo y *C. perfringens* y *C. difficile* Gram positivos.

Aunque la tinción de Gram es una técnica ampliamente utilizada en microbiología para diferenciar bacterias en dos grupos principales, es importante destacar que no todas las bacterias se tiñen de manera uniforme o efectiva con esta técnica. Algunas bacterias pueden mostrar resultados atípicos o inconsistentes en la tinción de Gram debido a diversas razones, como la variabilidad en la composición de su pared celular, la presencia de envolturas adicionales o el tiempo y la condición del cultivo bacteriano (Liechti et al., 2014).

3. Pruebas bioquímicas

El uso de pruebas bioquímicas para la identificación de bacterias implica la evaluación de la actividad enzimática, el metabolismo de carbohidratos, proteínas y otros sustratos por parte de los microorganismos. Estas pruebas proporcionan información sobre las características metabólicas de los microorganismos, ayudando a su identificación y diferenciación. Aunque el estudio de la fermentación de la lactosa o de lecitinasas mencionadas en el apartado de medios selectivos podrían considerarse pruebas bioquímicas, existen otras muchas opciones. Algunas de las pruebas bioquímicas más comúnmente utilizadas para distinguir entre diferentes especies bacterianas incluyen las pruebas de TSI (triple sugar iron agar), citrato de Simmons, SIM (sulfide, indole, motility), urea, oxidasa, catalasa e indol. Estas pruebas son fundamentales en microbiología clínica y de laboratorio para la identificación y caracterización de bacterias en muestras clínicas y ambientales. Cada una de estas pruebas evalúa diferentes características metabólicas o enzimáticas de las bacterias, lo que permite diferenciar entre especies y géneros bacterianos.

Entre estas técnicas, la prueba del indol es la que se ha utilizado con mayor frecuencia para la identificación de *E. coli*, típicamente positiva. Esta prueba se realiza para determinar su capacidad para descomponer el indol, derivado del aminoácido triptófano. Esta división molecular es llevada a cabo por una serie de enzimas intracelulares distintas, conjunto conocido comúnmente como triptofanasa. El indol se genera mediante una desaminación reductiva del triptófano a través de la molécula intermedia ácido indolpirúvico. Como ocurre con muchas pruebas bioquímicas, los resultados de la prueba de indol se interpretan mediante el cambio de color que sigue a la reacción, con una reacción positiva manifestándose como un viraje a color rosa (Darkoh et al., 2015).

4. Pruebas de susceptibilidad antimicrobiana

Estas pruebas evalúan la sensibilidad de los microorganismos a diferentes agentes antimicrobianos, como antibióticos y agentes quimioterapéuticos. La determinación de la susceptibilidad antimicrobiana es crucial para seleccionar el tratamiento más efectivo y evitar el desarrollo de resistencia bacteriana.

Las pruebas *in-vitro* de sensibilidad pueden realizarse siguiendo distintos protocolos, siendo los formatos más comunes la difusión en disco, la dilución en agar, la macrodilución en caldo, la microdilución en caldo y la prueba de gradiente de concentración. Cada uno de estos procedimientos tiene condiciones específicas, incluyendo tiempos de incubación, medios de cultivo usados o tipo de inoculo utilizado.

En el caso de *C. difficile*, la metodología recomendada para la realización de antibiogramas es la epsilometría. Esta técnica consiste en utilizar tiras de papel impregnadas con concentraciones en gradiente conocidas de antibióticos dispuestas en una placa de agar, donde se siembra de forma uniforme el microorganismo a estudiar a una concentración conocida.

Tras un período de incubación, se observa la formación de halos de inhibición alrededor de las tiras donde el antibiótico ha impedido el crecimiento bacteriano. Estos halos se forman debido a la difusión del antibiótico desde la tira hacia el agar, que impedirá crecer al microrganismo en el caso de que sea susceptible, creando una zona de inhibición alrededor de la tira (Figura 1).

Figura 1. E-test de vancomicina de un asilado de *C. difficile*, delante de la jarra de anaerobiosis utilizada para su crecimiento. La Concentración Mínima Inhibitoria está definida en 0.38 µg/L. En el halo central el crecimiento se encuentra inhibido por la presencia de antibiótico, mientras que en la zona periférica el crecimiento es normal.

La interpretación de los resultados se realiza observando la posición de corte del halo con la tira. Este resultado se compara con las tablas de referencia para determinar si el microorganismo es sensible, resistente o intermedio a la acción del antibiótico en cuestión.

En el caso de los antibiogramas para evaluar la sensibilidad del metronidazol en *C. difficile*, es crucial considerar el medio utilizado, ya que puede influir en los resultados al ser esta resistencia hemina dependiente. Por ello, para realizar los antibiogramas, se utilizó un agar Brucella comercial complementado con hemina vitamina, K y suplementado con un 5 % de sangre de oveja (Boekhoud et al., 2021).

5. VITEK2: identificación y antibiograma

El sistema de identificación VITEK2 es una herramienta diagnóstica desarrollada por la empresa bioMérieux para la identificación de bacterias, hongos y levaduras. Su metodología se basa en la utilización de reacciones bioquímicas y el consumo de nutrientes por parte de las distintas bacterias, cuyas modificaciones son detectadas por los instrumentos ópticos del propio equipo.

Este sistema tiene en cuenta la cantidad de inoculo inicial y utiliza los patrones de crecimiento para compararlos con su base de datos interna. Si los resultados obtenidos presentan una similitud del 85 % o más con los de la base de datos, se genera un resultado de identificación. En caso contrario, se indica un fallo en la identificación, por lo que es necesario buscar técnicas alternativas de identificación.

De manera similar, el sistema VITEK2 también ofrece la posibilidad de realizar antibiogramas. Utilizando las curvas de crecimiento y patrones conocidos, se calculan las concentraciones mínimas inhibitorias (CMI) para los distintos antibióticos probados. Es importante destacar que, previamente, es necesario indicar qué organismo se está analizando, ya que cada especie bacteriana tiene una curva de crecimiento característica y puede responder de manera diferente a los antibióticos (BioMérieux, SF).

El sistema VITEK2 es ampliamente utilizado en laboratorios clínicos y de microbiología para realizar pruebas de identificación bacteriana de manera rápida y precisa, especialmente debido a su fácil estandarización, lo que facilita el diagnóstico y tratamiento de enfermedades infecciosas. Su capacidad para detectar una amplia variedad de microorganismos lo convierte en una herramienta muy valiosa en la práctica médica y en la investigación microbiológica.

Metodología

La microbiología tradicional veterinaria, aunque es esencial para el diagnóstico de enfermedades infecciosas en animales, presenta tanto ventajas como limitaciones que deben considerarse.

En cuanto a sus ventajas, la microbiología tradicional está ampliamente disponible en clínicas y laboratorios veterinarios, lo que facilita un diagnóstico rápido y local. Además, la microbiología veterinaria desempeña un papel esencial en la vigilancia epidemiológica y el control de enfermedades zoonóticas. Asimismo, es relativamente económica en comparación con técnicas más avanzadas, lo que la hace accesible para una amplia gama de profesionales y propietarios de animales, tanto en animales de abasto como de compañía. Por último, ofrece la posibilidad de establecer perfiles fenotípicos de los microorganismos, lo que puede ser útil para su clasificación inicial y caracterización.

En cuanto a sus limitaciones, uno de los principales desafíos es el tiempo prolongado necesario para obtener resultados, ya que muchos microorganismos requieren períodos de incubación prolongados. Esto puede resultar en retrasos en el inicio del tratamiento, con las consecuencias que ello puede ocasionar. Además, algunas bacterias de difícil crecimiento o poco comunes pueden ser difíciles de identificar con las técnicas microbiológicas tradicionales, lo que puede llevar a errores diagnósticos o la necesidad de utilizar métodos más avanzados para su detección. Finalmente, la falta de sensibilidad en algunos casos puede ser un problema, ya que la microbiología tradicional puede no detectar niveles bajos de microorganismos en muestras clínicas, lo que puede resultar en falsos negativos o en una subestimación de la carga microbiana real.

Diagnóstico Anatomopatológico

El origen de la patología veterinaria es muy antiguo, pues históricamente la necropsia de animales se ha realizado desde la antigüedad (Gagea-Iurascu et al., 2012). No obstante, esta rama de la medicina veterinaria no comenzó a desarrollarse de una forma científica hasta finales del siglo XIX y principios del XX, cuando los avances en la microscopía y la comprensión de la patología humana impulsaron el interés por estudiar las enfermedades en animales. Hasta esta fecha, la mayor parte de las descripciones de los hallazgos eran únicamente macroscópicas, dejando de lado la histología (Cheville, 2013). Con el tiempo, esta disciplina ha evolucionado para convertirse en una herramienta indispensable para el diagnóstico, la investigación y la comprensión de las enfermedades que afectan a los animales, siguiendo aproximaciones mucho más completas que aúnan la información aportada por la patología macroscópica y microscópica.

Las técnicas utilizadas relacionadas con esta disciplina fueron:

1. Necropsia

Es el procedimiento post mortem que se realiza en el cadáver de los animales para valorar el estado de los órganos y tejidos con el fin de determinar la causa de la muerte o de evaluar el estado de salud de los animales. Durante la necropsia, se realizan observaciones macroscópicas detalladas de los órganos, seguidas de la toma de muestras para el análisis histopatológico o para otros estudios, tales como microbiológicos, parasitológicos, etcétera. En el caso del aparato digestivo, es importante resaltar que es especialmente sensible a los fenómenos de autolisis propios de las primeras horas tras la muerte del animal, por lo que las muestras deben tomarse lo antes posible tras su muerte, especialmente si se desea realizar histología de los tejidos.

Esta técnica es utilizada durante el trabajo 2, en el cual se tomaron conjuntamente las muestras para el estudio anatomopatológico y microbiológico. La toma de muestras es un momento crítico en el desarrollo de los estudios, ya que una ejecución incorrecta podría comprometer la interpretación de los resultados posteriores.

2. Frotis directo

Esta técnica consiste en la extensión de una muestra biológica sobre un portaobjetos transparente y su posterior fijación y tinción para su observación microscópica. Se puede utilizar, en ocasiones, durante la necropsia y permite observar la morfología y la disposición de los microorganismos en la muestra, lo que puede proporcionar pistas sobre su identidad y naturaleza. Además, facilita una cuantificación directa y simplificada de la cantidad de microorganismos presentes en la misma. Esto contrasta con el enfoque convencional de estudio microbiológico, donde las

bacterias aisladas pueden no ser representativas del estado real de la presencia de microorganismos en la muestra.

3. Fijación en formol y corte de muestras

El proceso inicial para la obtención de muestras histológicas implica la fijación de los tejidos en formol. Esta técnica se lleva a cabo con el propósito fundamental de preservar la estructura de los tejidos y prevenir su degradación. Por consiguiente, debe asegurarse una penetración adecuada del líquido en los tejidos, requiriendo la eliminación de la materia orgánica que pueda acumularse en ellos antes de su inmersión en el formol. El formol, una solución de formaldehído, penetra en los tejidos y establece enlaces cruzados con las proteínas, lo que conlleva a la estabilización de las estructuras celulares y previene su descomposición. Este proceso de fijación posibilita una conservación a largo plazo y asegura la integridad de las características histológicas, aspecto esencial para alcanzar un diagnóstico preciso y fiable (Ramos-Vara & Miller, 2014).

Una vez fijadas las muestras, el siguiente paso consiste en el corte de los órganos y su inclusión en parafina. Estos bloques resultantes son los que posteriormente se cortaran con micrótomo para generar las preparaciones a evaluar histológicamente.

4. Tinción hematoxilina-eosina

Esta tinción es una de las diversas técnicas que se pueden realizar para visualizar las preparaciones histológicas, y también es la más habitual. Está basada en el uso de dos colorantes: la hematoxilina tiñe los núcleos celulares de color azul o violeta, mientras que la eosina tiñe el citoplasma y otros componentes celulares de color rosa o rojo. Esta técnica permite visualizar la morfología celular y la arquitectura tisular bajo el microscopio, lo que facilita la identificación de anomalías histológicas, como inflamación, necrosis, fibrosis o degeneraciones, entre otros fenómenos.

5. Histología

La histología es la ciencia que estudia los tejidos biológicos y su estructura microscópica. Se basa en el análisis de secciones delgadas de tejido (llamadas cortes histológicos) que se tiñen con diferentes colorantes para resaltar las características morfológicas de las células y los componentes tisulares. Esta técnica proporciona una comprensión detallada de la organización celular, la composición de los tejidos y las alteraciones patológicas a nivel microscópico

El diagnóstico por anatomía patológica presenta una serie de ventajas en la medicina veterinaria, entre las cuales se destaca su capacidad para proporcionar una evaluación detallada de los tejidos y órganos afectados por enfermedades. Esta técnica permite identificar anomalías morfológicas y patológicas a nivel tanto macroscópico como microscópico, lo que facilita la detección temprana de enfermedades a nivel poblacional. Sin embargo, estas ventajas pueden estar limitadas por la necesidad de personal altamente capacitado para interpretar las muestras histopatológicas. Además, la obtención de resultados histológicos puede alargarse en el tiempo, lo que puede retrasar el inicio del tratamiento o la toma de decisiones clínicas. A pesar de estas limitaciones, el diagnóstico por anatomía patológica sigue siendo una herramienta indispensable en la práctica veterinaria, contribuyendo a la mejora del diagnóstico, tratamiento y comprensión de las enfermedades en los animales.

Diagnóstico Molecular

La biología molecular es, de las tres disciplinas desarrolladas en esta introducción, la disciplina más reciente. El término biología molecular fue acuñado en 1938 (Weaver, 1970), convirtiéndose actualmente en una disciplina que une conocimientos de campos muy diversos: bioquímica, genética, microbiología, virología y física. En el contexto de enfermedades bacterianas, los avances en biología molecular han sido fundamentales para mejorar el diagnóstico y el tratamiento de estas afecciones.

El verdadero desarrollo de esta disciplina aplicada al diagnóstico bacteriano se podría remontar a 1976, con el aislamiento de la primera Taq ADN polimerasa a partir de la bacteria *Thermus aquaticus*, base de la reacción en cadena de la polimerasa (PCR). Esta técnica proporcionó una herramienta para la detección de bacterias patógenas a partir de muestras clínicas, entre otros usos, lo que permitió diagnósticos más rápidos y precisos que los métodos tradicionales de cultivo.

Con el tiempo, la PCR se ha combinado con otras técnicas moleculares, como la secuenciación genómica y la hibridación de ácidos nucleicos, para desarrollar pruebas diagnósticas más avanzadas. Estas técnicas permiten, entre otras aplicaciones, la identificación específica de genes bacterianos y la caracterización de la resistencia a los antimicrobianos, lo que guía el tratamiento antibiótico de manera más precisa y ayuda a prevenir la propagación de cepas resistentes.

Desde entonces, los avances han seguido sucediéndose, destacando el rápido desarrollo de la disciplina en la última década, lo que han permitido el aumento de los datos obtenidos y el descenso de la carga económica. Los avances en la secuenciación de próxima generación (NGS) han ampliado aún más las capacidades de diagnóstico molecular, permitiendo la secuenciación rápida y a gran escala de genomas bacterianos completos. Esto ha facilitado la vigilancia epidemiológica de enfermedades bacterianas, la identificación de brotes y la trazabilidad de cepas patógenas en entornos clínicos y de salud pública.

1. PCR Convencional

   La PCR es una técnica fundamental en biología molecular que permite amplificar regiones específicas de ADN. Todas las bacterias analizadas durante esta tesis presentan doble hélice de ADN, por lo que se procederá a explicar las condiciones para este caso específico.

   La reacción de la PCR consta de tres fases que se repiten durante varios ciclos: la desnaturalización, el alineamiento de los cebadores y la extensión. La desnaturalización consiste en calentar la mezcla de la reacción a una temperatura elevada (alrededor de 95 °C). Esto rompe los enlaces de hidrógeno entre las dos cadenas de ADN, lo que resulta en la separación de

las hebras y la formación de dos cadenas simples de ADN. Posteriormente, se enfría la muestra a una temperatura más baja (alrededor de 50-60 °C, en función de la secuencia de los cebadores). A esta temperatura, se alinean los cebadores con las secuencias complementarias en las hebras de ADN simple. Finalmente, una vez que los cebadores están unidos a las hebras de ADN, se eleva la temperatura a alrededor de 72 °C, que es la temperatura óptima para la actividad de la enzima Taq polimerasa. Esta enzima sintetiza nuevas cadenas de ADN utilizando los cebadores como punto de partida y construyendo nuevas hebras complementarias a las hebras de ADN original. La Taq polimerasa agrega nucleótidos libres a la cadena, extendiéndola. Estos tres pasos se repiten durante varios ciclos, normalmente entre 20 y 40. Después de completar los ciclos, se obtienen millones de copias del fragmento de ADN específico que se desea amplificar (Khehra et al., 2024).

Para poder llevar a cabo la técnica, son necesarios varios componentes: el ADN de la muestra, cloruro de magnesio, Taq polimerasa, cebadores (también conocidos como primers), dNTPs, tampones y agua. La función que realizan los tampones y el agua es la de aportar un medio con las condiciones correctas para que se pueda llevar a cabo la reacción. El cloruro de magnesio se agrega a la reacción de PCR para proporcionar iones de magnesio, los cuales son cofactores para la actividad de la enzima Taq polimerasa, que es la encargada de realizar la reacción. Los dNTPs (desoxirribonucleótidos trifosfato) son los bloques de construcción básicos para la síntesis de ADN durante la PCR. Son moléculas que constan de un azúcar (desoxirribosa), un grupo fosfato (trifosfato) y una base nitrogenada (adenina, timina, citosina o guanina). Durante la PCR, los dNTPs se incorporan en la nueva cadena de ADN durante la extensión de la misma. La Taq polimerasa utiliza estos dNTPs para agregar nucleótidos complementarios a la cadena de ADN objetivo. Por último, los cebadores son oligonucleótidos cortos diseñados específicamente para hibridar con secuencias de ADN complementarias a ambos extremos de la región que se desea amplificar durante la PCR. Estos cebadores son fundamentales ya que determinan qué segmento de ADN se amplificará en la reacción. Aunque normalmente las reacciones incluyen únicamente un par de cebadores, que delimitan la región que será copiada durante la PCR al ser complementarios a cada una de las cadenas de ADN de la hélice, en ocasiones se pueden añadir más pares, lo que se conoce como PCR-Multiplex.

Una vez finalizados los ciclos de amplificación de la PCR, el resultado se revela en un gel de agarosa y colorantes fluorescentes en luz ultravioleta, aunque también existen otras técnicas de revelado. Al no poder ser evaluada la transcripción hasta este punto, a este tipo de reacción también se la conoce como PCR a punto final. Existen modificaciones de esta técnica, tal como la PCR a tiempo real, que permite la cuantificación durante el proceso de

amplificación de la Taq polimerasa, sin embargo, esta técnica no se ha utilizado durante el desarrollo de esta tesis doctoral.

2. Secuenciación Sanger

La secuenciación de Sanger es un método para determinar la secuencia de nucleótidos en un fragmento de ADN. Fue desarrollado por Frederick Sanger y su equipo en la década de 1970 (Sanger et al., 1977) y ha sido una herramienta clave en la investigación genética y molecular desde entonces. La muestra de partida debe ser un fragmento de ADN que puede tener distintos orígenes, pero debe estar en forma purificada y en cantidad suficiente para el análisis. En el caso de esta tesis, todos los fragmentos secuenciados procedían de amplificación por PCR convencional.

La técnica comienza de manera similar a la técnica habitual de PCR, pero la reacción de la transcripción es distinta. Una primera diferencia es que no se utilizan los cebadores en pareja, sino que solamente se incluye el cebador de la cadena de interés. A partir de la muestra con el cebador, en lugar de añadir dNTPs, se incluyen pequeñas cantidades de desoxinucleótidos marcados con fluorocromos de colores diferentes, uno para cada nucleótido (ddNTPs). Estos dideoxinucleótidos carecen del grupo hidroxilo del carbono 3', de manera que su incorporación en la cadena finaliza la elongación. Durante la reacción, la Taq polimerasa sintetiza una nueva cadena de ADN complementaria al fragmento de ADN original, utilizando los dNTPs presentes en la reacción. En esta reacción, se utilizarán tanto dNTPs normales como ddNTPs. Cuando se hace la lectura a través de electroforesis, un láser detecta la intensidad de la secuencia, creando "picos" con los colores de cada base. Como las cadenas tendrán distintos tamaños ya que los ddNTPs habrán cortado la cadena en distintos fragmentos, la electroforesis presentará distintas bandas. Identificar la posición de esos ddNTPs en la cadena permite secuenciar la cadena (Gomes & Korf, 2018).

La secuenciación de Sanger es una técnica ampliamente utilizada en la investigación científica, y en concreto en esta tesis se ha realizado para dos motivaciones distintas: confirmación de resultados de PCR y para la tipificación multilocus de secuencias (MLST). En el primer caso, la secuenciación de los genes permitió poder verificar resultados dudosos, y descartar falsos positivos. En el caso del MLST, esta técnica se emplea para caracterizar el perfil genético de bacterias mediante la secuenciación de múltiples genes constitutivos en su genoma. Estos genes han sido específicamente seleccionados por la comunidad científica para proporcionar información sobre la diversidad genética y las relaciones evolutivas entre diferentes cepas bacterianas. Al comparar las secuencias de estos genes con bases de datos existentes, se pueden analizar las relaciones evolutivas y epidemiológicas entre las bacterias, lo que permite comprender mejor su variabilidad genética y su importancia clínica y epidemiológica.

3. Secuenciación Completa

Aunque existen varias tecnologías que se pueden utilizar para secuenciar un genoma completo, actualmente destacan dos: la tecnología Nanopore e Illumina. Nanopore se basa en el análisis de moléculas simples, utilizando enzimas para detectar las bases que pasan a través de un poro. Illumina por otra parte, utiliza moléculas fluorescentes para realizar la secuenciación final (Qin, 2019).

En esta tesis, únicamente la tecnología Illumina ha sido utilizada. De forma más profunda, esta herramienta está fundamentada en la secuenciación Sanger, aunque mientras que la secuenciación Sanger puede reconocer fragmentos de hasta 1000 pares de bases (bp), las lecturas de Illumina son inferiores a 300 bp. En este caso, los fragmentos de ADN se unen a una placa mediante adaptadores específicos. Los fragmentos de ADN unidos son sometidos a una secuenciación por síntesis, donde se añaden nucleótidos fluorescentes marcados con diferentes colores uno por uno, y luego se detecta la emisión de luz para determinar el nucleótido incorporado en cada posición. Este proceso produce simultáneamente millones de lecturas cortas que son utilizadas para detectar la posición de los nucleótidos y construir la secuencia final. Estas lecturas cortas son después analizadas con diversos algoritmos informáticos para generar secuencias más largas.

Tal y como su nombre indica, el objetivo final de esta técnica es obtener toda la información genética de un organismo. Esto posteriormente puede tener varias aplicaciones, como puede ser la detección de resistencias antibióticas, el estudio epidemiológico de brotes o el análisis de genes de virulencia, entre otros.

4. Ribotipado Molecular

Esta metodología ha sido tradicionalmente utilizada para la caracterización de cepas de *C. difficile*, especialmente para la investigación de brotes y vigilancia epidemiológica. Se basa en las diferencias específicas y únicas en las regiones ribosómicas interespaciadoras 16-23S que cada ribotipo posee, ya que son específicas. La cantidad y longitud de estas regiones varía, por lo que la PCR de la región intergénica genera fragmentos de distintas longitudes, que pueden observarse a través del revelado en gel o con tecnologías específicas (por ejemplo, analizadores genómicos). Los patrones que generan las bandas se comparan con bases de datos existentes, lo que permite la identificación del ribotipo. Entre las limitaciones de este método se encuentra la baja reproductibilidad entre laboratorios debido a lo complejo de la puesta a punto, y sobre todo la imposibilidad de obtener los resultados a través de técnicas de secuenciación completa, ya que los operones rRNA incluidos en el tipado molecular no pueden ser

correctamente identificados por las herramientas de secuenciación actuales (Janezic, 2016; Seth-Smith et al., 2021).

La aplicación de técnicas de biología molecular en bacteriología permite aumentar la rapidez en la obtención de resultados, permitiendo un diagnóstico más ágil de las enfermedades bacterianas. Además, proporciona una cantidad considerable de información, ofreciendo una visión más profunda de la estructura genética y la diversidad bacteriana que no se puede obtener mediante métodos convencionales. Sin embargo, los fallos de identificación, tanto falsos positivos como negativos, pueden ocurrir y son más difíciles de detectar. Los altos costes de los reactivos y equipos necesarios para realizar estas técnicas pueden ser una barrera para su implementación generalizada en entornos con recursos limitados. Además, la presencia de inhibidores en las muestras y la posible necesidad de procesamientos adicionales pueden introducir artefactos y complicaciones en el proceso de análisis.

Trabajos de investigación

Trabajo 1

Multidrug resistance in pathogenic *Escherichia coli* isolates from urinary tract infections in dogs, Spain

ORIGINAL RESEARCH article

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Multidrug resistance in pathogenic *Escherichia coli* isolates from urinary tract infections in dogs, Spain

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Introduction

Urinary tract infections are one the most common causes of primary care veterinary supervision in dogs and a treatment challenge due to their high recurrence and therapeutic implications. *Escherichia coli* is the most common bacterium isolated in UTIs in dogs and humans (Flores-Mireles et al., 2015; Hall et al., 2013; Teh, 2022). In addition, *E. coli* bacteremia in humans (the most common cause of bacteremia in high-income countries) is caused by urinary tract infections in more than 50% of cases (Bonten et al., 2021). *Escherichia coli* has also been associated with an increase in antimicrobial resistance (Hall et al., 2013; Yousefi et al., 2017). Evidence suggests that dogs act as a reservoir of human infections with uropathogenic *E. coli* (UPEC) and are a source of spread of antimicrobial resistance (Jacob et al., 2015.

Escherichia coli is classified into various pathotypes based on the presence of virulence factors. Uropathogenic *E. coli* is included within the group of extraintestinal pathogenic *E. coli* (ExPEC) and are characterized by specific virulence factor. Some of these virulence factors include P-fimbriae (*papC*), α-hemolysis (*hlyA*) and cytotoxic necrotizing factor type 1 (*cnf1*) (Landraud et al., 2003). *Eae*, the gene that codifies for intimin and is associated with diarrheic strains, can also be found in uropathogenic strains (Abe et al., 2008). Other relevant *E. coli* virulent factors include Shiga toxins (*Stx*), also known as verotoxins and characteristic of Shiga toxin-producing *E. coli* (STEC), which are related to hemorrhagic diarrhea and hemolytic uremic syndrome, and have been described in several species, although natural infections are rarely described in dogs (de Brito et al., 1999; Dell'Orco et al., 2005). There is a potential zoonotic risk associated with the presence of these genetic elements in companion animals and other species (Persson et al., 2007; García et al., 2021). Hybrid strains have gained recent attention, especially those that harbor several virulence factors traditionally associated with different pathotypes. These new types of strains are considered "heteropathogen" or hybrid, such as STEC/UPEC strains (Yousefi et al., 2017), and are considered as able to produce both outcomes, diarrhea, or UTI (Gati et al., 2021).

Virulence genes are encoded by plasmids, bacteriophages, or pathogenicity islands (PAI). Pathogenicity islands are mobile and unstable fragments of DNA present in pathogenic strains, but absent in the related non-pathogenic strains, which can be shared by horizontal transmission. *PapC*, *hlyA* and *cnf1*, among other virulence genes, are

usually encoded simultaneously within PAIs in UPEC (Lloyd et al., 2007; Martin et al., 2009). P-fimbriae, encoded by *papC* gene, plays an important role in kidney adherence and the inflammatory response (Lane et al., 2007). α-Hemolysin is a toxin known to produce renal injuries, and even though the mechanism is still unclear, *cnf1* does not play a major role in the severity of the disease but it is usually associated with other virulence genes (Martin et al., 2009).

It has been previously demonstrated that some canine UPEC isolates are clonal with those isolated from humans, suggesting their zoonotic potential. It has also been proposed that dogs could act as a reservoir of this *E. coli* pathotype, hence the importance of the study of the potential implications of UTI in this animal species (Johnson et al., 2000; Johnson et al., 2001a).

In the last few decades there has been a rising concern about the increase in the number of *E. coli* isolates presenting a multidrug resistant (MDR) profile (Silwedel et al., 2016; ECDC et al., 2020). It has been described that the ownership of companion animals could be a risk factor in the spread of pathogenic *E. coli* strains between humans and pets, also favoring the dissemination of antimicrobial resistance in the community (Ukah et al., 2018; Köck et al., 2018; Zechner et al., 2020).

It is common to find antibiotic resistance in *E. coli* isolates from cases of UTI, which highlights the importance of monitoring the strain susceptibility to the antibiotic treatment, even if an experimental treatment has already been implemented. In fact, UPEC strains isolated from dogs have been described as MDR reservoirs in several countries (Rzewuska et al., 2015; Yudhanto et al., 2022) and as carriers of extended-spectrum beta-lactamase (ESBL) genes (Yousefi et al., 2017). ESBL-producing *E. coli* have been previously found in cats and dogs, and human-dog co-carriage in the same household has also been demonstrated in fecal samples (Costa et al., 2004; O'Keefe et al., 2010; van der Bunt et al., 2020). In general, ESBL and the presence of other antibiotic resistance mechanisms can difficult the treatment of infectious diseases and, therefore, result in complicated chronic infections.

Although microbiological culture and susceptibility testing are recommended before any antimicrobial therapy is established, empiric treatment is frequently established and the most common recommendations to treat these infections in companion animals include amoxicillin (without clavulanic acid) and trimethoprim-sulfonamides as a first approach (Weese et al., 2019). Therefore,

updated information about antimicrobial susceptibility patterns is highly needed.

The aim of this study was to determine the presence of *E. coli* in urine samples from a Spanish dog population presenting clinical signs of urinary tract infections and to characterize the isolates according to selected virulence factors and their antimicrobial resistance pattern, a research field scarcely investigated in Spain.

Materials and methods

Collection of *Escherichia coli* isolates

This study was conducted on a total of 52 *E. coli* isolates. This collection of isolates came from urine samples from dogs diagnosed with UTI. Samples were aseptically collected by cystocentesis as part of the **daily** activity of private veterinary practitioners in Zaragoza, Spain. The criteria followed to diagnose UTI were those used in everyday clinic, which include frequent urination, pain during urination, fever or vomiting, among others. The sampling period ranged from 2017 to 2019, and all urine samples were taken before any treatment was established. Mean age of the individuals was 8.97 years old (95% CI: 4.11–13.83%). In regard to the gender of these individuals, 21 of them were male and 31 were female. Isolates were identified using VITEK® (bioMérieux, France) and those confirmed as *E. coli* were then stored at −20°C for further analysis.

Virulence gene detection

DNA was extracted by boiling 3–5 colonies from pure cultures and then conventional PCR for the detection of virulence-related genes was performed. These genes included *eae* (intimin), *Stx*1 and *Stx* 2 (Shiga toxins 1 and 2), *papC* (P-fimbriae), *hlyA* (α-hemolysin) and *cnf1* (cytotoxic necrotizing factor type 1). Primers used in this study were those described in Table 1. PCR was performed in a BiometraTRIO 48 thermocycler (Analytik Jena, Germany), and PCR products were analyzed under UV light in 1.5% agarose gels stained with GelGreen® (Biotium, United States).

CECT 4783 strain was used as positive control for *eae*, *Stx*1 and *Stx*2 genes; C136b strain was the positive control for *hlyA* and *cnf1* genes, kindly provided by Dr. J. A. Orden, University Complutense of Madrid, Spain. A canine strain previously isolated by our research group (Pe8 strain, GenBank accession number MK034302) was used as positive control for *papC* gene.

Table 1. Primers used in conventional PCR performed in this study.

Gene	**Primer**	**Sequence (5'→3')**	**Annealing temperature (ºC)**	**Amplicon size (bp)**	**Reference**
eae	*eae*-common-F	CCCGAATTCGGCACAAGCATAAGC	55	881	(Oswald et al., 2000)
	eae-common-R	CCCGGATCCGTCTCGCCAGTATTCG			
Stx1	EC-vt1_2-F	CGTCTTTACTGATGATTGATAGTGGC	58	637	(Oh et al., 2014)
	EC-vt1_2-R	CGCGATGCATGATGATGAC			
Stx2	EC-vt2_2-F	TACCACTCTGCAACGTGTCG	58	297	(Oh et al., 2014)
	EC-vt2_2-R	CGATACTCCGGAAGCACATT			
papC	pap1	GACGGCTGTACTGCAGGGTGTGGCG	55	328	(Blanco et al., 1997)
	pap2	ATATCCTTTCTGCAGGGATGCAATA			
hlyA	*hlyA*-F	AACAAGGATAAGCACTGTTCTGGCT	55	1177	(Yamamoto et al., 1995)
	hlyA-R	ACCATATAAGCGGTCATTCCCGTCA			
cnf1	*cnf1*-A	GAACTTATTAAGGATAGT	54	543	(Blanco et al., 1997)
	cnf1-B	CATTATTTATAACGCTG			

Escherichia coli isolates were classified in pathotypes according to the presence of the virulence factor genes analyzed. Enterohemorrhagic *E. coli* (EHEC) are described as those *E. coli* strains that harbor both intimin and Shiga toxins (García et al., 2021). When only one of these virulence factors was present, isolates were classified as enteropathogenic *E. coli* (EPEC) or STEC, respectively. If any of the other virulence factor genes analyzed were found, that is *hlyA*, *papC* and/or *cnf1*, isolates were classified as extraintestinal pathogenic *E. coli* (ExPEC) (Osugi et al., 2014).

Antimicrobial susceptibility testing

Susceptibility to 74 different antimicrobials was determined using VITEK® (bioMérieux, France). The antimicrobial agents selected to test each isolate susceptibility depended on VITEK® guidelines, as clinically relevant antimicrobials recommended by VITEK® varied during the period in which the study was performed. Antimicrobials were classified in 12 categories: aminoglycosides, amphenicols, carbapenems, fluoroquinolones, nitrofurans, other β-lactams, penicillins, tetracyclines, sulfonamides, 1st–2nd generation cephalosporins, 3rd generation cephalosporins and 4th–5th generation cephalosporins, as shown in Table 2. All isolates were tested against at least one antibiotic of each category, except for 4th and 5th generation cephalosporins, which were added in the middle of the study. For those isolates having no information regarding 4th–5th generation cephalosporins, neither susceptibility nor resistance was included, and they were thus excluded from the prevalence analysis for that group. Resistance to a category of antimicrobials was defined as resistance to at least one of the agents in that category. MDR isolates were defined as those isolates with non-susceptibility to three or more antimicrobial categories (Magiorakos et al., 2012).

Additionally, VITEK 2 ESBL test (bioMérieux) was used in these isolates for rapid detection of extended-spectrum β-lactamase (ESBL) production, which is based on simultaneous assessment of the inhibitory effects of cefepime, cefotaxime, and ceftazidime, alone and in the presence of clavulanate.

Breakpoints for the interpretation of minimal inhibitory concentration (MIC) results were applied according to the criteria stablished by bioMérieux for small animals (AST-GN97, bioMérieux, France), which include natural resistance and breakpoints from the Clinical and Laboratory Standards Institute (CLSI, 2018).

Intermediate resistance category provides a flexible information in clinical practice. However, *E. coli* isolates have been previously found to harbor resistance genes (Zhang et al., 2018) For this reason, when they had to be categorized into dichotomic variants they were assessed as resistant.

Table 2. Antimicrobials tested in *E. coli* isolates and category classification.

Antimicrobial categories	Antimicrobials included in each category			
Aminoglycosides	Amikacin	Gentamicin	Neomycin	
	Isepamycin	Netilmicin	Tobramycin	
Amphenicols	Chloramphenicol			
Carbapenems	Doripenem	Ertapenem	Imipenem	Meropenem
1st and 2nd generation cephalosporins	Cephalexin	Cephalothin	Cefadroxil	
	Cefradine	Cefaclor	Cefonicid	
	Cefamandole	Cefotiam	Cefuroxime	
	Cefmetazole	Cefotetan	Cefoxitin	
3rd generation cephalosporins	Cefpodoxime	Ceftiofur	Cefsulodin	
	Cefditoren	Cefixime	Cefoperazone	
	Cefotaxime	Ceftazidime	Ceftizoxime	
	Ceftriaxone	Cefoperazone/ Sulbactam	Ceftazidime/ Avibactam	
	Cefpirome	Cefcapene	Cefdinir	

	Latamoxef	Cefmenoxime	Cefteram	
	Cefovecin			
4th and 5th generation cephalosporins	Cefepime	Cefozopran	Ceftobiprole	Ceftolozan/ Tazobactam
Fluoroquinolones	Enrofloxacin	Marbofloxacin		
	Pradofloxacin	Ciprofloxacin		
Nitrofurans	Nitrofurantoin			
Other beta-lactams	Loracarbef	Faropenem	Aztreonam	
Penicillins	Ampicillin	Temocillin	Oxacillin	
	Ampicillin/sulbactam	Carbenicillin	Amoxicillin	
	Amoxicillin/clavulanic acid	Mecillinam	Ticarcillin	
	Ticarcillin/ clavulanic acid	Piperacillin	Piperacillin/ Tazobactam	
	Azlocillin	Mezlocillin	Benzylpenicillin	
Sulfonamides	Trimethoprim/Sulfamethoxazole			
Tetracyclines	Doxycycline	Tetracycline	Minocycline	

Whole genome sequencing

Those isolates showing the highest rate of phenotypic resistance, that is resistance to six or more antimicrobial categories, were selected for further characterization through whole genome sequencing (WGS). A total of ten *E. coli* isolates were cultured for 24 h in Nutrient Agar (Oxoid, United Kingdom) and DNA was then extracted using Wizard Genomic DNA Purification Kit (Promega, Madison, WI, United States). Quality parameters for DNA were checked both on Qubit 4 (Invitrogen) and gel electrophoresis. Genome sequencing was performed on an Illumina Miseq platform with a paired-end read length of 150 bp. Sequences were trimmed on Galaxy (Version 0.3.8.1) and assembled with Unicycler (Galaxy version 0.5.0 + Galaxy 1). All sequencing data have been submitted to NCBI Genome Database under BioProject PRJNA1031085, and individual accession numbers are the following: SAMN37924970 (isolate 258.883), SAMN37926527 (isolate 262.947), SAMN37926528 (isolate 263.715), SAMN37926529 (isolate 266.493), SAMN37926530 (isolate 267.252), SAMN37926531 (isolate 269.901), SAMN37926532 (isolate 271.550), SAMN37926533 (isolate 271.758), SAMN37926534 (isolate 271.811) and SAMN37926535 (isolate 271.960).

Antibiotic resistance genes, virulence factors, serotypes and sequence types (ST) were assigned to these sequenced genomes using tools that included ResFinder 4.1 (Bortolaia et al., 2020; Camacho et al., 2009; Zankari et al., 2017), PathogenFinder 1.1 (Cosentino et al., 2013), VirulenceFinder 2.0 (Camacho et al., 2009; Clausen et al., 2018), MLST 2.0 (*E. coli* #1 and #2) (Camacho et al., 2009), Bartual et al., 2005; Griffiths et al., 2010; Jauregyu et al., 2008; Larsen et al., 2012; Lemée et al., 2004; Wirth et al., 2006), cgMLSTFinder 1.2 (Clausen et al., 2018; Alikhan et al., 2018), MGE v1.0.3 (Camacho et al., 2009; Carattoli et al., 2014; Joensen et al., 2014; Zankari et al., 2012) and SeroTypeFinder 2.0 (Joensen et al., 2015). Visualization of the genomic data was carried out using Proksee (Grant et al., 2023). A phylogenetic tree was created with Roary pipeline (Page et al., 2015) based on Prokka annotation (Seemann, 2014), and followed by use of IQ-TREE software (Nguyen et al., 2015).

Statistical analysis

Prevalence was calculated with 95% confidence intervals (CI). To test simple relationship between virulence factors and antibiotics, Fisher 's Exact Test was used, and the *p*-values determined, considering them statistically significant when value of $p \leq 0.05$. Numeric values were calculated

using Pearson's coefficient. Isolates showing intermediate antibiotic resistance were considered as resistant for statistical comparisons. All the analyses and calculations were performed using R version 4.1.1 and RCommander 2.7–1.

Results

Virulence factor analysis

According to the virulence factor analysis performed, the prevalence of the virulence-related genes was as follows: 1.92% for *eae* (95% CI: 0–5.66%), 1.92% for *Stx*2 (95% CI: 0–5.66%), 59.62% for *papC* (95% CI: 46.28–72.95%), 53.85% for *hlyA* (95% CI: 40.30–67.4%) and 32.69% for *cnf1* (95% CI: 19.97–45.44%).
However, *Stx*1 gene was not found in this study.

Regarding *E. coli* pathotype classification, 82.69% (95% CI: 79-87%) of isolates were classified as ExPEC, and around 20% (9/43) of them simultaneously harbored the three extraintestinal virulence factors analyzed. Additionally, 1.92% (95% CI: 0–5.66%) of isolates were defined as EPEC, and the same value was found for STEC. However, no EHEC isolates were detected. None of the virulence factors analyzed in this study were found in 13.46% (95% CI: 3–23%) of the isolates.

Prevalence of phenotypic antimicrobial resistance

According to the antimicrobial resistance profiles observed, only one out of 52 (95% CI: 0–5.66%) *E. coli* isolates was susceptible to all the antimicrobials tested. Also, all the antimicrobial categories presented resistant isolates, although in a variable percentage.

According to antimicrobial resistance levels defined by the European Food Safety Authority (EFSA & ECDC, 2020), an extremely high resistance level was found for the categories of 1st–2nd and 3rd generation cephalosporins, followed by very high resistance to penicillins and fluoroquinolones. These isolates also displayed a high resistance level to amphenicols (Figure 2). A low resistance level was found in 5 out of the 12 categories: carbapenems, nitrofurans, other β-lactams, 4th–5th generation cephalosporins and aminoglycosides.

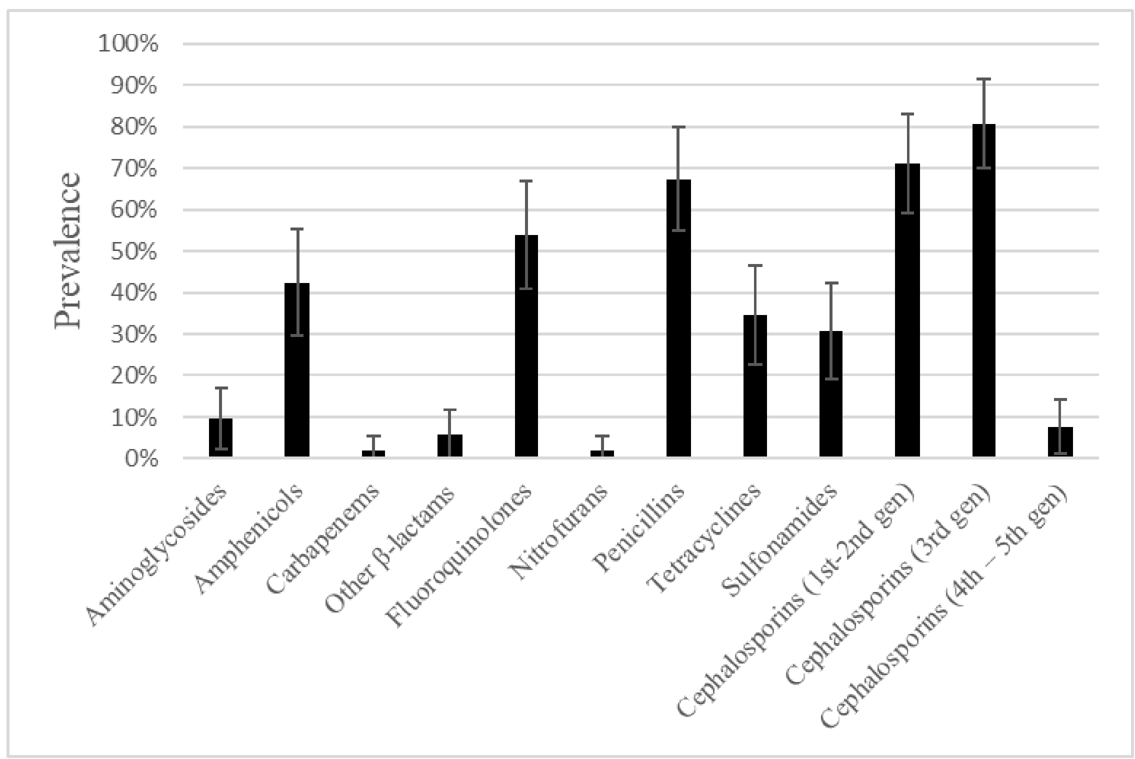

Figure 2. Prevalence of resistance to different antimicrobial categories found in *E. coli* isolates from dog urine.

Several isolates showed resistance to antibiotics which are considered critically important antimicrobials and are listed in category A (EMA et al., 2019). For example, three (out of 30) isolates were resistant to the β-lactam aztreonam, and there were several others found resistant to category A antibiotics from the penicillin group: three (out of 17) isolates were resistant to carbenicillin, 18 (out of 31) to ticarcillin, one (out of 33) to piperacillin and three (out of three) to mezlocillin. There was also one isolate showing intermediate resistance, and thus classified as resistant, to an agent from the carbapenem category (imipenem).

Apart from that, 13.46% (95% CI: 4.17–22.73%) of the isolates were considered ESBL-producers, and almost 60% (4/7) of them showed resistance to 9 or more out of the 12 antibiotic categories tested.

Multidrug resistant profiles

A total of 71.15% (95% CI: 58.84–83.46%) of the studied isolates were described as MDR.

Two main profiles of MDR, with a prevalence of 7.69% (95% CI: 0.45–14.93%) each of them, were observed. The isolates included in one of these profiles showed resistance to the following antimicrobial categories: penicillins, 1st–2nd and 3rd generation cephalosporins; while the other profile comprised those isolates resistant to amphenicols, fluoroquinolones, penicillins, and

1st–2nd and 3rd generation cephalosporins.

Genomic analysis of selected isolates

In silico molecular typing was performed in the sequenced genomes from those selected phenotypically resistant isolates (Table 3). Three different nomenclatures for sequence typing were assigned to each isolate according to Achtman's MLST scheme, Pasteur MLST scheme and core genome (cg)-MLST.

Table 3. Sequence types (ST) and serogroups of sequenced *E. coli* isolates.

Isolate ID	Serogroup	ST (Achtman)	ST (Pasteur)	cgMLST
267.252	O15H1	393	494	163,945
258.883	O5H20	6448	901	174,146
271.960	O4H31	372	490	135,819
262.947	O5H20	6448	901	174,146
263.715	O5H20	6448	901	174,146
266.493	O9aH30	224	479	143,321
269.901	O9H17	88	74	11,260
271.550	O8H25	58	24	207,634
271.758	O8H9	90	66	202,038
271.811	O183H18	117	48	187,123

Three of these isolates, that is 258.883, 262.947 and 263.715, shared the same serotype (O5H20), and the corresponding sequence type (Pasteur ST 901 / Achtman ST 6448) and core genome sequence type (cg-ST 174146), making this *E. coli* type the most prevalent one among the studied isolates. The rest of the isolates presented unique molecular types, although isolates 271.758 and 269.901 belonged to the same clonal complex (CC ST23) and were paired together in the phylogenetic tree (Figure 3). Annotated comparison of the isolates (Figure 4) showed no major missing regions.

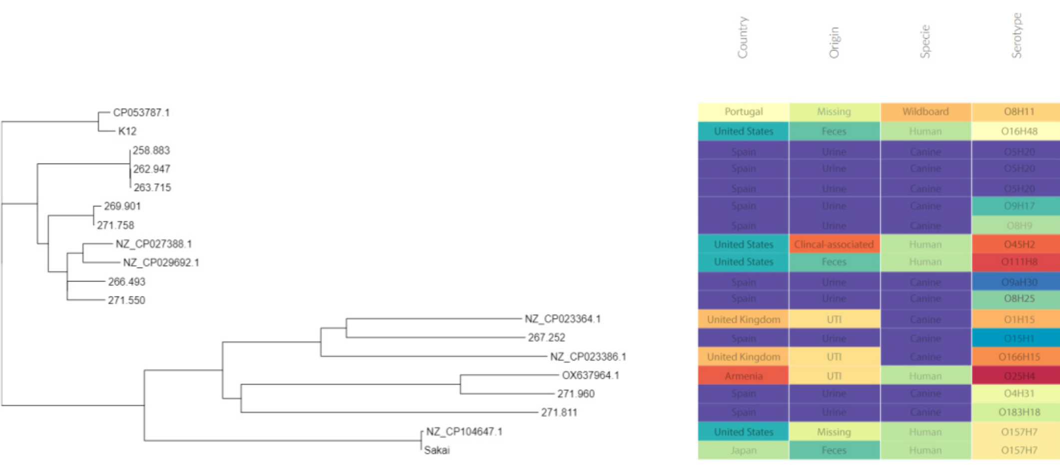

Figure 3. Phylogenetic tree including the sequenced *E. coli* isolates. Metadata was added using Phandango web application (Hadfield et al., 2018).

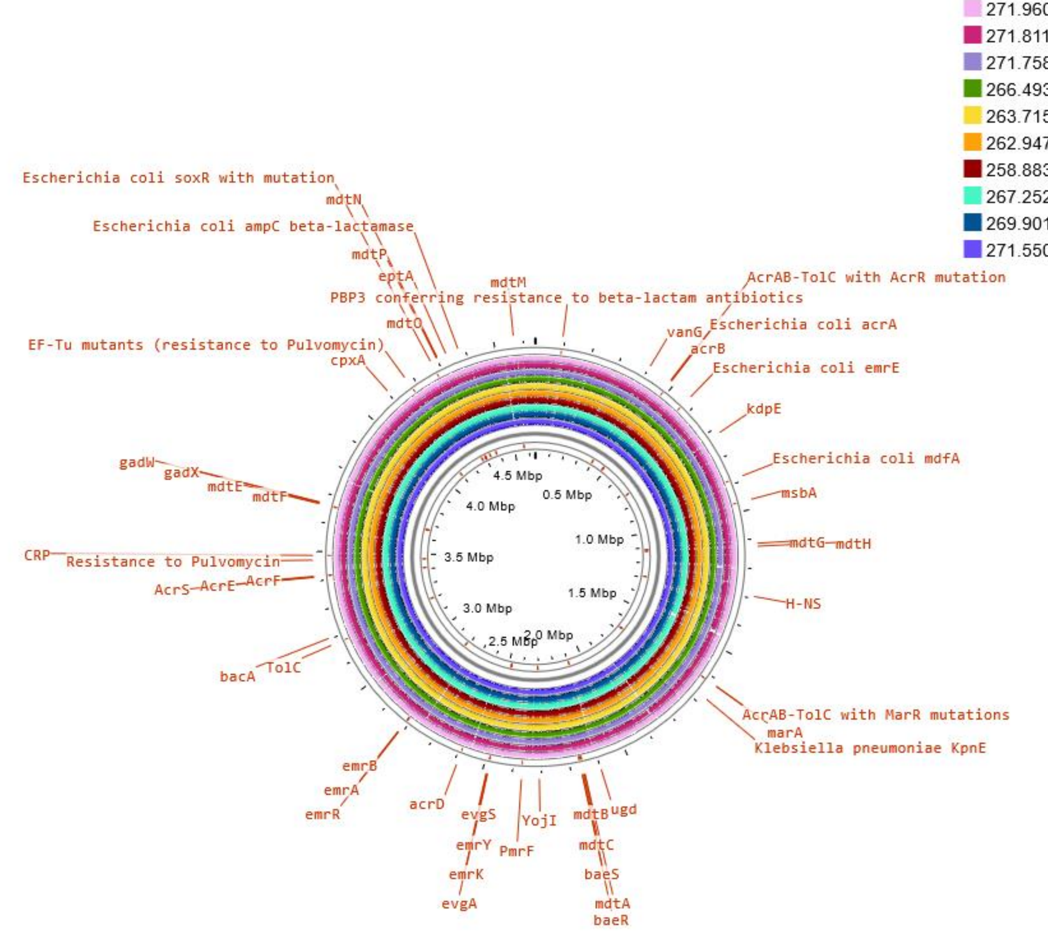

Figure 4. Comparison of *E. coli* K12 reference genome with sequenced isolates. The annotation of selected antimicrobial resistance genes was carried out on Proksee Server from the Stothard Research Group (University of Alberta, Canada) that uses BLAST analysis to illustrate conserved and missing genomic sequences (available online: https://proksee.ca/).

Several genes and mutations associated with resistance to different antimicrobial categories were detected in the sequenced isolates and are detailed in Table 4. In addition, some of these genes were associated to mobile genetic elements (MGE), which are described in Table 5.

Table 4. Antimicrobial resistance genes and mutations found in the sequenced genomes of the selected *E. coli* isolates, as well as the corresponding phenotypic resistance pattern showed in the susceptibility testing assay. A, Aminoglycosides; AN, Amphenicols; C, Carbapenems; B, Beta-lactams; FL, Fluoroquinolones; N, Nitrofurans; P, Penicillins; T, Tetracyclines; S, Sulfonamides; CP, Cephalosporins.

Isolate ID	Aminoglycosides	Phenicols	Beta-lactams	Fluoroquinolones	Tetracyclines	Sulfonamides	Other categories	Phenotypic resistance
258883	aph(6)-Id, aph(3'')-Ib, aadA5	floR	$bla_{CTX-M-55}$	parC:p.S80I, gyrA:p.S83L, gyrA:p.D87N	*tet*(A)	dfrA17		A-AN-FL-P-S-CP
262947	aadA1, aadA2b, aadA5	cmlA1	$bla_{CTX-M-55}$, bla_{TEM-1B}	parC:p.S80I, gyrA:p.S83L, gyrA:p.D87N	*tet*(A)	dfrA17, sul2, sul3	*qacL*	AN-B-FL-P-T-S-CP
263715	*aph(6)-Id, aph(3'')-Ib, aadA5*	*flor*R	$bla_{CTX-M-55}$, bla_Z	parC:p.S80I, gyr A:p.S83L, gyrA:p.D87N	*tet*(A), *tet*(M)	dfrA17		AN-B-FL-P-T-S-CP
266493	*aadA5*			parC:p.S80I, gyrA:p.S83L, gyrA:p.D87N, parE:p.S458A		dfrA17, sul1	*erm(B), mph(A), qacE, sitABCD*	AN-C-FL-N-P-T-S-CP
267252	*aph(6)-Id, aph(3'')-Ib*		bla_{TEM-1B}	parC:p.S80I, gyrA:p.S83L, gyrA:p.D87N, parC:p.S57T, parE:p.L416F	*tet*(B)	sul2	*sitABCD*	AN-B-FL-P-T-S-CP
269901	*aadA1, ant(2'')-Ia, 16S_rrsC:g.926_926del*	*floR, catA1*	bla_{OXA-1}	parC:p.S80I, gyrA:p.S83L,gyr A:p.D87N, parE:p.S458A	*tet*(B)	dfrA36, sul 1, sul2	*qacE*	A-AN-FL-P-T-S-CP
271550	*aph(6)-Id, aph(3'')-Ib, aph(3')-Ia*		bla_{TEM-1B}	gyrA:p.S83L	*tet*(A)	dfrA5, sul2	*sitABCD*	A-FL-P-T-S-CP
271758				parC:p.S80I, gyrA:p.S83L, gyrA:p.D87N, parE:p.S458A				AN-FL-P-T-CP
271811	*aph(6)-Id, aph(3'')-Ib*		bla_{TEM-1B}		*tet*(B)	dfrA8, sul2	*sitABCD*	AN-FL-P-T-S-CP
271960	*aph(6)-Id, aph(3'')-Ib, aadA1*	*cat86, catA1*		parC:p.S80I, gyrA:p.S83L, gyrA:p.D87G, parE:p.I355T	*tet*(A)	dfrA1, sul1, sul2	*qacE, sitABCD*	AN-FL-T-S-CP

Table 5. Presence of mobile genetic elements (MGE) in sequenced isolates, and antibiotic resistance and associated virulence genes. PL, plasmid; IS, insertion sequence; MIR, miniature inverted repeat; ICE, Integrative Conjugative Element.

Isolate ID	**MGE**	**Type**	**Coverage (%)**	**Identity (%)**	**Associated resistance or virulence genes**
258883	ISEc9	IS	100	100	*$bla_{CTX\text{-}M\text{-}55}$*
	MITEEc1	MIR	100	98.88	*terC, yehB, yehD, yehA, yehC*
	ISEc1	IS	99.77	96.74	*fdeC*
	IS26	IS	100	100	-
	IS421	IS	99.78	99.7	-
262947	ISVsa3	IS	100	100	*sul2*
	ISEc9	IS	100	100	*$bla_{CTX\text{-}M\text{-}55}$, terC*
	MITEEc1	MIR	100	97.56	*yehB, yehD, yehA, yehC*
	IncFIC	PL	100	100	*traT, anr*
	IS102	IS	100	92.72	*cma, cba*
	ISEc1	IS	99.77	96.74	*fdeC*
	IS640	IS	99.91	98.36	-
	IS421	IS	99.78	99.7	-
263715	ISEc9	IS	100	100	*$bla_{CTX\text{-}M\text{-}55}$*
	Tn6009	ICE	100	99.89	*tet(M)*
	MITEEc1	MIR	100	97.56	*terC, yehB, yehD, yehA, yehC*
	ISEc1	IS	99.77	96.74	*fdeC*
	IS421	IS	99.78	99.7	-
	IS26	IS	100	100	-
266493	IS6100	IS	100	100	*mph(A), qacE, dfrA17, sul1, aadA5*
	MITEEc1	MIR	100	100	*terC, nlpl, terC, yehB, yehD, yehA, yehC, csgA, hlyE*
	IS5	IS	100	99.75	*irp2, gad, fyuA*
	IncFII	PL	98.85	95.06	*traT*
	IncFIB	PL	100	98.93	-
	IncFIA	PL	100	99.74	-
	IncX1	PL	100	94.92	-

267252	IncQ1	PL	65.83	100	*aph(6)-Id, aph(3''), sul2*
	IncFII	PL	99.62	96.95	*anr*
	IncFIB	PL	100	99.22	-
	IncX4	PL	100	98.88	-
	IncFIA	PL	100	99.74	-
	ISEc45	IS	100	99.86	*iucC, papA, papC, iutA, sat, iha*
	ISEc46	IS	100	99.94	*fyuA, irp2*
	ISEc1	IS	100	98.06	*csgA, ompT*
	MITEEc1	MIR	99.19	97.56	*terC*
269901	Incl1	PL	100	100	*cia*
	ISEc78	IS	99.84	98.97	*fyuA, irp2*
	MITEEc1	MIR	100	98.37	*yehD, iss, fdeC*
271550	IncQ1	PL	65.83	100	*aph(6)-Id, aph(3''), sul2*
	IncFIB	PL	100	98.39	*cia, iroN, iss, mchF, etsC, cvaC, etsC, ompT, hlyF*
	IncFII	PL	100	100	*traT, anr*
	ISEc31	IS	99.28	92.73	*terC*
	MITEEc1	MIR	99.19	94.26	*iss, fdeC, terC*
	ISEc38	IS	100	94.6	*fyuA, irp2*
271758	IncFII	PL	100	100	-
	IncFIA	PL	100	99.74	-
	IS3	IS	100	99.92	*hlyE, csgA*
	MITEEc1	MIR	100	97.56	*yehB, yehD, yehA, yehC, terC, nlpl*
	ISEc1	IS	100	97.91	*fdeC*
271811	IncFII	PL	100	100	*sul2, aph(6)-Id, aph(3'')-Ib, anr*
	Incl1	PL	100	100	-
	Col(MG828)	PL	98.85	95.38	-
271960	IncQ1	PL	66.46	100	*dfrA1, aadA1, aph(6)-Id, qacE, tet(A), aph(3'')-Ib, sul1, dfrA1, catA1, sul2, dfrA1*

	IncFII	PL	98.85	98.05	*traT, anr, traJ*
	IncHI2A	PL	100	99.52	-
	IncHI2	PL	100	100	-
	ISKpn37	IS	99.68	97.3	*hlyA, cnf1*
	MITEEc1	MIR	100	100	*terC*
	ISEc38	IS	99.94	97.16	*cea*

Interestingly, there were two MGE of particular interest due to its association with important resistance genes or a high number of them, that is IS6100 and ISEc9, which can be seen in Figure 5.

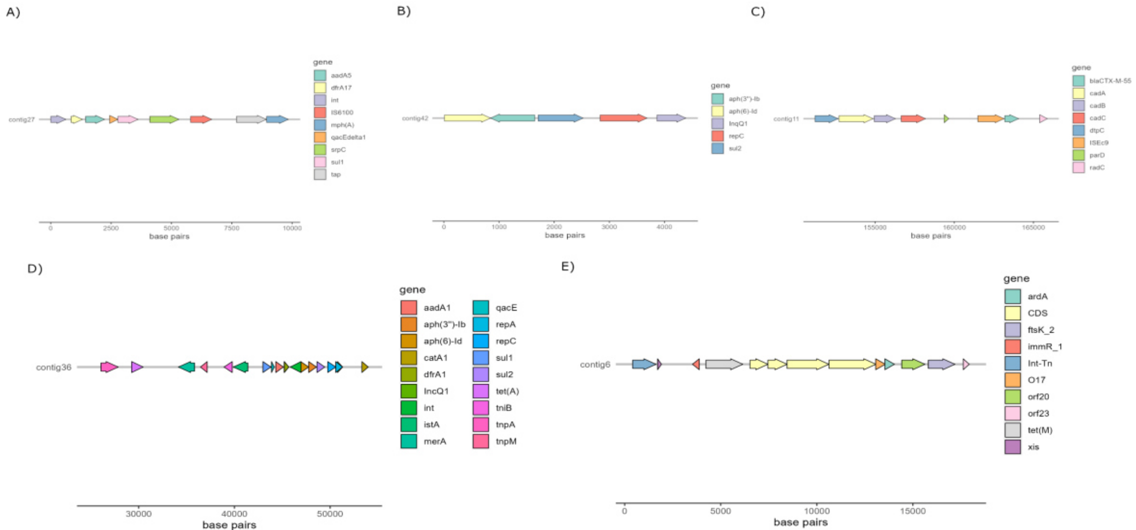

Figure 5. Selected contigs of different isolates containing antimicrobial resistance genes as well as MGE annotated with Prokka (Seemann 2014). (A) Organization of a fragment of contig 27 in isolate 266.493, which contains *aadA5, dfrA17, qacE, sul1*, and *mphA* genes. (B) Organization of contig 42 in isolate 269.901, which contains *aph(6)-Id, aph(3'')-Ib* and *sul2* genes. (C) Organization of a fragment of contig 11 in isolate 258.883, which contains $bla_{CTX\text{-}M\text{-}55}$ *gene*. (D) Organization of a fragment of contig 36 in isolate 271.960, which contains several resistance genes, such as *aadA1*, *sul2* and *tet(A)*. (E) Organization of a fragment of isolate 263.715, which contains *tet(M)* resistance gene.

Association between antimicrobial resistance and virulence factors

When testing for simple relationships between phenotypic antimicrobial resistance and presence of virulence factors in these isolates, the carriage of *cnf1* gene showed a significant association with resistance to several penicillins, as well as with the penicillin category itself (Value of $p = 0.01$). *Cnf1* gene also showed a significant association (Value of $p = 0.019$) with MDR category. Apart from that, age of individuals

was significantly associated with *E. coli* isolates showing resistance to various cephalosporins and to the 4th–5th generation cephalosporin category (Value of $p = 0.034$). Gender was associated with aminoglycoside resistant isolates (Value of $p = 0.007$).

ESBL production showed association with resistance to five out of the 12 antimicrobial categories tested (amphenicols, other β-lactams, 4th–5th generation cephalosporins, sulfonamides and fluoroquinolones). No significant relationship between the number of virulence-related genes and ESBL production was observed, however a negative relationship between the number of antimicrobial categories to which isolates showed resistance and number of virulence-related genes was found (Pearson coefficient = 0.33 value of $p = 0.014$).

Discussion

This study has evaluated both phenotypic and genotypic antimicrobial resistance as well as the presence of selected virulence genes in *E. coli* isolates from Spanish dogs with UTI and has shown that dogs may be reservoirs of resistant uropathogenic strains of *E. coli*.

Comparing with results obtained in a previous study (Sevilla et al., 2020) in which samples were collected in a similar time and geographical location, although they had a different origin (feces), we found that *E. coli* isolates from urine were more susceptible to aminoglycosides than those obtained from dog feces (phenotypic resistance found in 9.62% vs. 40% of isolates). In general, the prevalence of antimicrobial resistances found are similar to those found in *E. coli* isolated from UTI patients (Chang et al., 2015; Yu et al., 2020), except for the penicillin group, which was slightly higher in this study (67.31% vs. ~ 45–50%). Such high prevalence is also contrary to the decreasing trend of penicillin resistance in *E. coli* isolates in Europe (ECDC et al., 2020). Some hypotheses for this phenomenon could be the trend of increase of antibiotic resistance year to year or the fact that more antibiotics from the penicillin group were studied in this work.

Escherichia coli aminoglycoside resistance was the only type of resistance linked to the gender of the animal, being found exclusively in male individuals. In fact, male gender has previously been associated with aminoglycoside resistance in Gram-negative bacteria (Richter et al., 2019).

When taking into account some of the antibiotics considered clinically important for human and animal health by the European Medicines Agency (EMA et al., 2019), it is worth mentioning that *E. coli* isolates showing resistance to several of these antibiotics were detected in this study, even to antibiotics from category A ("Avoid," it includes antibiotics not authorized in veterinary medicine in the European Union), such as certain penicillins or carbapenems. Another relevant antibiotic category is category B ("Restrict"), which includes those listed as highest priority critically important antimicrobials (HP-CIAs) by the World Health Organization categorization, e.g., 3rd generation cephalosporins or fluoroquinolones. Indeed, as much as 80.77 and 53.85% of these isolates were considered resistant to 3rd generation cephalosporins and fluoroquinolones, respectively. The high amount of overall resistance found among all the categories could be biased by the fact that complicated UTI are more often requested for culture and antibiogram testing than simpler cases of UTI.

The treatment with amoxicillin or trimethoprim-sulfonamides as first-line agents is currently recommended for the management of bacterial UTI in dogs (Weese et al., 2019). However, considering these results, it seems that the use of these antimicrobials may be ineffective in a high percentage of cases, since 58.33% of isolates were found to be resistant to amoxicillin and 30.77% to trimethoprim-sulfonamides. Before suggesting any change in current guidelines for antibiotic treatment in canine UTI, it should be noted that *E. coli* is not the only pathogen responsible for UTI and that the data analyzed in this study might be overestimating the baseline resistance, mostly because of the selection of the patients. In any case, the use of antibiotic resistance testing as a routine allows not only the monitoring of the epidemiology of antibiotic resistance profiles but also the faster implementation of a treatment in case of failure of the empiric one.

It is worth mentioning the high percentage of MDR isolates found (71.15%). Among the MDR isolates found, more than 80% (30/37) were classified as ExPEC, and one of them corresponded to EPEC pathotype. This kind of strains possesses a potential zoonotic risk and can also serve as a reservoir of resistance genes (Johnson et al., 2001b), further contributing to the dissemination of antibiotic resistance and limiting the options for the treatment of infectious diseases in both humans and animals.

High antibiotic resistance has been associated with MGE in Enterobacteriaceae. Bacteria harboring these mobile elements can become a reservoir for antibiotic resistances and be transmitted then from pets to their owners or the environment. This phenomenon poses a serious health problem due to the spread of resistance and failure of current antibiotic treatments (Partridge et al., 2018).

Of special interest is the presence of transposon Tn6009 carrying *tet*(M) gene in isolate 263.715. This element has been previously described in other Gram-negative bacteria, such as *Enterococcus fecalis* (Tóth et al., 2021), and is associated with tetracycline resistance due to the presence of *tet*(M) (Figure 5E). This non-composite conjugative transposon is of clinical importance in Gram-positive bacteria and has a potential role in the dissemination of resistance (Rice et al., 1998, Soge et al., 2008). Resistance to beta-lactams was encoded by $bla_{CTX-M-55}$ gene in three isolates, which was located in a ISEc9 insertion sequence (IS1380-like). ISEc9 region has been previously associated with ESBL genes (Medugu et al., 2023) and has been described in other bacteria such as Vibrio vulnificus (Nakayama et al., 2023). In all cases, the gene and the insertion sequence were 46 bp away. One of them (isolate 263.715) also harbored a bla_Z gene, and another one (isolate 263.715) had a copy of bla_{TEM-1B}. Beta-lactamase-encoding genes such as bla_{TEM-1B} and bla_{OXA-1} were also identified in other isolates. For example, bla_{TEM-1B} was present in two isolates that displayed no phenotypic resistance to beta-lactams (isolates 271.550 and 271.811), and in one that did (isolate 267.252). There was only one isolate (269.901) containing a bla_{OXA-1} gene. As expected, this isolate was resistant to several antibiotics in the penicillin group (ampicillin, amoxicillin + clavulanic acid, amoxicillin), and it was negative in the ESBL production test Bla_{OXA-1} has been found in ST131 or associated with other genes in plasmids (Nicolas-Chanoine et al., 2014). Despite this gene being originally described in MGE (Evans & Amyes, 2014), we only identified IncI1 plasmid in this isolate, and it was not associated with any antibiotic resistance gene. Sulfonamide resistance genes (*sul* 1 or 2) were found in most of the sequenced genomes (7/10), and in five of them these genes were located in a MGE (IS6100 and IncQ1 for *sul1*, and ISVsa3, IncQ1 or IncFII for *sul2*). All these MGE also harbored other resistance genes, including those linked to streptomycin (*aadA5, aph(6)-Id, aph(3'')-Ib* and *aadA1* in IS6100, IncFII and IncQ1) (Sandvang, 1999; Meyer, 2009; Scholz et al., 1989; Hollingshead &

Vapnek, 1985), trimethoprim (*dfrA17* and *dfrA1* in IS6100 and IncQ1) (Sandalli et al., 2010, Sáenz et al., 2004), antiseptics (*qacL* and *qacE* in IS6100 and IncQ1, although all antiseptic resistance genes were incomplete) (Kazama et al., 1999), erythromycin (mph(A) in IS6100) (Pawlowski et al., 2018), doxycycline and tetracycline (*tet(A)* in IncQ1) (Roberts 2005) and chloramphenicol (*catA1* in IncQ1) (Alton & Vapnek, 1979).

The ISVsa3 transposase was found in one of the sequenced isolates (262.947) and contained the sul2 gene, which has also been identified in other enteropathogens (Lewis et al., 2023; García et al., 2011; Zhou et al., 2019). Isolate 266.493 harbored *dfrA17-aadA5-qacEdelta1*-IS6100-*mph(A)-sul1* integron structure, which is commonly identified in ExPEC pathotype (Jarocki et al., 2007).

Regarding the plasmids identified, IncFII plasmid has been previously documented in Spain as frequently linked to ESBL production (Novais et al., 2007). In this study, the plasmid was only identified in one isolate, although it was classified as non-ESBL producer.

Another plasmid identified was IncQ1, commonly found in *E. coli* and with ability to transfer between different bacterial species and strains, which facilitates the dissemination of antibiotic resistance in bacterial populations (Rawlings et al., 2023). This plasmid was detected in the genomes of three isolates (267.252, 271.550 and 271.960) and was found close to resistance genes linked to aminoglycoside resistance (aph(6)-Id and aph(3'')-Ib). All these three isolates were distant in the phylogenetic tree, which suggests that the plasmid has been likely acquired independently. One of these isolates harbored nine more resistance genes close to the detected plasmid (Figure 5D), indicating a potential hotspot for antibiotic resistance dissemination.

When studying antibiotic resistance genes in sequenced isolates, there was in general a consistent correlation between phenotypic and genetic resistance. However, there were two significant exceptions to this pattern. When examining aminoglycosides, several isolates exhibited susceptibility to this category despite carrying resistance genes related to both streptomycin and spectinomycin, which are included in this antimicrobial category. The second exception was observed with tetracycline, where the relationship between resistance genes and phenotypical resistance did not consistently align. This discrepancy in isolate 258.883 may be attributed to a nucleotide substitution at position 924 within the tet(A) gene (position 3,323,

GenBank: AF534183.1), specifically transitioning from cytosine (C) to thymine (T). Because of this alteration, there is a shift in the protein composition from alanine (Ala) at position 118 to threonine (Thr). Although these are not the first *E. coli* isolates that harbor this gene mutation (Vereecke et al., 2023), to the authors knowledge our study is the first that associates this mutation in tet(A) to a failure in phenotypic response. Also, in more than 40% (4/9) of the isolates considered phenotypically resistant to amphenicols, no resistance gene associated with this antimicrobial category was found.

The phylogenetic tree (Figure 4) showed that canine isolates clustered together, except isolate 271.960 (ST 372), which was grouped with a human isolate (assembly reference OX637964.1) that belongs to ST131. ST 131 is one of the predominant sequence types within the ExPEC pathotype worldwide (Nicolas-Chanoine et al., 2014; Bogema et al., 2020). In fact, *E. coli* O25b:H4/ST131 was described as a prevalent clone in Spanish human population. In accordance with bibliography, this canine isolate was not associated with ESBL resistance and had a similar resistance profile to human strains (López-Cerero et al., 2013; Dautzenberg et al., 2016).

According to the virulence factors analyzed, most of the *E. coli* isolates found in urine samples were categorized in the ExPEC pathotype, as expected. The most frequently detected virulence factors were *papC* and *hlyA*, followed by *cnf1*. *Cnf1* prevalence in these isolates was similar to that found in isolates from both dogs and humans, while *papC* and *hlyA* prevalence were higher in this study (Johnson et al., 2001a; LeCuyer et al., 2018; Torkan et al., 2016; Tramuta et al., 2014; Siqueira et al., 2019; Yuri et al., 1999). However, most of the sampled populations in these studies include healthy animals, which could lower the prevalence of *E. coli* virulence factors. The prevalence of these three virulence factors were higher in dog isolates than in those found in humans (Tramuta et al., 2014; Blum et al., 1995; Landraud et al., 2000; Johnson et al., 2001c). Some of these factors were found in MGE (Table 5), which highlights their potential of spread to other strains.

Almost 20% of these ExPEC isolates displayed in combination with the three extraintestinal virulence-related genes analyzed (*papC*, *hlyA* and *cnf1*), likely due to the presence of a PAI (Diard et al., 2010). This type of virulence factors is frequently found in *E. coli* strains causing extraintestinal disease in both humans and dogs, being thus

this animal species a possible reservoir for the ExPEC pathotype (Johnson et al., 2001a).

Apart from that there was one *Stx*2-positive isolate, that did not harbor any other virulence factor studied. Shiga toxin 2 is believed to be associated with the development of HUS (Persson et al., 2007) and is better produced when it is found in combination with other strains or bacteria (Xiaoli et al., 2018). However, there are also some descriptions of Shiga toxin-producing *E. coli* isolates associated with UTI cases, and it has been proposed that Shiga toxins can bind to receptors from urinary bladder epithelial cells and damage them (Toval et al., 2014b; Beutin et al., 1994). Additionally, an EPEC isolate was also detected. It is not the first time that an *eae*-positive isolate has been found among UTI-associated strains, although its frequency seems to be quite low as well (Abe et al., 2008; Toval et al., 2014a; Valiatti et al., 2020). The role of this gene product (i.e., intimin) in UTI pathogenesis is not fully understood and its significance remains to be studied (Abe et al., 2008).

Taking into consideration that fecal *E. coli* population might have a relationship with UTI pathogenesis (Moreno et al., 2008), it may be suggested that certain diarrheagenic pathotypes also have potential to cause UTI, although uncommon. However, the role of these strains in UTI development and the molecular and pathogenic causes behind it are still poorly understood, and more research in this field is needed in order to comprehend the mechanisms and epidemiological causes. Nevertheless, the ability of such strains to cause an extraintestinal infection in the host is not only dependent on their virulence-related genes but also on risk factors such as age or immunosuppression (Toval et al., 2014a).

It is also important to note that a wide variety of extraintestinal-associated virulence traits has been described in the literature. Thus, apart from these virulence genes typical of diarrheagenic strains, these two isolates might be also harboring some other extraintestinal virulence factors different from those analyzed in this study. In this regard, some *E. coli* strains have been recently classified as hybrids for harboring virulence factors usually associated with various pathotypes, e.g., STEC/UPEC strains (Gati et al., 2019). The genome plasticity of this microorganism promotes the exchange and combination of both intestinal and extraintestinal virulence determinants, resulting in an heteropathogenic potential (Toval et al., 2014a; Valiatti et al., 2020). The possible emergence of hybrid pathotypes not only in

humans but also in animals should therefore be surveilled.

The finding of eight isolates (15.38%) considered neither intestinal nor extraintestinal pathogenic isolates could be explained by the fact that only a selection of virulence factors was tested. Thus, these *E. coli* might harbor other different virulence-related genes not analyzed in this study. However, another explanation could be that the causal agent for UTI in these dogs was different from *E. coli*, or even a non-infectious cause. It is also worth noting that the detection of virulence factor genes does not mean that they are phenotypically expressed, so the severity of the disease could not be only assessed with this information. Nevertheless, it is known that the severity of the disease is not caused by a single virulence factor but a combination of them (Brzuszkiewicz et al., 2016; Dale & Woodford, 2015).

The most commonly isolated serotype in this study was O5H20. In this regard, O5:H(−) has been associated with STEC strains, and Shiga toxins have been also described in *E. coli* strains causing UTI (McLean et al., 2005; Misra, 1997). However, these isolates did not harbor any Shiga toxin gene. The rest of *E. coli* serotypes are distributed along different STs and antibiotic resistance patterns, showing a heterogenic distribution.

Interestingly, low antibiotic resistance patterns were linked to a higher number of virulence factors. There is some literature (Karam et al., 2018; Shah et al., 2019) that suggests a positive relationship between virulence factors and MDR. However, in isolates from this study only *cnf1* carriage showed a significant association with MDR, while a high virulence factor carriage was associated with low resistance profiles. The reason for this mechanism is still unclear, but it is hypothesized that the acquisition of MDR is “sacrificed” in exchange for virulence factors, or that the low presence of virulence factors facilitates the acquisition of antibiotic resistance (Wagner et al., 2014; Pootong et al., 2018). When analyzing correlation between all virulence factors found and the presence of antibiotic resistance genes in whole genome sequenced isolates, the relationship was non-significant ($p = 0.14$). However, there was a bias in selection of isolates, as only the more resistant ones were chosen.

Conclusion

Based on these data, a very high percentage of *E. coli* isolates found in urine samples from dogs suffering from UTI was considered MDR, the majority of them being classified as ExPEC. Phenotypic antimicrobial resistance to first-lines agents recommended in UTI management

was also frequently observed, which could be associated with a treatment failure. Furthermore, several antimicrobial resistance genes, some of them contained in MGE, were identified in the genome of selected resistant isolates. The use of WGS could identify some of the genetic mechanisms underlying antimicrobial resistance, although there were a few discordances between phenotypic resistance and genes found. Combining both phenotypic and genetic data enhances our understanding of antibiotic resistance and improves treatment selection efficiency.

Overall, these findings are of concern for both animal and public health, since dogs could act as reservoirs of MDR pathogenic *E. coli* and contribute to the spread of antimicrobial resistance. Surveillance of antimicrobial resistance and revision of therapeutic guidelines should be therefore continuously addressed in clinical veterinary settings.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found at: https://www.ncbi.nlm.nih.gov/bioproject; PRJNA1031085.

Ethics statement

Ethical approval was not required for the studies involving animals in accordance with the local legislation and institutional requirements because samples were collected as part of the daily activity of private veterinary practitioners. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

AA-F: Data curation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. ES: Data curation, Formal analysis, Supervision, Writing – original draft, Writing – review & editing. AO: Investigation, Writing – original draft. IM-B: Formal analysis, Supervision, Writing – review & editing. BM: Resources, Supervision, Writing – review & editing. MM: Conceptualization, Resources, Supervision. RB: Conceptualization, Funding acquisition, Resources, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Trabajo 2

Studies on the importance of *Clostridiodes difficile* in meat rabbits with diarrhea and comparison with other enteropathogens such as *E. coli* and *C. perfringens*

Introduction

In Spain, where the meat rabbit industry holds greater relative importance compared to other countries, the market faces numerous epidemiological challenges, particularly with regard to enteric diseases. Bacterial enteritis is one of the most important enteric diseases, with *Escherichia coli* and *Clostridium* spp. Being the most important causes (García-Rubio et al., 2017). Other bacterium species involved are *Salmonella*, *Klebsiella* or *Pseudomonas* spp., although they are frequently reported in isolated outbreaks. Parasites, such as coccidian, or viruses, likerotavirus or coronavirus, are also reported. Other pathologies, however, such as Epizootic rabbit enteropathy (ERE), previously known as mucoid enteropathy, are still poorly understood regarding their etiology or pathogenesis (García-Rubio et al., 2017).

E. coli is one of the most important enteropathogens in rabbits. In this species, compared to other animals, colibacillosis is caused by enteropathogenic *E. coli* (EPEC pathotype), whose main virulence factor is an intimin, codified by the *eae* gene (Krause et al., 2005). This intimin facilitates bacterial attachment and the formation of the characteristic attaching and effacing lesions (Puente & Finlay, 2001).

Clostridium species are also important rabbit enteropathogens and include three species: *Clostridium spiroforme*, *Clostridium perfringens* and *Clostridium difficile*. Although they are considered part of the normal flora in healthy rabbits, they are usually found only in small amounts (Varga Smith, 2023). Recently, a new *Clostridium* species, *C. cuniculi,* has been linked with ERE (Djukovic et al., 2018; Marlier et al., 2006). While the role of *C. spiroforme* is clearly recognized as a cause of diarrhea in rabbits, the importance of the other species remains unclear (Varga Smith, 2023). *C. perfringens* causes enteric disease in various species, including humans and rabbits (Kiu & Hall, 2018), although its role in the latter is still debated. Though it has been found in cases of ERE (Huybens et al., 2010; Licois et al., 2005), there is also evidence that its sole presence is not a good indicator as etiological cause (Romero et al., 2011).

Clostridioides difficile is an emerging pathogen of increasing concern due to its potential zoonotic capacity. Infection in humans has classically been considered a nosocomial infection, usually antibiotic-related; however, zoonotic infections are increasingly being suspected (Lim et al., 2020). Numerous animal species can be infected by *C. difficile*, although the pathogenesis and clinical and pathological consequences are very variable (Keel & Songer, 2006; Weese, 2020) Susceptibility to natural disease depends on the species, with Syrian hamsters being one of the most susceptible (Larson et al., 1980). A carrier status is important in *C. difficile* infection, which is highly

dependent on the host and age, even within the same species. In some species, the prevalence is high, although clinical signs are few, being important carriers. In others, such as horses, the carrier rate is low (Keel & Songer, 2006). However, as pathogenic *C. difficile* is toxinogenic, the determination of toxins is relevant as some carriers may harbor nontoxigenic isolates.

The importance of *C. difficile* in rabbits is still uncertain, with a few reports of natural infections (Drigo et al., 2015; Perkins et al., 1995; Taha et al., 2019) and experimental studies (Lima et al., 1988; Warren et al., 2012). Therefore, the objective of this study was to investigate the presence of *C. difficile* in normal and diarrheic rabbits of different ages, raised for consumption in Spain. Also, it aimed to molecularly classify the isolates and determine its antibiotic resistance patterns. Its presence and importance in diarrheic rabbits was evaluated in association with other pathogens such as *E. coli* or *C. perfringens*.

Materials and methods

Cases

The study population included samples of 190 farmed rabbits dedicated to meat consumption. All rabbits were from farms with a history of digestive problems. Health status was evaluated and rabbits were classified as healthy (n = 47), diarrheic (n = 127) or with nonspecific clinical signs (n = 16). Samples included digestive swabs from live animals and gastrointestinal tract organs from necropsied animals. Most of the samples were from diarrheic fattening rabbits and some (n = 15) from does. Some diarrheic rabbits were humanely euthanized. Necropsies were only carried out in diarrheic rabbits. The final distribution of samples taken can be seen in Table 6.

Table 6. Distribution of the samples. Necropsied animals included all animals from the first two rows and some of the last column.

n	*C. difficile* isolation	*C. perfringens* and *E. coli* isolation	Histopathology
30	Done	Done	Done
19	Done	Done	Not done
141	Done	Not done	Not done

Pathological studies

A complete necropsy was performed on 71 diarrheic rabbits. Some rabbits were necropsied in the farms, especially those recently dead. Other rabbits were taken alive to the Veterinary Faculty for a more detailed necropsy. Histopathological analyses were performed in 30 rabbits; they only included euthanized or recently dead rabbits, to avoid post-mortem changes (Table 6). Samples were taken from the jejunum, ileum, cecum, and colon. A portion of each digestive part was also taken for microbiological study. In rabbits showing post-mortem changes, only one swab was collected for *C. difficile*

isolation. For histopathology, samples were routinely processed, fixed in 10% buffered formalin for 48 hours, embedded in paraffin, and sectioned at 4 µm. Sections were stained with hematoxylin and eosin.

Microbiological studies

Microbiological studies were carried out on the contents of the different digestive organs and from swabs. Isolation of *C. difficile* was attempted in the 190 samples, while *E. coli* and *C. perfringens was only attempted to isolate in* 49 of these rabbits. *C. spiroforme* was also investigated in the 49 rabbits.

The digestive contents were plated on MacConkey agar (Panreac, United States), and Tryptose Sulfite Cycloserine agar supplemented with egg yolk (TSC) (Oxoid, United Kingdom) with a sterile loop; a swab from these contents was used for *C. difficile* isolation. After the content was plated, a direct smear was also performed.

For *E. coli* isolation, MacConkey agar plates were cultured for 24 h at 37 ºC in aerobic conditions, and then colonies compatible with *E. coli* were Gram stained and tested by indole production. Gram negative bacilli with compatible morphology and indole positive test were considered *E. coli*; then, a PCR for *eae* (intimin) gene detection was performed following a protocol described elsewhere (Yamamoto et al., 1995).

All swabs, regardless of origin, were processed for *C. difficile* determination. They were incubated for a week at 37 ºC under anaerobic conditions in Brain-Heat Infusion Broth (Oxoid, United Kingdom), supplemented with 6g/L of fructose, 1g/L of sodium-taurocholate (Sigma-Aldrich, United States of America) and a *C. difficile* selective supplement (CDMN, Oxoid, United Kingdom) following manufacturer instructions. The result was then mixed with 2 mL of ethanol in a 1:1 proportion and was let rest for 1h, after which it was centrifuged at 4000 g for 20 minutes and the pellet was anaerobically plated in a CLO agar (bioMérieux, France). After 48 hours, plates were read, and morphologically compatible colonies were chosen for further testing.

For *C. perfringens*, TSC plates were also incubated for 48 h at 37 ºC under anaerobic conditions. Then, black colonies with lecitinase activity were selected as suspected colonies of *C. perfringens*. These colonies were both Gram stained and tested for *cpa* toxin gene, following a modified version of a multiplex PCR (van Asten et al., 2009). Gram positive bacilli positive to the *cpa* gene were considered *C. perfringens*.

C. spiroforme was investigated by Gram staining of intestinal smears (Tuomisto et al, 2024; Varga Smith, 2023; Agnoletti, 2012).

Molecular studies

Both *E. coli* and *C. perfringens* DNA was extracted following the boiling method (Dashti et al., 2009), which included the preparation of a bacterial suspension in 200 µl of distilled water, ten minutes of boiling, ten of cooling and finally a 10 minutes' centrifugation at 13000g. Supernatant was collected in a different microtube and used as template for the PCRs.

For *C. difficile*, DNA was extracted with Wizard Omega DNA purification Kit (Omega, United States of America) following manufacturer instructions, and a multiplex PCR for toxins and *gluD* was performed (Paltansing et al., 2007; Persson et al., 2008). In addition, isolates were ribotyped according to Leeds-Leiden database (Fawley et al., 2015).

In the case of lactose negative *E. coli*, a further virulence factor characterization was performed. Analyzed genes included *cnf1, papC, hly, thermolabile toxin, thermostable toxin* and *Shiga-toxins V1* and *V2*. The protocols followed were developed by Oh et al. in 2014 and the Reference Laboratory for *Escherichia coli* (ECL) in 2013.

Antimicrobial resistance studies

Antibiograms were performed by epsilometry (E-test). Antimicrobial testing included clindamycin, erythromycin, metronidazole, moxifloxacin, tetracycline and vancomycin. Breakpoints established by the Clinical and Laboratory Standards Institute (CLSI) in 2023 were used for antimicrobial resistance categorization. If no breakpoint was available, as in the case of erythromycin and vancomycin, the cut off values suggested by EUCAST in 2023 were applied. Multidrug resistance (MDR) was defined as resistance against three or more antimicrobial classes.

Statistical analyses

The relationship between pathogens and the presence of diarrhea was studied using Fisher's exact test with a confidence interval of 95%. Statistical analysis was performed with R software, version 4.1.1.

Results

C. difficile was isolated in 6 of 190 samples (3.15%, CI 95% 0.67 - 5.64%). Five were from swabs and one from a first parturition doe. All positive samples were from diarrheic rabbits. Molecular studies revealed that all *C. difficile* isolates belonged to ribotype 126 and were positive for toxins A, B and binary toxin.

E. coli was isolated in 45 (45/49, 91.83%) diarrheic rabbits. Twenty-six of them (26/49, 53.06%) were positive to intimin. In 17 rabbits, intimin-positive *E. coli* was isolated as unique pathogen (Table 7). Five *E. coli* isolates were lactose negative and negative to all virulence factors studied.

C. spiroforme was observed by Gram staining in 9 rabbits with diarrhea (9/49, 18.36%), while *C. perfringens* was

isolated in 18 (18/49, 36.73%). *C. perfringens* was frequently isolated with *E. coli*, both intimin-positive and intimin-negative (Table 7).

Table 7. Association between pathogens in 49 rabbits studied.

Association	n
C. difficile, E. coli **negative to intimin**	**1**
C. perfringens, **no other pathogen**	1
C. perfringens, E. coli **negative to intimin**	5
C. perfringens, E. coli negative to intimin, C. spiroforme	3
C. perfringens, E. coli **positive to intimin**	8
C. perfringens, E. coli **positive to intimin, *C. spiroforme***	1
E. coli **with intimin**	14
E. coli **with intimin, *C. spiroforme***	3
E. coli **without intimin**	8
E. coli **without intimin, *C. spiroforme***	2
No pathogen	3
Total	49

Antibiotic resistances of *C. difficile* are shown in

Table 8. All isolates were resistant to erythromycin, while only two were resistant to clindamycin. None isolate was considered MDR.

Pathological findings were variable depending on the pathogens isolated. In the doe from which *C. difficile* was isolated, clinical signs corresponded with mild diarrhea. Necropsy did not show macroscopic lesion and microscopic examination only revealed generalized edema in the digestive system. *E. coli* was also isolated but was negative to intimin.

Table 8. Antibiotic resistance MIC (µg/mL), assessed by E-test. Concentrations considered resistant are in bold. CD: Clindamycin, E Erythromycin, MTZ Metronidazole, MXF Moxifloxacin, TE Tetracycline, VA Vancomycin

Isolate	CD	E	MTZ	MXF	TE	VA
TAHI 14	1	**256**	0,047	0,25	6	3
TAHI 16	**256**	**256**	0,125	0,38	1,5	0,75
TAHI -16	**256**	**256**	0,38	0,75	2	3
TAHI -17	3	**256**	0,25	0,5	2	2

Rabbits in which intimin-positive *E. coli* was isolated presented mild diarrhea and macroscopic lesions were mainly characterized by mild to moderate hyperemia in the small intestine and cecum, and fluid contents. Less frequently, severe cecal hyperemia and edema were observed in some rabbits. Two rabbits also presented mucous content in the intestine, compatible with ERE, and one rabbit also presented ascites. Microscopically, attaching and effacing lesions were observed and were characterized by effaced villus tips and attached bacteria (Figure 6).

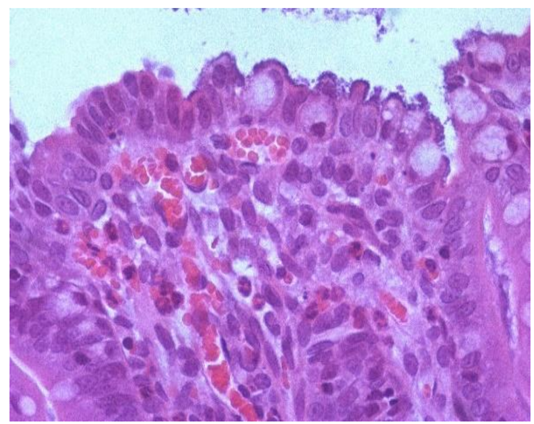

Figure 6. Attaching and effacing lesions characteristic of intimin-positive *E. coli*.x400.

In five rabbits, numerous parasites consistent with coccidian were observed (Figure 7).

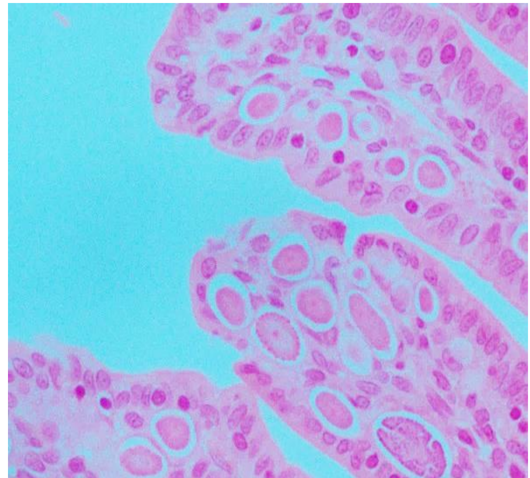

Figure 7. Presence of parasitic structures within the intestinal lamina propia. Animal was also positive to *C. perfringens*. x400.

In the rabbits with intimin-negative *E. coli* and with no other pathogens, mild typhlitis and greenish content in the jejunum and ileum was observed. These rabbits presented a generally better state of health. Histologically, submucosal edema and hyperemia was found, mostly affecting cecum.

In the rabbits from which *C. perfringens* (n = 18) was isolated the lesions were diarrhea, edema and intense hyperemia and hemorrhagic content (Figure 8). Histologically, hyperemia and hemorrhages were found in the jejunum and cecum. In those with positive-intimin *E. coli*, attaching and effacing lesions were also present.

Gross lesions in the remained rabbits were mainly observed in the cecum and corresponded with a dilated cecum with variable presence of gas, green to reddish fluid contents and moderate to intense hyperemia and edema. In some rabbits, the small intestine, mainly the jejunum and ileum, appeared mildly hyperemic, with greenish fluid content. Microscopically, a moderate to severe acute catarrhal typhlitis was observed in all rabbits and in some of them with jejunitis and ileitis. The lamina propia was infiltrated by a variable proportion of neutrophils and was edematous and hyperemic.

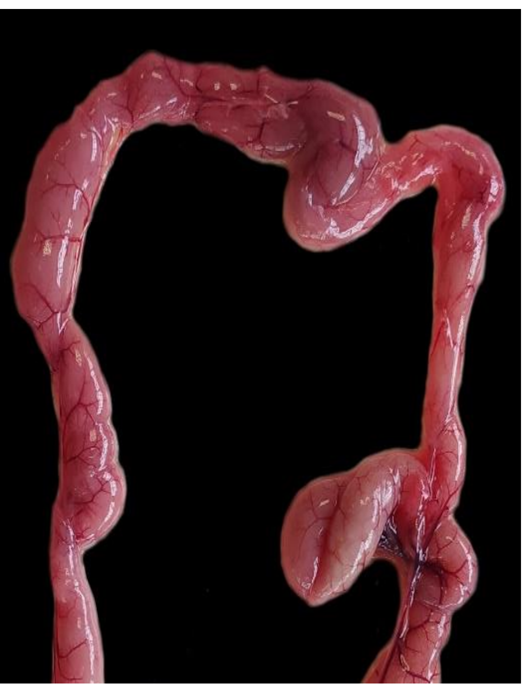

Figure 8. Hyperemia in the small intestine. Rabbit positive to *C. perfringens* and intimin-negative *E. coli*.

Discussion

Studies on the importance of *C. difficile* in meat rabbits are very scarce compared to other animal species. Only three reports of natural infection have been reported (Perkins et al., 1995; Drigo et al., 2015; Taha et al., 2019) and a few more on experimental infections (Warren et al., 2012). Rabbits have been used as animal model of antibiotic-associated *C. difficile* infection (Perkins et al., 1995). The first report of a natural case was in 1995 (Perkins et al., 1995); these authors isolated *C. difficile* from 3 of 4 specific-pathogen-free rabbits, suffering of diarrhea. However, the first report in meat rabbits was in Italy, in 2015 (Drigo et al., 2015). These authors isolated *C. difficile* in 3% of 1279 rabbits. In Egypt, Taha et al. (2019), detected *C. difficile* in 10% of 50 rabbits. The present study shows limited prevalence (3%) to those reported in natural cases and suggests that *C. difficile* is uncommon in meat rabbits and an unusual cause of diarrhea in young rabbits in Spain.

Molecular characterization of *C. difficile* is important as zoonotic ribotypes depends on the species and geographic region. Few studies have been performed in rabbits, one in Italy (Drigo et al., 2015) and a preliminary study in Spain (Andrés Lasheras, 2016). The present study revealed that all isolates showed low genetic diversity, and all belonged to ribotype 126. These results are in agreement with a previous study in Spain in which four out of 48 isolates were considered ribotype 126 (Andrés Lasheras, 2016), but clearly differs from that reported in Italy, where the diversity of ribotypes seems to be higher (Drigo et al., 2015). They found 16 different ribotypes among 38 isolates, but ribotype 126 was not detected. Although this difference could be explained by the small number of samples studied in the present study, the great number of rabbits studied by Driego et al., suggest that ribotype 126 is not frequent in Italy but usual in Spain. In Italy, however, ribotype 078 was frequently isolated, which is highly related to RT 126 (Knetsch et al., 2011). Ribotype 126 has been found frequently in humans in Greece (Kachrimanidou et al., 2022), thus rabbits may be a risk for humans in Spain (Kachrimanidou et al, 2022). In Taiwan, infection with ribotype 126 was associated with acquisition at the hospital, although the original source was not explored (Hung et al, 2014). In Sweden, human infections with ribotypes 078/126 have been commonly described and, interestingly, infections are clinically characterized by a high recurrence rate (Sandell et al, 2016). In some reports, ribotypes 078 and 126 are not distinguished from each other because sequence types are often reported instead of ribotypes (both belong to ST 11), or the band patterns are not correctly distinguished. Consequently, health implications of this ribotype in humans might be underestimated, given its high prevalence in animals (Baghani et al, 2020; Hung et al, 2014).

The carrier state is important in *C. difficile* infection in all species. In rabbits, it has been isolated from normal and diarrheic rabbits, although tend to be associated with digestive problems (Drigo et al., 2015). These authors, despite the not-significant differences, *C. difficile* was found in 0.7% of 143 rabbits without digestive problems and 3.3% of 1136 diarrheic rabbits. In our study, there was not either a significant statistical relationship between health status and the presence of *C. difficile*; however, all the positive samples came from animals with diarrhea, suggesting that there is a relationship between diarrhea and the presence of *C. difficile*. However, as some isolates were from swaps, the role of other pathogens cannot be ruled out.

Toxin determination of *C. difficile* is relevant due to the intestinal carriage of toxigenic and non-toxinogenic strains. In our study, all isolates were toxigenic and shared the same toxigenic profile. This agree with a preliminar study in Spain in which 4 ribotypes 126 were toxigenic (Andrés Lasheras, 2016). Drigo et al. (2015), found a 34% of non-toxinogenic strains; however, these authors studied a greater number of isolates.

Antibiotic resistances are important in *C. difficile*. A worrying increase resistance to the classic antibiotics used in human such as metronidazole and vancomycin is observed. In the present study, all isolates were not resistant to these antibiotics.

Enteropathies in rabbits are complex and difficult to diagnose as several pathogens are typically involved. In addition, some pathogens are commensal. Thus, it is important to correlate their presence with clinical signs, pathological findings and other pathogens. In the present study, necropsied rabbits frequently harbored *C. perfringens* and *E. coli*.

C. perfringens was frequently isolated together with *E. coli*. It was isolated mainly in the small intestine, eleven times in the jejunum, eight in the ileum and five in the cecum and colon. The jejunum has been proved to be the most susceptible to the damage caused by *C. perfringens* toxins (Vidal et al., 2008). Additionally, the lesion distribution of this bacteria has not been fully described, although our higher presence in jejunum is in agreement with the small intestine being the part of the digestive system more associated with this bacterium (Uzal & McClane, 2012). However, the mere presence of *C. perfringens* or alpha toxin is not enough to consider it as a causative agent of disease (Percy et al., 1993).

C. spiroforme is important in rabbits; however, its diagnosis is difficult if no molecular studies are performed. In our study the presence of this pathogen was limited and no gross or microscopic lesions were observed in rabbits with gram-coiled bacteria present in intestinal smears. The prevalence was also lower than in previous reports (Solans et al, 2019; Tuomisto et al,

2024). This discrepancy, however, might be attributed to the differences in the methodologies used, particularly the absence of molecular biology techniques in our study.

E. coli was isolated very frequently. Interestingly, it was found as the only pathogen in 27 diarrheic rabbits, although 10 of them were intimin-negative. Pathogenic *E. coli* in rabbits is usually intimin-positive and was common in our study, confirming that EPEC is one of the main causes of digestive problems in rabbits (Stakenborg et al., 2006). The meaning of the presence of numerous cases of diarrheic rabbits in which only intimin-negative *E. coli* was detected is unclear, especially because no other pathogens were found. These *E. coli* strains could be commensal, or other undetected pathogens may be responsible for the symptoms; nevertheless, it is interesting to continue studying this phenomenon. The isolation of five intimin-negative *E. coli* that did not fermented lactose, associated with signs of colibacillosis and without other pathogens was also intersting. Lactose-negative *E. coli* are strains greatly underestimated in most microbiology laboratories; however, they are important pathogens in humans, often isolated from intestinal and extraintestinal sites (Mazumder et al., 2022). In animals, information is limited with sporadic reports in dogs and poultry (Siqueira et al., 2021; Zanella et al., 2000). These strains may represent emerging high-risk clones with atypical biochemical phenotypes that should not be neglected in epidemiological and One Health studies (Mazumder et al., 2022; Santos-Neto et al., 2022). In humans, this kind of strains have been gaining recent attention, and have been described as capable of causing disease in both children and adults, although the pathogenesis behind its pathogenicity is still unknown (Kaczmarek et al, 2017; Sharma et al, 2023). The present study suggests that these *E. coli* strains might be important in some rabbits, but further studies are needed.

Pathological findings in necropsied rabbits corresponded to varying degrees of edema and hyperemia in both the small intestine and cecum and fluid content, as typically reported (Okumu et al., 2014; Uzal et al., 2016). However, no etiology could be suspected and microbiological studies were necessary. Unfortunately, only one necropsied rabbit was positive for *C. difficile* in our study and the lesions were nonspecific, dominated mainly by vascular changes. Only one report has described *C. difficile*-associated lesions in naturally occurring cases of enteritis in rabbits (Perkins et al., 1995). They described necrotic enteritis with edema and hemorrhages, especially in the ileum; however, the description is confusing since it was based only on the presence of toxins and no other pathogens were ruled out. In our study, *C. difficile* was

isolated together with intimin-negative *E. coli*, so interpreting the findings is even more complicated. Furthermore, some gross changes described by Perkins et al., such as gastric rupture in two of the four rabbits studied, suggested that the time between death and necropsy was too long to adequately assess the intestinal tract microscopically. In our study, a small number of rabbits were used for microscopic analysis because only euthanized diarrheic rabbits or rabbits that died ten minutes before necropsy were used.

Epizootic rabbit enteropathy was observed in some rabbits, but microbiological findings were inconsistent. All were negative for *C. difficile* and isolation of *C. perfringens* and *E. coli* did not show significant relationship. *C. perfringens* has been previously linked to this disease (Huybens et al., 2010; Licois et al., 2005; Marlier et al., 2006); however, their association is contradictory at the moment (Romero et al., 2011). The role of other pathogens in this disease remains unknown at present, with a suspected multicausal bacterial origin in which altered microbiota and management conditions also play a role (Puon Peláez et al., 2018).

Conclusions

This study shows that *C. difficile* is infrequent in rabbits in Spain, although some animals may carry toxigenic strains of the ribotype 126 and represent a risk to humans. The role of *C. difficile* in enteric disease of rabbits has not been finally demonstrated due to the low number of isolations, although all positives were from diarrheic animals, suggesting a possible clinical association. This study has also shown that *E. coli* is important in digestive pathology of rabbits, although the high detection of intimin-negative and lactose-negative strains deserves further studies.

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Trabajo 3

Characterization and antimicrobial resistance analysis of *Clostridioides difficile* and *Clostridium perfringens* isolated from wastewater

Introduction

In 1880, the genus *Clostridium* was proposed. Ever since, a myriad of spore forming, anaerobic rods, most of them Gram-positive stained, have been classified in this genus, some of great clinical importance, like *Clostridium perfringens* or *Clostridium difficile* (Cruz-Morales et al., 2019). This genus has ever since suffered important changes in nomenclature and structure, mostly due to 16S rRNA sequencing (Lawson & Rainey, 2016). These modifications included the change of nomenclature and genus of *Clostridium difficile* to *Clostridioides difficile* (Lawson et al., 2016). In this study, we focused on the isolation of *C. difficile* and *C. perfringens* in wastewater. Both bacteria have something in common: they are capable of forming spores that are highly resistant in the environment, but also of causing severe illness in humans and animals.

C. difficile has historically been associated with antibiotic treatment in hospital settings, while toxicoinfections and gangrene has been associated with *C. perfringens* (Azimirad et al., 2019; Mullish & Williams, 2018).

Particularly interesting is the surge of cases of community acquired *C. difficile* infection (CA-CDI), and the role that animals and environment can play in its transmission (Lim et al., 2020). Ever since, *C. difficile* has been described in both in animals and the environment, reinforcing the idea of the need of a One Health approach, as the exact impact of environmental presence of the spores is still under discussion (Bjöersdorff et al., 2021; Fu et al., 2021; Kim et al., 2019; Lim et al., 2020).

C. perfringens has been used as a marker for fecal contamination in water (Bisson & Cabelli, 1980; L. Sorensen et al., 1989; Miller-Pierce & Rhoads, 2019; Vierheilig et al., 2013; Wiedenmann et al., 2006). It has also been associated with illness in humans and animals, including bacteremia or food poisoning (Kiu & Hall, 2018; Stabler et al., 2020). Toxins take a huge role in the onset of this illness. In accordance with the distribution of the major toxins, different toxinotypes have been proposed (Table 9). Toxin α gene is considered to be present in all strains (Mehdizadeh Gohari et al., 2021) although there are descriptions of strains without the *cpa* gene (Bendary et al., 2022). Type A strains with no other virulence factors are usually not able to cause illness in the host (Li et al., 2013). β-toxin action creates small pores in the cells, inducing swelling and later cell death (Nagahama et al., 2015). ϵ toxin gets it activation from protease activity after its release in the intestine.

Table 9. *C. perfringens* toxin-based typing scheme (Rood et al., 2018).

Toxino-type	Toxin					
	α	β	ϵ	ι	CPE	Net-B
A	++	-	-	-	-	-
B	+	+	+	-	-	-
C	+	+	-	-	±	-
D	+	-	+	-	±	-
E	+	-	-	+	±	-
F	+	-	-	-	+	-
G	+	-	-	-	-	+

Both type B and D are related to ruminants (Camargo et al., 2022). Type D is usually found in the soils and environment, and generally causes sudden death in its acute form in lambs, most likely caused by changes in the digestive tract that cause the overgrowth of bacteria (Popoff, 2011). ι toxin is a binary toxin, such as *C. difficile* binary toxin, and part of the group of iota-like binary toxins (Beer et al., 2018). It is associated with type E, which has been described to cause enterotoxaemia in animals, like calves, lambs or rabbits. However, the understanding of this type of enterotoxin is still very limited (Billington et al., 1998; Sakurai et al., 2009). Cpe toxin (enterotoxin) causes cell morphological damage, apoptosis, and it is implicated in cases of foodborne disease (Alouf, 2006; Freedman et al., 2016). Enterotoxin is usually associated with type F, although it can be also present in other toxinotypes, such as C, D or E. Type F *C. perfringens* used to be called type A *cpe*-positive strains, however it acquired the condition of type with the aim to create a clearer epidemiological analysis in the future. The expansion of the toxinotype from 5 to 7 was proposed in 2018 (Rood et al., 2018).

Lastly, the other toxin studied is not part of the toxin scheme and corresponds to β2 toxin. Its prevalence varies in the different animal species (Park & Rafii, 2019) and its role in disease is still not fully understood, as it has also been isolated in healthy animals. It is believed to be associated with piglet diarrhea (Klaasen et al., 1999; Waters et al., 2003). Some studies suggest that this toxin associates and interacts with major toxins to cause disease (Freedman et al., 2015; Gangwar et al., 2022).

Type G toxinotype, associated with disease in chickens (Rood et al., 2018) has not been studied in this work.

In contrast, *C. difficile* main toxins are two: toxin A and B. Some strains are also capable of producing a third toxin, the binary toxin. Although the place of action varies across species (Keel & Songer, 2006), in general they cause harm by disrupting the epithelial tight junctions and inducing epithelial cell death, subsequently producing

inflammatory responses, mucosal damage, and severe diarrhea with colitis (Chandrasekaran & Lacy, 2017). Toxin A and Toxin B can occur separately or together, with their respective toxicities acting independently of one another. (Kuehne et al., 2010). Binary toxin can also be present alone in some strains, though this is uncommon and predominantly associated with asymptomatic carriage. Nevertheless, this toxin has been frequently isolated in animals and from strains responsible for hypervirulent outbreaks (Gerding et al., 2014).

In this study, the presence of these two anaerobic bacteria in wastewater was investigated, and obtained isolates were characterized by the identification of the toxin genes. A selection of these isolates was further studied according to their genomic relatedness and antimicrobial resistance patterns.

Materials and methods

Sample preparation

Wastewater samples from four neighborhoods and two hospitals from Zaragoza, Spain, were collected for analysis in eight different days, from the end of June to the start of August 2020. Hospital samples were not collected on the first day, and from the sixth sampling to the end a new neighborhood was incorporated to the study. Hospitals' samples came from various drainages. In total, 49 wastewater samples were analyzed. Distribution of samples is further detailed in Table 10.

Table 10. Sample quantity according to time and origin. ND: Not done

Date	**Origin**	
	Hospital	Neighbourhood
23/06/2020	ND	4
25/06/2020	2	4
02/07/2020	2	4
07/07/2020	2	4
15/07/2020	2	4
21/07/2020	2	5
28/07/2024	2	5
04/08/2020	2	5

When samples arrived at the laboratory, they were homogenized into one unique flask. They were centrifuged at maximum speed (3300 rpm) for ten minutes and then the supernatant was transferred to another flask. A total of 250 mL of each sample was filtered through a 0.45 µm/pore filter of 90 mm of diameter (Merck KGaA, Darmstadt,

Germany). Afterward, 100 mL of the resultant filtrate were filtered again through a 0.45 μm/pore filter of 47 mm of diameter, and then the filters were plated on TSC and CLO agar. To avoid cross contamination, the instrumental and the filtering device were washed with a 10% solution of bleach between samples, and then rinsed with water. At the end of the process, a tap water sample was filtered and plated in both agar plates as a negative control.

Plates were incubated at 37 °C for 48 hours in anaerobic jars. Afterwards, colonies morphologically compatible with *C. difficile* and *C. perfringens* were re-plated for isolation. A Gram stain test was performed in these suspected colonies, and morphologically compatible Gram positive bacilli were selected for further molecular testing. For the morphological description, *C. difficile* colonies in CLO agar are grey, irregular-edged and non-hemolytic. *C. perfringens* colonies on TSC are black and present an opalescence around the colonies (lecithinase activity).

PCR analysis and MLST

DNA from the selected isolates was extracted using a commercial kit (Tissue Genomic DNA Extraction Mini Kit, favorgen, Taiwan). DNA concentration was measured using Nanodrop (Thermo Scientific, United States of America), and when concentration was low, a more reliable kit for DNA extraction was used (Wizard® Genomic DNA Purification Kit, Promega Biotech, United States of America). For *C. difficile* identification, *gluD* and toxin A, B and binary genes were studied. The presence of *gluD* and the amplification of housekeeping genes were used as a positivity confirmation for *C. difficile* (Lemée & Pons, 2010). Amplification of a fragment of *16S* gene was used as a positive control for the DNA extraction. PCR testing for these genes was performed as described elsewhere (Paltansing et al., 2007; Persson et al., 2008).

For *C. perfringens*, toxin genetic profile was studied in all positive samples, with *cpa* and a fragment of *16S* gene used as a double positive control of the samples. Toxins α, β, β2, ϵ, ι and enterotoxin were studied by PCR (van Asten et al., 2009). Afterwards, a total of 8 housekeeping genes (*colA, groEL, sodA, plc, gyrB, sigK, pgk* and *nadA)* were amplified by PCR for MLST analysis in 13 selected isolates (Xiao et al., 2012). The resulting PCR products were then sent to Stab Vida (Caparica, Portugal) for Sanger sequencing, and sequences were submitted to the MLST PubMed database for characterization and sequence type allocation (http://pubmlst.org/cperfringens/). The sequences used for reference were chosen based on phylogroups proposed elsewhere (Abdel-Glil et al., 2021).

Antimicrobial susceptibility testing

The testing was performed in four *C. difficile* isolates and in six *C. perfringens* isolates. E-test method was used against clindamycin (CD), erythromycin (E), metronidazole (MTZ), moxifloxacin (MXF), tetracycline (TE) and vancomycin (VA). Plates were incubated for 48h in Brucella agar in an anaerobic jar, and *C. difficile* metronidazole plates were also read after five days in order to detect heteroresistant strains (Peláez et al., 2008). CLSI breakpoints were used in both bacterial species when possible (CLSI, 2023), and other references were used when the breakpoint was not established (Keessen et al., 2013; EUCAST, 2023; Wei et al., 2020).

Statistical analysis

Prevalence was calculated with 95% confidence intervals (CI). To test simple relationship between type of sample, virulence factors and other associations, Fisher 's Exact Test was used, and the *p*-values determined, considering them statistically significant when *p*-value ≤ 0.05. All the analyses and calculations were performed using R version 4.1.1.

Results

From the 49 samples included in this study, a total of 12 were positive for *C. difficile* while 37 tested positive for *C. perfringens*. In total, nine samples were positive to both bacteria, three were only positive to *C. difficile*, and the rest of samples (n = 28) were only positive to *C. perfringens*. A detailed summary of these isolation results can be seen in Table 11.

The toxin genetic analysis allowed to classify *C. perfringens* isolates (n = 37) in different molecular toxinotypes (A-F), which resulted in 56.76% (IC 95%; 39.48 – 72.9%) of them being toxinotype A and 43.24% (IC 95%; 27.1 – 60.51%) of toxinotype F. No other toxinotypes were found. As for the β2 toxin, which is not associated with a specific toxinotype, 48.64% (IC 95%; 31.92 – 65.6%) were positive, with eight of them being toxinotype A and 10 being toxinotype F.

The majority of our findings regarding to the sequence type profiles revealed novel STs, despite the absence of descriptions for new alleles. Previously documented ST found were 72, 207 and 546. A comprehensive depiction of the samples, phylogenetic relationships, and metadata can be found in Figure 9. New isolates were assigned to STs from 838 to 845.

Table 11. Table of positive samples to each bacterium, grouped by date. Negative samples (n = 8) to both are not included. YP, SK, NP, KG and FR: Neighborhoods; JV and DN: Hospitals

Sampling date	Localization	Positivity to *C. difficile*	Positivity to *C. perfringens*	Sample type
23-jun.	KG	No	Yes	Neighbourhood
23-jun.	NP	No	Yes	Neighbourhood
23-jun.	YP	No	Yes	Neighbourhood
23-jun.	SK	No	Yes	Neighbourhood
25-jun.	DN	Yes	Yes	Hospital
25-jun.	NP	Yes	Yes	Neighbourhood
25-jun.	SK	Yes	Yes	Neighbourhood
25-jun.	KG	No	Yes	Neighbourhood
25-jun.	YP	No	Yes	Neighbourhood
25-jun.	JV	No	Yes	Hospital
2-jul.	NP	No	Yes	Neighbourhood
2-jul.	KG	Yes	Yes	Neighbourhood
2-jul.	DN	No	Yes	Hospital
2-jul.	YP	Yes	Yes	Neighbourhood
2-jul.	JV	No	Yes	Hospital
2-jul.	SK	No	Yes	Neighbourhood
7-jul.	SK	No	Yes	Neighbourhood
7-jul.	NP	Yes	Yes	Neighbourhood
7-jul.	KG	No	Yes	Neighbourhood
7-jul.	YP	No	Yes	Neighbourhood
7-jul.	JV	No	Yes	Hospital
15-jul.	SK	No	Yes	Neighbourhood
15-jul.	NP	No	Yes	Neighbourhood
15-jul.	KG	No	Yes	Neighbourhood
15-jul.	DN	Yes	No	Hospital
15-jul.	YP	Yes	No	Neighbourhood
21-jul.	SK	No	Yes	Neighbourhood
21-jul.	NP	No	Yes	Neighbourhood
21-jul.	KG	No	Yes	Neighbourhood
21-jul.	YP	No	Yes	Neighbourhood
28-jul.	SK	Yes	Yes	Neighbourhood
28-jul.	NP	No	Yes	Neighbourhood
28-jul.	YP	No	Yes	Neighbourhood
28-jul.	JV	No	Yes	Hospital
28-jul.	FR	Yes	No	Neighbourhood
4-aug.	KG	No	Yes	Neighbourhood
4-aug.	YP	Yes	Yes	Neighbourhood
4-aug.	FR	No	Yes	Neighbourhood
4-aug.	JV	No	Yes	Hospital
4-aug.	SK	Yes	No	Neighbourhood
4-aug.	DN	No	Yes	Hospital

Figure 9. Tree visualization of the studied *C. perfringens* isolates alongside selected reference strains, incorporating metadata such as date, source, clinical outcome, country, toxinotype, and sequence type (ST). Studied isolates names from Neighborhoods are in red and Hospital origins are in purple. Abbreviations: Env.: environmental.

In regard to *C. difficile*, only four out of 12 isolates were considered positive for toxins, specifically 2 July – YP to A and B, 15 July - DN and 4 august - SK to A, B and binary and 4 august YP to toxin A.

Statistical analysis between bacteria and type of sample (neighborhood/hospital), presence of type A/F *C. perfringens* and β2 toxin were studied. In all cases, associations resulted not significant except for the association between type of sample and presence of bacteria.

The antimicrobial susceptibility profiles for *C. difficile* revealed two isolates resistant to tetracycline, one susceptible to all tested antimicrobials, and a multidrug-resistant (MDR) isolate (CD-E-TE). In the case of *C. perfringens*, the profiles were more variable, with only one demonstrating multidrug resistance (CD-MTZ-TE), and another one remaining susceptible to all tested antimicrobials. Detailed information on the resistance results can be found in Table 12.

Discussion

With a prevalence of approximately 75% of positive samples for *C. perfringens* and 24% for *C. difficile*, the prevalence, especially for *C. perfringens*, was high. Similar studies have found a prevalence between 36 and 50% (Yanagimoto & Haramoto, 2021; Mueller-Spitz et al., 2010). Even in lower concentrations, *C. perfringens* has been associated with an increased risk of human disease and the analysis of its isolation has been proposed as fecal indicator and quality standards (Wiedenmann et al., 2006).

Table 12. Minimum Inhibitory Concentrations (MIC) associated with the studied isolates in µg/mL, with concentrations in bold indicating resistance to the corresponding antimicrobial agents.

Isolates	Clindamycin (CD)	Erythromycin (E)	Metronidazole (MTZ)	Moxifloxacin (MXF)	Tetracycline (TE)	Vancomycin (VA)
02july- YP ***C. difficile***	0.75	2	0.75	0.38	**16**	1.5
25june DN ***C. difficile***	2	3	1.5	0.5	**48**	2
04august YP ***C. difficile***	3	1	0.125	1	0.064	1
25june SK ***C. difficile***	**12**	**256**	1.5	0.5	**16**	1
28july JV **C. perfringens**	0.064	0.38	0.25	0.5	0.047	1.5
23june JV ***C. perfringens***	**16**	0.75	0.023	0.047	0.064	12
23june YP ***C. perfringens***	**4**	8	**256**	0.75	**32**	4
07july YP ***C. perfringens***	**8**	**24**	0.5	0.75	0.064	8
02july JV ***C. perfringens***	0.064	4	**256**	0.5	3	8
23june SK ***C. perfringens***	0.38	0.125	1.50	0.38	**8**	2

Regarding *C. perfringens*, when contrasting to studies from existing literature, this investigation reports a prevalence of type F in wastewater samples of 43.24%, indicative of a slightly lower occurrence compared to similar studies (Mueller-Spitz et al., 2010; Yanagimoto et al., 2020). It is noteworthy, however, that the prevalence observed in this study remains substantial, suggesting variability in *C. perfringens* prevalence across different settings.

In our investigation, we detected a notable prevalence of *C. perfringens* isolates carrying the enterotoxin gene, corroborating the significance of these enteropathogens in the context of environmental samples. This finding aligns with a prior study (Mueller-Spitz et al., 2010) where a similar trend was observed in wastewater samples. Despite the older classification system employed in that study, which did not differentiate between types A-F, the consistency in the prevalence pattern emphasizes the enduring presence and potential impact of these enteropathogens.

Although the prevalence of enterotoxin positive isolates has been described as low as 5% (Smedley et al., 2004), similar prevalence has been found in soils, once again indicating the great

diversity that this bacterium can exhibit (Park & Rafii, 2019). In contrast, some studies have reported minimal occurrences of the *cpe* gene in environmental samples. Our findings propose that the origin of sewage may play a role in the dissemination of *C. perfringens* in contaminated water. It is important to note that the presence of the *cpe* gene alone is insufficient to ascertain its pathogenic potential (Kokai-Kun et al., 1994), yet it signifies a possibility that these strains may function as a reservoir.

Since all samples came from human-related environments, it is not surprising that none of them were from toxinotypes associated with animals. Type A isolates can usually be considered as non-toxigenic, since toxin α is considered a housekeeping gene (Mehdizadeh Gohari et al., 2021). However, type F isolates harbor the potential of causing human disease (Freedman et al., 2016).

The presence of the enterotoxin in isolates from environmental water samples poses a significant threat to public health. When strains with this type of toxin get in contact with human, it can lead to waterborne infections when ingested or coming into contact with wounds. However, the highest risk is when the spores enter in contact with food, where they germinate and multiply (Cortés-Sánchez, 2018). These infections can range from mild gastrointestinal discomfort to severe illness, particularly impacting vulnerable populations. Moreover, *cpe*-positive strains may cause more severe illnesses and contribute to outbreaks.

Despite the relationship between type of *C. perfringens* and type of sample being not significant, one possible explanation for the predominance of type A isolates in hospital samples is that they could be of environmental origin, possibly originating from sources like rats or other urban pests commonly found in wastewater canals. Isolates containing enterotoxin can serve as a potential source of future human illnesses and could also indicate the presence of *C. perfringens* cases in the population. However, the fact that only one isolate from a hospital was confirmed to be type F (with the others being type A) can also mean that the prevalence of *C. perfringens* cases in hospital settings might be low. This aligns with previous research in Spain, where *C. perfringens* infection cases are infrequent but highly lethal (Lopez-Fabal et al., 2018). The only statistical relationship found was between the presence of bacteria and the type of sample (p = 0.033), which can be explained by the frequent use of disinfection measures before the discharge of wastewater in hospitals that might be limiting the isolation of these more hazardous pathogens, further contributing to the predominance of type A isolates. The association between 'Type A' and 'Type F' and the origin ('Neighborhood' or 'Hospital') was analyzed, and the statistical tests did not reveal a

significant relationship between these variables.

In our investigation, the frequency of β2 toxin was determined to be 48.64%. This outcome is notably comparable to analogous studies, like Yanagimoto et al. in 2020 which reported a 52% positivity rate to *cpb2* or Álvarez-Pérez et al. in 2016 with a 42% prevalence. While the specific role of β2 toxin in infection remains under debate, our results contribute valuable insights. The observed prevalence aligns with other studies conducted in both wastewater and water sources linked to animal environments. This consistency underscores the need for further exploration to unravel the potential implications and significance of β2 toxin within the context of *C. perfringens* dynamics.

The identified STs that were not novel encompassed ST 72, previously isolated in the USA and Japan (clinical source); ST 207, previously identified in France (food source); and ST 546, isolated in China and Finland (both from clinical sources). Notably, isolates tended to cluster based on their sources, with those of nosocomial origin displaying a tendency to group with food poisoning isolates. Conversely, isolates from animal sources appeared more distant on the branches of the phylogenetic tree. These observations carry implications for public health, emphasizing the need for targeted strategies and interventions in different sources to manage and mitigate the potential spread of these pathogens.

Apart from that, two *C. perfringens* isolates were considered resistant to metronidazole. The description of metronidazole resistance is still not common, although it has been previously reported (Baghani et al., 2020; Slavíc et al., 2011; Álvarez-Pérez et al., 2016). While a correlation between the absence of β2 toxin and metronidazole resistance has been reported in the literature (Álvarez-Pérez et al., 2016), our study revealed that only one of the metronidazole resistant isolates harbored this toxin gene, while the other did not. These isolates, originating from distinct sources (neighborhood and hospital), displayed significant phylogenetic divergence, implying independent events of resistance acquisition. The observed high variability in resistance patterns within *C. perfringens*, with the set of isolates as a whole showing resistance to four out of six tested antibiotics, raises concerns. This is particularly troubling given the genomic openness of *C. perfringens*, which facilitates the exchange of antimicrobial genes with phylogenetically distant bacterial species, as documented in previous research (Álvarez-Pérez et al., 2016).

As for *C. difficile*, its prevalence, while relatively low, is not insignificant, aligning with earlier reports suggesting the pathogen's capacity to withstand disinfection procedures when not specifically targeted (Freier et al., 2023). The

observed prevalence of *C. difficile* (12/49 = 24%) exceeds rates reported in certain studies but falls below those in others (Chisholm et al., 2023). The variability in prevalence across the literature is likely attributable to disparities in the isolation procedures employed, emphasizing the necessity of a standardized methodology in the isolation of this pathogen from wastewater. The isolation of *C. difficile* exhibited no significant association with the source of isolation, indicating that the presence of *C. difficile* was not notably linked to hospital settings. Possible explanations for this phenomenon include the effectiveness, albeit imperfect, of disinfection procedures, or the possibility that the origin of the isolates is unrelated to the specific source, suggesting that the identified isolates may have a community rather than clinical origin.

On the other hand, our investigation revealed the absence of detectable toxin genes in *C. difficile*. Despite multiple attempts using diverse extraction methods, the DNA extraction process exhibited suboptimal quality. This challenges the interpretation of our findings, suggesting that the lack of toxin detection may stem from natural factors, given the typically lower toxin levels in environmental samples compared to clinical ones (Janezic et al., 2016), or from unresolved technical issues.

Antibiotic resistance in *C. difficile* was found to be heterogeneous among the isolates. One isolate was MDR (E-CD-TE), a second was susceptible to all antimicrobials tested and the remaining two were resistant only to tetracycline.

The tetracycline resistance in the two isolates might be attributed to the presence of specific resistance genes such as *tet*(M), which are commonly located on mobile genetic elements, facilitating their transfer between bacteria (Spigaglia, 2016; Spigaglia et al., 2006). On the other hand, resistance to macrolide-lincosamide-streptogramin B is often associated with gene *erm(B)*. This gene could explain the resistance to erythromycin (a macrolide) and clindamycin (a lincosamide) observed in the 25june-SK isolate (Farrow et al., 2000; Spigaglia & Mastrantonio, 2002). Importantly, none of the isolates demonstrated resistance to metronidazole, vancomycin, or moxifloxacin. This finding, along with the fully susceptible isolate, indicates that not all *C. difficile* strains present a therapeutic challenge, as these antibiotics remain effective. The heterogeneity in antibiotic resistance profiles among *C. difficile* isolates highlights the complexity of bacterial adaptation to antibiotic pressure. Continuous monitoring and genetic studies are essential to understand the mechanisms driving resistance.

Conclusion

In conclusion, this study reinforces the idea that sewage can be a reservoir for bacteria, especially those that are very resistant in the environment such as sporulated anaerobes. Our results show that *C. perfringens* toxinotypes usually associated with humans are present in urban wastewaters. On the other hand, *C. difficile,* albeit in smaller quantities, was also isolated from wastewater, which carries potential public health implications. However, the fact that less bacteria were isolated in wastewater samples with a hospital origin shows that a good disinfection is key to the control of the spread of this pathogen.

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Trabajo 4

Clostridioides difficile in pig farms from farrowing to weaning, characterization and resistance profiles

Introduction

Clostridioides difficile, formerly known as *Clostridium difficile* (Lawson et al., 2016), is an anaerobic, Gram positive, spore-forming bacterium that can be found in multiple species and in the environment (Bolton & Marcos, 2023). It has been traditionally described as a nosocomial pathogen in humans, but more recently another form of infection has been recognised, that is community associated *C. difficile* infection (CA-CDI) (Otten et al., 2010). The search for sources of human CDI as an effort to explain the recent changes in the epidemiology (emergence of new hypervirulent strains as well as increase of CA-CDI cases) has pointed the search of diverse sources of infection, highlighting the investigation of animal reservoirs (Cookson, 2007; Turner et al., 2019; Zanichelli et al., 2020). Particularly, it has been suggested that swine could have a role in CA-CDI (Brown & Wilson, 2018; Squire & Riley, 2013; Tsai et al., 2021), due to the zoonotic potential of strains commonly found in pigs such as *C. difficile* sequence type 11 (Knight & Riley, 2019).

However, there are still numerous characteristics that remain to be fully clarified in swine, such as aspects concerning susceptibility to infections, the mechanisms of pathogenesis, and the factors associated with increased risk (Moono et al., 2016). Notably, a remarkable observation is that animal isolates, particularly in porcine populations, are often associated with hypervirulent ribotypes. These ribotypes typically include RT 078 and, more broadly, 078-like ribotypes, such as RT 126 (Álvarez-Pérez et al., 2017).

Neonatal diarrhoea causes important economic losses in the pig industry and *C. difficile* is considered an important cause of enteric disease worldwide (Moono et al., 2016), however its role as a cause of swine diarrhoea is still debated (Alvarez-Perez et al., 2009). The presence of *C. difficile* in pigs is usually associated with young animals (Proctor et al., 2021) and decreases with age, although it has also been described in adult pigs or at carcasses in slaughterhouses (Hopman et al., 2011b; Rodriguez et al., 2016; Wu et al., 2017). Notably, in experimental challenges, no clinical signs were observed after one week of age (Proctor et al., 2021).

C. difficile is also increasingly associated with multidrug resistance (MDR) (Fuzi et al., 2020; Ramírez-Vargas et al., 2017), defined as non-susceptibility to three or more antimicrobial categories (Magiorakos et al., 2012). The description of strains with reduced susceptibility to first-line antibiotics, such as metronidazole, could complicate the treatment of CDI in humans (Darkoh et al., 2022; Phanchana et al., 2021). In swine populations, the isolation of a significant number of MDR strains is a common occurrence (Andino-Molina et al., 2019; Lührmann et al.,

2023; Peláez et al., 2013), emphasizing its potential as a reservoir for MDR.

Consequently, a One Health perspective for this infection is required (Squire & Riley, 2013), and an adequate understanding of the epidemiology in swine may thus contribute to the improvement of CDI management and prevention.

This study, therefore, focuses on describing the prevalence of *C. difficile* in three farms, from farrowing to weaning. In addition, the characterization of the strains isolated and relatedness among isolates from different farm sources is also studied, along with the phenotypical resistance patterns of selected isolates.

Materials and methods

Animal population and sample collection

Samples were collected in three different farms: one farm was sampled in December 2016 (farm A). Another farm was sampled in November 2020 (farm B) and the third one, in March 2021 (farm C). The production facilities were situated in the northeastern region of Spain, a primary hub for pig farming within the country.

As sows were grouped by parturition date, a batch of 8 sows from the same unit were chosen for each sampling. Faecal samples from each sow were collected directly from the rectum to sterile containers, one month before farrowing and at the same time as the piglet sampling, except for farm A, in which samples from sows were only taken at the pre-farrowing stage. A total of 87 samples from sows were analysed.

After parturition, 5 piglets from each sow were sampled using rectal swabs at four different times: 24h-48h after parturition and then weekly until the end of the lactation stage, i.e. 3 weeks. Extra piglets were identified to choose if a piglet died during the sampling period. At the end, a total of 475 samples from piglets were studied.

The general health status of the animals, including whether or not they had diarrhoea and any antibiotic treatment received, were also recorded. In farm A, piglet litters were treated when a surge of digestive disorders occurred. On the other hand, farms B and C followed a protocol that involved administering amoxicillin and toltrazuril on day 3, with male piglets receiving a second dose of amoxicillin on day 5.

Additionally, samples from the surface of the sows' teats and different areas of the farm environment were also collected along the sampling period. A sterile cloth dampened in Brain Heart Infusion Broth (Oxoid, Basingstoke, Hampshire, UK) was used to sample these surfaces: sow teats, floors of the individual

farrowing crates, aisle of the farrowing unit, entrance to the farrowing unit, the gestation pen and the workers' changing rooms. Additionally, samples of the drinking water provided to the animals and the animal feed were collected in sterile containers during the pre-farrowing stage. In this case 115 samples were collected, making it a total of 676 samples analysed in this study including all types of samples. Sample distribution regarding origin and collection time can be seen in Table 13 (results section).

Upon arrival to the facility, they were stored at a temperature of -30 °C until further processing.

Isolation of *C. difficile*

Bacteriological culture was conducted following the procedure outlined by Andrés-Lasheras et al. (2017). In summary, samples were diluted 1:10 in 9 mL of BHI (Oxoid) supplemented with *C. difficile* selective supplement (C.D.M.N. Selective supplement; Oxoid), 6 g/L fructose (Sigma-Aldrich, St. Louis, Missouri, US) and 1 g/L sodium taurocholate (Sigma-Aldrich), and then anaerobically incubated at 37 °C for 6 days. In the case of cloth, a sterile container with 45mL of the enriched BHI was used. After incubation, 2mL of broth were mixed with 2mL of ethanol 96°, and incubated at room temperature for 1 hour. The result was centrifuged (4000 rpm for 15 minutes) and the precipitates were plated on cycloserine-cefoxitin agar (CLO agar; bioMérieux, Marcy L'Etoile, France). Plates were incubated under anaerobiosis at 37 °C for 48 hours, and a second reading was performed at the 72 hours when no *C. difficile* growth was initially observed.

Identification of *C. difficile* was performed based on colony morphology (characterized by grey, irregular shape, and absence of hemolysis, as depicted in Figure 10) and Gram stain. Moreover, bacterial DNA was extracted from pure cultures utilizing the FavorPrep Tissue Genomic DNA Extraction Minikit (favorgen). Confirmation of isolates as *C. difficile* was achieved through the amplification of the specific glutamate dehydrogenase (*gluD*) gene (Paltansing et al., 2007). Three colonies per positive sample were isolated and preserved at -30 °C for subsequent analysis.

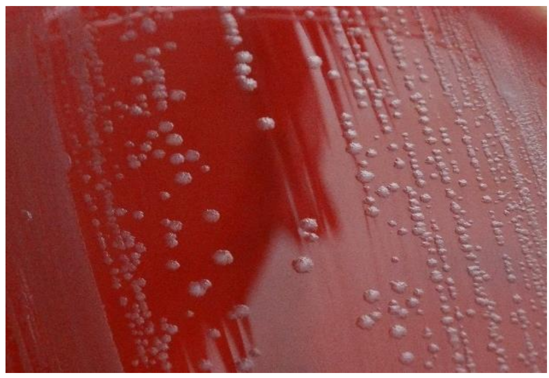

Figure 10. Morphologic details of *C. difficile* isolates in blood agar.

Detection of toxin genes, PCR-ribotyping and MLVA

To detect toxin A (*tcdA*), toxin B (*tcdB*), and the binary toxin (*cdtA*/*cdtB*) genes, multiplex PCR was performed on one isolate from

each positive sample (Persson et al., 2008). *C. difficile* isolates underwent additional characterization through capillary ribotyping, following previously established methods (Fawley et al., 2015). PCR-ribotypes were assigned using the Leeds-Leiden database.

Additionally, those isolates from farms A and B were also chosen to perform multiple locus variable analysis (MLVA). The primers and conditions used for MLVA analysis have been described elsewhere (van den Berg et al., 2007). Markers B, E, F and H were taken into account to create the initial matrix, and UPGMA (*unweighted pair group method with arithmetic mean)* was used for clustering. Source, ribotype and time were included as metadata.

Antimicrobial susceptibility testing

A total of 41 *C. difficile* isolates were selected for this purpose, 33 of them from farm A, and the 8 from the other two farms, B and C. This selection included isolates from sows and piglets of their corresponding litters, as well as isolates from environmental samples and from teats of the sows. The primary objective of this selection was to evaluate potential changes in antimicrobial resistance patterns over time. To achieve this, whenever feasible, isolates obtained during the initial phase of sampling were compared with isolates from the same individual or environmental location collected during the last sampling phase.

Antimicrobial susceptibility testing was conducted against six agents—clindamycin, erythromycin, metronidazole, moxifloxacin, vancomycin, and tetracycline—using the E-test method (Liofilchem, Teramo, Italy) on Brucella blood agar supplemented with hemin and vitamin K1 (Sigma-Aldrich). The plates were incubated at 37 °C for 48 hours under anaerobic conditions. In the case of metronidazole, plates were further incubated up to 5 days to detect slow-growing resistant subpopulations (Peláez et al., 2008). Antimicrobial resistance categorization was determined using breakpoints established by the Clinical and Laboratory Standards Institute (CLSI, 2023). If no breakpoint was available, as in the case of erythromycin and vancomycin, those cut off values suggested by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) were applied (EUCAST, 2023). Multidrug resistance (MDR) was defined as resistance against three or more antimicrobial classes.

Also, plasmid-mediated metronidazole resistance was genetically analysed following the conditions described by Boekhoud et al. (2020). Primers used in these PCR reactions were oBH-1 (5'-CCTCGTAGAATCCGGTGCAA-3') and oBH-2 (5'-TATTTCCTTGCCGCTGAGGT-3').

Statistical analyses

Prevalence estimates were constructed along with their respective 95% Confidence Intervals (95% CI). The Chi-squared test with Yates' continuity correction was utilized to compare differences in prevalence across various sampling times and the presence of diarrhea with a significance threshold set at a *p*-value of 0.05. When expected frequencies were lower than 5 in any category tested, Fisher's Exact Test was used instead. All the analyses were performed R software (R version 4.1.1).

Results

Isolation and characterization of *C. difficile*

Out of the 676 samples collected, 146 resulted positive to *C. difficile* isolation, including all types of samples and stages studied (general prevalence of 19.93% (95% CI: 16.87-23.01%)).

In piglets, the prevalence of *C. difficile* showed a similar trend in all studied farms. Specifically, in farm A *C. difficile* prevalence reached its highest value at the end of the first week, with around 50% of positive piglet samples, and then tended to decrease (Figure 11). In the other two farms, B and C, the prevalence was highest at farrowing (with 16.94% and 18.4% positive samples, respectively), and then the percentage of piglets shedding *C. difficile* started to decrease. Overall, a total of 475 piglet fecal samples were collected, and 99 of them were found positive to *C. difficile* (20.84%; 95% CI: 17.19-24.49%).

In general, prevalence of *C. difficile* in sows reached its highest value between 1st and 2nd week, with very few positive samples being positive during farrowing or pre-farrowing. These patterns were consistent with the results observed in environmental isolates, which also peaked during 1st and 2nd week, although in general positivity was lower. *C. difficile* was not isolated neither from animal feed nor water samples. A detailed summary of *C. difficile* positivity in each type of sample can be found in Table 13.

A total of 30 piglets suffered from diarrhoea at any time during lactation, with 8 of them being positive to *C. difficile* at some point in the sampling period. Specifically, the number of piglets that presented at the same time diarrhoea and *C. difficile* positivity was two, while the number of piglets in which *C. difficile* was detected after an episode of diarrhoea was seven. On the contrary, the number of piglets that presented diarrhoea after having been diagnosed with *C. difficile* was six.

No significant differences were found between healthy piglets and those with diarrhoea regarding *C. difficile* presence (*p*-value = 0.43). The relationship was also non-significant in neonatal period (farrowing and first week, *p*-value = 0.22)

Table 13. Samples analysed in the study and positivity to *C. difficile*.

Type of sample	Pre-farrowing	Farrowing	1st week of lactation	2nd week of lactation	Weaning
Farm A					
Piglets	nd	11/48	25/48	23/52	10/45
Sows	0/11	nd			
Farm B					
Piglets	nd	4/35	1/31	1/28	3/28
Sows	0/8	0/7	0/7	3/6	3/6
Sow teats	0/1	0/1	0/1	0/1	0/1
Farrowing crates	nd	1/1	1/1	0/1	1/1
Floors*	2/3	4/4	3/3	2/3	2/3
Water and food	0/2	nd			
Farm C					
Piglets	nd	12/36	7/46	2/37	0/41
Sows	1/8	1/8	7/9	1/8	3/8
Sow teats	0/1	0/8	3/9	1/8	0/8
Farrowing crates	nd	0/8	2/9	2/8	0/8
Floors*	1/3	0/4	2/4	0/4	1/4
Water and food	0/2	nd			

(nd) not done. * Samples from the farm floors were collected in four different locations at each sampling time: aisle of the farrowing unit, entrance to the farrowing unit, gestation pen and the workers' changing room.

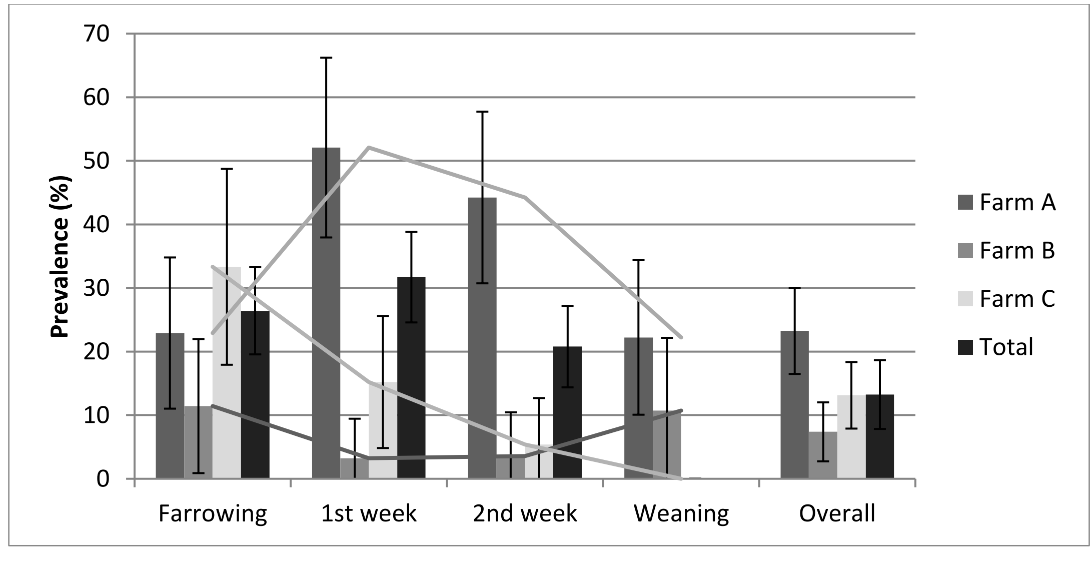

Figure 11. Prevalence of *C. difficile* in the piglet population from each sampled farm.

All the *C. difficile* isolates analysed in this study were positive for toxin A (*tcdA*) and B (*tcdB*) and the binary toxin (*cdtA / cdtB*) genes, with the exception of one isolate, which was considered non-toxigenic. Most of the isolates were assigned to RT 078 (n = 116), and the rest were classified as RT 126 (n = 29).

The non-toxigenic isolate corresponded to a different ribotype, that is RT 731. Interestingly, one of the piglets was *C. difficile* positive thrice along the sampling period, but isolates obtained belonged to different ribotypes: RT 078 those from farrowing and second week of lactation, and RT 126 the isolate from the first lactation week.

MLVA

MLVA analysis showed various profiles in the strains: 9 different patterns among 58 studied strains. Visualization of the clustering can be found in Figure 12

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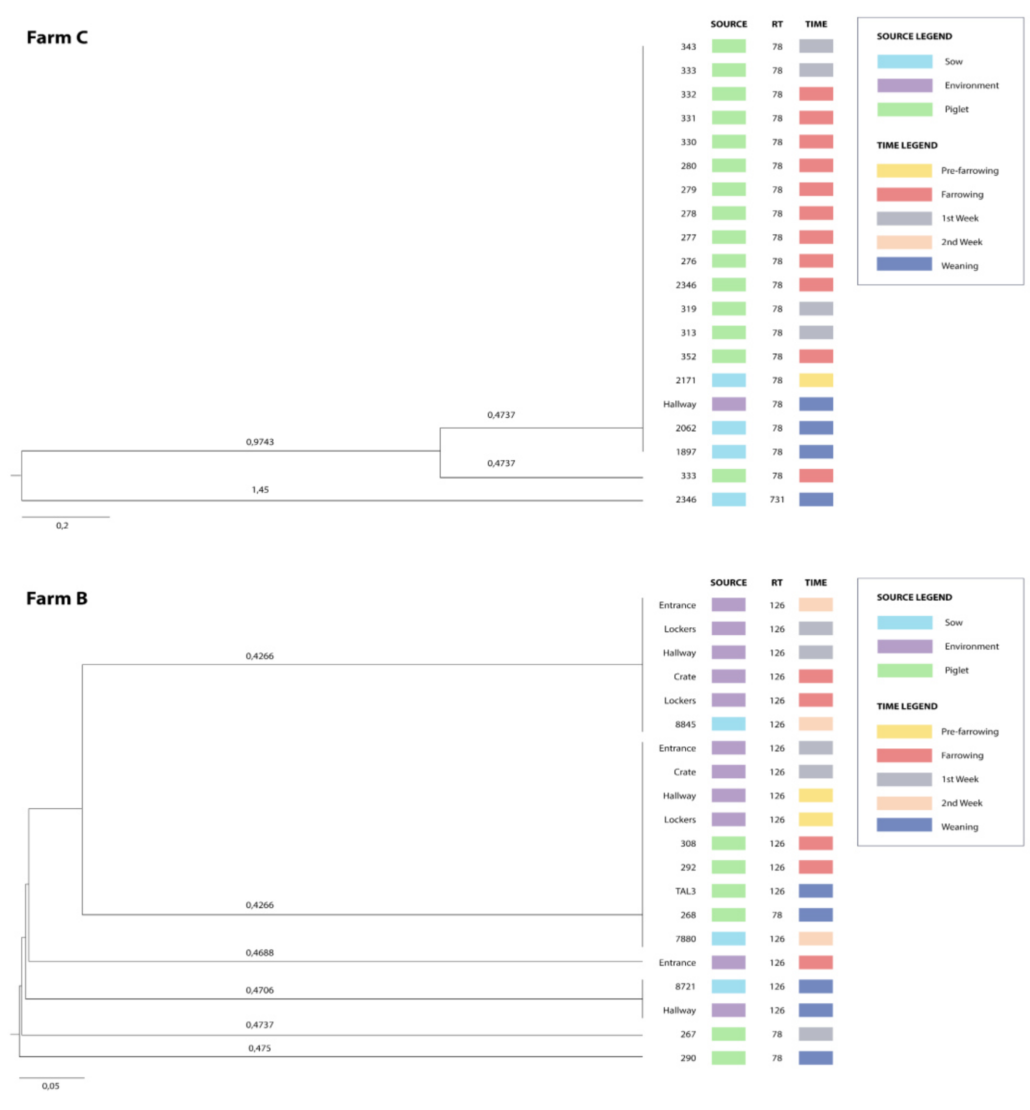

Figure 12. MLVA tree of farms B and C. Metadata includes source from which the stain was isolated, time of isolation and RT.

Antimicrobial resistance

All isolates tested from farm A (n = 33) were phenotypically resistant against erythromycin and moxifloxacin. Also, 24.24% of these isolates presented resistance against clindamycin. All isolates were susceptible to tetracycline, although 16 out of 33 showed intermediate resistance against this antimicrobial agent. No antimicrobial resistance was detected for metronidazole or vancomycin.

In farm A, a MDR profile was found in 24.24% of isolates, presenting all of them the same pattern: fluoroquinolones-lincosamides-macrolides (F-L-M). In isolates from farm B (n = 4), the resistance patterns observed were F-M, L-T and F-L-M, the latter in one of the isolates and thus considered MDR. Isolates from farm C (n = 4) were mostly susceptible to all antimicrobials, with resistance or intermediate resistance to tetracycline, and one of them being also resistant to lincosamides. No MDR profiles were found in this case.

There were two cases of animals having two different ribotypes at different sampling times, all found in farm A. The antimicrobial resistance patterns of these ribotypes were different, with F-M pattern for RT 078 and F-L-M for RT 126 in both cases.

In general, antimicrobial resistance to moxifloxacin and erythromycin was very high (above 90%) in all these *C. difficile* isolates (Figure 13Figure 5). Also, clindamycin and tetracycline, both bacteriostatic agents, were related to high percentages of resistant isolates (including intermediate resistance). No isolates were considered resistant to metronidazole and pCD-METRO was not detected in any of the samples tested.

A comparative analysis of resistance prevalence in isolates obtained during the initial sampling stages (farrowing and first week of lactation) versus later stages (second week of lactation and weaning), revealed no notable differences. This result suggests a phenotypic antimicrobial resistance stability in *C. difficile* isolates over the lactation period.

Discussion

In this study, patterns in *C. difficile* isolation in pig farms were analyzed from farrowing to weaning, including piglet, sow and farm environmental samples. Isolates obtained were further characterized genetically and evaluated in relation to their antimicrobial susceptibility profile. According to these results, piglets exhibited colonization as early as 24-48 hours after farrowing. This finding is in agreement with previous research, as newborn piglets typically get colonized in the first hours of life (Alves et al., 2022; Hopman et al., 2011a; Proctor et al., 2021).

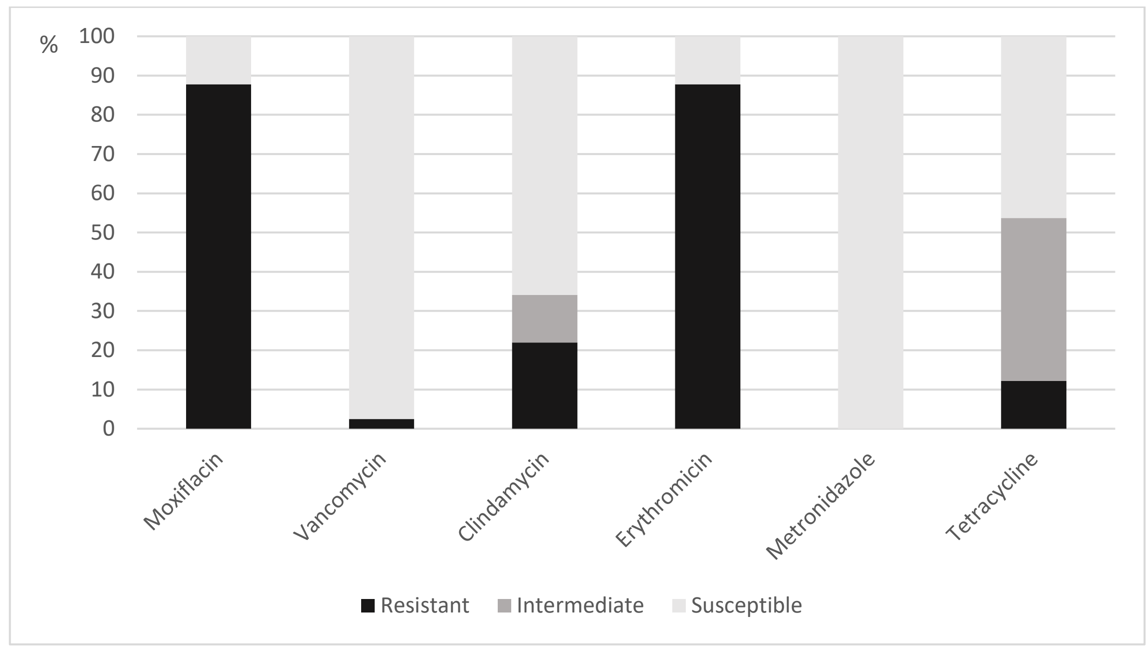

Figure 13. Antimicrobial resistance prevalence in analysed *C. difficile* isolates from piglet, sow and environmental samples.

Sows showed minimal colonization ante partum or during farrowing, with prevalence peaking during the first week post-farrowing, one week later than the piglets, whose highest prevalence in general found at farrowing.

These results were in general lower than those from similar studies, which usually found a prevalence close to 50% or higher. However, the pattern of colonization, that is high at farrowing and then decreasing with age, was consistent with current literature (Knight et al., 2015; Norman et al., 2009; O'Shaughnessy et al., 2019; Schneeberg et al., 2013). Interestingly, a previous study conducted in piglets from Spanish pig farms reported similar prevalence to ours (Alvarez-Perez et al., 2009). High colonization rates up to second week post-farrowing have been also previously described (Schneeberg et al., 2013).

The prevalence of *C. difficile* in both diarrheic and healthy animals did not show significant differences (*p*-value = 0.43). Although there are some studies that reported differences between these groups (Krutova et al., 2018; Waters et al., 1998; Yaeger et al., 2002), most studies do not indicate an appreciably higher prevalence in either group (Knight et al., 2015; Schneeberg et al., 2013; Spigaglia et al., 2023). In neonatal pigs, diarrhoea is typically associated with a range of other factors (Songer & Anderson, 2006).

Considering the results of this study and the existing body of research, it can be concluded that the presence of *C. difficile* alone may not be sufficient to trigger outbreaks of

neonatal diarrhoea, despite its potential to cause this disease. The extent of *C. difficile* involvement in neonatal diarrhea remains incompletely understood, with additional factors such as gut microbiota composition and immunity status potentially playing significant roles. Passive immunization through colostrum is considered essential, although its exact mechanisms and interactions within the context of neonatal diarrhea warrant further investigation (Kongsted et al., 2013; Moono et al., 2016). According to other study (Andino-Molina et al., 2019), piglets frequently exhibit subclinical colonization with *C. difficile*, which may be attributed to an apparent physiological predisposition.

Several potential explanations may account for the variability in prevalence observed across the different sampled farms. One hypothesis for this is that the absence of significant transmission within farm B could be attributed to a minimum threshold of positive animals required for successful population colonization. Another possibility revolves around differences in farm management practices as a potential influencing factor. In the other farms (B and C), animals were given antibiotics as a general rule. Although these antibiotics are not effective towards *C. difficile* (amoxicillin, with *C. difficile* usually harbouring β-lactamase-like proteins, and toltrazuril, a coccidiostat), previous research has suggested that toltrazuril can help prevent clostridial infections in piglets (Mengel et al., 2012). It is plausible to contemplate that the reduction of cases of neonatal diarrhoea, attributable to the administration of antibiotics, may have obstructed the establishment of gut colonization, consequently limiting the spread of this infection.

Apart from that, *C. difficile* isolates were detected in piglets earlier than in sows. The increase in piglet *C. difficile* prevalence was subsequently followed by a similar increase in sows. Environmental samples also followed the same trend as piglets, meaning that *C. difficile* prevalence decreased with time. In general, both environmental and sow samples presented very low levels of *C. difficile* positivity before the first lactation week. Considering these findings, it appears that neither sows nor the environment played a significant role in the dissemination of *C. difficile* during farrowing. Instead, piglets were colonized shortly after farrowing and emerged as key factor in the amplification and dissemination of *C. difficile*. To explore alternative sources of *C. difficile*, feed and water samples were analyzed, yet no presence of *C. difficile* was detected in these samples, as could be expected.

While *C. difficile* infection in piglets has been linked to various sources beyond the sow, such as the farm environment (Hopman 2011a; Weese et al., 2010), this study found relatively low levels of *C. difficile* in

environmental samples before farrowing. Although there was not a clear pattern in environmental prevalence, it generally mirrored the patterns observed in piglets and sows, with a higher prevalence the week following a peak in animal prevalence. A possible explication for the limited positivity of environmental samples may be attributable to disinfection procedures that had been performed in pens before sows entered the farrowing crates. These procedures could effectively reduce environmental contamination to a level that is challenging to detect but still effective for piglet colonization. Nevertheless, other factors could have influenced *C. difficile* transmission within the farm. For example, there is a suggestion that pest species, such as wild birds, rodents, and insects, may contribute to the dissemination of *C. difficile* within pig farms. (Andrés-Lasheras et al., 2017; de Oliveira et al., 2018; Krijger et al., 2019). Similarly, to the established role of the hands of healthcare workers in the transmission of *C. difficile* in hospitals (Gerding, 2009), farm workers have been described as a mean of transmission of *C. difficile* (Büchler et al., 2022), and therefore might have also acted as vectors.

Due to the rapid spread of *C. difficile* within the farm, the implementation of strategies to control infection is essential. General management measures should include a deep disinfection of the farm environment with chlorine-based products, wearing gloves and shoe covers and pest control (Moono et al., 2016; Squire & Riley, 2013). Possible preventive strategies have been also proposed in pigs, such as monoclonal antibodies, vaccines, probiotics or the administration of non-toxigenic strains, although there has been no availability of a specific commercial method (Oliveira Júnior et al., 2019).

The most common ribotype found across all farms was RT 078, followed by RT 126, which is in line with current literature (Hopman et al., 2011a; Schneeberg et al., 2013). While some studies have reported more heterogeneous isolates (Knight et al., 2015; Norén et al., 2014), the distribution of ribotypes in this study was very homogeneous. In farm A, only ribotype 078 was detected in fact. Similarly, all isolates from farm C corresponded to RT 078, except one of them that was a non-toxigenic strain. On the other hand, in farm B there was a predominance of RT 126 isolates. Interestingly, isolates from the non-prevalent ribotype were found towards the end of lactation.

Sows were only colonized by the most predominant ribotype, even when the litter was colonized by another one. Our study is in accordance with previous research that suggests that PCR ribotype variability within farms is low (Avbersek et al., 2009; Hopman et al., 2011a; Schneeberg et al., 2013; Spigaglia et al., 2023). The emergence of different ribotypes during lactation, not found in sows

neither the environment, suggests multiple sources of infection within the piglet population.

Overall, MLVA revealed significant clustering, particularly notable in farm B, where most isolates could be classified as clones. Farm C exhibited two bigger cluster groups; however, these were not discernibly differentiated by the metadata utilized, as they shared identical ribotypes and included isolates from various time points and sources. The analysis concludes that multiple clones coexist within the same farm Concurrently, occasionally exhibiting high abundance and sometimes sharing ecological niches.

C. difficile shows a great ability to develop antimicrobial resistance, which could be involved in driving epidemiological changes and treatment failure (Spigaglia, 2016). According to this study, phenotypic resistance to several antimicrobials was remarkably common, with very high rates of resistance to moxifloxacin (92.75%) and erythromycin (92.75%). These results were higher than those from other previous analyses performed in swine isolates (Spigaglia et al., 2015; Thakur et al., 2010). In similar studies made in swine, treatment with fluoroquinolones resulted in a higher presence of *C. difficile*, which is coherent with the results obtained as naturally high rates of resistance to fluoroquinolones could be cause of the higher prevalence (Lührmann et al., 2023). Fluoroquinolone resistance has been associated in *C. difficile* with mutations in the *gyrA* and *gyrB* genes (Spigaglia et al., 2010).

No resistance was found to vancomycin, despite using the breakpoint suggested by EUCAST instead of a clinical cutoff breakpoint. However, reduced sensitivity to vancomycin, although uncommon, is a trend that has already been described in Spain (Peláez et al., 2002) and other parts of the world (Adler et al., 2015; Freeman et al., 2015; Peng et al., 2017), and therefore should be closely surveilled. In addition to this, although no resistance to metronidazole was found, therefore a continuous surveillance should not be discarded, since metronidazole, as vancomycin, has been an antibiotic of interest in the treatment of CDI in humans (Krutova et al., 2022).

Another objective of the antimicrobial resistance analysis was to assess if there was any difference between isolates from the beginning of the sampling period (farrowing and first week of lactation) and those from the end (second week of lactation and weaning). No differences in antimicrobial resistance profiles were observed, except in the case of two piglets that had isolates from different ribotypes. The analysis of the piglet that was positive thrice along the sampling showed that both RT 078 isolates shared the same resistance pattern (F-M), while RT 126 isolate did not (F-M-T). The fact that both ribotypes were found in the same

animal can imply that colonization with several strains is possible, and, considering that resistance patterns were associated with a certain ribotype, it could imply that that there are more factors than the mere coexistence to unify resistance patterns.

When analysing the three farms together, *C. difficile* prevalence was generally lower in those farms routinely treated with amoxicillin (that is, farm B and C), contrary to previous research in which a significant positive relationship between penicillin treatment and isolation of *C. difficile* was found (Lührmann et al., 2023). A hypothesis for our result could be that the general lack of diarrhoea and a good health status of the animals could result in a better status of the gut, and therefore a lower proliferation of *C. difficile*.

There was no difference in antimicrobial resistance profiles observed that could not be explained by epidemiological circumstances, like the recolonization of the piglet with a different ribotype, and, most likely, with different strains. While MLVA was not conducted on farm A, the emergence of distinct clonal groups within the same ribotype on farm B implies a similar scenario might be occurring on farm A. In this case, isolates exhibiting varied antibiotic resistance patterns but sharing the same ribotype could represent distinct strains.

Conclusion

To sum up, this work studied the dynamics of *C. difficile* prevalence, transmission and antimicrobial resistance patterns in three pig farms. These findings are in agreement with the early colonization shortly after birth, followed by a peak in prevalence in the first week of age. The prevalence in healthy and diarrheic animals did not significantly differ, which supports the idea that other factors contribute to neonatal piglet diarrhoea.

The study also explored potential sources of *C. difficile* transmission, including sow colonization and environmental contamination. Results highlighted the role of piglets in amplifying and spreading the pathogen. Additionally, the study raised the possibility of other transmission routes involving farm pests or even human vectors, as environmental samples were less positive than expected. Antimicrobial resistance analysis revealed concerning trends, with a significant proportion of isolates exhibiting resistance to moxifloxacin and erythromycin.

Despite these findings, this study underscores the need for comprehensive infection control strategies in pig farms, including biosecurity measurements, environmental disinfection, and pest control. As we understand more about how *C. difficile* interacts with farms and how resistance to antibiotics develops, continued surveillance is essential, especially regarding the emergence of reduced susceptibility to vancomycin and other antibiotics. These measures are crucial for minimizing risks to animal and human health.

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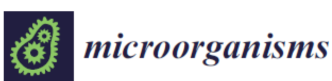
Trabajo 5

Update on Commonly Used Molecular Typing Methods for *Clostridioides difficile*



Review

Update on Commonly Used Molecular Typing Methods for *Clostridioides difficile*

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Introduction

Purpose of Work

The objectives of this review are to describe some of the most important molecular typing techniques currently used in the *Clostridioides difficile* research and medical community and to highlight the epidemiological characteristics of the most prevalent strains worldwide. In addition, this review will also explore prospective research directions in the field that will allow to enhance our understanding of this intricate bacterium.

Clostridioides difficile Infection

Clostridioides difficile, formerly known as *Clostridium difficile* (Lawson et al., 1938), is an anaerobic, Gram-positive bacterium known for its slow growth and unique characteristics. Most strains are capable of producing two major toxins: toxin A and toxin B. These toxins exhibit potent cytotoxic effects on host cells and are encoded by *tcdA* and *tcdB* genes, respectively, located in a locus of pathogenicity (PaLoc) (Cohen et al., 2000). Some strains are also capable of producing a binary toxin (CDT), but its role in the disease development is still uncertain (Martínez-Meléndez et al., 2022).

This bacterium is primarily associated with a healthcare-associated infection known as *Clostridioides (Clostridium) difficile* infection (CDI). CDI can manifest as mild to severe diarrhea and, in severe cases, as pseudomembranous colitis, which can be life-threatening. It can also have a recurrent nature, causing a significant burden on healthcare facilities and patients' quality of life (Garey et al., 2023).

Historically, CDI was predominantly considered a nosocomial infection, acquired especially in hospitals and long-term care facilities. However, there has been a noticeable shift towards community-acquired CDI cases in the last decade (Gupta et al., 2014). Additionally, certain strains of *C. difficile* have exhibited increased virulence, leading to severe and recurrent infections. This phenomenon, often referred to as hypervirulence, has raised concerns in healthcare settings, making clear the necessity of developing more effective preventive measures (Fatima & Aziz, 2014). Furthermore, *C. difficile* is known to be associated with antimicrobial resistance to a wide range of antimicrobial agents, further complicating treatment options and contributing to the challenges in managing CDI (Spigaglia, 2016).

Epidemiology of CDI

C. difficile is recognized as the leading cause of healthcare-associated infective diarrhea,

particularly among patients receiving antibiotic therapy. *C. difficile* can persist in the environment for extended periods thanks to its ability to sporulate, making contaminated surfaces and equipment potential sources of infection. It is transmitted through the fecal–oral route, with contaminated hands and surfaces serving as vehicles for transmission (Durovic et al., 2018).

Several studies have identified *C. difficile* in a great variety of animal species, including livestock, pets, and wildlife (Hain-Saunders et al., 2022; Lim et al., 2018; Orden et al., 2018; Squire & Riley ,2013; Rabold et al., 2018; Andrés-Lasheras et al., 2017). This fact has raised concerns about potential zoonotic transmission, where animals may serve as reservoirs and a source of infection for humans through direct contact or consumption of contaminated animal products. It is believed that animals may acquire *C. difficile* through exposure to contaminated environments, contaminated food, or through contact with infected humans. Several studies have demonstrated high genetic similarities between *C. difficile* isolates from humans and animals (Kinght et al., 2015; Schneeber et al., 2013; Koene et al., 2012), providing evidence for potential transmission between species. However, the exact mechanisms and extent of zoonotic transmission are still not fully understood and require further investigation as there are also cases that show limited risk of transmission (Bandelj et al., 2018; van Dorp et al., 2017; Rodriguez et al., 2015). Nevertheless, a One Health approach is needed to better understand *C. difficile* epidemiology.

Molecular Typing Techniques in *C. difficile*

In the field of *C. difficile* research and healthcare, a wide range of techniques and approaches are employed to study various aspects of this bacterium, including its virulence factors, epidemiology, and interactions with the host.

In Europe, PCR ribotyping is regarded as the preferred typing method for *C. difficile*, providing valuable information about the genetic diversity and relatedness of *C. difficile* strains and enabling effective surveillance and outbreak investigations (Gürtler, 1993). On the other hand, in North America, pulsed-field gel electrophoresis (PFGE) is more commonly employed for molecular characterization of *C. difficile*. This technique allows the comparison of banding patterns to identify genetic variations among strains (Alonso et al., 2005).

While PCR ribotyping and PFGE are the predominant methods in Europe and North America, respectively, it is important to note that there are other available molecular characterization techniques that provide valuable insights into *C. difficile*. Each method offers unique advantages in terms of resolution, accuracy, and the ability to detect specific genetic markers. In the following sections, we will explore a selection of the most common techniques that have greatly contributed to the understanding of *C. difficile*, considering their respective attributes and potential applications. A brief summary of each technique can be found in Table 14. In addition, Figure 14 represents a concise overview of the protocols of these techniques.

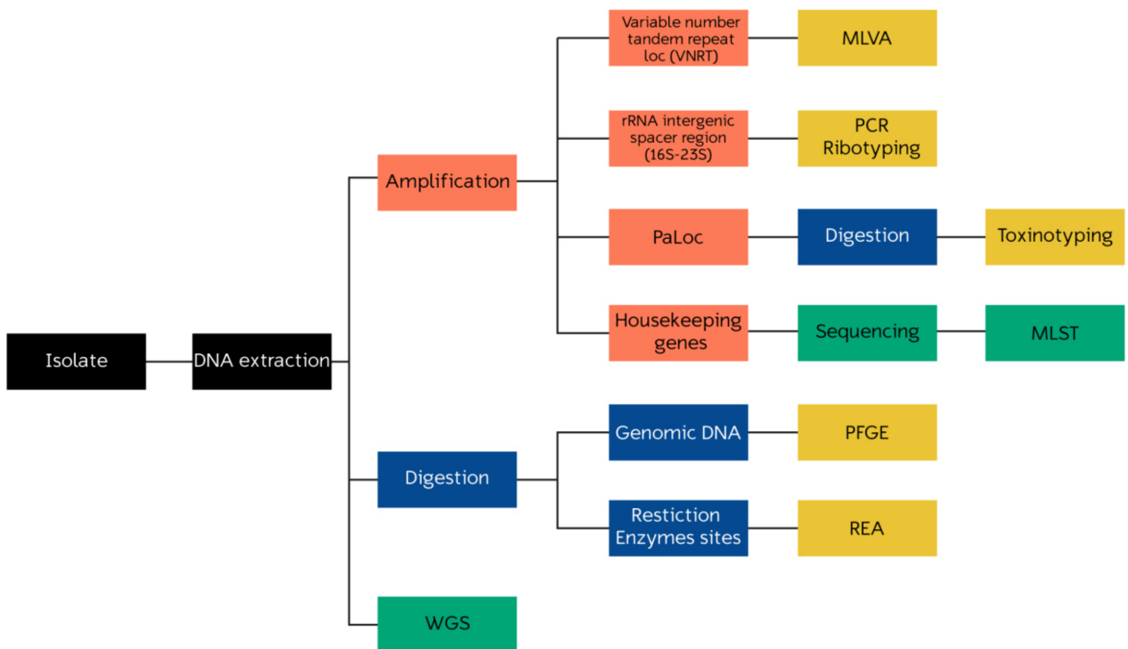

Figure 14. Overview of the protocols of molecular typing techniques in *C. difficile*. Each color represents a different technique: blue for enzymatic digestion, orange for PCR amplification, green for DNA sequencing, and yellow for electrophoresis and pattern analysis.

Table 14. Comparison of molecular techniques used for *C. difficile* characterization. Most common advantages and disadvantages are listed.

Technique	Description	Advantages	Disadvantages
Banding pattern-based analysis			
PCR Ribotyping	It analyzes the diversity of the 16S-23S intergenic spacer region through gel electrophoresis	-Widely used and well-established technique -Standardized protocols available -Rapid and cost-effective -Good inter-laboratory comparability	-Moderate discriminatory power compared to other methods -Interpretation of complex banding patterns can be challenging and subjective -Limited resolution at strain level for some closely related isolates -It cannot be obtained through WGS
PFGE (Pulsed-field gel electrophoresis)	It analyzes the banding patterns of fragmented DNA with an alternating electric field to separate larger fragments	-High discriminatory power -Widely used and well-established technique -Standardized protocols available -Compatibility with existing databases	-Time-consuming and labor-intensive -Skilled personnel and specialized equipment are required -Interpretation of complex banding patterns can be subjective -It cannot be obtained through WGS

Technique	Description	Advantages	Disadvantages
REA (Restriction endonuclease activity)	It analyzes patterns of restriction enzyme digested DNA	-High discriminatory power -Suitable for outbreak investigations	-It requires optimization for each target gene or region -High manual labor and subjectivity in pattern interpretation -Lack of standardization -It cannot be obtained through WGS
MLVA (Multilocus variable-number tandem-repeat analysis)	It analyzes variations in tandem repeats within multiple loci	-High discriminatory power -Suitable for outbreak investigations -Rapid and cost-effective -Easy interpretation of results	-Relatively low resolution compared to other methods -Dependent on the choice of loci and primers
Toxinotyping	Analyzes the polymorphisms in fragment length of PaLoc (REFL-PCR)	-It provides information on the toxin variants of *C. difficile* -Well-stablished technique	-Limited discriminatory power compared to other methods -Interpretation can be challenging due to the presence of multiple toxin genes and variants -Irregular distribution of PaLoc across phylogenetic groups -It cannot be obtained through NGS

Technique	Description	Advantages	Disadvantages
Sequence-based analysis			
MLST (Multilocus sequence typing)	It analyzes sequences of selected housekeeping genes	-Highly portable results across laboratories -It allows comparison of data between studies -Phylogenetic studies are possible	-It requires DNA sequencing, which can be costly -Limited discriminatory power compared to other methods -Targeted loci may not reflect the entire genome diversity
WGS (Whole-genome sequencing)	It analyzes sequencing of large amounts of DNA	-High-resolution genomic analysis -It can trace phylogenetic relationships -It enables identification of genetic variants and resistance markers -Potential for discovering new virulence factors	-Costly -Specialized bioinformatics analysis is required -Longer turnaround time for analysis -Data storage and management can be challenging

PCR Ribotyping

One of the widely used molecular typing techniques for *C. difficile* is PCR ribotyping. PCR ribotyping involves the amplification of a specific region of the bacterial genome, known as the 16S-23S rDNA intergenic spacer region. Each pattern of DNA fragments corresponds to a ribotype (RT), and it is based on the differences in size and distribution of the amplified fragments. This allows the differentiation and classification of *C. difficile* strains into distinct

ribotypes (Fawley et al., 2015). Harmonization and standardization of this methodology have been validated by a multicenter study comprising Europe, North America, and Canada, allowing its spread as a high quality technique with comparable results (Smits et al., 2016). Also, the existence of public databases has improved inter-center comparisons (Frentrup et al., 2020).

The use of capillary gel electrophoresis in PCR ribotyping, instead of the conventional agarose gel electrophoresis, has improved this technique as it offers several advantages, including a high discriminatory power, reproducibility, and ease of interpretation (Fawley et al., 2015). Ribotyping data can be easily shared among laboratories and integrated into global databases, helping the comparison and tracking of *C. difficile* strains across different regions and time periods.

Moreover, PCR ribotyping has revealed the existence of specific ribotypes that are associated with different clinical outcomes, virulence characteristics, and antibiotic-resistance profiles. Global geographical prevalence has also been previously discussed (Mengoli et al., 2022). Understanding the distribution and prevalence of specific PCR ribotypes can provide valuable insights into disease transmission, the emergence of hypervirulent strains, and the impact on public and animal health.

Pulsed-Field Gel Electrophoresis (PFGE)

PFGE is a molecular typing method that consists of digesting bacterial DNA with a restriction enzyme, followed by separation of the resulting fragments by electrophoresis using an alternating and cross field, which allows the separation of large DNA fragments (Sharma-Kuinkel et al., 2016; Gebreyes & Adkins, 2015). PFGE was adopted as the primary typing method for *C. difficile* by both CDCs from Canada and the USA, although it was later on substituted by other methods such as MLST or WGS (Collins et al., 2015; CDC, 2012).

PCR ribotyping and PFGE have been the most popular methods for strain characterization. Despite having similar utilities and a good correlation (Oka et al., 2012), PFGE can discriminate some strains better than ribotyping (Brazier 2001), while it is complicated to be used in others such as RT 1 strains (Corkill et al., 2000), although this issue has been already investigated and solved through an increase of lysis time, lysozyme concentration, and proteinase K, among other changes in protocol (Gal et al., 2005).

PFGE has been used to study the epidemiology and transmission

of *C. difficile* as it can provide high-resolution molecular typing with good discriminatory power. PFGE has been shown to be effective in identifying outbreaks and tracking transmission of *C. difficile* strains within hospitals and other healthcare facilities (Pasanen et al., 2011).

However, PFGE has some limitations, including the requirement for highly skilled personnel and specialized equipment as well as potential technical variability between laboratories. Additionally, PFGE results can be difficult to interpret due to the high degree of genetic diversity within *C. difficile* strains, which can make it challenging to establish standardized interpretation criteria.

Overall, although PFGE has been a valuable tool for studying the epidemiology of *C. difficile*, like other techniques its use is being reduced in favor of newer, higher-resolution molecular typing methods such as whole-genome sequencing (Gebreyes & Adkins, 2015).

Restriction Endonuclease Activity (REA)

Restriction endonucleases are enzymes that cleave DNA molecules at specific recognition sites, resulting in fragments of different lengths. The REA technique involves the digestion of DNA with one or more restriction enzymes, followed by separation of the resulting fragments using gel electrophoresis. By comparing the DNA fragment patterns of different samples, REA can be used for a variety of applications, such as strain typing, identification, and phylogenetic analysis (Sambol et al., 2016). An extensive protocol was developed by Clabots et al. in 1993 and showed reproducible results consistent with other typing methods. However, the highly demanding protocol made it a technique rarely used in other laboratories.

Also, REA has been used as a way to determine relatedness in the case of outbreaks as it is capable of distinguish between strains of the same type, as opposed to ribotyping or PGFE (Clabots et al., 1993). REA and PFGE have been shown to have similar discriminatory power and higher than PCR ribotyping (Kristjánsson et al., 1994).

Although REA analysis is a useful technique for studying the epidemiology of *C. difficile* and management of hospital outbreaks, it has not been widely used within the *C. difficile* research community (Rafferty et al., 1998).

There is little information on REA profiles, especially when compared with other techniques. In general, most studied RT 027/NAP1 strains are associated with REA group BI, while other important RT 078-like

strains are linked to REA group BK, and RT 014/020 is linked to group Y (Cheknis et al., 2018). The strong correlation between ribotyping and REA suggests that the results obtained through ribotyping may provide valuable insights into REA analysis (Figueroa et al., 2012).

It should be noted that REA requires a high manual labor as well as involves subjectivity in interpretation of the banding patterns in the gel. This along with the lack of standardization and its cost may be the reasons why this kind of analysis lacks popularity within the *C. difficile* research community.

Multilocus Variable-Number Tandem-Repeat Analysis (MLVA)

In MLVA, a set of predetermined genetic loci are selected for analysis. These loci consist of repeated DNA sequences where the number of repeats varies between strains of *C. difficile*. The variations in the number of repetitions are measured using PCR amplification and fragment analysis techniques (van der Berg et al., 2007).

By comparing the lengths of the amplified fragments in the loci among different *C. difficile* strains, MLVA generates unique genetic profiles for each strain, which can be used to differentiate and classify different isolates, allowing for strain identification, tracking of outbreaks, and understanding the genetic relatedness among isolates.

MLVA has emerged as a valuable technique for differentiating strains during CDI outbreaks. It has been described that MLVA, as well as REA, offer high resolution in genotyping, while other methods fail.

To ensure the comparability of MLVA results across different laboratories, the establishment of standardized protocols and the use of reference strains as controls are essential. This will promote consistency and reproducibility of MLVA analysis across laboratories, ensuring the reliability of the technique.

In summary, MLVA has demonstrated its effectiveness in characterizing and subtyping *C. difficile* strains during outbreaks. Its ability to provide high-resolution genotyping along with its simplicity and cost-effectiveness make it a valuable tool in the study of *C. difficile* epidemiology, especially since it is easily standardizable and results can be shared between laboratories. As research progresses and technologies advance, the integration of MLVA with other molecular techniques, such as genome sequencing, may further enhance our understanding

of *C. difficile* strains and their transmission dynamics.

Toxinotyping

Another historically relevant typing technique, although less used nowadays, is toxinotyping. This method is based on the use of PCR-restriction fragment length polymorphisms to assess the differences in the PaLoc region (Rupnik & Janezic, 2016).

The significance of toxinotyping lies in its ability to characterize the genetic variability of *C. difficile* toxins, particularly toxins A and B, which are considered the primary virulence factors and play crucial roles in the pathogenesis of *C. difficile*-associated diseases. Toxinotypes, therefore, represent a diverse group of strains that exhibit alterations in the toxin A and B coding regions. The differences in the patterns after the use of restriction enzymes, and its comparison with standardized patterns, allow the assignment of the strains into one of the 34 toxinotypes, which are labeled from I to XXXIV in Roman numerals.

The toxinotyping method was initially described in 1998 and has since undergone updates to incorporate new knowledge and advances in molecular techniques (Janezic et al., 2020). It provides a reliable and standardized approach for characterizing *C. difficile* strains based on toxin gene variations, facilitating epidemiological investigations, and informing the development of preventive strategies. The continuous discovery of new toxinotypes and improvements in toxinotyping protocols contribute to our comprehensive understanding of the genetic landscape and pathogenic potential of *C. difficile*.

Multilocus Sequence Typing (MLST)

MLST is a molecular typing method that studies molecular diversity among different strains of a bacterial species. It is based on the sequence of a selected set of housekeeping genes, which in the case of *C. difficile* are the following: *adk* (adenylate kinase), *atp*A (ATP synthase subunit alpha), *dxr* (1-deoxy-d-xylulose 5-phosphate reductoisomerase), *gly*A (serine hydroxymethyl transferase), *rec*A (recombinase A), *sodA* (superoxide dismutase), and *tpi* (triose phosphate isomerase) (Griffiths et al., 2010). These genes are present in all strains of *C. difficile* and can be therefore compared between strains. When these gene sequences are compared, allelic profiles are generated based on the specific variants found at each gene. These allelic profiles are then used to define sequence types (STs), which identifies each strain. A curated database can be found at https://pubmlst.org/organisms/clostridioides-difficile (accessed on

30 May 2023), which allows comparisons between laboratories (Frentrup et al., 2020; Griffiths et al., 2010).

This tool has been used to track epidemiology changes in bacterial populations, allowing the identification of clonal complexes and the tracing of their spread over time and across geographical locations. MLST data can be used not only to identify the strains but also to infer the evolutionary relationship among strains and to investigate the mechanisms of bacterial adaptation and evolution.

Studies have shown that the majority of STs in *C. difficile* are in accordance with their RTs (Byun et al., 2019). Therefore, MLST and ribotyping can provide complementary information that can be used to identify and track the spread of *C. difficile*. Exploring the prevalence and distribution of *C. difficile* ribotypes has been a topic of recent interest, with a particular focus on the most common ribotypes as well as emerging ones, and the revisions on the topic can be found elsewhere (Martínez-Meléndez et al., 2020). The relationship between ST and RT of some of the most important *C. difficile* strains can be found in Table 15.

Based on MLST, the population structure of *C. difficile* can be divided into five distinct clades and three cryptic ones, each of them with its own characteristics (Knight et al., 2021). Clade 1 represents the largest and most heterogeneous group, displaying significant genetic diversity within its members. Clades 2 and 5, which have been identified as harboring hypervirulent strains, have lower recombination rates compared to the other clades. In general, the recombination rates of *C. difficile* are moderate (Zhao et al., 2021). Clade 3 has garnered attention due to its unique characteristic of harboring a clade-specific PaLoc with a *Tn6218* insertion, which serves as a distinguishing feature from other *C. difficile* strains (Chen et al., 2017). Additionally, Clades 3 and 5 share genetic similarities in their *tcdC* sequences (Dingle et al., 2011).

Clade 4 is distinguished from the rest by its high prevalence of multidrug resistance (MDR) (Imwattana et al., 2021). Clade 5 encompasses various strains, including those associated with animals, such as ST 11 strains. This clade has been previously associated with community-acquired *C. difficile* infection (CA-CDI). Notably, when considering long-term outcomes, Clades 2 and 5 consistently exhibit higher mortality rates, while Clades 1 and 3 display lower mortality rates, even after adjusting for differences in biomarkers (Walker et al., 2013).

Table 15. Ribotype and sequence type relationship of commented *C. difficile* strains, including the reference sequence type (in bold) for each clade. Cryptic clades do not have a reference type.

Clade	Sequence Type	Ribotype	References
1	2	005, 020/014, 015, 069, 076, 095, 220	(Rodriguez et al., 2015; Dingle et al., 2011; Knetsch et al., 2012)
	3	001, 009, 055, 072, 077, 115, 262, 305	(Dingle et al., 2011; Knetsch et al., 2012)
	10	015	(Dingle et al., 2011)
	17	018, 052	(Dingle et al., 2011; Knetsch et al., 2012)
	33	014/020, 064, 216, 369	(Dingle et al., 2011; Knetsch et al., 2012; Ngamskulrungroj et al., 2015)
	34	056	(Dingle et al., 2011)
	35	002, 046, 220	(Dingle et al., 2011; Janezic & Rupnik, 2015)
	42	106, 118, 174	(Dingle et al., 2011; Knetsch et al., 2012)
	44	015, 062	(Dingle et al., 2011; Knetsch et al., 2012)
	45	013, 017	(Dingle et al., 2011; Ngamskulrungroj et al., 2015)
	54	012, 014/020	(Dingle et al., 2011; Knight et al., 2023)
2	**1**	002, 003, 016, 027, 036, 176	(Dingle et al., 2011; Knetsch et al., 2012; Knight et al., 2023)
	41	46, 106, 156, 164, 194, 208, 209, 244, 321	(Dingle et al., 2011; Knetsch et al., 2012; Ngamskulrungroj et al., 2015; Stabler et al., 2012)
3	**5**	023, 063, 069, 122, 438	(Knetsch et al., 2012; Chen et al., 2017; Krutova et al., 2016)
	22	023	(Chen et al., 2017)
4	**37**	017, 047	(Knetsch et al., 2012; Imwattana et al., 2019)
	81	PKI-017, A	(Wang et al., 2018b; Cheng et al., 2018b; Jolley et al., 2018)

Clade	Sequence Type	Ribotype	References
5	**11**	033, 045, 066, 078, 126, 127, 193, 193, 237, 280, 281	(Dingle et al., 2011; Knetsch et al., 2012; Stabler et al., 2012; Gu et al., 2021; Lim et al., 2020)
I	200	Not studied	(Knight et al., 2021)
I and II	946, 947 and 948	151	(Ducarmon et al., 2022)
II	181	Not studied, phylogenetically similar to NML211	(Knight et al., 2021; Ramírez-Vargas et al., 2018)
III	369	Not studied	(Knight et al., 2021)

More recently, cryptic Clades C-I, C-II, and C-II have been described and are associated with atypical variants of all four toxin-related genes (*tcdA*, *tcdC*, *cdtA*, *cdtB*), although they have been poorly investigated thus far (Williamson et al., 2022).

It has been reported that MLST also fails to identify the problematic RT 014/020 (Berger et al., 2020) and to correctly assign phylogenetic relationship between some Clade 1 and 2 isolates (Cabal et al., 2018). Clade 4 has also presented problems in China (Cheng et al., 2018a) as well as some discordances in ST 3 (Byun et al., 2019; Seth-Smith et al., 2021). The interpretation of MLST profiles can potentially lead to the erroneous diagnosis of reinfection as the identification of the same ST within the same patient does not invariably indicate relapse (Eyre et al., 2012). On the contrary, in RT 015 strains, MLST can further identify the differences between ST 44 and ST 10, and a lower mortality has been described in ST 44 (Walker et al., 2013). Among the limitations of MLST, some clades have more differences between them than others. Clades 3 and 4 have shown to have a high inter-RT allele differences, while Clades 1 and 5 have lower (Baktash et al., 2022).

Hence, it should be noted that the homology between STs and RTs is not always perfect. Some RT correlate with more than one ST, and vice versa. In this regard, MLST has been proposed as a tool for strains identification in cases where ribotyping fails to fully detect differences.

Based on this sequencing methodology, core genome (cg-) MLST and whole genome (wg-) have been also developed, using NGS technology. CgMLST is a variant of the traditional MLST technique that focuses on the sequencing of the core genes within bacterial genomes. The core

genome refers to the set of genes that are present in all members of a given bacterial species and are typically conserved in their sequence and function. In opposition to the traditional approach, more information is generated and analyzed. Compared to traditional MLST, cgMLST provides a higher level of discrimination between bacterial strains and can be used to identify even closely related isolates, which makes cgMLST an optimal tool for outbreak investigations. Additionally, the use of a standard cgMLST scheme across laboratories allows comparison of results and data sharing between different institutions and countries (Wang et al., 2023; Uelze et al., 2020). As with traditional MLST, limitations include, in some specific strains, not being able to distinguish between outbreak strains and regular ones, especially in ribotypes with low intra-RT allele differences. Therefore, caution should be exercised when making epidemiological affirmations based on that information alone (Baktash et al., 2022).

A few hash-based methods have been proposed based on cg MLST (Frentrup et al., 2020; Eyre et al., 2019a; Deneke et al., 2021; Werner et al., 2020; Bletz et al., 2018). This type of method involves generating unique numerical identifiers, or "hashes," for each allele in the targeted genes, instead of focusing on the core genome. These hashes are generated by converting the nucleotide sequence of each allele into a numerical value using a hashing algorithm.

In opposition to cgMLST, wgMLST analyses both the core genome and accessory genes providing a comprehensive view of its genetic building. It has the ability to detect variations that can be missed by cgMLST, giving a more accurate vision of genetic relatedness between strains. However, it should be noted that the computational power needed is much higher, and standardization between studies is harder. By tracking the genetic changes that occur over time, wgMLST can help to identify the sources of infection and the routes of transmission and can provide valuable information for infection control and prevention efforts (Blanc et al., 2022; Janezic & Rupnik, 2019).

When compared to other approaches, such as cgMLST and wgMLST, most strains have the same MLST profile as wgMLST. However, in certain cases, wgMLST can provide additional useful information for further strain identification and outbreak investigations, making it a valuable strategy in these scenarios (Kamboj et al., 2021). As wgMLST also includes accessory genes, it can provide a higher resolution than cgMLST, being a more powerful

tool when studying outbreak situations. Nevertheless, the amount of computational work needed is also much more demanding and usually does not give more detailed information than cgMLST (Uelze et al., 2020; Janezic & Rupnik, 2019).

Whole-Genome Sequencing (WGS)

WGS has been proposed as an alternative for traditional methodologies thanks to the development of next-generation sequencing techniques (NGS).

The single nucleotide polymorphism (SNP) technique has emerged as a powerful tool for molecular epidemiology and strain characterization. SNPs are single base-pair variations in the DNA sequence that can serve as genetic markers to differentiate strains and track their transmission dynamics (Baktash et al., 2022). The application of SNP analysis in *C. difficile* involves sequencing and comparing specific genomic regions among different isolates. By comparing the sequence variations at specific SNP sites across the genome, the relatedness and evolutionary relationships between *C. difficile* strains can be determined.

The main advantages of SNP are its high resolution and discriminatory power, which allows the differentiation of closely related strains. It can also be used to assess the phylogenetic relationships and population structure of *C. difficile* isolates and detect genomic changes associated with virulence and antibiotic resistance (Monteford et al., 2021).

However, SNP analysis requires advanced bioinformatics tools and expertise for data analysis and interpretation. Additionally, the cost and time required for sequencing whole genomes may limit its widespread adoption in routine surveillance, in addition to its difficulty in standardization. In contrast, the tools used for cgMLST typing are often user-friendly, and there is no need of selection of phylogenetically related strains to perform the analysis, which is needed for SNP typing.

As discussed previously, the use of cgMLST in investigating local *C. difficile* epidemiology has shown comparable results to SNP analysis, providing a significant advancement. The ability of cgMLST for *C. difficile* to identify closely related isolates and infer genomic distances is inferior to SNP analysis due to errors introduced during de novo assembly and a lack of per-base quality control. However, when applied to a large dataset of *C. difficile* genomes from hospital patients, both cgMLST and SNP analysis have shown to discriminate between epidemiologically related and

unrelated isolates (Frentrup et al., 2020).

Similarly, to SNP analysis, molecular sequencing studies of virulence factors have been proposed as a method for studying short-term evolution, as opposed to housekeeping MSLT techniques which are more suited to long-term evolution (Lemée et al., 2005).

WGS provides a more extensive dataset compared to other types of analyses. Average amino acid identity (AAI) analysis can be performed, which compares conserved protein-coding genes among genomes. This approach clusters strains based on sharing more than amino acid content, demonstrating higher resolution at the species level compared to 16S rDNA or MLST, as it assesses a larger fraction of the genome (Cabal et al., 2018). STs and RTs do not seem to predict toxins, although toxin diversity can be predicted within the clades (Li et al., 2020). For this reason, toxin genetics are best investigated with WGS than with conventional approaches when gathering epidemiological information about toxins and toxin production.

Additionally, WGS has emerged as a valuable tool in approaching treatment strategies for CDI, particularly in relation to the identification of antimicrobial resistance genes. While not all resistance genes are currently known, WGS allows a comprehensive analysis of the genome, enabling the detection of known resistance mechanisms. However, genomic studies combined with phenotypic assays are still necessary to detect resistance mechanisms not described yet.

In conclusion, WGS offers valuable insights into *C. difficile* epidemiology, particularly in understanding resistance mechanisms and strain relatedness. While challenges and limitations exist, ongoing advancements in genomic analysis methods will continue to enhance our understanding of this pathogen and inform future research paths.

Molecular Epidemiology of *C. difficile*

Hospitals play a crucial role in the transmission of *C. difficile*, and extensive research has focused on unraveling the genetic profiles and transmission dynamics within healthcare facilities. Beside the nosocomial environment, CA-CDI is becoming an emerging threat. As CA-CDI refers to cases whose source of infection is not linked to healthcare facilities, they have not been as profoundly investigated as healthcare-associated CDI. Exploring the molecular

epidemiology of CA-CDI helps us understand the sources and transmission routes of *C. difficile* in community settings.

Additionally, molecular epidemiology of *C. difficile* in the environment is also an interesting topic of research. This includes investigating the presence and persistence of *C. difficile* in water sources, wastewater treatment plants, and other environmental reservoirs. Understanding the genetic characteristics and behavior of *C. difficile* in the environment allows us to assess potential dissemination pathways and evaluate the associated public health risks. Furthermore, the presence of *C. difficile* in animals adds another dimension to its molecular epidemiology. The evaluation of its zoonotic potential and understanding the transmission dynamics through a One Health perspective becomes crucial.

The investigation of various isolates belonging to the same ST has revealed that antibiotic resistance and virulence factors can coexist within the same molecular profile (either ST or RT). Consequently, it is imperative to exercise caution as relying solely on molecular evolutionary distances may not be sufficient in comprehending the epidemiology of *C. difficile*. It is essential to consider other factors that may impact the pathogenesis and transmission of the bacterium. This underscores the importance of adopting a comprehensive approach that accounts for all relevant variables when studying the epidemiology of *C. difficile* (Muñoz et al., 2017).

Epidemiological Characteristics in Healthcare Setting of Commonly Isolated Sequence Types

The molecular characteristics of *C. difficile* strains exhibit a wide range of diversity across different countries, continents, hosts, and risk factors. A comprehensive literature review (Markovska et al., 2023) provides updated insights into the geographical distribution of these strains, along with their associated characteristics, particularly focusing on commonly identified ribotypes (Martínez-Meléndez et al., 2020). Nevertheless, it is important to note that alternative typing methods have also yielded valuable information that may be overlooked due to their relatively lower popularity in the research community.

In studies performed in human populations, CDI cases belonging to Clade 2 were more common among older patients and those with multiple risk factors. Clade 2 and 5 infections had a significantly higher mortality rate compared to Clade 1, with a trend towards higher mortality for Clade 5 compared to Clade 2 CDI. Despite Clades 3 and 5 showing genetic similarity in

several PaLoc genes, they have been found to have different mortality rates. In Clade 1, ST 44 showed a lower 14-day mortality risk, despite being highly similar to ST 10 (one nucleotide difference in the housekeeping analyzed). Both ST 10 and ST 44 are usually identified as RT 15. However, Clade 3 appeared different from other clades as it had significantly higher neutrophil, similar to Clades 2 and 5, despite significantly lower mortality (Walker et al., 2013).

One of the most common studied strains, mostly because of its hypervirulent characteristics, is known as North America PFGE type 1, or NAP-1, and is associated with certain attributes. It also correlates with RT 027 and belongs to Clade 2. NAP-1 produces high levels of toxins A and B as well as an additional binary toxin, which is linked to increased morbidity and a poor response to antibiotic treatment (McMaster-Baxter & Musher, 2007). For these reasons, this ribotype, along with RT078, has been considered hypervirulent (couturier et al., 2018). Furthermore, a mutation in the *tcdC* gene, a negative regulator of toxin production known as the anti-sigma factor TcdC, is also considered a key factor in the hypervirulence development of RT027 strains. This naturally occurring mutation is thought to contribute to a substantial increase in toxin production (Carter et al., 2011). However, studies have shown that not all NAP1/027 isolates are consistently associated with a more severe disease, regardless of their ability to produce larger amounts of toxins. This suggests that other factors may also contribute to the clinical outcomes (Sirad et al., 2011). When taking into account antibiotic resistance, *C. difficile* isolates of PFGE types P1, P3, P4, P8, and P10 have been associated with high-level resistance against clindamycin, ceftriaxone, erythromycin, and ciprofloxacin (Oka et al., 2012).

In a 10-year study in a Chicago tertiary care hospital (Belmares et al., 2009), they found REA technique to be able to detect clusters associated with higher incidence of *C. difficile* infection. However, despite the ever-changing strain population, some epidemic groups repeated themselves in time, concluding that the change in the bacterial population was caused by the introduction of new patients to the institution. This idea has been previously proposed in other studies including REA analysis, although there has been in general a switch to more recent techniques, such as PFGE (Clabots et al., 1992).

In another study based on North America, no dominant sequence types (STs) were identified, suggesting a diverse population of *C. difficile* circulating in the healthcare facilities of both regions.

The absence of large clusters of closely related isolates further suggests the effectiveness of infection control practices in preventing widespread transmission within these settings. However, different sequence types (STs) showed associations with distinct forms of CDI, with ST1, ST53, and ST43 being more likely associated with HCA-CDI and ST3 and ST41 commonly isolated from CA-CDI cases (Pecora et al., 2022). Notably, the distribution of specific STs varied between the two surveillance regions. In the northwest, ST1 was found to be more prevalent, while in Minnesota, ST41 was more commonly observed. These regional differences in ST distribution may reflect variations in the local epidemiology, patient populations, or healthcare practices. In fact, less than half of the isolates showed clear epidemiological links, indicating that a significant proportion of CDI cases may have originated from sources not directly linked to previously identified cases. This is a trend that has already been observed in other occasions (Walker et al., 2012). The identification of diverse STs among epidemiologically unlinked isolates suggests the potential for multiple introduction events from various sources, including asymptomatic carriers and contaminated environmental reservoirs.

The distribution of *C. difficile* STs varies across continents, with some STs being more commonly isolated in certain regions compared to others. While STs 1, 2, and 3 are frequently reported across all continents, they are rarely the most commonly isolated ST in any given region (Martínez-Meléndez et al., 2020).

For instance, in Asia, STs 37 and 54 are more commonly isolated compared to other STs. Meanwhile, in Europe, ST 11 is frequently isolated, but its presence in Asia is rarely reported (Martínez-Meléndez et al., 2020; Zhao et al., 2021; Wang et al., 2014; Li et al., 2018; Shoaei et al., 2019; Eyre et al., 2019b). In America, both ST 11 and ST 42 are commonly isolated (Paulick et al., 2016). ST 42 has been highly prevalent in the United States adult population. It is widely characterized by the deletion of a single nucleotide in the *tcdC* negative regulator and the lack of binary toxin. It has been associated with higher virulence. It also possesses several genes that encode proteins described in other bacterial species related to intestinal mucosal adhesion, sporulation, and protection from oxidative stress and foreign DNA (Kociolek et al., 2018). ST 54 has also been found in healthy patients and in those with CDI (Tian et al., 2016). Some STs, such as ST 37, have contradictory information. While one study showed a higher

prevalence of ST 37 in diarrhea in adults but not in healthy individuals (Tian et al., 2016), in other cases, it has been found to be one of the most prevalent types in patients colonized with *C. difficile* but not presenting active CDI (Yan et al., 2017).

In a separate study, *C. difficile* ST 17 (RT 018) isolates exhibited distinct pulsotypes across different hospitals. However, when studying other hospitals, diverse STs were detected, while some types were unique to each hospital. This epidemiological study suggests that *C. difficile* infections in hospitals are associated with the persistence of endemic clones along with the emergence of unique clones. Combining MLST with PFGE or ribotyping could be a valuable approach for monitoring epidemic *C. difficile* strains and the emergence of new clones in hospital settings (Nicholas et al., 2017).

In addition, the application of MLVA in investigating nosocomial *C. difficile* infections has yielded promising results (Marsh et al., 2006). When analyzing isolates from several outbreaks caused by the hypervirulent RT 027 (ST 1), MLVA identified 13 distinct clusters. Additionally, MLVA analysis of 29 toxin A-negative, toxin B-positive isolates belonging to RT 017 (ST 37) from eight different countries revealed the presence of eight country-specific clusters. This highlights the ability of MLVA to subtype and differentiate newly emerging variants of *C. difficile* (van der Berg et al., 2007).

In regard to the relationship between non-toxigenic isolates and disease, it has been proposed that the presence of non-toxigenic *C. difficile* isolates could have a significant role in the context of CDI prevention and control (Natarajan et al., 2013). These non-toxigenic strains, which do not produce the toxins associated with *C. difficile*, have emerged as potential protective agents against toxigenic *C. difficile* strains, yielding intriguing possibilities for mitigating the burden of CDI in healthcare facilities, and are seen as a possible route of study for preventive treatments in the future (Hughes et al., 2022).

In conclusion, *C. difficile* strains exhibit significant diversity, and the use of various typing methods, including ribotyping, MLST, PFGE, and MLVA, has provided valuable insights into strain distribution, pathogenicity, and risk factor associations. Furthermore, the identification of specific ribotypes, such as NAP-1/027/ST2, associated with hypervirulence highlights the need for a comprehensive understanding of the complex factors contributing to disease severity. Geographic differences in strains prevalence

reinforces the need of surveillance as changes in bacterial populations, specially the emergence of hypervirulent strains, can have an impact on human health. The use of alternative techniques, especially in the case of outbreaks or geographically limited locations, can provide meaningful information about transmission routes and aid in identifying potential sources of infection, such as persistent endemic clones or the emergence of unique ones.

Epidemiological Characteristics in Community-Acquired *C. difficile* Infection (CA-CDI)

In general, CA-CDI has been associated with younger patients with no prior antibiotics exposure or healthcare association (Kim et al., 2017). This kind of patients also have a lower mortality rate compared to traditional CDI. The sources of infection can be diverse, including other humans, animals, the environment, or food sources, among others (Fu et al., 2021).

ST 11 has been associated with CA-CDI and animals. This ST has been found to contain strains from RT 078, 078-like, and 126 and also RT 045 and 033. Interestingly, Krutova et al. found that, among ST 11 isolates, only those characterized as RT 033 had a different toxigenic profile (Krutova et al., 2018). In other studies, ST 11 was found to be associated with reduced sensibility to erythromycin and moxifloxacin, but all the isolates found were from RT 78 (Weber et al., 2013).

As an example of the application of WGS in *C. difficile* epidemiology, Xu et al. (Xu et al., 2021) used geotemporal analysis to investigate strains from China. Their findings revealed that the initial introduction of ST 37 into China occurred through multiple independent importation events, and some of them likely transmitted through household acquisition. The multiple importation events from various geographic regions and subsequent household transmission and interprovincial spread suggest that CA-CDI cases in China may involve diverse sources and have the potential for widespread dissemination.

In Australia, a lower occurrence of healthcare-
associated *C. difficile* infection (HA-CDI) was observed compared to surveillance reports from North America and Europe. Seasonal patterns were apparent, with declining rates of HA-CDI observed during the analyzed period. Furthermore, severe infections were more prevalent in community-associated CDI (CA-CDI), underscoring the need for heightened surveillance in community settings in the future as the cause of this increase in severity when compared to other locations is still not understood (Worth et al.,

2016). Similar findings have been found in other countries in Europe (Karlström et al., 1998; Banks et al., 2016). In America, hypervirulent NAP1 (ST 2) was found more commonly in healthcare-associated cases, while in CA-CDI cases, NAP6 (ST 8) was found in almost double the cases of nosocomial CDI. Interestingly, NAP7 (ST 11/RT 078) was not significantly different in neither of the settings, although prevalence was a bit higher in the CA-CDI group (Lessa et al., 2015).

ST 3 strains have been found present in abundance in healthy children but not in adults. This type of strains has been classified as non-toxigenic several times by their lack of toxigenic genes, but they are also capable of being toxigenic (Tian et al., 2016; Stone et al., 2016). However, they have been described in cases of CA-CDI, usually associated with fluoroquinolone resistance (Collins et al., 2017).

Other ribotypes found to be associated with CA-CDI include RT 001 (ST 3), RT 002 (ST 8), RT 015 (ST 2), RT 017 (ST 37), and RT 018 (ST 17). In some cases, the introduction of a specific strain in the hospital was linked to the admission of patients with asymptomatic *C. difficile,* and, as with CA-CDI, these patients tend to be younger. RT diversity was also higher in CA-CDI, compared with HA-CDI. These findings imply variations in pathogenicity or survival fitness among distinct ribotypes as the higher proportion of prevalent ribotypes in CDI cases, specifically CA-CDI cases, implies that these specific ribotypes have characteristics that promote their ability to cause symptomatic infections (Kim et al., 2019).

In conclusion, the emergence of CA-CDI poses a significant challenge in healthcare settings and the community. The increasing incidence of CA-CDI highlight the need for heightened surveillance and preventive measures. The unique epidemiological characteristics of CA-CDI, such as patient characteristics, strain diversity, and potential for transmission in community settings underscore the importance of understanding its risk factors and transmission dynamics.

Epidemiological Characteristics in the Environment

The epidemiology of *C. difficile* extends beyond the clinical setting and includes its presence and dynamics in the environment. Understanding the environmental epidemiology of *C. difficile* is crucial for comprehending the transmission pathways, reservoirs, and potential sources of infection.

In a study about wastewater, ST 2 had four different RTs: 878, 879, 020, and 014. However, when

analyzed by WGS, RT 878 and 879 had 0–2 SNP differences between them, suggesting a genetic relationship between both ribotypes. In the case of the other RTs, notable differences between the strains were found, despite sharing RT and ST, highlighting the differences that these methodologies can have (Rivas et al., 2020).

Through whole genome comparisons, more diverse STs were found in wastewater than in the clinical setting. However, 13 STs were common to both. Additionally, highly similar isolates were identified in both clinical and wastewater samples, indicating potential extensive release of toxigenic *C. difficile* into surface waters (Moradigaravand et al., 2018).

Regarding surface water samples, another study revealed the presence of diverse RTs and STs of *C. difficile*, including both toxigenic and nontoxigenic strains. Remarkably, one isolate exhibited resistance to metronidazole, which was attributed to the presence of a plasmid called pCD-METRO (Boekhoud et al., 2020). Although the resistant isolate was a nontoxigenic ribotype and could not be associated with CDI, the potential transmissibility of the plasmid and metronidazole resistance phenotype could happen (Cizek et al., 2022).

Furthermore, a study conducted in Iran identified toxigenic *C. difficile* (toxinotype V) in wastewater. The isolated strain showed genetic similarities to the hypervirulent *C. difficile* RT078. The presence of multidrug-resistant strains and a *C. difficile* isolate carrying a Tn*916*-like transposon in the treatment plant outlet raises concerns about the efficacy of wastewater treatment processes. Discharge of the treated wastewater into the environment could contribute to the dissemination of *C. difficile* beyond hospital settings, potentially leading to CA-CDI in human and animal populations (Baghani et al., 2020). It has also been described that *C. difficile* can survive treatment process, in addition to those isolates being associated with those found in hospitals in the same area (Chisholm et al., 2023).

Overall, these findings suggest that *C. difficile* can survive the treatment processes of wastewater and be released into the environment, serving as a potential source and reservoir for CA-CDI. The overlap between *C. difficile* genotypes found in wastewater and those isolated from hospital patients indicates a possible connection and highlights the importance of understanding the environmental epidemiology of *C. difficile* for effective prevention and control strategies.

Epidemiological Characteristics in Animals

As a bacterial pathogen associated with gastrointestinal infections, *C. difficile* is not limited to human populations and also has an impact on animals. The epidemiology of *C. difficile* in animals is an important area of study that focuses on understanding its presence, distribution, and dynamics within various animal species. Investigating the epidemiology of *C. difficile* in animals provides valuable insights into the transmission pathways and reservoirs of this bacterium. It helps to identify potential sources of infection and assess the risk of zoonotic transmission to humans.

The fact that *C. difficile* RT 078 and 078-like isolates (usually associated with ST 11) have been identified both in humans and animals has bolstered the hypothesis of a zoonotic origin of the infection. Although the transmission routes still remain unknown, there has been studies that provide evidence of the relationship of human and animal strains (Knight et al., 2015; Schneeberg et al., 2013). For instance, a high rate of *C. difficile* contamination with phylogenetically related strains was found both in the food chain and in farms, indicating a possible source of infection for humans (Heise et al., 2021). In other study, the spread of clones included different continents, sometimes without any connection to healthcare facilities, suggesting that they were disseminated in the community through zoonotic or anthroponotic means over long distances. ST11 strain has a large pan-genome and contains various clinically significant antimicrobial resistance elements and prophages, which likely play a role in the successful global dissemination of this lineage that is of One Health significance (Knight et al., 2019).

However, the application of NGS techniques has allowed for the detection of subtle differences in strains that were previously considered indistinguishable using traditional typing methods. This kind of studies point in the direction of, rather than direct transmission, other shared infection sources being the origin of the infection. Like van Dorp et al. pointed out (van Dorp et al., 2017), even if direct transmission between hospital cases and animals are not related, community-acquired or foodborne transmission cannot be ruled out (Weese, 2010). The existence of common sources of infection is another possibility that might explain these transmission events, making animals a reservoir for *C. difficile*.

C. difficile has been linked to livestock and companion animals on several occasions. *C. difficile* is considered an important cause of diarrhea in neonatal pigs. In particular,

hypervirulent *C. difficile* RT 078 (ST 11) has been the most commonly isolated strain in pig farms, thus raising a public health concern (Squire & Riley, 2013). *C. difficile* has also been documented in poultry, with notable occurrences of its presence in the fertilized grounds of specific chicken farms. This phenomenon led to the enduring colonization of the soil by *C. difficile* spores that exhibit a high degree of similarity to those commonly encountered in human patients. This scenario underscores the potential public health implications of the persistent presence of *C. difficile* in poultry and its potential role as a source of transmission to humans (Frentrup et al., 2021).

In horses, there have been inconsistencies in the reported prevalence and perceived impact of *C. difficile*, with prevalence ranging from 5% to 90%, although it seems to be higher in foals. At a molecular level, strains not associated with other species as well as those identified in other animals, the environment, and humans have been isolated (Hain-Saunders et al., 2022).

Although less studied, *C. difficile* has also been studied in several wild (Rodriguez Diaz et al., 2018) and exotic animals (Andrés-Lasheras et al., 2018). In general, these studies found a high prevalence of RT 078 and of uncommon RTs, varying between species (Cizek et al., 2022; Bandelj et al., 2011). The knowledge regarding the presence of *C. difficile* in wildlife and exotic pets is currently limited, mostly because of the diversity of these animal populations. The potential role of proximity to humans or other animals in the acquisition of *C. difficile* remains largely unexplored. Further studies should consider investigating whether certain animal species could serve as reservoirs of this bacterium or potential sources of infection. At present, the understanding of this aspect is lacking, and additional research is needed to shed light on the epidemiology of *C. difficile* in relation to wildlife and exotic pets.

Another important issue is the presence of *C. difficile* in the food chain. Although the possibility of *C. difficile* being a foodborne disease has already been studied without reaching a clear conclusion (Weese, 2010; Lund & Peck, 2015; Marsh, 2013), new evidence suggests that *C. difficile* is present in the environment and along the food chain (Bolton & Marcos, 2023). *C. difficile* contamination in slaughterhouses has been described (Wu et al., 2017; Rodriguez et al., 2013) as well as in other products, like shellfish (Agnoletti et al., 2019; Candel-Pérez et al., 2020). Furthermore, molecular relationship between strains isolated from animal feces and the strains found as

contamination in the processing plant has been found (Abay et al., 2022).

While current evidence does not strongly indicate a high risk of infection, the possibility that slaughterhouses and the food chain may serve as a source of infection or act as reservoirs for *C. difficile* cannot be entirely disregarded. Although the exact contribution of the food chain to *C. difficile* transmission remains uncertain, the presence of characterized strains associated with animals and their occurrence in human CA-CDI cases warrants careful investigation. These analyses will help shed light on the potential role of slaughterhouses and other parts of the food chain in *C. difficile* epidemiology, ultimately leading to a better understanding of the dynamics of this pathogen and the implementation of appropriate preventive measures.

While our understanding of *C. difficile* epidemiology in animals is still evolving, recognizing the potential role of animals as reservoirs or sources of infection is crucial. Further research is needed to explore the transmission dynamics, host specificity, and impact of *C. difficile* in animal populations. By expanding our knowledge in this area, we can enhance the overall management and control of *C. difficile* infections, both in animal health and public health contexts.

Virulence Factors and Toxin Production

There has been previous research on the relationship between the levels of toxins produced by different strains of *C. difficile* and disease severity, but it has yielded contradictory results. However, a recent study that investigated the impact of different STs (Hamo et al., 2021) found that, although there was no significant association between toxin levels and disease severity, ST 42 and ST 104, which produce high levels of toxins, had the most significant effects on cell proliferation, causing a reduction when compared to control cells. Additionally, ST 37 had a more severe impact on cell morphology. This highlights that not only strain characterization and toxin production but also host response play an important role in the onset of symptoms. Furthermore, it was found that stool samples containing *C. difficile* strains with ST 42 and ST 104 had higher levels of toxins.

The phylogenetic topology can be reflected in the PaLoc, which encodes toxins A (*tcdA*) and B (*tcdB*) and the negative regulator of toxin production, *tcdC*.
Specifically, *tcdC* exhibited identical mutations across a group of analyzed ST 1 strains, as indicated by

1 and 18 bp deletions. In addition, fluoroquinolone resistance genes, particularly *gyrA*, exhibited uniformity in their sequences among analyzed ST 1 strains. On the other hand, these genes showed variations in their sequences when compared to those present in other STs. These findings suggest coevolution of the MLST genes with toxin genes and *gyrA*, indicating that the evolution of virulence and antimicrobial resistance in *C. difficile* may be linked to the evolution of the core genome (Zhou et al., 2014).

In general, ST and virulence factors other than antibiotic resistance do not seem to be associated (Rohana et al., 2020). However, as for virulence genes, *agr* genes have been observed to have implications beyond toxin and virulence factors' production regulation, although they have been found both in toxigenic and nontoxigenic strains. This type of gene has been implicated in the regulation of several characteristics related to *C. difficile* virulence, including flagellar biosynthesis, production of the toxin TcdA, and signaling proteins involved in cyclic dimeric GMP (c-di-GMP) signaling (Martin et al., 2013). Notably, the distribution of *agr* gene variants differs among clades, with *agr2R*-positive strains primarily found in Clades 1 and 2, and STs carrying *agr2M* exclusively identified within Clade 4 (Okada et al., 2020). It has also been hypothesized that host response, rather than ST or strain characteristics, plays a major role in the development of the disease (Rousseau et al., 2011).

In a recent study (Lemée et al., 2005), the analysis of monolocus dendrograms revealed that the topologies derived from various housekeeping genes and certain virulence-associated genes, including *fbp68, groEL, tcdA, tcdB,* and *tcdB* loci, exhibited a consistent pattern, suggesting a possible co-evolutionary relationship. This implies that these genes have likely evolved together over time. However, the analysis also identified two discrepancies in the dendrograms. The *fliC* and *fliD* loci showed a global clustering pattern like that of the housekeeping genes, except for three isolates that were closely related to A-B+ isolates. On the other hand, the *cwp66* and *slpA* loci displayed a higher level of polymorphism compared to the other virulence-associated genes, resulting in a distinct dendrogram topology. Notably, certain isolates showed close relatedness in the *cwp66* and *slpA* trees but were distant in the other trees, indicating potential recombinational events or strong selective pressures affecting these gene clusters. As a conclusion of the study, the variability of the *slpA* gene, known to be

associated with serogroups, seems to be influenced by both recombinational events and selective pressures. This suggests that the formation of serogroups in *C. difficile* is a result of the interplay between genetic recombination, environmental selection, and the polymorphic nature of the variable domain of the *slpA* gene.

In conclusion, the co-evolutionary relationship observed among certain housekeeping and virulence-associated genes suggests their concerted evolution over time. Discrepancies in dendrogram topologies indicate potential genetic recombination and selective pressures impacting gene clusters.

Antibiotic Resistance

Antibiotic resistance is a growing concern as it poses significant challenges to the effective treatment of CDI. Molecular typing techniques, such as whole-genome sequencing, PCR-based assays, and gene expression profiling, are essential tools for studying genetic resistance mechanisms and its link with phenotypical expression and the transmission of those genes. These techniques enable the identification of resistance genes, genetic variations, and mobile genetic elements, providing valuable insights into the molecular characteristics of antibiotic-resistant strains and aiding in the surveillance and control of resistant strains.

Regarding antibiotic resistance, the topic remains controversial as different studies have pointed in different directions. For example, some studies have found no relationship between ST and antimicrobial resistance, like no association between the presence of *erm*(B) and *tet*(M), MLST, and toxin phenotypes (Wang et al., 2018c). In other study, non-toxigenic *C. difficile* isolates had a lower presence of antibiotics resistance than its toxigenic pairs (Zheng et al., 2017). ST 2 isolates have been found more susceptible to ceftriaxone than other STs, suggesting a possible relation between ST type and ceftriaxone resistance (Wang et al., 2018a). However, in other studies, they found that the rates of clindamycin, tetracycline, and ampicillin resistance among ST 2 isolates were lower than those in other STs (Meng et al., 2021). ST 81, which is closely related to ST 37, is capable of generating more spores and toxins and presents higher resistance rates to fluoroquinolones (Wang et al., 2018b).

In China and other parts of Asia, ST 54 and ST 35 have been extensively associated with CDI as well as ST 37 (Li et al., 2018; Yan et al., 2017; Chen et al., 2018). ST 35 isolates have been found to be associated

with tetracycline and erythromycin (Zheng et al., 2017) and capable of causing outbreaks (Tang et al., 2018). Additionally, genetic mutations related to glycometabolism, amino acid metabolism, and biosynthesis were associated with the transcription of *tcd*R and the expression of toxin repressor genes, *ccpA* and *codY*, suggesting their impact on protein activity stability, as this could cause instability and loss of function due to alterations in the protein structure (Xu et al., 2021). Interestingly, in ST 37, despite exhibiting low toxin production (toxin A negative), there has been an alarming increase in antibiotic resistance within this particular strain, which raises concerns about its potential for widespread dissemination. The emergence of higher resistance rates in ST 37 strains underscores the need for vigilance and monitoring as these strains may pose an increasing threat and contribute to the spread of more resistant *C. difficile* strains (Dong et al., 2014). High resistance to clindamycin has been described in this ST 37 (Zheng et al., 2017).

Samples from Clades 1, 2, and 3 have been found to be more likely to exhibit reduced susceptibility to vancomycin, and moxifloxacin resistance was more frequently observed in samples collected from Europe and Asia compared to other continents, with no instances of moxifloxacin resistance identified in samples from Oceania (Zhao et al., 2021).

The prevalence of multidrug resistance (MDR), defined as those isolates with non-susceptibility to three or more antimicrobial categories (Magiorakos et al., 2012), was highest in Clade 4, with a rate over three times higher than in Clade 2. Three major epidemic *C. difficile* STs showed a strong association with specific antimicrobial resistance (AMR) determinants. ST 1 (Clade 2) was associated with fluoroquinolone resistance, ST 11 (Clade 5) with tetracycline resistance, and ST 37 (Clade 4) with macrolide–lincosamide–streptogramin B (MLSB) resistance and MDR. Among Clade 2 strains, a transposon labeled as *Tn6944* that carried *tetM* was identified (Imwattana et al., 2021). In ST 42 (Clade 1), significantly higher rates of gene *erm(B)* (macrolide resistance) have been found compared with the others STs (Girão et al., 2021). ST 3, also from Clade 1, was associated with a high rate of resistance to erythromycin and to moxifloxacin simultaneously. Association with decreased sensitivity to antibiotics is likely to favor its dissemination (Weber et al., 2013). It is of special interest in the possibility of spread of pCD-METRO, a plasmid known to cause resistance to metronidazole, an antibiotic of special interest in the

treatment of CDI. This plasmid has been found in strains isolated from the environment, as previously discussed (Cizek et al., 2022).

In summary, the analysis of molecular characteristics of *C. difficile* strains reveals diverse patterns across different regions, genetic loci, and sequence types. The prevalence of specific STs varies geographically, highlighting regional differences in strain distribution, antimicrobial resistance profiles, and virulence factors. The association between STs and antibiotic resistance remains a topic of debate, with conflicting findings reported.

Conclusions and Future Directions

To sum up, the variety of methods used for the study of *C. difficile* allow researchers to adapt its projects to their resources and circumstances. However, there is a trend of shifting towards NGS methodologies as this technology becomes more affordable and provides high-resolution strain typing and detailed analysis, which can be easily integrated with epidemiological data to enhance outbreak investigations, track transmission routes, and identify potential virulence factors or antimicrobial resistance determinants.

Furthermore, integrating NGS data with phenotypic studies can provide a comprehensive understanding of *C. difficile*. By combining genetic information with phenotypic traits such as toxin production, sporulation ability, or antibiotic susceptibility, new information about correlations between genotypes and phenotypes would be obtained. This information could be used to improve treatments, shorten diagnosis times, and, in general, improve possible patient outcomes.

Besides, it would be also advisable to conduct studies in countries that do not have established *C. difficile* surveillance systems. By expanding research efforts to understudied regions, new insights into the global burden and distribution of *C. difficile* infections would be gained as well as into the identification of regional variations and antimicrobial resistance patterns, supporting the development of prevention and control measures.

In addition to NGS, other areas of research can contribute to our understanding of *C. difficile* epidemiology. Microbiota studies represent a promising field given the nature of *C. difficile*. The composition and dynamics of the gut microbiota play a crucial role in modulating susceptibility to *C. difficile* infection and disease outcomes. This kind of approach can give new insights into

host–microbe interactions, identify potential biomarkers of susceptibility or resistance, and explore novel approaches for preventing or treating CDI. It can also be linked to new ways of treatment, like fecal microbiota transplantation.

In addition, adopting a One Health approach is another promising take for future studies. *C. difficile* is not limited to human populations and can be found in animals, food, and the environment. There is evidence that the epidemiology of *C. difficile* has changed, therefore making it important to investigate new routes of transmission, which include a possible zoonotic element or the existence of higher rates of CA-CDI than traditionally reported. Investigating the epidemiology of *C. difficile* across different species and environments can help elucidate transmission dynamics, reservoirs, and potential sources of infection.

Lastly, studying host–pathogen interactions in CDI could prove to be an important area of investigation. Understanding the mechanisms underlying host susceptibility, immune response, and disease severity can help with the development of targeted therapeutics and better treatments. As exposed previously, strain characterization alone does not explain the development of CDI, suggesting that host response may play a significant role in the disease process.

By focusing on these future perspectives, researchers can contribute to a deeper understanding of *C. difficile* epidemiology, enhance our ability to prevent and control CDI, and improve patient outcomes.

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Data Availability Statement

No new data were created or analyzed in this study. Data sharing is not applicable to this article.

Conflicts of Interest

The authors declare no conflict of interest.

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Trabajo 6

Comparative genomics of *Clostridioides difficile* ribotype 126 isolated from several species

Introduction

Clostridioides difficile is a major nosocomial pathogen responsible for a wide spectrum of gastrointestinal diseases in humans, ranging from mild diarrhea to life-threatening pseudomembranous colitis. However, since 2005, an increase in the incidence of *C. difficile* infection (CDI) has been observed. Further investigations led to the conclusion that a new ribotype, RT078, had emerged as a cause of human disease, causing a shift in the epidemiological landscape known until that moment (Goorhuis et al., 2008). This particular ribotype exhibits a tendency of affecting younger demographics and demonstrates a propensity for community-associated transmission. One possible source for the origin of these community associated cases has been hypothesized to be animals, specially linked to production animals (Koene et al., 2012; Marcos et al., 2021; Weese, 2020).

Similarly, RT126 is closely related to RT078, differing only in the lack of a band in the PCR ribotype pattern (Martínez-Meléndez et al., 2020). RT126 has been implicated in cases of pseudomembranous colitis and recurrent CDI in humans (Hung et al., 2014). Furthermore, descriptions of heteroresistance to metronidazole have surfaced in association with this ribotype (Álvarez-Pérez et al., 2017). In animals, it has been described in a myriad of species, including swine (Knight et al., 2015; Clara Tramuta et al., 2021; Tsai et al., 2016), rats (Andrés-Lasheras et al., 2017), calves (Blasi et al., 2021; Knight et al., 2013; Zidaric et al., 2012) or sea life (Pasquale et al., 2012; Troiano et al., 2015), among others.

Currently, *C. difficile* is classified into five major clades (denoted as 1 to 5) and five cryptic clades (designated with roman numerals, I to V) (Williamson et al., 2022). However, these cryptic clades are typically encountered in environmental settings rather than clinical contexts, with infrequent isolation from animals or humans. Clade 1 exhibits the highest degree of heterogeneity and clinical relevance, especially due to it being the more numerous. Clade 5, to which RT126 belongs, also holds importance. Its representative is RT078, frequently associated with both animals and community-acquired CDI (Debast et al., 2009; Knight & Riley, 2019; Tramuta et al., 2021). Additionally, within Clade 5, atypical Pathogenicity Locus (PaLoc) structures have been observed (Elliott et al., 2014).

The PaLoc constitutes a critical region within the genome of *C. difficile*, housing genes responsible for encoding toxins. Besides the genes for toxin A and B, this region also includes three accessory genes, *tcd*R, *tcdE* and *tcdC*, with different possible organizations (Figure 15).

The other major toxin of *C. difficile*, binary toxin, is encoded in other region, known as CDTLoc (Janezic et al., 2020).

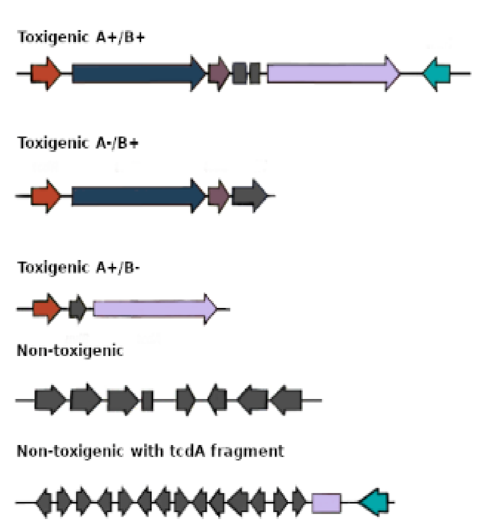

Figure 15. Examples of different possibilities of PaLoc organization. Adapted from Monot et al., 2015 and Elliott et al., 2014. Each gene within the PaLoc region is represented by a distinct color for clarity: *tcd*R is depicted in red, toxin B in blue, *tcdE* in brown, toxin A in purple, and *tcdC* in turquoise.

PaLoc region is one of the most important in regions the genome of the bacterium, as it is responsible for the toxins that is the cause of the development of clinical signs in the host. Alterations within the PaLoc can have significant implications, potentially leading to diagnostic challenges. Many diagnostic tests rely on detecting these toxins or their corresponding genes to confirm CDI, including PCR assays and rapid tests. Consequently, changes in the PaLoc structure can compromise the accuracy of such diagnostic methods, impacting patient care and management. Moreover, while the evolutionary dynamics of the PaLoc may differ from overall phylogenetic evolution, specialized studies focusing on this region offer insights into the genetic relationships among *C. difficile* strains within a population. Such investigations enable a deeper understanding of the genetic diversity and evolution of this pathogen, aiding in the development of more effective diagnostic and therapeutic strategies.

As a result of advances in genomic technology, Whole Genome Sequencing (WGS) has emerged as a powerful tool for studying the genetic structure of bacterial strains. By conducting WGS on a set of RT126 isolates from distinct species and environments, this study aims to provide insights into the genetic determinants that make this ribotype unique, contributing to the broader understanding of CDI.

Materials and methods

Sample collection and DNA isolation

A total of 13 isolates were selected for this study, all meeting the inclusion criterion of prior confirmation as RT126 by earlier investigations (Andrés Lasheras, 2016; Sevilla Romeo, 2019). These isolates were chosen to include a range of host species. The dataset comprised three isolates from rabbits collected between 2017 and 2018, four from rabbits collected between 2018 and 2022, one from a rat, one from an environmental sample associated with rat habitats,

two from piglets, and one originating from the environment of a pig farm and are detailed in Table 16. In addition, for phylogenetic analyses, genomic data sourced from other databases such as Enterobase were incorporated. The selection criteria focused on *C. difficile* isolates belonging to RT126. As of November 26, 2023, a total of 115 genomes meeting these criteria were identified and included in the study. Metadata associated with these isolates was gathered, including information such as the year of isolation, country of origin, host organism, and source of the isolate whenever available.

Table 16. Name, year and source of selected isolates. The country of origin for all isolates is Spain.

Identification	**Source**	**Year**
PHRT21	Environment-Rat	2011
CFarrLock	Environment-Swine	2020
C1	Livestock-Rabbit	2012
C2	Livestock-Rabbit	2012
C6	Livestock-Rabbit	2013
A2115	Livestock-Swine	2016
AD134	Livestock-Swine	2016
TAHI-16	Livestock-Rabbit	2019
TAHI-17	Livestock-Rabbit	2019
TAHI14	Livestock-Rabbit	2021
TAHI16	Livestock-Rabbit	2021
R1	Wildlife-Rat	2011
Bu33	Wildlife-Vulture	2011

The DNA extraction process began by directly plating the selected isolates on blood agar plates (OXOID, United Kingdom). These isolates were incubated under anaerobic conditions at 37 °C for 24 hours. Subsequently, they were cultured in LB broth until they reached the exponential growth phase, which typically required around 8 hours. Following this, 2 mL of incubated broth, with optical density value within the range of 1 to 2 McFarland units, underwent DNA extraction using a commercial kit (E.Z.N.A Bacterial extraction kit, Omega Biotek, United States), following the manufacturer's instructions. The extracted DNA integrity was verified through 1% agarose gel electrophoresis, performed for 40 minutes at 120 V, and DNA concentration was quantified using Qubit 4 (Invitrogen, United States of America).

Whole Genome Sequencing

All DNA samples underwent sequencing using an Illumina MiSeq platform, conducted by an external service provider (Stab Vida, Portugal). Following sequencing, the sequences were subjected to trimming using Galaxy (Version 0.3.8.1) and assembled using Unicycler (Galaxy version 0.5.0 + Galaxy 1). To analyze the sequenced genomes, various bioinformatics tools were employed. Antibiotic resistance genes, virulence factors, and sequence types (ST) were assigned using tools such as ResFinder 4, PathogenFinder 1.1, VirulenceFinder 2.0, MLST 2.0, cgMLSTFinder 1.2 and MGE v1.0.3 (Bartual et al., 2005; Bortolaia et al., 2020; Camacho et al., 2009; Clausen et al., 2018; Cosentino et al., 2013; Frentrup et al., 2020; Griffiths et al., 2010; Jaureguy et al., 2008; Joensen et al., 2014; Johansson et al., 2021; Larsen et al., 2012; Lemee et al., 2004; Wirth et al., 2006; Zankari et al., 2017; Zankari et al., 2012). A phylogenetic tree was constructed utilizing the Roary pipeline (Page et al., 2015) based on Prokka annotation (Seemann, 2014). Subsequently, IQ-TREE software (Nguyen et al., 2015) was employed for further analysis and visualization of the phylogenetic relationships among the sequenced genomes. Best fit model was found with model finder (Kalyaanamoorthy et al., 2017), which was selected according to AIC: Tamura Nei DNA model, with empirical base frequencies and allowing for a proportion of invariable sites.

The PaLoc phylogeny tree was manually created isolating the region of interest and aligning it using MEGA X (Kumar et al., 2018). The evolutionary history was inferred by using the Maximum Likelihood method and General Time Reversible model (Nei & Kumar, 2000). Initial tree for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.1448)).

Data analysis tools and visualization

For the visualization and analysis of the data, several tools were employed. Firstly, visualization of the genomic data was carried out using Proksee (Grant et al., 2023). Annotation was performed with Bakta (Schwengers et al., 2021). PaLoc analysis and visualization were conducted using Easyfig (Sullivan et al., 2011). *Clostridioides difficile* strain S-0253 served as reference when the analysis required one (BioSample Number: SAMN19491759). Circular visualization of the tree was created in R using the package ggtree (Xu et al., 2022; Yu, 2020, 2022; Yu et al.,

2018; Yu et al., 2017). Additionally, gene ontology analysis was conducted using GO Enrichment Analysis (Ashburner et al., 2000; Gene Ontology Consortium, 2023; Thomas et al., 2022) to further elucidate the functional significance of the identified genes.

Results

This study analyses the genome of 13 isolates of *C. difficile* obtained from different sources. The probability of being a human pathogen was assessed to be 81.9%. Despite all isolates belonging to ST 11, the cgMLST analysis revealed some differences: while most of them corresponded to cgST 96, the rat environmental isolate was from cgST 21615, and 3 rabbit isolates were from cgST 1311.

During the analysis, core genomes were found to be relatively consistent, comprising approximately 3240 genes out of a total of 6038 potential genes identified. Among the 1109 accessory genes with known ontologies associated, 43.2% were categorized under cellular processes, while 35% were involved in regulating metabolic processes. The remaining genes were distributed across various functional categories: localization, biological regulation, response to stimulus, homeostatic process, locomotion, and reproduction, in descending order of abundance.

The phylogenetic analysis of all available RT126 genomes was conducted, resulting in the generation of a phylogenetic tree (Figure 16). This tree provided insights into the genetic relatedness and evolutionary relationships among the RT126 isolates included in the study.

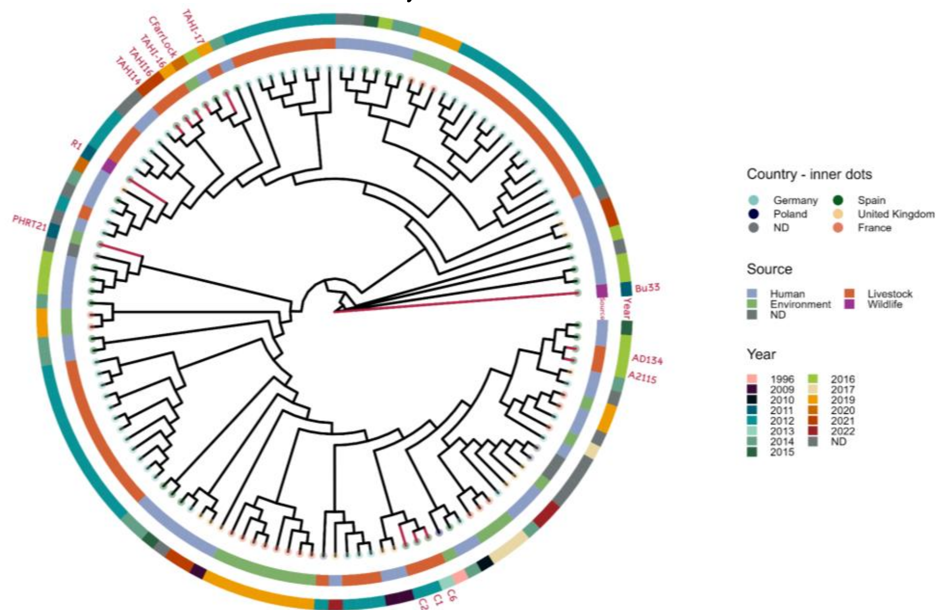

Figure 16. Phylogeny tree of all RT126 genomes from this study, with its source, year of origin and country. Each branch of the tree represents a distinct lineage of *C. difficile*, with branch lengths indicating the degree of genetic divergence between isolates.

Two distinct phylogenetic trees were constructed using the 13 isolates from the study: one based on the PaLoc region (Figure 17a) and another one based on WGS data (Figure 17b). The phylogenetic tree generated from the PaLoc region exhibited minimal divergence among the samples, indicating a high degree of genetic conservation within this specific genomic region across the sampled isolates. In contrast, the phylogenetic tree derived from WGS data displayed greater divergence among the samples, suggesting a higher level of genomic variability across the entire genome.

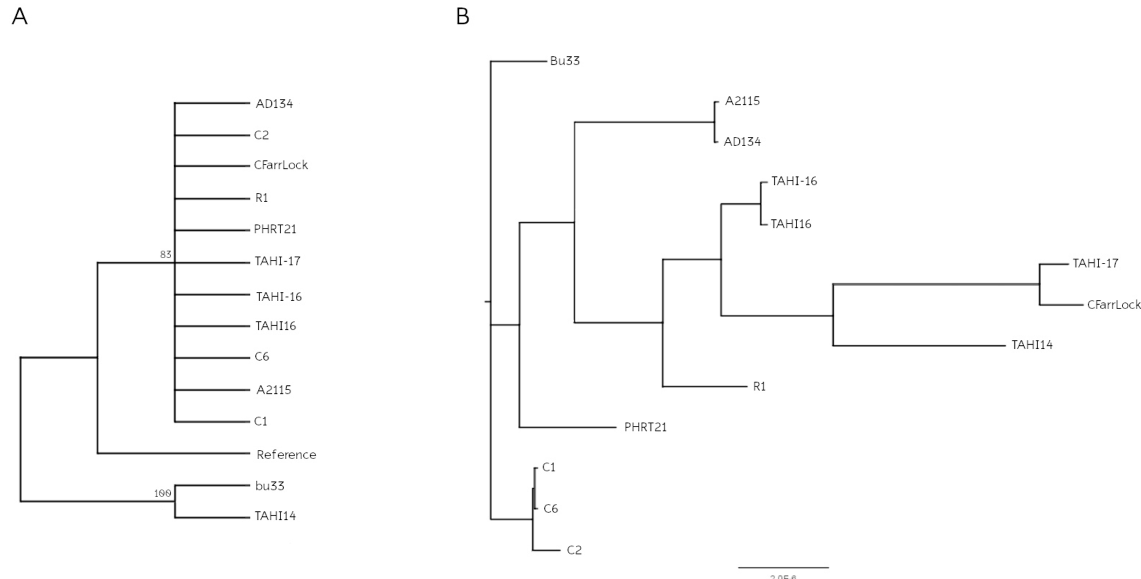

Figure 17. Side comparison of PaLoc phylogeny tree and whole genome phylogeny tree. (A) The evolutionary history was inferred by using the Maximum Likelihood method and General Time Reversible model. As there is no correlation between branch length and genetic divergence, no scale was used. (B) Tree is unrooted although outgroup taxon 'Bu33' is drawn at root. Scale indicates close genetic proximity between isolates.

Variation within the PaLoc was low, with most of the isolates showing the same toxin pattern, which was described as *tcdR*, *tcdB*, *tcdE*, *tcdA* and *tcdC*, with minor genes or different sequences in between each identified gene. PaLoc sequence from isolate TAHI14 was incomplete. Further details and comparisons can be found in Figure 18.

All isolates presented resistance genes *tet*(M*)*, *tet*(40) and *aph(3´)III, ant(6)-la* as well as plasmid repUS43. Only two isolates harbored the *erm(B)* gene, associated to Tn6194. The other identified genes were *aph(2'')-If*, *aac(6')-Im*, *aph(2'')-Ib* and *cfr(B)*. Results are further detailed in Table 17.

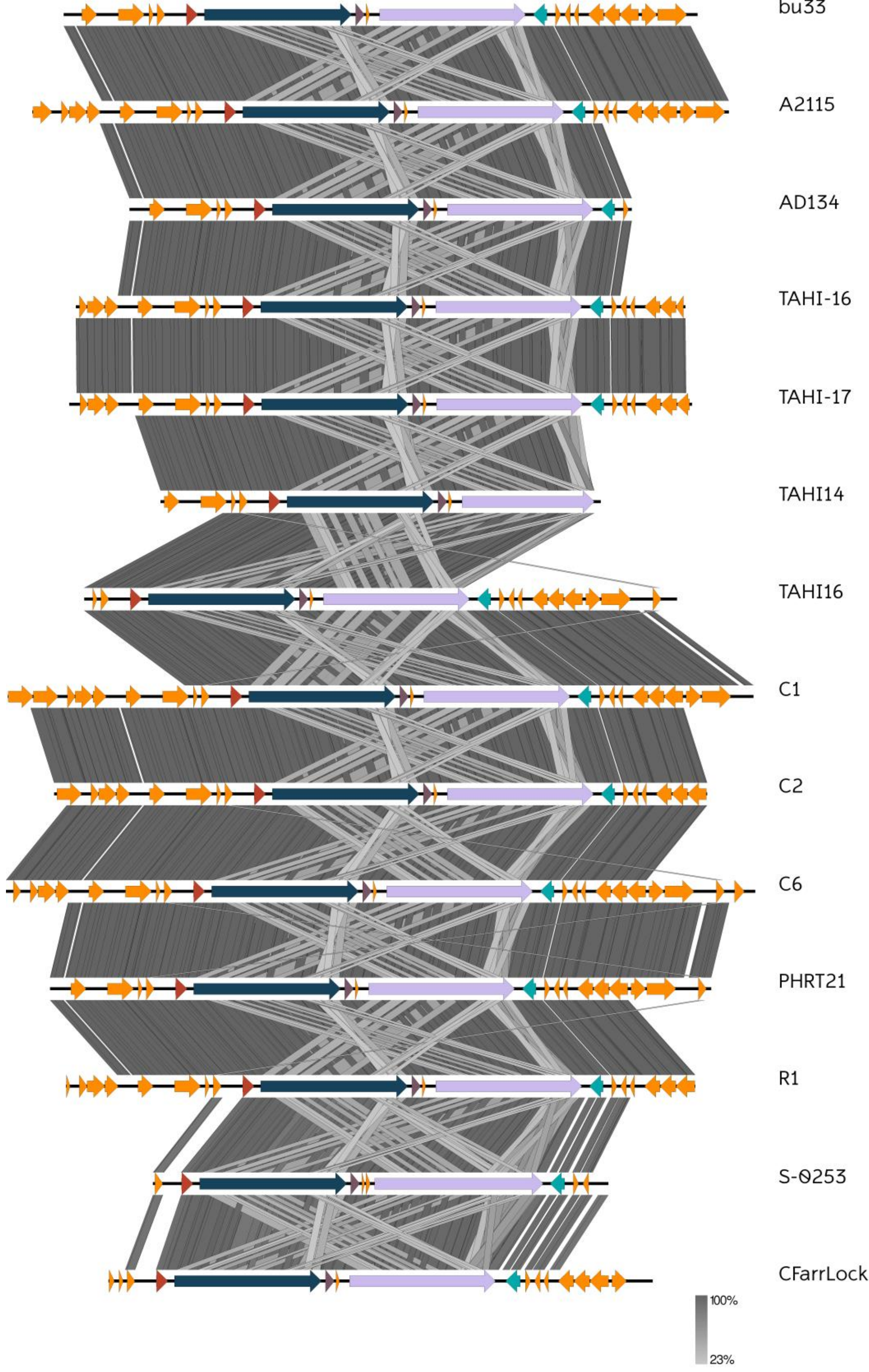

Figure 18. Comparison of PaLoc region in all the isolates and the reference strain. In this figure, the PaLoc region of all isolates examined in the study, along with the reference strain, is compared. Each gene within the PaLoc region is represented by a distinct color for clarity: *tcdR* is depicted in red, toxin B in blue, *tcdE* in brown, toxin A in purple, and *tcdC* in turquoise.

Table 17. Antibiotic resistance genes found in each isolate, coverage and identity.

Gene	Predicted phenotype	Coverage (%)	Identity (%)	MGE	Presence in isolate
tet(M)	doxycycline, tetracycline, minocycline	100	98.89	repUS43	Bu33, CFarrLock, A115, AD134, C1, C2, C6, TAHI14, TAHI16, TAHI-16, TAHI-17, PHRT21, R1
tet(40)	doxycycline, tetracycline	100	99.75	No	Bu33, A2115, AD134, C2, C6, TAHI14, TAHI16, TAHI-16, TAHI-17, PHRT21, R1
aph(3´)III	isepamicin, amikacin, kanamycin, butirosin, paromomycin, ribostamycin, lividomycin, neomycin	100	100	No	Bu33, CFarrLock, AD134, C1, C2, C6, TAHI14, TAHI16, TAHI-16, TAHI-17, PHRT21, R1
ant(6)-la	streptomycin	100	100	No	Bu33, CFarrLock, A2115, AD134, C1, C2, C6, TAHI14, TAHI16, TAHI-16, TAHI-17, PHRT21, R1
erm(B)	clindamycin, lincomycin, pristinamycin, quinupristin,	100	100	Tn6194	TAHI16, TAHI-16

	virginiamycin, erythromycin				
aph(2'')-If	tobramycin, amikacin, kanamycin, gentamicin	95.08	94.15	No	Bu33, AD134, C2, C6
		95.08	93.56		PHRT21
		100	100		TAHI14
aac(6')-Im	kanamycin, tobramycin, amikacin	100	96.27	No	CFarrLock, TAHI-17
aph(2'')-Ib	kanamycin, gentamicin, tobramycin, amikacin	100	98.78	No	CFarrLock, TAHI-17
cfr(B)	florfenicol, chloramphenicol, dalfopristin, pristinamycin, virginiamycin, linezolid, clindamycin, lincomycin, tiamulin	100	99.90	No	A2115

Discussion

The analysis of isolates from RT126 revealed a similarity percentage in the genome of 53.66%. This proportion is higher than the expected core genome, estimated to be around 20%, attributable to the notable diversity within the *C. difficile* species (Scaria et al, 2010).

Upon comparison with previous studies encompassing strains from various clades, not limited to clade 5, a notable difference was observed: this predicted core genome contained approximately 1000 more genes. This discrepancy suggests a level of stability specific to the ribotype under examination. Furthermore, this stability is corroborated by the consistent retention of a high percentage of identified accessory genes across all strains within RT126. Clade 5, albeit to a lesser extent than the cryptic clades, is recognized for introducing considerable variability to the *C. difficile* species, a finding that aligns with our observations of accessory genes (Golchha et al., 2022; Knight et al., 2021).

Despite the expansive nature of the pangenome, the observed diversity in accessory genes within this ribotype was comparatively modest, totaling approximately 2500 genes.

This could be attributed to two plausible hypotheses. Firstly, the relatively limited number of isolates included in the analysis may have contributed to this reduced diversity. Secondly, focusing exclusively on a single ribotype likely resulted in higher similarities among the analyzed isolates, as it is expected that intraribotype comparisons would reveal more similarities due to shared evolutionary history and genetic characteristics (Knight et al., 2021).

According to the phylogenetic analysis incorporating all the strains (Figure 16), the greatest similarities were observed among strains originating from the same source, followed by strains from the same country, and then by the year of isolation. This pattern suggests that the genetic composition of *C. difficile* strains is relatively stable over time, with variations more closely associated with geographical and ecological factors rather than temporal changes. This finding aligns with previous studies indicating that *C. difficile* exhibits a considerable degree of genomic stability, being primarily shaped by selection pressure (He et al., 2010). Therefore, the observed distinctions based on source and geographical origin are consistent with the notion of localized microbial populations maintaining distinct genetic signatures. In a more detailed view, the 13 isolates from this study also tend to group together according to species (Figure 17b), with the exception of CFarrLock, which comes from an environmental swine sample but groups with samples from rabbits.

The structure of the PaLoc remained consistent across all analyzed isolates, with no observed changes in the position or orientation of known genes (Figure *18*). Similarly, comparative analysis of phylogenetic trees constructed from the PaLoc sequences revealed a high degree of conservation within this region, as most isolates clustered closely together without significant divergence. However, three exceptions were observed, including one strain isolated from a rabbit, the reference strain, and one from a vulture. The divergence of the reference strain is rationalized by its origin from a different ribotype, and context compared to the rest of the strains. Similarly, the divergence of TAHI14 isolate is attributed to the initial incompleteness of the PaLoc sequence. While truncation in the PaLoc could explain such discrepancies, deeper analysis suggested poor sequence quality rather than structural alterations. Interestingly, the vulture isolate exhibited differences in the region immediately following the last identified gene (*tcdC*) and displayed 100% identity when compared to PaLoc sequences from other previously identified ribotypes, specially RT078. This divergence might indicate a distinct evolutionary event in the PaLoc region of this strain, possibly linked to its origin from wildlife rather than livestock. When contrasting with the

phylogenetic tree constructed from whole genome sequences, it becomes apparent that the strains are not entirely homologous, underscoring the stability of the PaLoc region despite genomic variations. This finding aligns with previous literature supporting the stability of the PaLoc across *C. difficile* strains (Janezic et al., 2020; Janezic & Rupnik, 2015).

The discovery of tetracycline resistance genes in this isolate collection was anticipated, given their widespread prevalence within the species (Spigaglia et al., 2008). However, our investigation also identified two isolates that tested positive for the *erm(B)* gene. According to a previous hypothesis, the evolutionary divergence of RT126 from RT078 might have been driven by the selective pressure exerted by fluoroquinolones on *erm(B)*-negative RT078 strains (Spigaglia et al., 2010). Therefore, the presence or absence of *erm*(B) gene in the studied isolates suggests the existence of distinct genetic variants within this ribotype. It's plausible that the *erm(B)*-positive strains may have acquired this resistance determinant through horizontal gene transfer or other evolutionary mechanisms, thereby conferring resistance to specific antibiotics, especially considering that one of the isolates had the gene associated with a genetic mobile element.

Conclusion

In conclusion, our study provides insights into the genetic characteristics and stability of *C. difficile* RT126 strains. Through genomic analysis, we identified a higher core genome percentage than expected, indicating a degree of stability specific to this ribotype. The retention of a proportion of accessory genes across strains within RT126 further underscores this stability. Additionally, the presence of *erm(B)* gene in a subset of strains suggests the existence of distinct genetic variants within this ribotype, potentially acquired through evolutionary mechanisms such as horizontal gene transfer.

Furthermore, the phylogenetic analysis including also other RT126 genomes revealed that genetic similarities among strains are primarily influenced by source and geographical origin, rather than temporal changes. The conserved structure of the PaLoc region across all strains indicates a stable genetic landscape despite genomic variations.

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Discusión

Discusión

Esta tesis doctoral parte desde una filosofía One Health para estudiar distintos enteropatógenos. El objetivo es, a través de diversas técnicas y su análisis desde la epidemiologia, clínica y patología, valorar las relaciones que existen entre los patógenos, las especies seleccionadas y el medio ambiente y su posible relación con la especie humana. Para ello, se eligieron tres especies bacterianas: *E. coli*, *C. perfringens* y *C. difficile*, importantes en animales y en el hombre.

La memoria de resultados de esta tesis comienza con un estudio de *E. coli* en orina de la especie canina, reflejado en el trabajo 1. En este caso, se analizó la presencia de este patógeno en otra de sus formas de infección (cuando causa patología urinaria) en vez de la habitual patología digestiva. Entre los hallazgos más relevantes de este trabajo se encuentra la presencia de dos cepas pertenecientes al patotipo EPEC, frente a la mayoría de cepas que fueron ExPEC. La aparición de cepas de origen entérico en orina puede suponer una vía de trasmisión de estos patógenos (Bélanger et al., 2011; Nielsen et al., 2014).

Tanto en este enteropatógeno como en todos los estudiados a lo largo de los trabajos de investigación, cobra especial importancia la presencia de resistencia a los antibióticos. A este respecto, otro hallazgo relevante fue la presencia de varios genes de resistencia asociados a elementos móviles. Estos MGE son capaces de pasar con facilidad a otras bacterias y diseminar las resistencias que posean, por lo que representan una clara amenaza para la salud pública. De especial interés es el caso de la cepa 271.960, que se encontró genéticamente cercana a otra cepa humana, del ST 131 (Nicolas-Chanoine et al., 2014). Las posibles implicaciones de este hecho sugieren que puede haber una relación entre cepas humanas y animales, incluyendo la posibilidad de trasmisión zoonótica entre animales de compañía y personas (Debast et al., 2009).

En el segundo trabajo también se investigó sobre la presencia de *E. coli*, esta vez como agente causal de diarrea, y se incluyó también el análisis de *C. perfringens* y *C. difficile*, en este caso en la especie cunícola. *E. coli* estuvo implicada en la mayoría de los casos de diarrea estudiados, especialmente asociado a la presencia del gen de la intimina, causando lesiones de "adhesión y borrado". Además de ello, aunque previamente se ha relacionado la presencia de *C. perfringens* con la enteropatía mucoide (Puon Peláez et al., 2018), no se apreció ninguna relación clara en este aspecto. Por último, la presencia de *C. difficile* fue baja (aproximadamente del 3 %), lo que parece indicar una limitada importancia en esta especie. A pesar de ello, cabe destacar que todos los aislados positivos se encontraron en animales con diarrea, sugiriendo la importancia de continuar haciendo estudios en esta especie. Los patrones de resistencia a antibióticos también fueron los esperables para esta especie bacteriana. Por último, fue destacable la presencia del ribotipo 126 —relacionado con el 078 —, que,

aunque suele estar vinculado a animales, ha sido descrito en humanos recientemente (Azimirad et al., 2020; Rodríguez-Pardo et al., 2013). Por ello, incluso a pesar de la baja prevalencia que hace que *C. difficile* no sea uno de los principales agentes causales de diarrea en conejos y por tanto su posible implicación como zoonosis sea limitada, sí es necesario tener en cuenta a este agente como diagnóstico diferencial de diarrea en conejos, ya que su asociación con la expresión clínica puede ser relevante en brotes por *C. difficile.*

En los trabajos de investigación de esta tesis también se ha estudiado la presencia de *C. difficile* y *C. perfringens* en aguas residuales. La positividad de las muestras a *C. perfringens* fue elevada, aproximadamente del 75%, destacando también la alta positividad de estos aislados a enterotoxina (aproximadamente el 43 %). Esta toxina está habitualmente asociada al ser humano (Heikinheimo et al., 2006), lo que supone un riesgo de diseminación al medio ambiente, y que posteriormente podría implicar un foco de infección para el ser humano por distintas vías de trasmisión. La presencia de *C. difficile*, aunque inferior (aproximadamente 24 %), es también importante ya que varios de estos aislados presentaron genes de producción de toxinas. Además, la presencia de aislados resistentes a antibióticos también puede suponer un riesgo para la salud pública, ya que en muchas ocasiones y especialmente en esta bacteria, estas resistencias están ligadas a genes presentes en elementos genéticos móviles, y por lo tanto fácilmente trasmisibles a otras bacterias, tanto de la misma especie como de otras (Kartalidis et al., 2021). Recientemente, el agua residual urbana ha adquirido gran relevancia como un factor clave en la transmisión de genes de resistencia antimicrobiana (ARGs), debido a su papel como un importante reservorio y fuente crítica de su diseminación en el medio ambiente (Reddy et al, 2022). Los resultados obtenidos en esta tesis refuerzan esta tendencia.

Los siguientes trabajos se centraron únicamente en *C. difficile*, siendo el trabajo 4 su valoración en la especie porcina, el 5 una revisión bibliográfica sobre métodos de caracterización genética de *C. difficile* y, finalmente, un estudio sobre la caracterización genética de esta bacteria procedente de diversos orígenes.

En el caso del porcino, a diferencia de lo observado en conejos, la prevalencia fue elevada (22,7 %). Esto demuestra que, aunque *C. difficile* tiene la capacidad de afectar a varias especies, no a todas les afecta por igual. Apoyando a esta idea, mientras que en los conejos todos los aislados positivos se encontraron en animales enfermos, en el trabajo sobre la especie porcina no se encontró una asociación significativa entre la presencia de diarrea y el aislamiento de *C. difficile*. Incluso si consideramos a la especie humana, mientras que en los hospitales la edad elevada siempre ha sido un factor de riesgo para la presentación de CDI, en porcino se asocia mayoritariamente con la fase neonatal (Uzal et al., 2023). Como conclusión, existen diferencias importantes en los hospedadores que

hacen que la bacteria se comporte de una manera u otra. Aunque es cierto que estas diferencias podrían deberse también a cambios en la bacteria, el hecho de que los mismos ribotipos/STs se encuentren tanto en la especie humana como en animales, con el riesgo zoonótico que eso puede implicar (Koene et al., 2012; Tsai et al., 2021), parece apoyar la idea de que la respuesta del hospedador es el factor más importante a la hora de valorar la enfermedad o los signos que esta pueda generar. En concreto, en ambos casos se aislaron bacterias procedentes de los ribotipos 078 y 126. Ambos ribotipos son genéticamente similares, siendo la hipótesis más habitual que el ribotipo 126 deriva del 078 (Spigaglia et al, 2010). Ambos ribotipos tienen un alto potencial zoonótico, por lo que su estudio en animales y su posible relación con la cadena de transmisión es de vital importancia (Hensgens et al., 2012).

Siguiendo esta idea, en el estudio 6 se incluyeron cepas de los trabajos 2 y 4, además de otras procedentes de la colección del laboratorio y cepas obtenidas a partir de repositorios públicos. Este estudio se basó principalmente en el ribotipo 126, y se encontró una homogeneidad de cepas del 53,66 %, superior a lo esperado para la especie bacteriana. Las cepas se alinearon principalmente por especie y localización, aunque algunas de ellas fueron asociadas con cepas de origen humano, lo que refuerza la idea de que es necesario seguir investigando posibles rutas alternativas de trasmisión, especialmente aquellas que implican a animales.

De forma global, la alta prevalencia de resistencia antimicrobiana encontrada, especialmente en genes asociados a elementos móviles, resalta la amenaza que estas cepas representan para la salud pública. En concreto, los valores aproximados de multirresistencia fueron del 71 % para *E. coli* en el trabajo 1; 25 % para *C. difficile* y 16 % para *C. perfringens* en el trabajo 3 y 24 % para *C. difficile* en el trabajo 4 (aumentando hasta el 54 %, si se consideran como resistentes los resultados intermedios). Estos hallazgos destacan la necesidad de abordar la resistencia antimicrobiana desde un enfoque interdisciplinario, integrando medidas de control tanto en medicina veterinaria como en la humana (Andino-Molina et al., 2024). Sin embargo, también merece destacar que, a pesar de compartir ciertas tendencias epidemiológicas, los patrones de resistencia antimicrobiana observados y los genes asociados a dicha resistencia han revelado diferencias entre las tres especies bacterianas examinadas en este estudio. Por ejemplo, mientras que en *C. difficile* es frecuente encontrar los genes *erm*(B), *tet*(40) o *tet*(M), en *E. coli* fue frecuente aislar genes de resistencia frente a betalactámicos (*bla*$_{Z}$, *bla*$_{CMTX-M-55}$) u otros genes de resistencia a tetraciclinas distintos a los aislados en *C. difficile,* como *tet*(A) o *tet*(B). Estas discrepancias reflejan la complejidad intrínseca de la resistencia antimicrobiana y señalan la diversidad genética y evolutiva que caracteriza a cada especie bacteriana.

Aunque existe cierta concordancia entre las tendencias epidemiológicas generales de cada especie según los estándares del Comité Europeo de Antimicrobianos (EUCAST, 2023), los patrones encontrados subrayan la importancia de considerar las particularidades de cada patógeno, independientemente de la especie en la que se haya aislado, y destacan la existencia de factores intrínsecos que influyen en la resistencia antimicrobiana.

La detección de modificaciones en estos patrones de resistencia es una herramienta que puede ser empleada en la vigilancia epidemiológica. Este enfoque, comúnmente utilizado en la especie humana, podría tener aplicaciones en el ámbito de la medicina veterinaria, particularmente para monitorizar la aparición de nuevos genes de resistencia y su potencial diseminación. A lo largo de esta tesis doctoral, se han descrito distintos patrones de resistencia en los diferentes trabajos, destacando una elevada variabilidad. En el trabajo 1, se destaca la elevada cantidad de agentes a los que son resistentes las muestras de *E. coli*, mientras que en los trabajos 2 y 4 se destaca la elevada variabilidad que existe en los patrones de resistencia que pueden presentar las bacterias. En concreto, en el trabajo 2, las cepas de *C. difficile* presentan únicamente resistencia a clindamicina y eritromicina, mientras que en el trabajo 4 aparecen varios patrones de resistencia, incluyendo patrones multirresistentes. El trabajo 3 es el que presenta mayor variabilidad en sus resultados, incluyendo aislados susceptibles a todos los antibióticos estudiados. La detección temprana de estos patrones y el estudio de su trasmisión podría abrir la posibilidad de establecer conexiones de transferencia que, hasta el momento, han sido poco exploradas. Esto se considera especialmente relevante en el caso de bacterias con una alta capacidad de supervivencia en el medio ambiente, como es el caso de *C. difficile* o *C. perfringens*. En estas situaciones, es fácil perder la trazabilidad de los límites de transmisión que puedan existir entre el entorno, los animales y los seres humanos. El uso de esta herramienta en medicina veterinaria no solo contribuiría a la comprensión más profunda de la dinámica de resistencia antimicrobiana, sino que también podría ser fundamental para la identificación y prevención temprana de posibles riesgos de zoonosis y la adopción de medidas preventivas adecuadas. Además, algunas bacterias pueden exhibir genes de resistencia más estrechamente asociados que otros, evidenciando la necesidad de un enfoque personalizado al abordar la resistencia antimicrobiana en el ámbito clínico y veterinario.

También se ponen de manifiesto las variaciones observadas tanto entre especies bacterianas como entre los mamíferos analizados en esta investigación. Por ello, se destaca la necesidad de adoptar una aproximación individualizada en la comprensión de la dinámica patogénica. La diversidad en las respuestas inmunológicas, las características genéticas de los patógenos y los factores

ambientales específicos de cada especie sugieren que las estrategias de prevención y control previamente discutidas no pueden adoptarse de manera universal, sino que deben adaptarse a las particularidades de cada especie y sus patógenos asociados especialmente en el contexto de las zoonosis. La transferencia de patógenos entre animales y humanos se ve influenciada por esta diversidad, destacando la importancia de un enfoque One Health para prevenir y controlar la propagación de enfermedades zoonóticas

Consideraciones y limitaciones

En el trabajo de investigación número 3 se mencionan ciertos errores de procesamiento de las muestras que se van a aclarar a continuación:

Las cepas de *C. difficile* fueron identificadas por los métodos habituales que se han explicado a lo largo de los distintos trabajos de investigación, es decir, identificación por morfología compatible en agar CLO (bioMérieux, Francia) y posterior tinción Gram, con la observación consiguiente de bacilos Gram positivos. Se realizó una extracción de ADN con un kit de extracción comercial (FavorPrep Tissue Genmic DNA Extraction Kit with proteinase K, favorgen, Estados unidos). A estas cepas se les realizo una PCR multiplex (Figura 19) para la toxina A, B y binaria junto con el gen *GluD*.

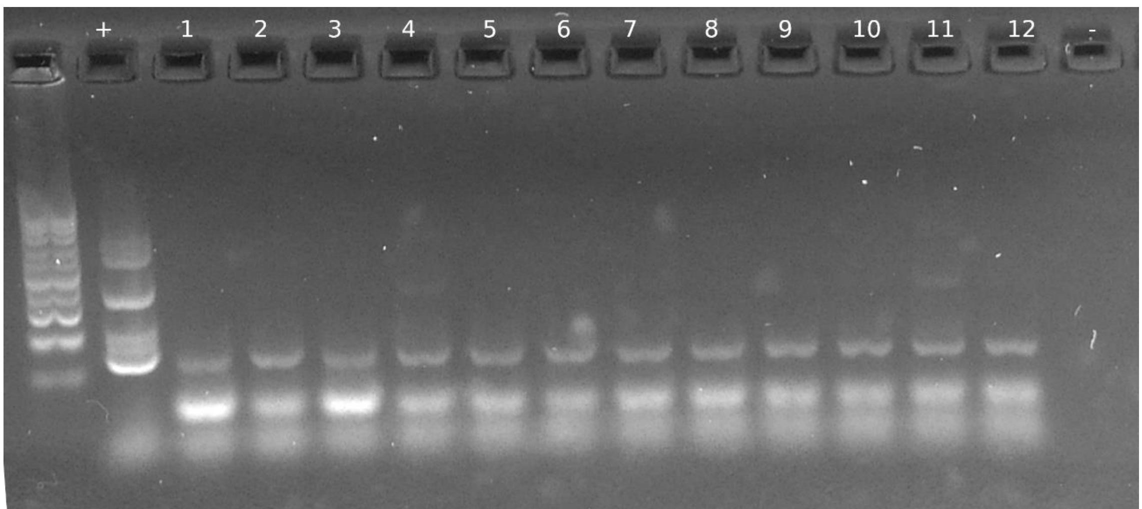


Figura 19: Resultado de PCR multiplex. + es el control positivo, mientras que de 1 a 12 son muestras. – representa al control negativo. El orden de las bandas, de abajo a arriba, incluye *gluD* (158pb), *cdtA* (221pb), *cdtB* (262pb), *tcdB*/toxina B (410pb) y tcdA/toxina A (629pb).

En la imagen se puede apreciar la banda positiva del gen constitutivo (*GluD*) en las muestras de la 1 a la 12, además de bandas positivas para la toxina B (410 bp) en las muestras 4 y 11. En el gel original también se podía apreciar la banda de la toxina A en ambas muestras (629 bp), pero las restricciones técnicas de los equipos de captación de imagnes disponibles en el momento de realización

del experimento limitan su visualización sin manipular digitalmente la Figura 19.

En vista de los resultados con tan baja resolución y mala calidad de los resultados, se intentaron caracterizar las cepas inicialmente por MLST. Los resultados de las PCR fueron limitados e inconsistentes, especialmente considerando que los genes housekeeping analizados están inherentemente presentes en todas las cepas de *C. difficile* (Janezic & Rupnik, 2019). Sin embargo, únicamente amplificaban algunos genes y no en todos los aislados.

Ante estos resultados conflictivos, se procedió a intentar mejorar la calidad de la extracción de ADN. Inicialmente, se volvió a realizar la extracción bajo las mismas condiciones, con idéntico resultado. Frente a este hecho, se repitió una vez más la extracción, pero cambiando la concentración inicial de la bacteria: desde 3 colonias para las muestras poco concentradas hasta todo el crecimiento de la placa sembrada en masa. La modificación de las concentraciones iniciales tenía como objetivo abordar posibles fuentes de error en la adherencia de la muestra a la membrana de la columna de extracción, buscando resolver los problemas asociados. Las concentraciones elevadas se implementaron con el fin de compensar los bajos rendimientos que podrían deberse a una extracción insuficiente de ADN de cada cepa. Por otro lado, las concentraciones reducidas se diseñaron para mitigar la posibilidad de errores causados por un exceso de nucleasas o el colapso del poro de las columnas en alguna etapa del procedimiento. En ambos casos se obtuvieron bajos rendimientos de concentración y fallos en las PCR de los genes *housekeeping*.

Ante estos resultados, y teniendo en cuenta que las extracciones de las cepas control, que se incluyeron en cada proceso simultáneamente, no presentaron ningún problema, se intentaron otros métodos de obtención de ADN con las concentraciones habituales: el método de cocción, utilizado con éxito en los trabajos de esta tesis para la extracción de *C. perfringens* y *E. coli*, y la extracción con un kit de más riguroso (Wizard Genomic DNA Purification Kit, Promega, Estados Unidos). Los resultados de la cocción fueron de mala calidad, incluyendo los controles, presentando estas muestras degradación al realizar la PCR de los genes *housekeeping*. En cuanto a los resultados obtenidos por la extracción por kit, aunque la calidad fue buena, las concentraciones no fueron satisfactorias. La calidad fue evaluada con el índice 260/280 de las lecturas del NanoDrop (Thermo Scientific, Estados Unidos; valores considerados aceptables entre 1 y 2), los valores de concentración (Qubit 4 Fluorometer, Invitrogen, Estados Unidos) y la prueba de integridad de las bandas en gel. Al ser *C. difficile* resistente a la lisozima (Kaus et al., 2020) se realizaron dos extracciones distintas: la primera, siguiendo las instrucciones del fabricante, y la

segunda adaptando las condiciones de una metodología propuesta por Chiak Sim et al (Sim et al., 2015), lo que incluyó un aumento de la concentración de lisozima (de 10 µl concentrado a 45 µg/ml a 70µl), un aumento de tiempos y sobre todo, las incubaciones pasaron a realizarse en agitación. Estos últimos ajustes fueron los que mejor resultado obtuvieron.

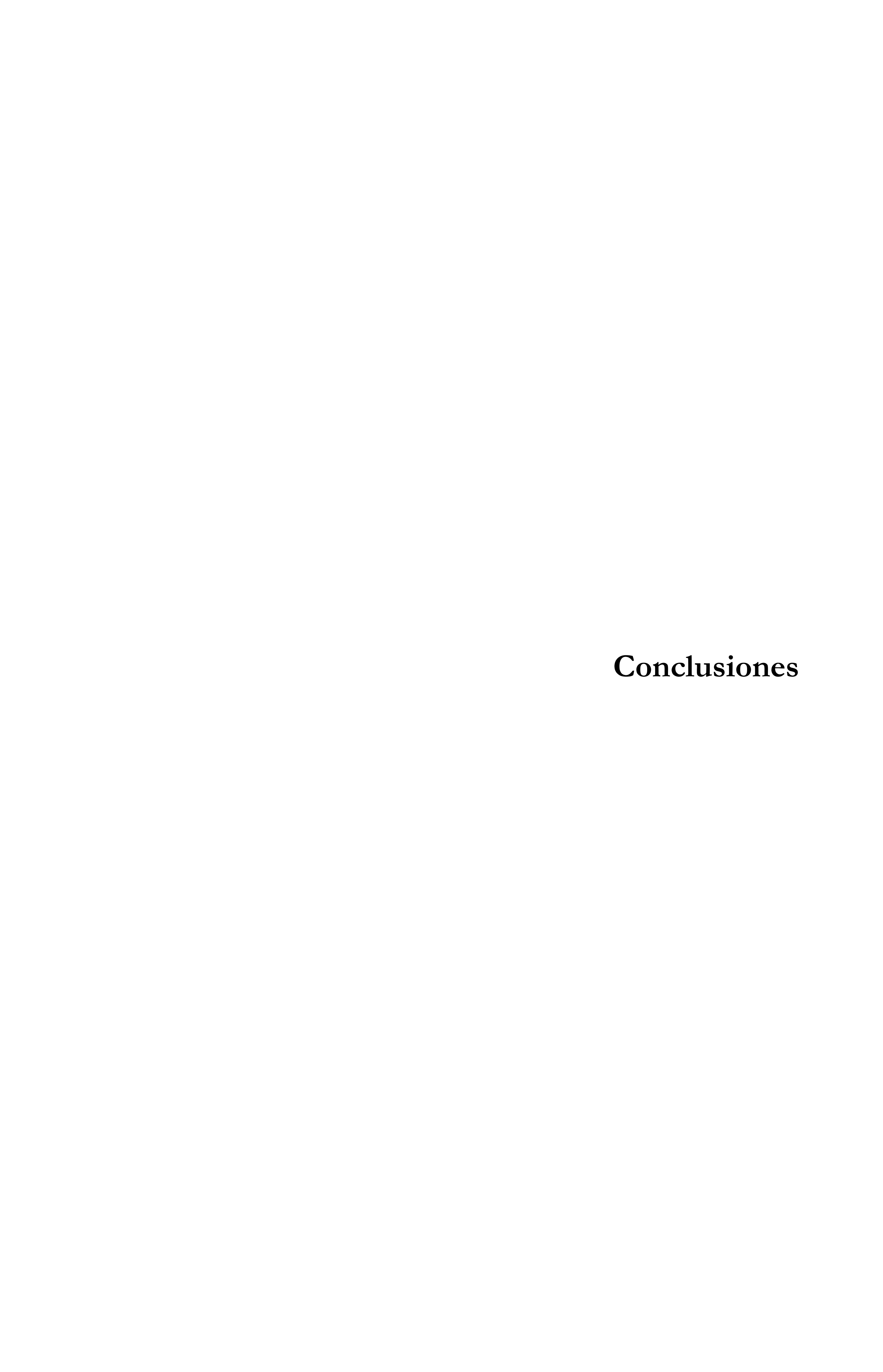
Una de las hipótesis que se planteó fue que el fallo de extracción pudiese ser debido al origen de estas. Al ser las muestras procedentes de cepas ambientales, estas pueden esporular antes que otras cepas obtenidas de animales, y esto dificultar la extracción. Por ello, se repitieron las extracciones con el método que mejor había funcionado hasta el momento (Wizard Genomic DNA Purification Kit), con cultivos recogidos a las 4 y las 8 horas, es decir, en fase exponencial de crecimiento, tanto desde placa como desde cultivo líquido. Todos los resultados fueron infructuosos.

Paralelamente, se comenzó a trabajar con una taq polimerasa más sensible (sensiFAST, Probe No-ROX, bioline, Reino Unido), con la que se obtuvieron algunos resultados, aunque igualmente inconsistentes entre muestras.

Durante la estancia realizada en el Leiden University Medical Center, las mismas cepas se enviaron para su estudio. Allí, se extrajo el ADN de forma automática (ABI automated DNA Extraction System, Applied biosystems, Estados Unidos). Aunque no se comprobó la calidad y concentración de las muestras, sí que se ribotiparon y se les realizo una PCR multiplex para toxinas. Se obtuvo información únicamente de dos muestras: RT 126 para la muestra 7 (157DN), con presencia de toxinas A, B y binaria y RT 1 con presencia de toxina A para la muestra 12 (48YP).

De hecho, esta muestra del ribotipo 126 se planteó inicialmente para ser incluida en el trabajo 6, por lo que se sometió al tratamiento con otro kit distinto que no se intentó con el resto (E.Z.N.A Bacterial extraction kit, Omega Biotek, United States). Aun así, tras varias repeticiones con distinta concentración inicial de bacteria, la mejor concentración obtenida fue de tan solo 9.44 µg/ml, llegando a 13.3 µg/ml en la mejor lectura, menos de la mitad de la cantidad recomendada para la secuenciación completa, por lo que se excluyó del estudio.

Ante la falta de resultados, se decidió derivar los esfuerzos hacia la tipificación por MLST de *C. perfringens*, cuyos resultados se pueden encontrar en el trabajo 3. Aun así, se obtuvieron diversas secuencias que permitieron la confirmación por secuenciación de la identificación de *C. difficile*, depositadas en el anexo 1 de esta tesis.

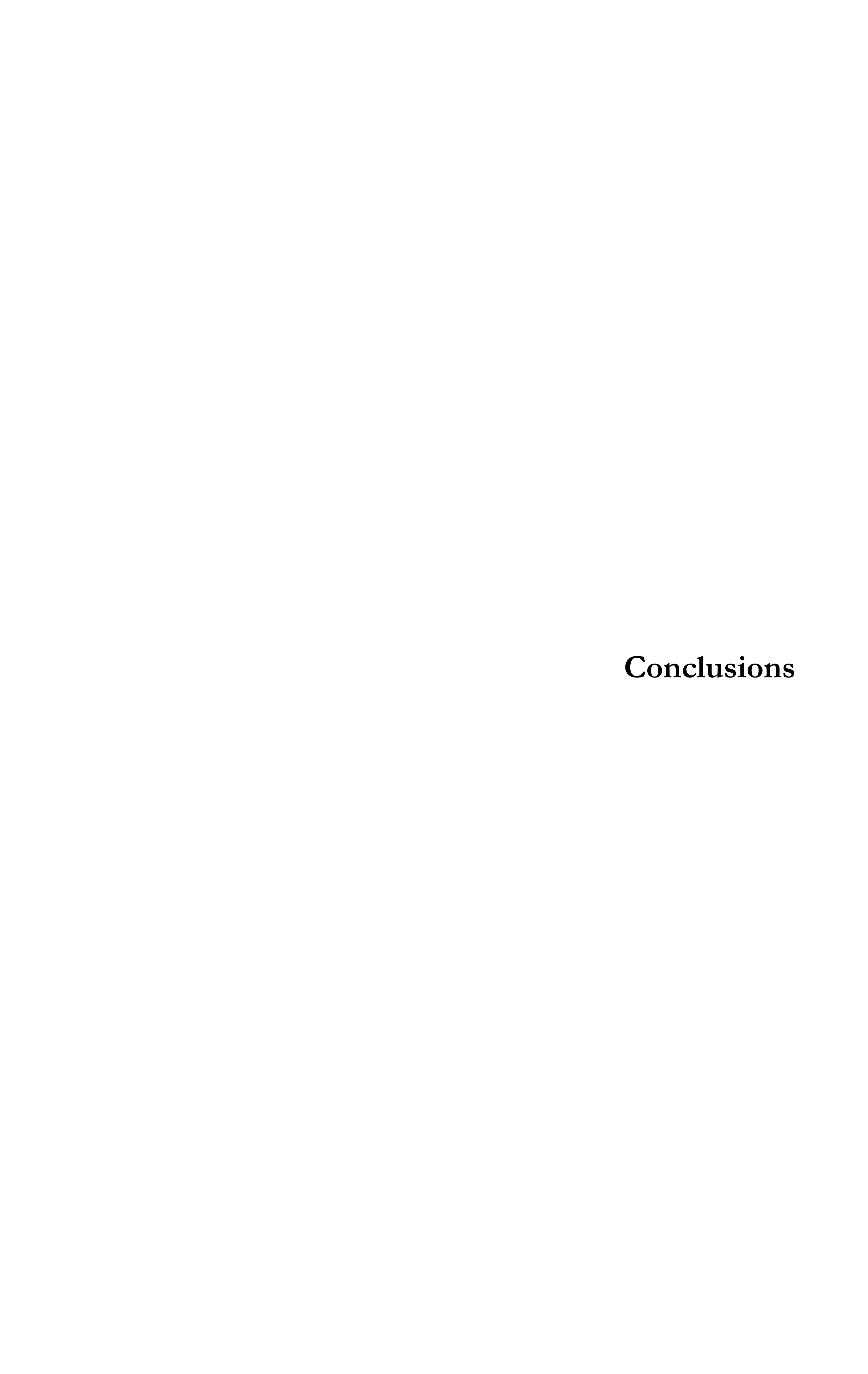


Conclusiones

1. En perros, las infecciones urinarias se asocian frecuentemente a cepas de *Escherichia coli* multirresistentes, especialmente frente a antibióticos comúnmente usados en el tratamiento de estas infecciones, por lo que es necesaria una constante vigilancia epidemiológica de la resistencia antibiótica. Además, la especie canina podría actuar como reservorio de *E. coli* multirresistente.
2. En conejos, *Clostridioides difficile* no parece representar un problema importante en los procesos digestivos, sin embargo, pueden suponer un riego zoonótico ya que todos los aislados fueron del ribotipo 126, detectado en humana y, además, todos fueron toxigénicos.
3. En conejos, se han detectado varias cepas de *E. coli* negativas a la intimina o que no fermentan lactosa, hallazgos de significado incierto y que merecen ser estudiados.
4. Las aguas residuales son reservorios importantes de bacterias anaerobias, tales como *C. perfringens* o *C. difficile*, siendo testigos de contaminación ambiental. Además, algunas de estas bacterias pueden presentar toxinas, pudiendo suponer un riesgo de salud pública.
5. La desinfección del agua residual antes de su asimilación con la red de agua urbana, como se ha observado en aquella procedente de hospitales, es eficaz para disminuir la presencia de bacterias.
6. En la especie porcina, la infección por *C. difficile* ha sido frecuente; aunque no se ha encontrado relación alguna con la presencia de diarrea, ni siquiera en el periodo neonatal, en el cual los procesos digestivos son más frecuentes. Sin embargo, sí se ha encontrado un papel fundamental de los lechones de esta edad en la transmisión de la infección.
7. En ganado porcino, se han detectado varias cepas diferentes de *C. difficile* en una misma granja, incluso afectando al mismo animal.
8. Los estudios de resistencias antimicrobianas han mostrado una amplia variabilidad por especies animales, siendo menor en conejos y mayor en cerdos, sugiriendo que el ganado porcino puede representar un mayor riesgo de transmisión de resistencias antibióticas al ser humano.
9. El estudio genético del ribotipo 126 de *C. difficile* ha mostrado un porcentaje de genoma central superior al esperado, lo que indica una alta estabilidad del ribotipo. Además, esta estabilidad también se ha reflejado en el PaLoc.
10. La presencia del gen *erm(B)* en algunas cepas del ribotipo 126 de *C. difficile* sugiere la existencia de variantes genéticas dentro del ribotipo,

probablemente adquiridas a través de mecanismos evolutivos como la transferencia horizontal de genes.

11. Aunque hay ciertas tendencias epidemiológicas compartidas, los patrones y genes de resistencia antimicrobiana difieren entre las tres especies bacterianas estudiadas (*E. coli*, *C. perfringens* y *C. difficile*), resaltando la complejidad intrínseca de la resistencia antimicrobiana.
12. La relación existente entre la salud animal, el medio ambiente y la salud humana refuerza la importancia del enfoque One Health para prevenir y controlar zoonosis, reconociendo las interacciones específicas entre patógenos, hospedadores y factores ambientales para maximizar la eficacia de las medidas preventivas.



Conclusions

1. In dogs, urinary tract infections are frequently associated with multidrug resistant *Escherichia coli* strains, especially against antibiotics commonly used in the treatment of these infections, therefore a constant epidemiological vigilance of antibiotic resistance is needed. In addition, canines could act as reservoirs of multidrug resistant *E. coli*.
2. In rabbits, *Clostridioides difficile* does not seem to represent a major problem in the digestive processes; however, it can constitute a zoonotic risk as all isolates were classified as ribotype 126, also detected in the human species, and all of them were toxigenic.
3. In rabbits, several isolates of non-lactose fermenting or intimin negative have been isolated associated with digestive symptoms, findings of uncertain significance that deserve to be studied.
4. Wastewater is an important reservoir of anaerobic bacteria, such as *C. perfringens* or *C. difficile*, being good detectors of environmental contamination. Besides, some of these bacteria can harbor toxins, representing a risk for public health.
5. Waste water disinfection prior to its liberation to urban network, as has been observed in that of nosocomial origin, is effective to diminish the presence of bacteria.
6. In swine, *C. difficile* infection has been frequent. However, no relationship between the presence of bacteria and diarrhea has been found, even at neonatal period, when digestive processes are more common, although piglets of this age have been described as having a fundamental role in the transmission of the infection
7. In swine, different strains of *C. difficile* in the same farm have been isolated, even affecting the same animal.
8. Antibiotic resistance studies have shown great variability between species, being less in rabbits than in pigs, suggesting that swine can represent a higher risk of antibiotic resistance transmission to humans.
9. The genetic study of *C. difficile* ribotype 126 has revealed a shared genome higher than expected according to its core genome, which indicates a high stability within the ribotype. In addition, this stability is also present in PaLoc structure.
10. The presence of *erm(B)* gene in some strains of *C. difficile* ribotype 126 suggests the existence of variants within the ribotype, probably acquired through evolutionary mechanisms such as gene horizontal transfer.
11. Although there are epidemiological tendencies shared among the three bacterial species studied (*E. coli*, *C. perfringens* and *C. difficile*), patterns and

antimicrobial resistance genes differ, highlighting the complexity of the latter.

12. The relation between animal, human and environmental health reinforces the importance of the One Health approach to prevent and control zoonosis, recognizing specific interactions between pathogens and environmental factors to maximize the efficiency of preventive measurements.

Anexo 1

> 25-June ND dxr Query cover 94% Identity 99.79%

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> 25-June SK sodA Query cover 90% Identity 99.41%

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> 25-June YP sodA Query cover 87% Identity 99.80%

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> 2-July YP sodA Query cover 84% Identity 99.79%

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> 15-July ND sodA Query cover 94% Identity 99.62%

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> 15-July YP sodA Query cover 92% Identity 99.62%

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> 28-July SK sodA Query cover 90% Identity 98.44%

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> 28-July NP sodA Query cover 94% Identity 98.69%

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> 28-July NP dxr Query cover 96% Identity 99.79%

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TAGCACAAAGAGTTGGA

> 4-Aug SK dxr Query cover 97% Identity 99.79%

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> 4-Aug YP sodA Query cover 95% Identity 99.26%

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> 4-Aug YP atpA Query cover 93% Identity 99.35%

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> 4-Aug YP recA Query cover 97% Identity 99.35%

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> 4-Aug YP dxr Query cover 97% Identity 99.79%

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Apéndice

Apéndice

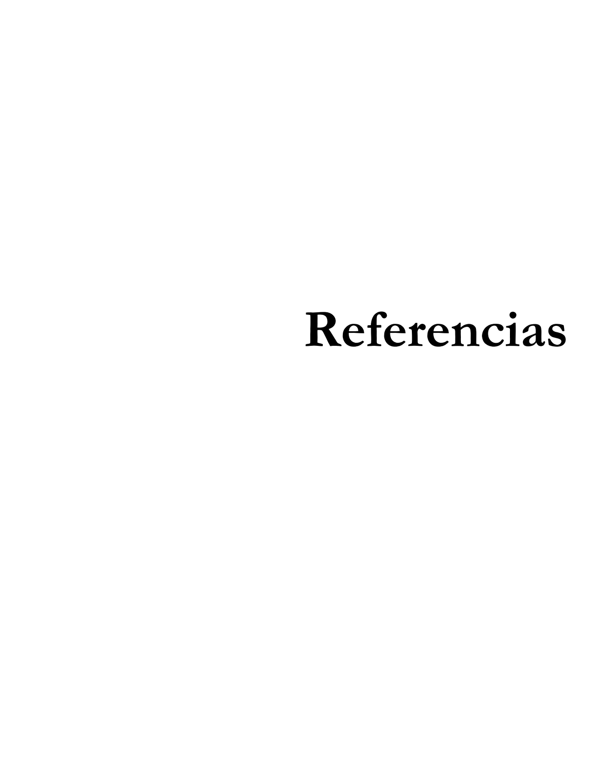
Esta tesis doctoral incluye dos trabajos publicados en revistas bajo licencia Open Access. A continuación, se detallan las características de las publicaciones. Los datos como el factor de impacto o la posición de la revista en el cuartil hacen referencia a la última versión disponible online de los datos a fecha 09 de agosto de 2024. Los datos de los parámetros se han obtenido del Journal Citations Reports disponible en la página web del ISI Web of Knowledge.

Título: Multidrug resistance in pathogenic *Escherichia coli* isolates from urinary tract infections in dogs, Spain

Autores: **Ana Abad-Fau**, Eloisa Sevilla, Ainara Oro, Inmaculada Martín, Bernardino Moreno, Mariano Morales y Rosa Bolea
Revista: FRONTIERS IN VETERINARY SCIENCE
Editorial: Frontiers
Año: 2024
Indexada en base de datos (ISI-WoS): Sí
DOI: 10.3389/fvets.2024.1325072
Número de citas: 0
Factor de Impacto (JCR): 2.6
Q: VETERINARY SCIENCES (22/167) - Q1
Contribuciones: **AA-F**: base de datos, análisis de datos, investigación, escritura—preparación del borrador inicial, escritura—corrección y edición. ES: base de datos, análisis de datos, supervisión, escritura—preparación del borrador inicial, escritura—corrección y edición. AO: investigación, escritura—preparación del borrador inicial. IM-B: análisis de datos, supervisión, escritura—corrección y edición. BM: recursos, supervisión, escritura —corrección y edición. Mm: conceptualización, recursos, supervisión. RB: conceptualización, adquisición de fondos, recursos, supervisión, escritura—corrección y edición

Título: Update on Commonly Used Molecular Typing Methods for *Clostridioides difficile*

Autores: **Ana Abad-Fau**, Eloísa Sevilla, Inmaculada Martín-Burriel, Bernardino Moreno y Rosa Bolea
Revista: Microorganisms
Editorial: MDPI
Año: 2023
Indexada en base de datos (ISI-WoS): Sí
DOI: 10.3390/microorganisms11071752
Número de citas: 4
Factor de Impacto (JCR): 4.1
Q: MICROBIOLOGY (49/161)-Q2
Contribuciones: Conceptualización, **AA-F**, ES y BM; escritura—preparación del borrador inicial**, AA-F** Y ES; escritura—corrección y edición, **AA-F**, ES, IM-B, BM y RB; figuras, **AA-F** y ES; supervisión, BM y RB. Todos los autores han leído y están de acuerdo con la versión publicada del manuscrito.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