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Caracterización fenotípica, genotípica y genómica de los atributos de virulencia y la resistencia a los antibióticos de una colección de aislamientos de *Salmonella enterica* de origen bovino de Uruguay

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Contenido

RESUMEN

Salmonella es una bacteria que afecta diferentes especies de animales y al ser humano en el mundo. Es una bacteria Gram negativa, intracelular facultativa que constituye un género muy extenso con más de 2500 serovares descriptos. Particularmente en bovinos las enfermedades que produce son la enteritis seguido de septicemia y muerte, afectando terneros jóvenes, mientras que en adultos la enfermedad cursa con diarrea sanguinolenta. En este trabajo se obtuvo información epidemiológica, clínica y patológica de brotes de mortalidad de terneros afectados por *Salmonella enterica* y se realizó una caracterización genómica de atributos de virulencia y resistencia que presentan los aislamientos que afectan al rodeo bovino uruguayo. En el trabajo realizado en 15 brotes de mortalidad de terneros se determinó que los serotipos actuantes eran *S.* Typhimurium y *S.* Dublin y que el motivo de consulta a los veterinarios era porque los animales presentaban diarrea seguida de muerte. Por otro lado, en los brotes la morbilidad, mortalidad y letalidad oscilaron entre 4,8-100%, 3,8-78,9% y 10-100% respectivamente. La diarrea, el signo clínico más frecuente (14 casos), se asoció con el serotipo Typhimurium (p=0,01), especialmente en terneros ≤ 30 días de edad con enteritis fibrinosa como principal hallazgo de autopsia. El serotipo Dublin afectó a terneros ≥ 50 días de edad y se asoció con esplenitis fibrinosupurativa (p = 0,01) y nefritis tubulointersticial (p=0,01). Mediante PCR, 17 genes que codifican para proteínas con funciones importantes en la virulencia que en diferentes etapas de la infección se detectaron en alta frecuencia en los aislamientos de *Salmonella* que comprendieron estos brotes. La portación del gen *pef*A se asoció con el serotipo Typhimurium (p = 0,04), la enteritis macroscópica (p = 0,03) y la esplenitis fibrinosupurativa microscópica (p = 0,04). En un enfoque genómico que incluyó una colección de 75 aislamientos, se detectaron los serotipos Typhimurium, Dublin, Newport, Anatum, Montevideo, Agona y IIIb 61:i:z53, los cuales representaron los secuenciotipos ST19, ST10, ST45; ST64, ST13, ST138 y ST 430 respectivamente. La búsqueda de genes de resistencia arrojó que los genes que confieren resistencia a aminoglucósidos son los más frecuentes, seguidos por los que codifican para resistencia a las tetraciclinas y sulfonamidas. En menor medida se detectaron genes de resistencia a β- lactámicos y quinolonas y en muy baja frecuencia a fenicoles, fosfomicina, amonio cuaternario y trimetoprim. Se detectaron 12 diferentes grupos de incompatibilidad de plásmidos y sólo Integrones de tipo I. Se detectaron 14 diferentes islas de patogenicidad y el 100% de los aislamientos presento las islas de patogenicidad SPI-1, SPI-2, SPI-3 y SPI-9. *Salmonella* Dublin presentó la SPI-10 que estuvo ausente en el resto de los serotipos. La detección de genes de virulencia por medio de la evaluación del genoma completo arrojó la presencia de múltiples genes con diferentes funciones y además genes que se encuentran en otras especies bacterianas, todos ellos, involucrados en la patogénesis bacteriana. Los genes de adherencia fimbrial fueron los más abundantes, seguidos por los de adherencia no fimbrial, genes inducidos en macrófagos, genes de captación de nutrientes y

supervivencia en el suero. Se observaron asociaciones estadísticas entre el serotipo Dublin y Typhimurium con los genes *sodCI*. Por su parte *S.* Typhimurium se asoció con *pef*, *stc*, *mig-5*, *rck* y *spvB*, mientras que *S*. Newport se asoció a *peg* y *ste* de adherencia fimbrial. Además, de acuerdo con el origen de los aislamientos se observó asociación en aquellos aislados de heces y los genes de adherencia fimbrial y afimbrial; mientras que los aislamientos obtenidos a partir de órganos y secreciones se encuentran específicamente asociados con los genes *mig-5*, *spvB* y *sodCI* relacionados con procesos sistémicos. El análisis filogenético logró discriminar y agrupar los diferentes serotipos involucrados en este trabajo. Estos resultados nos permitieron generar información local y científica relativa a las características epidemiológicas, de virulencia, resistencia antibiótica y los hallazgos patológicos relacionados a brotes de salmonelosis en Uruguay, pudiendo determinar que los aislamientos presentan los atributos necesarios para generar daño en el rodeo bovino.

INTRODUCCIÓN Y ANTECEDENTES

Etiología

Salmonella es una enterobacteria de forma bacilar Gram-negativa, intracelular facultativa, anaerobia facultativa, generalmente móvil. Dentro del género se reconocen dos especies, *Salmonella bongori* y *Salmonella enterica*. Dentro de *S*. *enterica* se encuentran descriptas 6 subespecies, la más importante que afecta a los mamíferos es la subespecie *enterica* dentro de la cual y de acuerdo con los antígenos somáticos, flagelares y capsulares se describen más de 1600 serotipos (Tindall, 2005, Issenhuth-Jeanjean et al. 2014).

Salmonella spp. presenta requerimientos simples para su desarrollo óptimo. La temperatura a la cual se puede desarrollar varía entre 5,2°C a 46,2°C. El pH de desarrollo se encuentra entre 3,8 y 9,5 siendo el valor óptimo de 7-7,5, mientras que el valor de actividad de agua (a_w), es de 0.99. Las bajas temperaturas reducen el número de microorganismos, pero no garantiza su destrucción. Por otro lado, *Salmonella* se hace más resistente a temperatura altas cuando la a_w decrece. Además, las temperaturas también afectan el valor del pH al cual *Salmonella* spp. puede desarrollar (Keerthirathne et al. 2016)

En una de las tantas clasificaciones de este género, la diferenciación entre salmonelas tifoideas y no tifoideas hace referencia al tipo de enfermedad, serotipo y los hospederos involucrados. En el primer grupo se encuentran las bacterias *S.* Typhi y *S.* Paratyphi, restringidas a los humanos, en el segundo grupo a las no tifoideas que infectan a numerosos grupos de vertebrados incluidos también los humanos. Ambos grupos dependiendo de diversos factores pueden producir en su hospedero desde una enfermedad gastroentérica hasta la muerte (Ajmera & Shabbir 2022, Zhang et al. 2003). Los serotipos no tifoideos se denominan generalistas porque pueden infectar amplia variedad de hospederos, y algunos pueden causar enfermedad dependiendo de la especie animal, serotipo, la dosis infectante y el estado inmunitario del hospedero. Por otro lado, hay serovares especializados como los tifoideos que afectan a humanos, *S*. Gallinarum y *S*. Pullorum que afectan a las aves y *S*. Abortusovis afectando a ovinos. Un tercer grupo son los adaptados al hospedero como *S.* Dublin y *S.* Cholerasuis que pueden afectar ocasionalmente a los humanos generando septicemia (Gal-Mor, 2018).

Epidemiología de la salmonelosis

Las afecciones por *Salmonella* se describen mundialmente, pero los países en desarrollo son los que presentan mayores tasas de morbilidad y mortalidad (Crump et al. 2004). Salmonelosis es la principal causa de las enfermedades transmitidas por alimentos (ETA) a nivel mundial, con 153 millones de casos anuales de salmonelosis no tifoidea y más de la mitad de estos asociados a ETA (Kirk et al. 2015). Particularmente los diferentes serovares de *Salmonella enterica* subespecie *enterica* provocan enfermedad según la especificidad del hospedero, generalmente se caracteriza por una gastroenteritis autolimitada.

En bovinos la salmonelosis suele presentarse en terneros de menos de 10 días de vida con enteritis fibrinonecrotizante y septicemia y también en bovinos mayores de 3 semanas (Maxie 2016 a). Los animales que sobreviven a la enfermedad pueden permanecer por un tiempo como portadores asintomáticos contribuyendo a la contaminación del agua, alimento y ambiente a partir de la excreción intermitente de *Salmonella enterica*. En ciertos casos bajo condiciones de estrés se puede generar una bacteriemia donde los animales pueden nuevamente presentar signos y comportarse como excretadores. La morbilidad y mortalidad son considerables, sobre todo donde la carga bacteriana es alta y la edad de los animales es menor y se encuentran en confinamiento. En adultos se caracteriza por pérdida de peso por diarrea esporádica y a veces presentan aborto (Nielsen LR, 2013, Maxie 2016 b).

Patogenicidad y virulencia de *Salmonella* spp.

La capacidad de un patógeno para lograr su sobrevida en el hospedero depende de sus características particulares de virulencia y de la respuesta inmunitaria que el hospedador pueda desarrollar. Andrews-Polymenis y colaboradores describen particularmente en *Salmonella enterica*, que “cualquier determinante de virulencia es aquel que permita que un serotipo de *Salmonella* ingrese a un hospedero, encuentre un nicho único para multiplicarse, evite o subvierta las defensas del hospedero, provoque una enfermedad y se transmita al siguiente hospedero susceptible” (Andrews-Polymeris et al. 2006).

Salmonella enterica presenta un rango óptimo de desarrollo a temperaturas de entre 32°C a 35°C y un pH que oscila entre 4 y 9 (Jajere, 2019). Sin embargo, exposiciones moderadas de pH 5,8 en fase logarítmica de desarrollo de la bacteria y luego expuestas a pH 3,3 permiten a *Salmonella* adaptarse y sortear un ambiente ácido extremo (Foster 1995, Álvarez-Ordóñez et al. 2012).

Luego de la ingestión, *Salmonella* sobrevive al pH ácido del estómago gracias a la activación de la respuesta de tolerancia al ácido (ATR) que le permite lograr una homeostasis entre el pH que la rodea y el intracelular (Foster & Hall, 1991 Sutar et al 2023). Posteriormente, las bacterias sobrevivientes alcanzan el pH básico del intestino delgado donde encuentran y atraviesan el mucus intestinal para ponerse en contacto con las células intestinales (Fàbrega & Vila, 2013, dos Santos et al. 2019). Los cambios ambientales que atraviesa activan y favorecen la expresión de genes localizados en islas de patogenicidad (SPI). La infección comprende 3 etapas, adhesión, invasión y maduración y replicación en las denominadas vacuolas que contienen *Salmonella* (SCV), del inglés *Salmonella- containing-vacuole* (López et al. 2012, dos Santos et al. 2019).

Salmonella presenta diversas estructuras de adhesión fimbrial y no fimbrial (Wagner & Hensel, 2011, Zhou et al. 2023) que le permite superar la peristalsis intestinal y lograr la colonización del intestino. Las fimbrias o pili son orgánulos delgados con función de adhesión (Klemm &Schembri, 2000). Se han descrito más de 15 operones fimbriales con diferentes combinaciones dependiendo los serovares implicados que se unen a distintas proteínas de membrana, residuos de azúcar o estructuras lipídicas presentes en el hospedador (Wagner & Hensel, 2011, Elpers et al. 2020).

El operón *fim* se encuentra entre los principales operones fimbriales de *Salmonella,* al codificar la subunidad *fimH* le permite a la bacteria adherirse a receptores de manosa de la célula hospedadora. El operón *lpf*, se une a las células M en las placas de Peyer, las fimbrias *"thin aggregative fimbriae"* (Tafi) incluyen dos operones *ags* y son responsables de la autoagregación, la formación de biopelículas y la adhesión de *Salmonella* a varias superficies (Gonzales et al. 2017, Mejía et al. 2020). Por su parte los pili tipo IV presentes sólo en *S.* Typhi, actúan de forma sinérgica con el operón *pef* en la adherencia e invasión de este microorganismo. (van der Velden et al. 1998, Ellison et al. 2022*).* Otras adhesinas fimbriales en *S.* Typhimurium y *S.* Enteritidis son *Bcf, Saf, Stb, Stc,Stf, Sth, Sti y* además *Stj y Stj* específica para el serotipo Enteritidis (Wagner & Hensel, 2011).

Las adhesinas no fimbriales *SiiE* (codificado en SPI-4) y *BapA* se secretan a través del sistema de secreción tipo 1 (SST1) al espacio extracelular, actúan propiamente como adhesinas uniéndose a la envoltura bacteriana después de ser secretadas formando biopelículas agregativas (Wagner & Hensel, 2011, Azimi et al. 2020). Otro grupo de adhesinas no fimbriales son las adhesinas autotrasportadoras como *ShdA, MisL y SadA*. La adhesina *ShdA* está codificada en un gen de la isla CS54, presente en los serovares patógenos de mamíferos y aves, mientras que *MisL*, está codificada en un gen presente en la SPI-3, ambas presentan un efecto sinérgico y son necesarias para la colonización agregativa y persistencia de *S.* Typhimurium en el intestino (Kingsley et al. 2004, Wang et al. 2018).

Salmonella posee la capacidad de invadir, por diferentes vías, a células fagocíticas y no fagocíticas. Para esto activa diferentes mecanismos de entrada a las células, entre ellos, los denominados *"trigger"* y "zipper" (ver más abajo). además, puede atravesar basolateralmente las células, puede ser transportada a través de células dendríticas que captan muestras desde la luz intestinal e ingresar a través de células M especializadas en las placas de Peyer (Biedzka-sarek & El Skurnik 2006). El mecanismo de invasión de células no fagocíticas implica la inducción de modificaciones del citoesqueleto de la célula hospedadora que culminan en la formación de "*ruffles*" que son proyecciones de la membrana citoplasmática (Ibarra et al. 2009).

El mecanismo "*trigger*" se activa debido a la acción de efectores eyectados a partir del sistema de secreción tipo 3 (SST3) de la SPI-1, generando los reordenamientos de actina del citoesqueleto constituyendo prolongaciones largas de la membrana que finalmente engloban a la bacteria (Velge et al. 2012, Boumart et al. 2014)

A través del SST3, los efectores bacterianos *sipA, sipB, sopB, sopE* y *sopE2* entre otras proteínas efectoras, se inyectan en la célula eucariota. Estas moléculas activan dos caminos de modificación de la actina. Las proteínas *SipA* y *SipB* se unen directamente a ella, donde *sipB* nuclea el ensamblaje de actina y *sipA* polimeriza la actina, estabiliza los filamentos, previene la despolimerización y reconstituye filamentos. Por otro lado, los factores *sopE*, *sopE2* y *sopB* no se unen a la actina, pero activan las enzimas RhoGTPasas Rac/Cdc42/RhoG las cuales a su vez activan las proteínas reguladoras WASP/Scar/WAVE/WASH, que activan el complejo Arp2/3 que lleva al ensamblaje del citoesqueleto (McGhie et al. 2009, Velge et al. 2012).

El segundo mecanismo descripto en *Salmonella* llamado "*zipper*", constituye una entrada mediada por el reconocimiento de proteínas de membrana externa como *Rck,* que expresa la bacteria y reconocido por medio de receptores de superficie de las células hospedadora. Estas proteínas de membrana generalmente se encuentran en serovares no adaptados al huésped (Boumart et al. 2014). Este mecanismo es independiente del SST3 y forma reordenamientos menores de membrana por activación de proteínas del citoesqueleto similares a una cremallera que absorben la bacteria (Rosselin et al. 2010, Mambu et al. 2017).

PagN es una proteína de membrana externa que es activada por PhoP (Azimi et al. 2020). PagN actúa en la adhesión e invasión a través de la unión a proteoglicanos de sulfato de heparina extracelular de la célula hospedadora, induciendo la invasión de *Salmonella* (Boumart et al. 2014).

Otro mecanismo importante de invasión de *Salmonella* enterica es a través de células fagocíticas. Una de estas son las células dendríticas (CD) que se encuentran en la lámina propia o placas de Peyer donde emiten prolongaciones para captar antígenos desde la luz intestinal y fagocitarlos (Swart& Hensel 2012). Dentro de las células la

bacteria se aloja en vacuolas que contienen *Salmonella* (SCV), estas últimas maduran al utilizar los componentes del endosoma para formar extensiones membranosas denominadas filamentos inducidos por *Salmonella* (SIF) (Kehl et al. 2020). Posterior a la fagocitosis la CD procesa y presenta antígenos en la superficie de las moléculas MHC de clase I y II a los linfocitos T en los ganglios linfáticos. De esta manera sufre un proceso de maduración perdiendo la capacidad de captación de antígenos (Zanna et al. 2021).

Luego de la invasión celular, *Salmonella* sobrevive y se replica en los fagosomas modificados SCV. Los factores que involucran la biogénesis, supervivencia y replicación en las SCV están codificados en islas de patogenicidad SPI-1 y SPI-2 a través de sus sistemas de secreción (Steele-Mortimer, 2008). La maduración de las SCV consta de tres etapas, temprana intermedia y tardía. La etapa temprana comprende los primeros 30 minutos post-infección (PI). En esta etapa se translocan proteínas del SST3-1, *SopB* contribuye al cierre de la copa fagocítica y como enzima permite la acumulación de fosfatidil inositol 3 fosfato (PI3P). *SopD* coopera con *sopB* en la formación inicial del fagosoma y *sipA* induce el movimiento tardío del endosoma y coopera con *sifA* para mantener la SCV en etapa tardía. Luego, los SCV presentan marcadores de la vía endocítica temprana EEA1, y rab5. Posteriormente, estos marcadores son reemplazados por marcadores de la vía endocítica tardía como Lamps y ATPasa y el SCV se localiza de forma yuxtanuclear. Estas actividades junto con la formación de una red de actina alrededor del SCV y la prolongación anterógrada de filamentos inducidos por *Salmonella* (SIFs) están reguladas por efectores de SST3-2.

La poca cantidad de nutrientes y la acidez dentro de la vacuola dispara la activación de SST3 de la SPI-2, que comienza a translocar efectores a través de la membrana con funciones como adquirir nutrientes, propagarse, inducir la muerte celular y suprimir la respuesta inmune (Heggie et al. 2021). Durante la infección activa las células expresan quimioquinas como CCR7 que induce la quimiotaxis y migración a sitios sistémicos como el bazo y ganglios linfáticos (Li, 2022).

Resistencia a los antibióticos

La resistencia a los antibióticos se reconoce a partir de la década de 1930 cuando se identifica la resistencia a las sulfonamidas. Rápidamente en los sucesivos años se detectó la resistencia a diferentes grupos de drogas entre ellas las penicilinas, la estreptomicina e incluso hacia 1950 la resistencia genética transferible (Davies & Davies, 2010). En términos generales, la resistencia a los antimicrobianos (RAM)

ocurre en bacterias, virus, parásitos y hongos cuando estos mismos no responden a las drogas creadas para su tratamiento generando así el riesgo de propagación de enfermedades y muerte (WHO, 2021).

Hacia el año 2015 en la Asamblea Mundial de la Salud, los estados miembros de la OMS aprobaron el Plan Global de Acción para la contención de la Resistencia Antimicrobiana donde se establecen los objetivos a cumplir para mitigar el incremento de la RAM. En sintonía con las medidas a nivel mundial, Latinoamérica a través de la OPS y la OMS trabaja en la creación de la "Red Latinoamericana para la Vigilancia de la Resistencia a los Antimicrobianos" (ReLAVRA), a través de laboratorios de referencia centrales en cada país y laboratorios centinelas.

En el año 2016, se publica el reporte realizado por el macroeconomista Jim O´Neill y colaboradores a pedido del entonces primer ministro inglés David Cameron donde se destaca entre otros puntos, que la mortalidad estimada vinculada a la RAM para el año 2050 ascendería a 10 millones de personas si no se tomaban medidas para paliar esta tendencia (O'Neill, 2016). Actualmente, casi 5 millones de muertes son atribuibles a la RAM y las mismas se distribuyen de forma desigual según la región del mundo, afectando principalmente zonas de bajos recursos, detectando valores de 27,3 muertes cada 100 000 habitantes en África subsahariana occidental y 6,5 muertes cada 100000 habitantes en Australasia. Los principales síndromes asociados a muerte por RAM son los respiratorios inferiores, infecciones del torrente sanguíneo y de cavidad abdominal. Los antibióticos betalactámicos y fluoroquinolonas son utilizados en forma empírica para infecciones graves. Estos representan el 70% de las muertes atribuibles a la RAM (Murray et al. 2022).

En Uruguay en salud animal, hacia el 2017 se logra el "Plan Nacional de contención de la resistencia antimicrobiana de Uruguay con enfoque en salud animal y cadenas productoras de alimentos", luego de varias reuniones con diferentes instituciones lideradas por el Ministerio de Ganadería, Agricultura y Pesca (MGAP). Hacia el 2018, esta última junto con el Ministerio de Salud Pública (MSP) constituyen el "Grupo Técnico para la prevención de la Resistencia antimicrobiana" con el fin de vigilancia, monitorización y contención de la resistencia antimicrobiana bajo el concepto de "Una Salud" (MSP, 2018).

En Uruguay los hallazgos relacionados con la resistencia bacteriana a los antibióticos se vienen detectando hace varias décadas principalmente en patógenos de origen humano. Entre los principales reportes de infecciones se encuentran las diarreas en niños, las infecciones urinarias y las neumonías. Los patógenos involucrados en estas infecciones fueron *E. coli*, *Shigella* spp., *Salmonella* spp., *Staphylococcus* spp., *Streptococcus* spp. entre otros. En general, las resistencias detectadas fueron a los principales antibióticos usados de rutina, como diferentes generaciones de betalactámicos, trimetoprima/sulfametoxazol (Torres et al. 2001, Mota et al. 2010, Robino at al. 2014, Hortal et al. 2000), y más recientemente carbapenemes,

quinolonas, colistina. Además, se ha detectado la presencia de elementos genéticos movilizables capaces de transferir la resistencia mencionada (Papa-Ezdra et al. 2021, Marquez et al. 2014, García- Fulgueiras 2011, Cordeiro et al. 2016, Papa- Ezdra et al. 2020).

En salud animal, los primeros datos reportados de resistencias en bacterias aisladas de animales estuvieron relacionados a mastitis (Gianneechini et al. 2014) y posteriormente además del estudio de la resistencia en *Staphylococcus* spp. (de los Santos et al. 2017, Meyer et al. 2017) fueron sumándose otras especies bacterianas como *E. coli, Salmonella* spp. *Campylobacter* spp. (Umpierrez et al. 2017, Casaux et al. 2019, Coppola et al. 2020, Dorsch et al. 2022, y además numerosos resultados de resistencia en peces (Perretta et al. 2018, Carnevia et al. 2013, Perretta et al. 2019).

Hacia el año 2010, los hallazgos obtenidos por Betancor y col. de la resistencia a antibióticos en *Salmonella* de origen aviar mostraron la presencia de aislamientos resistentes a ácido nalidíxico y en menor medida a betalactámicos. En estudios publicados en años posteriores de aislamientos de *Salmonella* de origen humano, encontraron resultados similares, pero con la detección de enzimas betalactamasas y genes *qnr* (Cordeiro et al. 2016, Bado et al. 2012).

En los sucesivos años las publicaciones sobre aislamientos resistentes portadores de nuevas enzimas betalactamasas fueron incrementándose (Cordeiro et al. 2013, Cejas et al. 2014, Cordeiro et al. 2018) así como la investigación de la resistencia en *Salmonella* de origen animal. En este contexto, durante el año 2019 se reportamos el perfil de susceptibilidad a antibióticos en *Salmonella* aisladas de terneros lecheros detectando una alta frecuencia de resistencia a estreptomicina y tetraciclina, alta frecuencia de aislamientos con sensibilidad intermedia a ciprofloxacina y una baja frecuencia de aislamientos resistentes a betalactámicos. Además, encontramos diferencias en los serotipos detectados de acuerdo con la resistencia, se observó que los aislamientos de *S*. Dublin eran sensibles a los antibióticos ensayados en oposición con lo que sucedía con *S.* Typhimurium (Casaux et al. 2019). Estos antibióticos como las tetraciclinas, fluoroquinolonas aminoglucósidos, son utilizados en producción animal como terapia frente a infecciones bacterianas por lo que el hallazgo de resistencia refleja un estado de situación de la resistencia en las granjas lecheras.

A continuación, se mencionarán brevemente los principales mecanismos de resistencia a tetraciclinas, fluoroquinolonas y aminoglucósidos

1. Resistencia a tetraciclinas

Las tetraciclinas son un grupo de drogas que actúan para inhibir la síntesis proteica y que se comenzaron a utilizar a partir de la década de 1940. La droga clásica es la clortetraciclina de la cual derivaron otros compuestos en los sucesivos años (Nguyen at al. 2014). Su acción consiste en impedir la síntesis de proteínas al unirse sitio del aminoacil- ARN de transferencia de la subunidad 30S del ribosoma de forma reversible

(Roberts, 1996, Wilson et al, 2020). Son 4 los mecanismos que confieren resistencia a las tetraciclinas en bacterias 1) eflujo; 2) protección ribosomal, 3) degradación y 4) mutaciones en el ARN ribosómico. Se han descrito 62 familias de genes de resistencia a la tetraciclina, donde predominan los genes que codifican proteínas de eflujo, seguidos por proteínas de protección ribosomal y enzimas inactivadoras de tetraciclinas (Berglund et al. 2020) Los genes de resistencia más frecuentes en *Salmonella* son *tet*A seguido de *tet*B (Santos Pavelquesi et al. 2021) y lo mismo ocurre en los reportes de genes en salmonellas de origen bovino (French et al. 2000).

2. Resistencia a aminoglucósidos

Los aminoglucósidos actúan al inhibir la síntesis de proteínas y la resistencia a los mismos se produce por diferentes mecanismos, como la prevención de la entrada de fármacos, la expulsión activa de antibióticos, alteración de la diana (modificación mutacional del rRNA 16S, modificación mutacional de proteínas ribosómicas y alteración post transcripcional del sitio blanco mediante la acción de 16S metiltransferasas e inactivación enzimática (Jana & Deb et al. 2006). El principal mecanismo descripto de resistencia a aminoglucósidos son las enzimas modificadoras de los mismos que se agrupan en 3 clases: las nucleotidiltransferasas (ANT), las aminoglucósidos fosfotransferasas (APH) y los aminoglucósidos acetiltransferasas (AAC) (Magnet & Blanchard, 2005). En su acción estas enzimas inhiben el proceso de traducción sobre el sitio A de la subunidad 30S ribosomal provocando lecturas erróneas impidiendo la síntesis de proteínas (Magnet & Blanchard, 2005). Los genes que codifican estas enzimas se encuentran en plásmidos, integrones o transposones por lo que estos se pueden diseminar a diferentes géneros bacterianos (Jana & Deb, 2006). En el mundo los reportes de resistencia en *Salmonella* a aminoglucósidos son frecuentes incluso en aislamientos bovinos (Otto et al. 2018, Srednik et al. 2021).

3. Resistencia a quinolonas

Los antibióticos de este grupo actúan sobre las topoisomerasas tipo II bacteriana, la ADN girasa y la topoisomerasa IV (Bush et al. 2020). Los mecanismos de resistencia a quinolonas están representados por mutaciones en las topoisomerasas, resistencia mediada por plásmidos, bombas de eflujo e impedimento de la entrada del antibiótico (Hooper & Jacoby, 2015). Las mutaciones en los genes *gyrA* y *parC* de la ADN girasa y la topoisomerasa IV respectivamente son el tipo de resistencia más comúnmente reportada que resultan en la sustitución de aminoácidos (Redgrave et al. 2014) Hopkins & Threlfall, 2005). La resistencia de alto nivel a quinolonas es poco frecuente en *Salmonella*, pero se sostiene que la aparición de la resistencia es consecuencia de la utilización del antibiótico (Hopkins & Threlfall, 2005). Otro mecanismo identificado es aquella en la que se involucran plásmidos que transportan genes *qnr* (Johnning et al. 2015) y al igual que las mutaciones confieren un bajo nivel de resistencia (Jacoby,

2006). Las proteínas codificadas por estos genes actúan protegiendo el sitio diana de las quinolonas (Adriaenssens et al. 2011). Hay más de 100 variantes de genes qnr que se agrupan en 5 familias principales *qnrA*, *qnrB*, *qnrC*, *qnrD* y *qnrS* (Salah et al. 2019). Los aislamientos de *Salmonella* de bovinos en los últimos años presentan los genes *qnrB* seguido de *qnrA* (Delgado-Suárez et al. 2021, Barrera et al. 2023).

Actualmente, las detecciones de mecanismos de resistencia se incrementan día a día con múltiples reportes en todo el mundo de resistencia a diferentes grupos de antibióticos (Gelalcha & Kerro, 2022, Tayyaba & Ahmed,2022, Aleri et al. 2022).

Dentro de los elementos genéticos movilizables, *Salmonella* alberga plásmidos, fagos y transposones los cuales permiten a la bacteria desarrollar una función específica (Frost et al. 2005). En este género bacteriano se describen pequeños y grandes plásmidos, algunos de ellos relacionados a la virulencia, la resistencia bacteriana o ambas entre otras funciones Todos los plásmidos de virulencia independientemente del serotipo poseen los genes genes *spv*RABCD, además de poseer otros genes con diferentes funciones de virulencia (Rychlik et al. 2006).

No menos importantes son los plásmidos asociados a genes de resistencia a antibióticos los cuales a través de la transferencia horizontal de genes pueden diseminarse a diferentes especies bacterianas (McMillan et al. 2019). Se describen 23 grupos de incompatibilidad de plásmidos de acuerdo con la tipificación de estos. Los plásmidos se tipifican basándose en la incompatibilidad con otros plásmidos que es la incapacidad de dos plásmidos para mantenerse juntos en la misma línea celular (McMillan et al. 2019, Rozwandowicz et al. 2018).

Los integrones también son elementos genéticos que pueden portar genes asociados a resistencia antibiótica o virulencia, los cuales a diferencia de los plásmidos no poseen la estructura para movilizarse de forma independiente (Krauland et al. 2019). Los plásmidos e Integrones son frecuentes en *Salmonella* y son importantes porque contribuyen a la diseminación de la resistencia a los antibióticos (Carattoli, 2003). Se describen 5 diferentes tipos de Integrones y en *Salmonella enterica* los Integrones de clase 1 son los más frecuentemente descriptos tanto en aislados de origen animal y sus derivados como en aislamientos clínicos humanos (Rao et al. 2019).

OBJETIVOS

General

El objetivo de este trabajo es caracterizar, con diferentes abordajes, una colección de *Salmonella enterica* aisladas de muestras obtenidas de brotes de salmonelosis bovina y del entorno productivo de establecimientos lecheros de la cuenca lechera.

Específicos

1- Realizar una caracterización de aislamientos de *Salmonella* obtenidos de 15 brotes de salmonelosis bovina en base a los determinantes de virulencia y la descripción de las lesiones patológicas causadas debido a la infección.
2- Determinar el acervo genético que presentan los aislamientos de *Salmonella* de acuerdo con la resistencia a los antibióticos y correlacionarlo con el fenotipo de susceptibilidad.
3- Determinar la asociación entre la presencia de genes de virulencia y el origen y serotipo de los aislamientos de *Salmonella* spp.

ESTRATEGIA DE TRABAJO

Para llevar adelante este trabajo se utilizaron 75 aislamientos de *Salmonella enterica* de una colección obtenidos de casos de diarrea, de mortalidad de terneros, un aborto y muestreos ambientales entre los años 2016 y 2020.

De estos, se seleccionaron 22 aislamientos obtenidos a partir de tejidos de autopsias de 20 terneros que pertenecían a 15 brotes de mortalidad caracterizados epidemiológicamente. En los aislamientos se buscaron por PCR genes de virulencia y a partir de las autopsias se registraron los hallazgos patológicos de cada animal. Luego se buscaron asociaciones entre lesiones observadas y la carga de genes de virulencia.

En un segundo enfoque, se secuenció el genoma competo de los 75 aislamientos de *Salmonella enterica.* Se realizó un análisis para detectar los genes de resistencia a antibióticos, la búsqueda de plásmidos e integrones. Finalmente se realizó la caracterización de las islas de patogenicidad y de los genes de virulencia que se identificaron mediante diferentes herramientas genómicas.

CAPÍTULO I

Estudio epidemiológico de brotes de salmonelosis bovina y la virulencia de los aislamientos obtenidos

INTRODUCCIÓN

En el mundo, la salmonelosis bovina se presenta en mayor medida en aquellos predios en los cuales la producción es intensiva. Existe una amplia diversidad de serotipos de *Salmonella* y si bien se sabe que la enfermedad en bovinos es causada por principalmente dos serotipos, *Salmonella* Dublin y *Salmonella* Typhimurium, hay otros serotipos que se describen en menor medida que pueden afectar a diferentes especies animales y al hombre como son los serovares Enteritidis, Heidelberg y Newport (Holschbach & Peek, 2018; Ferrari et al. 2019, Jajere et al. 2019). En el contexto de la salmonelosis, los animales afectados pueden ser de diferentes categorías y los mismos pueden excretar y eliminar la bacteria por diferentes vías como leche, saliva, heces y contaminar productos primarios derivados (Constable, 2017).

En los sistemas de producción animal, los alimentos derivados sufren riesgo de contaminación debido a diferentes factores. Debido a esto es necesario evaluar el estatus sanitario del rodeo e incrementar la bioseguridad tanto para proteger a los animales de enfermedades como para impedir las enfermedades de transmisión alimentaria a través de la inocuidad de los alimentos (Fossler et al. 2005).

La categoría más susceptible es la de terneros, los cuales deben enfrentar una carga microbiológica ambiental bajo una etapa de desarrollo inmunológico insuficiente, con mayores posibilidades de enfermarse. Por otro lado, las vacas previo al parto y post parto enfrentan un proceso de estrés en donde sus defensas se ven disminuidas incrementando la probabilidad de enfermar. En caso de haber sido infectadas por *Salmonella* ésta puede volver a circularizarse y ser excretada por diferentes secreciones, fluidos y en las heces (Fossler et al. 2005).

Los animales afectados por *Salmonella* pueden presentar diarrea, signos respiratorios, deshidratación y fiebre. Los animales adultos pueden cursar la enfermedad de forma asintomática presentar diarrea con o sin sangre e incluso las hembras en gestación pueden sufrir abortos (Constable, 2017).

Dependiendo de los serotipos actuantes en cada brote de salmonelosis la epidemiología y la presentación de la enfermedad puede variar, causando altos valores de morbilidad y mortalidad. En la presentación de la enfermedad, *Salmonella* Typhimurium afecta con más frecuencia terneros en sus primeros días formando parte del síndrome de diarrea neonatal con una alta morbilidad. Por otro lado, *Salmonella* Dublin causa muerte aguda en terneros de mayor edad presentando a veces sintomatología respiratoria (Mollossi et al. 2021, Kudirkiene et al. 2020, Nielsen et al. 2004).

Las presentaciones de la enfermedad se encuentran relacionadas con los atributos de virulencia que presenta la bacteria, su expresión y el estado inmunitario del huésped, por lo que en ciertos casos puede generar una enfermedad entérica o tener la capacidad debido a los genes que presenta, de alcanzar tejidos más allá de la barrera intestinal logrando una enfermedad sistémica (Casadevall & Pirofski, 2001). Estos factores se encuentran implicados en la adherencia a la pared intestinal, invasión celular, supervivencia dentro de células inmunitarias, captación de hierro y magnesio, entre otros (Eng et al. 2015).

JUSTIFICACIÓN

La monitorización de la presencia de *Salmonella* en establecimientos ganaderos contribuye a identificar animales positivos potenciales excretores de la bacteria al ambiente y de los inconvenientes que implica tener la enfermedad. A través de la caracterización del estatus que presentan los rodeos es posible obtener datos de la distribución de *Salmonella* en el país y de las características de los aislamientos circulantes. El conocimiento de la situación sanitaria de nuestros rodeos con respecto a la salmonelosis y su caracterización nos permite tomar decisiones para el futuro productivo y para la creación de nuevas tecnologías que puedan aplicarse al control de la enfermedad.

OBJETIVOS

Describir datos epidemiológicos, signos clínicos y hallazgos patológicos en brotes espontáneos de salmonelosis en granjas lecheras de Uruguay causados por *S.* Typhimurium y *S.* Dublin y sus genes de virulencia (VG). En segundo lugar, exploramos posibles asociaciones entre estas variables.

RESULTADOS

El producto de este capítulo es el artículo **"Epidemiological and clinicopathological findings in 15 fatal outbreaks of salmonellosis in dairy calves and virulence genes in the causative *Salmonella enterica* Typhimurium and Dublin strains"** el cual incluye los resultados epidemiológicos y hallazgos patológicos de 15 brotes de *Salmonella* y caracterización de los atributos de virulencia que presentan los aislamientos. El mismo fue publicado en la revista *Brazilian Journal of Microbiology*. Se adjunta el original doi: 10.1007/s42770-022-00898-9

ARTÍCULO CIENTÍFICO CAPÍTULO I

RESUMEN EN ESPAÑOL

Salmonella enterica es un importante agente patógeno de transmisión alimentaria que afecta a los sistemas de cría de ganado bovino en todo el mundo. Se dispone de poca información sobre la epidemiología y patología de la salmonelosis y los genes de virulencia (VG) que porta *Salmonella* en los brotes espontáneos en el ganado. Describimos los hallazgos epidemiológicos en 15 brotes mortales de salmonelosis en explotaciones lecheras uruguayas y la edad, signos clínicos y patología en 20 terneros afectados. A su vez se determinaron los serotipos y frecuencia de 17 genes de virulencia en los aislamientos de *Salmonella* causantes y exploramos sus asociaciones con los hallazgos epidemiológicos, clínicos y patológicos. Se identificaron *Salmonella* Typhimurium y Dublin en 11/15 y 4/15 brotes, respectivamente. El motivo de consulta más frecuente fue la enfermedad digestiva (8 brotes causados por *S*. Typhimurium), seguida de la muerte súbita (4 brotes, 3 causados por *S*. Dublin). La morbilidad, mortalidad y letalidad oscilaron entre 4,8-100%, 3,8-78,9% y 10-100%, sin diferencias significativas entre los serotipos. La diarrea, el signo clínico más frecuente (14 casos), se asoció con el serotipo Typhimurium (OR = 26,95), especialmente en terneros ≤ 30 días de edad con enteritis fibrinosa como principal hallazgo de autopsia. El serotipo Dublin afectó a terneros ≥ 50 días de edad y se asoció con esplenitis fibrinosupurativa (p = 0,01) y nefritis tubulointersticial (OR = 48,95). Las probabilidades de la enfermedad por el serotipo Dublín aumentaron significativamente con la edad. La variabilidad de genes de virulencia entre serotipos fue baja. El gen *pef*A se asoció con el serotipo Typhimurium (OR = 21,95), la enteritis macroscópica (p = 0,03) y la esplenitis fibrinosupurativa microscópica (p = 0,04). Comprender la epidemiología, patología y virulencia de *S. enterica* en las explotaciones es clave para delinear estrategias de prevención y control que mitiguen su impacto en la salud animal y humana.

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VETERINARY MICROBIOLOGY - RESEARCH PAPER

Epidemiological and clinicopathological findings in 15 fatal outbreaks of salmonellosis in dairy calves and virulence genes in the causative *Salmonella enterica* Typhimurium and Dublin strains

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Abstract

Salmonella enterica is a major food-borne pathogen that affects cattle-rearing systems worldwide. Little information is available on the epidemiology and pathology of salmonellosis and the virulence genes (VGs) carried by *Salmonella* in spontaneous outbreaks in cattle. We describe epidemiological findings in 15 fatal outbreaks of salmonellosis in Uruguayan dairy farms and the age, clinical signs, and pathology in 20 affected calves. We also describe the serotypes and frequencies of 17 VGs in the causative *Salmonella* strains and explore their associations with epidemiological, clinical, and pathological findings. *Salmonella* Typhimurium and Dublin were identified in 11/15 and 4/15 outbreaks, respectively. The most frequent reason for consultation was digestive disease (8 outbreaks caused by *S*. Typhimurium), followed by sudden death (4 outbreaks, 3 caused by *S*. Dublin). Morbidity, mortality, and lethality ranged 4.8–100%, 3.8–78.9%, and 10–100%, without significant differences between serotypes. Diarrhea, the most common clinical sign (14 cases), was associated with the Typhimurium serotype (OR = 26.95), especially in ≤ 30-day-old calves with fibrinous enteritis as the main autopsy finding. The Dublin serotype affected ≥ 50-day-old calves and was associated with fibrinosuppurative splenitis ($p = 0.01$) and tubulointerstitial nephritis (OR = 48.95). The chances of the Dublin serotype increased significantly with age. There was low variability of VG across serotypes. The *pef*A gene was associated with the Typhimurium serotype (OR = 21.95), macroscopic enteritis ($p = 0.03$), and microscopic fibrinosuppurative splenitis ($p = 0.04$). Understanding the epidemiology, pathology, and virulence of *S. enterica* at the farm level is key to delineating prevention and control strategies to mitigate its impact on animal and human health.

Keywords *Salmonella enterica* · Calves · Epidemiology · Pathology · Virulence genes · Uruguay

Introduction

Salmonella is an important pathogen worldwide that affects different animal species, including humans. The *Salmonella* genus comprises 2 species, *Salmonella bongori* and *Salmonella enterica.* This last species is made up of 6 subspecies with more than 2500 reported serotypes and is responsible for 93.8 million human cases of diarrhea and 155,000 deaths per year [1–3].

Circulating serotypes differ among geographic regions, although little information is available from South American countries. A systematic review that summarizes the serotypes reported in apparently healthy cattle throughout the world from 2000 to 2017 indicates that Montevideo, Typhimurium, Kentucky, Meleagridis, Anatum, Cerro, Mbandaka, Muenster, Newport, and Senftenberg were the most frequent. *Salmonella* Montevideo was the most

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frequent in North America, *S.* Dublin in Europe, and *S.* Typhimurium in Africa, Asia, and Australasia [4]. Surprisingly, with regard to South American countries, this review included information from only one Venezuelan study and claimed that Javiana and Weltevreden were the only serotypes identified, although this information is not readily available in the original reference [5].

In a recent study involving 50 dairy farms in Argentina, *S. enterica* was detected in 36% of the farms, and the most prevalent serotypes were Mbandaka, Anatum, Typhimurium, Dublin, and Montevideo [6]. In Uruguay, *S.* Typhimurium was the most frequently isolated serotype in a study performed in 30 dairy farms with spontaneous outbreaks of neonatal calf diarrhea, while the serotype Anatum was isolated in a small subset of diarrheic and healthy calves [7]. In this country, the Dublin serotype was identified in calves with fatal septicemia [8], as well as in humans [9]. The identification of the bovine-adapted serotype Dublin in invasive infections in humans in Uruguay [9] raises concerns about direct or indirect bovine-to-human transmission.

The clinical outcome and severity of salmonellosis in cattle depend on the age, physiologic status of the animals, infecting serotypes, and infective dose [10, 11]. *Salmonella* Dublin and Typhimurium frequently cause clinical disease in cattle, although various other serotypes, including Montevideo and Newport, can also be involved in other clinical presentations, such as abortion [12, 13]. In calves, these serotypes can cause morbidity higher than 50% with lethality up to 100% if no medical treatment is applied. The economic impact of salmonellosis is based on reduced productivity, animal deaths, diagnostic expenses, and treatments [12].

Bovine salmonellosis is frequent in intensive farming systems, and calves have a higher risk of infection. In calves, *S.* Typhimurium usually causes diarrhea, fever and, less frequently, septicemia. Calves infected with *S.* Dublin can show diarrhea, fever, septicemia, respiratory distress, dry gangrene in the legs, tail or pinnae, and sudden death [10, 14]. In pregnant cows and heifers, *S.* Dublin can cause abortion in the presence of pyrexia or without any other obvious clinical signs [15, 16].

After digestive transmission, *S. enterica* unfolds a battery of virulence attributes that lead to the invasion and colonization of many organ systems. After crossing the intestinal barrier, Salmonellae can colonize the liver and gallbladder, spleen, lungs, brain, urinary tract, and bone marrow. Macroscopic lesions can be absent, or there could be enteritis/typhlitis/colitis with petechiae in the submucosa and serosa of the intestine, mesenteric lymphadenomegaly, fibrinous peritonitis, splenomegaly, and hepatomegaly. The lungs can have congestion, edema, or embolic interstitial pneumonia with exudation of fibrin and neutrophils in the lumen of the alveoli [12, 17]. *Salmonella* Typhimurium frequently causes enteric lesions, including necrotizing enteritis/colitis with watery, bloody, or fibrinous, malodorous intestinal contents [10].

Clinical and pathological findings in cases of salmonellosis can be explained by the presence of genetic virulence determinants of the pathogen. Once in the host, these mechanisms are activated and allow bacterial survival and further replication in the tissues. Many of these mechanisms are encoded by genes in *Salmonella* pathogenicity islands (SPIs), which are involved in cellular invasion, survival, and replication inside phagocytic cells, and their protein products are responsible for the lesions and the clinical signs [18].

Here, we describe epidemiological data, clinical signs, and pathological findings in spontaneous outbreaks of salmonellosis in dairy farms in Uruguay and their virulence genes (VGs) in the causative *S. enterica* Typhimurium and Dublin isolates. Second, we explore possible associations among these variables.

Materials and methods

Epidemiological data and clinical signs

Fifteen outbreaks of salmonellosis with fatal outcomes in dairy calves were studied upon request from field veterinary practitioners at the Instituto Nacional de Investigación Agropecuaria (INIA) veterinary diagnostic laboratory from 2016 to 2018. All outbreaks occurred spontaneously at commercial dairy farms in Uruguay. The primary reason for consultation (herd-level health problem) indicated for each outbreak by the referring veterinarian was recorded. Epidemiological data (number of exposed calves, number of clinically affected calves, and number of deceased calves in the exposed group) were obtained and used to calculate the morbidity, mortality, and lethality risks for each outbreak. Additionally, the clinical signs that the affected calves subjected to autopsy (see below) showed before dying, as reported by the referring veterinarians, as well as their age in days, were collected in all cases.

Pathologic examination

Autopsies of 20 naturally deceased calves from all 15 outbreaks were performed, and the macroscopic lesions were recorded. Tissue samples from all autopsied carcasses were collected and immersion-fixed in 10% neutral buffered formalin (pH 7) for a minimum of 48 h and processed routinely for histology [19]. Briefly, tissues were dehydrated through immersion in a series of solutions of ethanol and xylol and embedded in paraffin wax (Histoplas, Sakura) in an automated vacuum infiltration tissue processor (Tissue-Tek VIP Jr., Sakura). Paraffin-embedded tissue blocks were made in

a tissue embedding console (Tissue-Tek TEC 5, Sakura) and then sectioned at 4–5 µm in a rotatory microtome (Accu-Cut SRM 200, Sakura). Tissue sections were mounted on glass slides and stained with hematoxylin and eosin (Sigma Aldrich) in an automated slide stainer (Tissue-Tek Prisma, Sakura). Slides were examined under an optic microscope (Axio Scope. A1, Carl Zeiss) by an anatomic veterinary pathologist, and microscopic lesions were recorded.

Salmonella enterica isolate sources and serotyping

Selected tissue or fluid samples from all autopsied calves were collected aseptically at autopsy and subjected to selective culture for *S. enterica*, as described by Casaux et al., 2019. A total of 20 *S. enterica* isolates, one from each autopsied calf, were serotyped following the Kauffman-White-LeMinor scheme [20] at the bacteriology service of the "Instituto de Higiene, Universidad de la República (UdelaR)" in Montevideo, Uruguay.

Virulence gene detection

All *S. enterica* isolates were previously stored (− 80 °C) in trypticase soy agar (TSA)-glycerol (20%), grown in TSA (24 h, 37 °C), and later analyzed for the detection of 17 virulence genes by PCR. After incubation, two colonies of a pure culture were picked and suspended in 100 µl of distilled water. DNA extraction was conducted by boiling the suspension for 15 min, followed by centrifugation, and the supernatant was stored at − 20 °C. The presence of the virulence gene *spv*B associated with growth within the host; genes *spi*A, *pag*C, and *msg*A related to survival within macrophages; genes *inv*A, *prg*H, *org*A, *tol*C, *lpf*C, *sop*B, and *pef*A related to host recognition/invasion; genes *iro*N and *sit*C, the latter as a representative of the *sit*ABCD operon and both responsible for iron acquisition; gene *sif*A associated with filamentous structure formation; and genes *spa*N and *sip*B linked to entry into non-phagocytic cells and killing of macrophages, were assessed by PCR following procedures and primers described by Skyberg et al. [21]. The *stn* gene, which is responsible for the enterotoxicity of *Salmonella*, was amplified according to Murugkar et al. [22] using primers described by Gharieb et al. [23].

The amplification reactions were performed in a ProFlex PCR System (Applied Biosystems, Weiterstadt, Germany), and PCR products were run in 1% agarose gels stained with GoodView (SBS, China). To confirm their identities, representative amplicons for every gene were purified and sequenced at Macrogen Inc. (Seoul, South Korea) using the same primers used for PCR. The obtained sequences were compared with those in the database of the "National Center for Biotechnology Information" (NCBI, https://blast.ncbi.nlm.nih.gov/Blast.cgi) using the BLASTN tool [24] and later used as positive controls for PCR. A tube containing the PCR mix without DNA addition was used as a blank for every reaction.

Statistical analyses

The available information was used to conduct exploratory statistical analyses, despite the small sample size. Reasons for consultation and epidemiological data (morbidity, mortality, and lethality) for all 15 outbreaks were recorded. Similarly, the age, clinical signs, and gross and microscopic pathological findings for each of the 20 calves, as well as the serotypes and virulence genes for all *S. enterica* isolates, were stored in LibreOffice Calc (Supplementary Table 1) and later analyzed in R software (R Foundation for Statistical Computing, Vienna, 2011, version 3.6.2). Variables with < 20% variability, such as the occurrence of the clinical sign coughing/tachypnea, some macroscopic lesions (those involving the rumen, urinary bladder, and brain/rete mirabile), microscopic lesions (enteritis and hepatitis), and most virulence genes (all except for *iro*N and *pef*A), were excluded from posterior analyses.

Pearson's chi-square test was implemented to assess the association between the VGs as well as between serotypes, clinical signs, pathological findings, and virulence genes. The permutation test was used as an approximation of Fisher's exact test to address the small sample size. Additionally, to test the hypotheses of relationships between epidemiological data (morbidity, mortality, and lethality), the reasons for consultation, age, clinical signs, pathological findings, the involved *S. enterica* serovars, the occurrence of virulence genes, and generalized linear regression models were fitted, and odds ratios (ORs) and 95% confidence intervals (95% CIs) were calculated. For all tests, the results were regarded as significant if $p < 0.05$.

Results

Salmonella enterica serotypes, reasons for consultation, and epidemiological data

Information on the sample of origin, the *S. enterica* serotypes, and the age of each calf in each outbreak is summarized in Table 1. The number of exposed, sick, and deceased calves in each outbreak, as well as the calculated morbidity, mortality, and lethality risks, are shown in Table 2.

Digestive disease was the most common reason for consultation (8 outbreaks), followed by sudden death (4 outbreaks), depression (2 outbreaks), and respiratory disease (1 outbreak) (Table 2). Seventeen calves from 11 outbreaks were infected with *S.* Typhimurium, and 5 calves from 4 outbreaks were infected with *S.* Dublin (Tables 1 and 2).

Table 1 Sample of origin (source) and serotype of the *Salmonella enterica* isolates obtained from 20 deceased dairy calves in 15 spontaneous outbreaks of salmonellosis

Outbreak number	Serotype	Calf ID	Age (days)	Source
I	Typhimurium	1	14	Mesenteric lymph node
	Typhimurium	2	16	Intestinal content
II	Typhimurium	1	20	Mesenteric lymph node
	Typhimurium	2	20	Mesenteric lymph node
III	Typhimurium	1	15	Mesenteric lymph node
IV	Typhimurium	1	5	Mesenteric lymph node
V	Typhimurium	1	10	Mesenteric lymph node
	Typhimurium	1	45	Mesenteric lymph node
VII	Typhimurium	1	10	Mesenteric lymph node
VIII	Typhimurium	1	8	Mesenteric lymph node
IX	Typhimurium	1	90	Intestine
X	Typhimurium	1	15	Mesenteric lymph node
XI	Dublin	1	50	Lung
	Dublin	2	50	Mesenteric lymph node
XII	Dublin	1	64	Mesenteric lymph node
XIII	Typhimurium	1	30	Mesenteric lymph node
	Typhimurium	2	30	Liver
	Typhimurium	3	30	Mesenteric lymph node
XIV	Dublin	1	60	Mesenteric lymph node
XV	Dublin	1	60	Kidney

All 8 outbreaks with digestive disease as the main reason for consultation were caused by *S.* Typhimurium, while 3 of the 4 outbreaks with sudden death as the main reason for consultation were caused by *S.* Dublin. In the generalized linear regression model, the serotype Dublin was a significant predictor of sudden death as a reason for consultation (OR = 30, 95% CI 2–1282, $p = 0.03$).

For the two most frequent reasons for consultation (digestive disease and sudden death), the average morbidity and mortality rates were 51.67% (range: 10–100%) and 25.4% (range: 3.29–78.9%) for digestive disease ($n = 8$ outbreaks) and 17.76% (range: 3.29–30%) and 17.32% (range: 1.97–30%) for sudden death ($n = 3$ outbreaks, no data = 1 outbreak) (Table 2). The average lethality risk for outbreaks with digestive disease as the reason for consultation was 51.4% (range: 10–100%) and 86.67% (range: 60–100%) for sudden death. The average morbidity and mortality risks for the outbreaks involving the Typhimurium ($n = 11$ outbreaks) and Dublin ($n = 3$ outbreaks, missing data = 1 outbreak) serotypes were 42.35% (range: 10–100%) and 23.02% (range: 3.85–78.95%) for the Typhimurium serotype and 9.35% (range: 3.29–20%) and 8.46% (range: 1.97–20%) for the Dublin serotype, respectively. The average lethality risk for the outbreaks involving the serotypes Typhimurium and Dublin was 62.38% (range: 10–100%) and 77.14% (range: 71.43–100%), respectively. Morbidity, mortality, and lethality risks did not differ significantly among these reasons for consultations or serotypes.

Clinical signs, age, and serotypes

The clinical signs observed in the autopsied calves before death and reported by the veterinarians included diarrhea (14 cases), neurological signs/depression (4 cases), and coughing/tachypnea (3 cases). Two calves had diarrhea and neurological signs/depression (1 for each serotype), and 1 calf (infected with *S.* Typhimurium) had diarrhea and coughing/tachypnea. The most common clinical sign in the15 cases infected with *S.* Typhimurium was diarrhea (13 cases), followed by neurological signs/depression (1 case) and coughing/tachypnea (1 case), while the 5 calves infected with the Dublin serotype most frequently manifested neurological signs/depression (2 cases), followed by coughing/tachypnea (1 case) and diarrhea (1 case). One of the *S.* Dublin-infected calves was reported to have died suddenly without observable clinical signs.

The average age of the 5 calves affected by *S.* Dublin was 55 days (min. 50 d, max. 60 d), while 15 calves affected by *S.* Typhimurium had an average age of 23.9 d (min. 5 d, max. 90 d; Table 1); this difference was statistically significant (Fig. 1). In the generalized linear regression model, age was a significant predictor of the infecting serotype; for each one-day increment in age, the odds of *S.* Typhimurium (vs. *S.* Dublin) infection decreased by 0.1 (OR = 0.9, 95% CI 0.8–1, $p = 0.03$).

The average age of the 14 calves that had diarrhea as a clinical sign before death was 22.4 days, while the 6 calves that did not have diarrhea averaged 53.3 days; this difference was statistically significant (Fig. 2). Similarly, in the generalized linear regression model, age was a predictor of diarrhea; for each one-day increment in age, the odds of diarrhea decreased by 0.08 (OR = 0.92, 95% CI 0.8–1, $p = 0.02$). In calves with diarrhea, the age was 60 days for the single case infected with the Dublin serotype and averaged 19.46 days for the calves infected with *S.* Typhimurium.

Table 2 Reasons for consultation and epidemiological data in 15 outbreaks of salmonellosis in dairy calves

Reason for consultation		Serotype	Number of exposed calves	Number of sick calves	Morbidity (%)	Number of dead calves	Mortality (%)	Lethality (%)
Digestive disease	Outbreak **I**	Typhimurium	20	2	10.0	2	10.0	100
	Outbreak **II**	Typhimurium	5	4	80.0	2	40.0	50.0
	Outbreak **III**	Typhimurium	26	10	38.5	1	3.8	10.0
	Outbreak **IV**	Typhimurium	41	11	26.8	3	7.3	27.3
	Outbreak **V**	Typhimurium	20	4	20.0	1	5.0	25
	Outbreak **VI**	Typhimurium	19	19	100.0	15	78.9	78.9
	Outbreak **VII**	Typhimurium	5	5	100.0	1	20.0	20.0
	Outbreak **VIII**	Typhimurium	42	16	38.1	16	38.1	100
Respiratory disease	Outbreak **IX**	Typhimurium	200	20	10.0	15	7.5	75.0
Depression	Outbreak **X**	Typhimurium	40	5	12.5	5	12.5	100
	Outbreak **XI**	Dublin	147	7	4.8	5	3.4	71.4
Sudden death	Outbreak **XII**	Dublin	152	5	3.29	3	1.97	60.0
	Outbreak **XIII**	Typhimurium	50	15	30.0	15	30.0	100
	Outbreak L **XIV**	Dublin	100	20	20.0	20	20.0	100
	Outbreak **XV**	Dublin	ND	ND	-	ND	-	-

[A]ND: No data

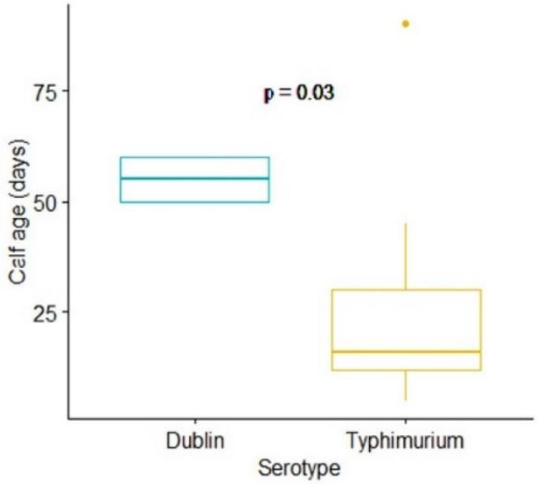

Fig. 1 Boxplot distribution of the age of the calves with salmonellosis caused by *S.* Dublin (average = 55 days, $n = 5$ calves) and *S.* Typhimurium (average = 23.9 days, $n = 15$ calves)

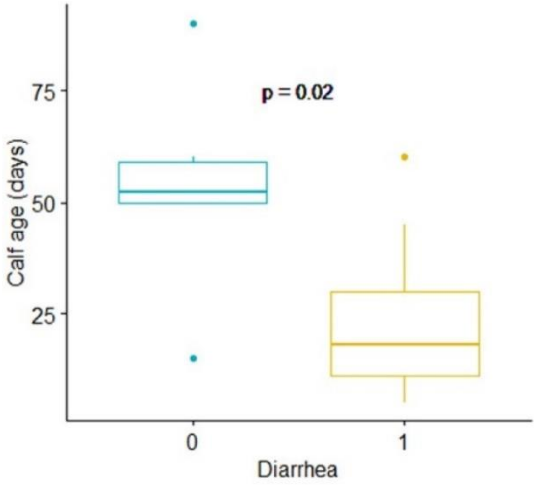

Fig. 2 Boxplot distribution of the age of the calves with salmonellosis without (average = 53.3 days, $n = 6$, left plot) or with (average = 22.4, $n = 14$ calves, right plot) manifestation of diarrhea as a clinical sign before death

Macroscopic and microscopic lesions

Gross lesions were recorded in 20 autopsied calves (Table 3; Fig. 3). The anatomic location of the lesions included serosae or joints (11/17), the intestinal tract (10/20), the liver (7/18), the adrenal glands (6/13), the lungs (6/16), the mesenteric lymph nodes (8/10), the spleen (5/16), the abomasum (4/16), and the cerebral meninges (1/16). No gross lesions were identified in the heart or kidneys. One calf (number 2 of outbreak XI) had jaundice and fibrinous cholecystitis.

All 10 calves with macroscopic lesions in the intestinal tract were infected with the Typhimurium serotype. None of the 3 calves infected with the Dublin serotype for which there were data on gross examination of the intestinal tract had grossly visible enteric lesions.

Table 3 Macroscopic lesions in 20 calves with salmonellosis subjected to autopsy

Outbreak	Animal ID, serotype[a]	Enteritis[b]	Hepatitis[c]	Adrenocortical hemorrhage	Pneumonia[d]	Mesenteric lymph node [e]	Splenomegaly	Abomasum[f]	Rumenitis	Urinary bladder [g]	Serositis or arthritis[h]	Brain[i]
I	1, T	+	+, A, B, C	+	+	+, B	-	-	-	-	+, B	-
	2, T	+	+, A, B	+	-	ND	-	-	-	-	+, A	-
II	1, T	-	-	+	+	ND	+	-	-	ND	+, C	-
	2, T	+	-	ND	-	ND	-	+, A	-	ND	+, C	-
III	1, T	-	-	ND	ND	+, A	-	-	+	-	-	-
IV	1, T	+	+, A, B, C, D	-	+	+, A	+	-	-	+, A, B	+, E	-
V	1, T	+	-	-	-	+, A	-	-	-	-	-	-
VI	1, T	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
VII	1, T	+	+, B	-	-	+, A	+	+, B	ND	-	+, B	-
VIII	1, T	ND	ND	ND	ND	+, A	ND	ND	ND	ND	ND	ND
IX	1, T	+	-	ND	-	+, A	-	-	ND	-	-	ND
IX	1, T	-	-	-	-	-	ND	-	-	-	+, E	-
XI	1, D	-	-	+	-	ND	+	-	-	ND	+, E	-
	2, D	-	+, B, C, D	+	+	+, A	+	ND	-	+, A	+, B	-
XII	1, D	ND	+, A, B, D	ND	+	ND	-	+, A, B	ND	ND	+B, C, E	+
XIII	1, T	+	-	+	-	ND	-	+, B	-	-	-	-
	2, T	+	-	-	-	ND	-	-	-	-	-	-
	3, T	+	-	-	-	ND	-	-	-	-	-	-
XIV	1, D	-	-	-	+	-	-	-	-	-	+, E	-
XV	1, D	ND	+, A	ND	ND	ND	ND	ND	ND	ND	ND	ND

[a]*T*, Typhimurium; *D*, Dublin. [b]Includes fibrinous, erosive/ulcerative enteritis, colitis, typhlitis, or combinations (enterocolitis, enterotyphlitis, typhlocolitis, or enterotyphlocolitis). [c]Includes hepatomegaly (A) with or without diffuse ochre/yellowish discoloration of the parenchyma (cholestasis, B), with or without multifocal disseminated hepatitis (C), with or without fibrinous cholecystitis (D). [d]Includes multifocal disseminated embolic interstitial pneumonia and/or pulmonary edema. [e]Includes lymphadenomegaly (A) and/or fibrinosuppurative lymphadenitis (B). [f]Includes ulcerative abomasitis (A) or abomasal edema (B). [g]Includes petechiae on the mucosa (A) and/or fibrin in the urine (fibrinous urocystitis, B). [h]Includes fibrinous arthritis (A), fibrinous peritonitis (B), fibrinous pericarditis (C), fibrinous pleuritis (D), or serosal petechiae (E). [i]Includes congestion, edema, and cloudy cerebrospinal fluid. *ND*, not determined

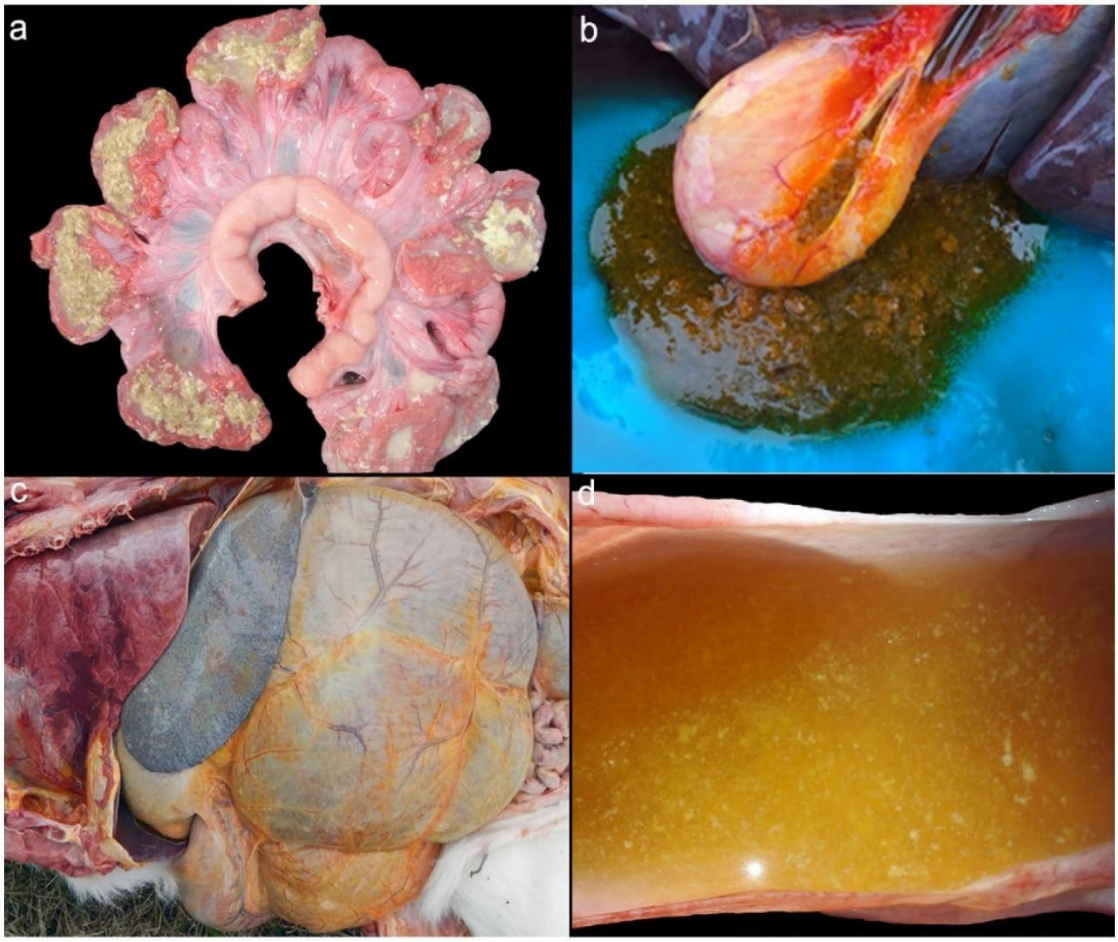

Fig. 3 Gross lesions in dairy calves with salmonellosis. (**a**) Calf 1, outbreak XIII. Segment of jejunum with abundant yellowish fibrillar material (fibrin) adhered to the mucosa (severe fibrinous enteritis); the mesenteric lymph nodes are enlarged and swollen (mesenteric lymphadenitis). (**b**) Calf 2, outbreak XI. The gallbladder is distended and contains bile admixed with abundant fibrin (fibrinous cholecystitis). (**c**) Calf 2, outbreak XI. Left side view of abdominal and thoracic organs in situ. The spleen is enlarged (splenomegaly), and there is yellow discoloration of the omentum, serosal surfaces of the forestomachs and adipose tissue (jaundice). The lung failed to collapse and showed rib imprints on the pleural surface and dark red discoloration of multiple lobules (interstitial pneumonia). (**d**) Calf 1, outbreak IV. The urinary bladder contains abundant urine with granular to fibrillar yellowish clots (fibrinous urocystitis)

The results of the histopathologic examination of various tissues of the 20 carcasses subjected to autopsy are shown in Table 4. The most consistent microscopic lesions were necrotizing enteritis in 19 of 19 calves, multi-focal random necrotizing and/or portal hepatitis in 18 of 19 cases, interstitial pneumonia in 13 of 17 cases, lymphadenitis in 12 of 15 cases, splenitis in 7 of 17 cases, abomasitis in 6 of 8 cases, adrenocortical hemorrhage in 7 of 14 cases, tubulointerstitial nephritis in 5 of 18 cases, and meningitis or perivasculitis in the rete mirabile in 4 of 15 cases. Selected microscopic lesions are shown in Fig. 4. There were no cardiac lesions observed. Splenitis was more frequent in calves infected with *S*. Dublin (4/5) than in calves infected with *S*. Typhimurium (2/13), while tubulointerstitial nephritis affected 4/5 of calves infected with *S*. Dublin and 1/13 of calves infected with *S*. Typhimurium.

Detection of virulence genes

There was < 20% variability in the detected virulence genes across isolates, except for the *pef*A and *iro*N genes. The absolute and relative frequencies for each of the 17 virulence genes detected in all *S*. Typhimurium and *S*. Dublin isolates are shown in Table 5. Eleven virulence genes (*inv*A, *sit*C, *org*A, *lpf*C, *sip*B, *msg*A, *sif*A, *tol*C, *sop*B, *prg*H, and *stn*) were detected in all *S*. Dublin isolates, while the *spa*N,

Table 4 Microscopic lesions in 20 calves with salmonellosis subjected to autopsy

Outbreak	Animal ID, Serotype[a]	Enteritis[b]	Hepatitis[c]	Adrenocortical hemorrhage	Pneumonia[d]	Fibrinous/suppurative lymphadenitis	Fibrinous/suppurative splenitis	Abomasitis[e]	Rumenitis/reticulitis	Urinary bladder[f]	Kidney[g]	Brain or rete mirabile[h]
I	1, T	+, A, B	+, A, C	+	+, A	+	-	+, A, B	ND	ND	-	-
	2, T	+, A, B	+, B, C	+	+, A	+	-	ND	ND	ND	-	-
II	1, T	+, A, B	+, B	+	+, A	-	-	+, A, B	-	-	-	-
	2, T	+, A, B	+, B	-	+, A, B	-	-	ND	+	ND	-	-
III	1, T	+, A, B	+, B	-	+, A, C	ND	-	ND	+	ND	-	+, B
IV	1, T	+, A, B, C	+, A	-	+, A, B	+	+	-	ND	+, A	-	-
V	1, T	+, A, B	+, A	ND	-	+	-	ND	-	-	-	ND
VI	1, T	+, A, B	+, B, C	ND	ND	+	ND	ND	ND	ND	ND	ND
VII	1, T	+, A, B	+, A, B	ND	-	+	+	ND	ND	ND	-	-
VIII	1, T	+, A, B, C	+, A	ND	ND	+	ND	ND	ND	ND	ND	ND
IX	1, T	+, A, B	+, A, B, C	-	+, A, B, C	+	-	+, A	-	-	-	-
X	1, T	+, A, B, C	+, B	-	+, A	ND	+	ND	ND	ND	-	-
XI	1, D	+, A, B	ND	+	+, A, B, C	+	+	-	-	+, B	-	+, B
	2, D	+, A, B	+, A	+	+, A, B, C	ND	+	ND	-	+, A	+	ND
XII	1, D	+, A, B, C	+, A, B, C	-	+, A, B, C	+	+	+, A, B	-	ND	+	+, B
XIII	1, T	+, A, B, C	-	+	-	ND	-	ND	ND	ND	+	-
	2, T	+, A, B, C	+, B	+	-	+	-	+, A, B	ND	ND	-	-
	3, T	+, A, B	+, B	ND	+, A	+	-	ND	ND	ND	-	-
XIV	1, D	+, A, B	+, A	-	+, A, B	-	+	+, A, B	-	ND	+	+, A
XV	1, D	ND	+, A, C	ND	ND	ND	ND	ND	ND	ND	+	ND

[a]*T*, Typhimurium; *D*, Dublin. [b]Includes fibrinous, suppurative, or fibrinosuppurative enteritis-colitis-typhlitis (A) or combinations thereof with epithelial necrosis (erosions/ulcers), necrotizing cryptitis (B), and microthrombosis of mucosal or submucosal blood vessels (C). [c]Includes focal or multifocal random neutrophilic/histiocytic necrotizing hepatitis (A), portal hepatitis (B), or cholestasis in the canaliculi or bile ducts (C). [d]Includes multifocal neutrophilic/histiocytic interstitial pneumonia (A), with alveolar edema or fibrinous/neutrophilic alveolitis/bronchiolitis (B), and microthrombosis in alveolar capillaries (C). [e]Includes inflammation in the lamina propria (A) with necrosis of the glandular or superficial epithelium (B). [f]Includes microhemorrhages in the mucosa/submucosa (A) and multifocal neutrophilic/histiocytic urocystitis (B). [g]Multifocal neutrophilic/histiocytic tubulointerstitial nephritis. [h]Includes meningitis (A) or fibrinous/neutrophilic perivasculitis in the rete mirabile (B). *ND*, not determined

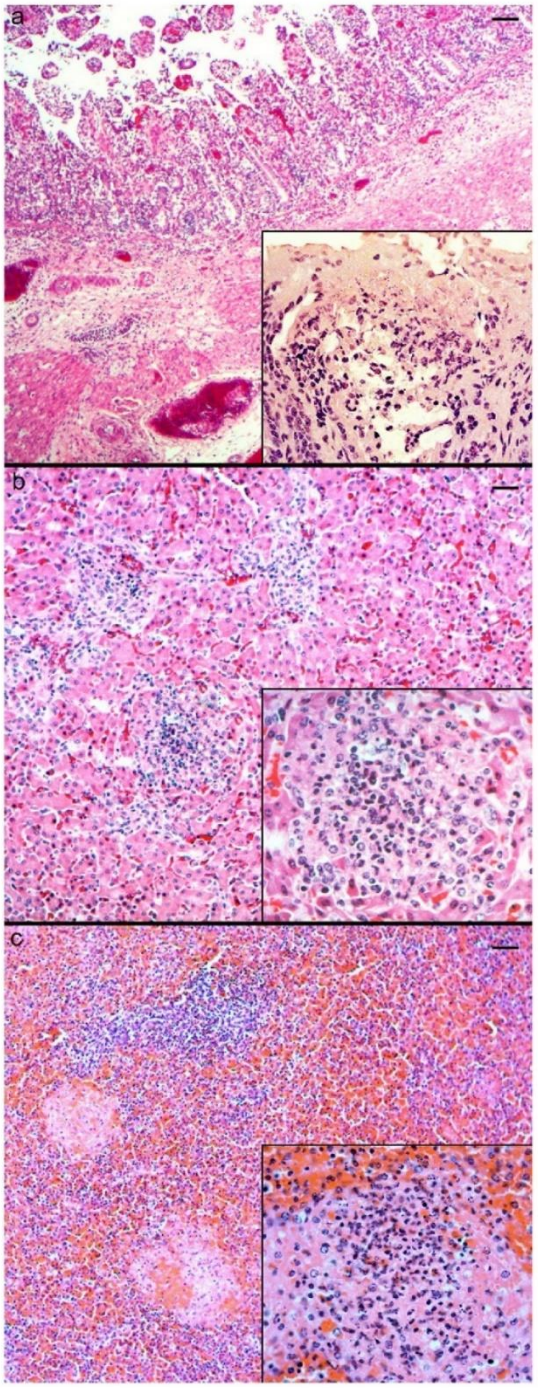

Fig. 4 Microscopic lesions in dairy calves with salmonellosis. (**a**) ▸ Calf 1, outbreak IV. The small intestinal histoarchitecture is effaced, and the lamina propria is expanded by degenerate neutrophils, proteinaceous material, and necrotic debris (inset). (**b**) Calf 1, outbreak IV. Multifocal random necrotizing hepatitis, characterized by infiltration of macrophages and neutrophils admixed with necrotic hepatocytes (inset). (**c**) Calf 1, outbreak XII. There is multifocal extravasation of fibrin entrapping macrophages and neutrophils (inset) in the red pulp of the spleen (splenitis). Hematoxylin and eosin stain. Bars = 100 µm

*sip*A, and *pag*C genes were detected in four isolates. The *spv*B gene was detected in 3 isolates, and *pef*A and *iro*N were detected in two isolates.

The genes *inv*A, *sit*C, *org*A, *sif*A, *sop*B, and *stn* were detected in 15 isolates of *S*. Typhimurium. Fourteen isolates had the *spv*B, *spa*N, *lpf*C, *sip*B, *msg*A, *pef*A, *tol*C, and *prg*H genes, while the *sip*A and *pag*C genes were present in 13 isolates. The gene *iro*N was present in only 2 isolates. Nine isolates of *S*. Typhimurium presented 16 genes, 5 isolates presented 15 genes, and 1 isolate presented 11 genes.

Analysis of virulence gene clinical signs, pathological findings, and epidemiological data

Pearson's chi-square tests were performed to determine the associations between the two virulence genes that had > 20% variability (*iro*N and *pef*A) among them and between serotypes, clinical signs, pathological findings, and these virulence genes. These two genes did not show a significant association between them. Regarding clinical signs, serotype, and gross lesions, the Typhimurium serotype was significantly associated with diarrhea ($p = 0.01$) and enteritis ($p = 0.03$), mesenteric lymphadenitis was associated with neurological signs ($p = 0.02$), and arthritis/serositis was associated with hepatitis ($p = 0.04$). When clinical signs, serotypes, and microscopic lesions (histology) were tested, the Dublin serotype was associated with tubulointerstitial nephritis ($p = 0.01$) and fibrinosuppurative splenitis ($p = 0.01$). For the clinical signs, serotype, and the two virulence genes subjected to analysis, the pefA gene was associated with the serotype Typhimurium ($p = 0.04$).

Regarding macroscopic and microscopic lesions, enteritis observed grossly was associated with microscopic meningitis or fibrinous/neutrophilic perivasculitis in the rete mirabile ($p = 0.03$), grossly visible arthritis/serositis ($p = 0.03$) and splenomegaly ($p = 0.04$) were associated with fibrinosuppurative splenitis, and gross and microscopic adrenal hemorrhage were associated ($p = 0.03$). Although microscopic enteritis and hepatitis were excluded from the statistical analysis due to low variability, all calves with grossly visible enteritis had microscopic enteritis, and all of the cases with grossly visible hepatitis had microscopic hepatitis, as expected. Regarding lesions and virulence genes, the *pef*A

Table 5 Frequency of 17 virulence genes detected in 20 isolates of *Salmonella enterica*

Gene	Function	*S.* Typhimurium	*S.* Dublin	Absolute frequency	Relative frequency (%)
*inv*A	Host recognition and invasion	15	5	20	100
*org*A		15	5	20	100
*prg*H		14	5	19	95
*tol*C		14	5	19	95
*sop*B		15	5	20	100
*lpf*C		14	5	19	95
*pef*A		14	2	16	80
*spa*N	Macrophage invasion	14	4	18	90.9
*sip*B		14	5	19	95
*iro*N	Iron acquisition	14	5	19	95
*sit*C		15	5	20	100
sifA	Filament formation	15	5	20	100
*pag*C	Intramacrophage survival	13	4	17	85
*msg*A		14	5	19	95
*spi*A		13	4	17	85
stn	*Salmonella* enterotoxin	15	5	20	100
spvB	Growth within host	14	3	17	85

gene was associated with macroscopic enteritis ($p=0.03$) and with microscopic fibrinosuppurative splenitis ($p=0.04$).

Univariable generalized linear regression analyses revealed that the Typhimurium serotype was associated with diarrhea (OR = 26, 95% CI 2–695, $p=0.01$). The Dublin serotype was associated with tubulointerstitial nephritis (OR = 48, 95% CI = 3.5–1979, $p=0.01$). Finally, the *pef*A gene was associated with the Typhimurium serotype (OR = 21, 95% CI 2–578, $p=0.03$).

Discussion

Calf morbidity and mortality lead to significant productive and economic losses in dairy farms [25]. Morbidity and mortality values of 25% and 5%, respectively, have been regarded as acceptable in dairy calf rearing systems [26]. However, in Uruguay, the annual dairy calf mortality risk between birth and weaning is 15.2%, with neonatal diarrhea and respiratory disease being the main farmer-reported clinical signs shown by the calves before death [27].

Salmonella enterica is one of the main causative agents of enteritis, septicemia, and respiratory disease in rearing calves [28, 29]. In the 15 outbreaks of salmonellosis evaluated in this manuscript, the average morbidity, mortality, and lethality risks were 35.3%, 19.9%, and 65.6%, respectively, which exceed acceptable values and the average mortality risk reported in 225 dairy farms in Uruguay [27]. This finding is in concert with a previous study by our group in which enteric *S. enterica* infection was associated with an increased risk of mortality, especially in diarrheic calves [30], and provides evidence for the virulence of the acting strains. Morbidity, mortality, and lethality risks did not differ significantly in outbreaks caused by either serotype in our study.

The clinical signs of salmonellosis in calves can vary from diarrhea, fever, loss of appetite, respiratory signs, encephalitis, swollen joints, gangrene of the limbs, or tips of the pinnae, to decreased production (reduced weight gain or weight loss) [31]. In this document, the reported clinical signs were mainly diarrhea and, to a lesser extent, respiratory and nervous signs. However, in Uruguay, health data recording in dairy farms is generally scarce [27], and therefore, there might have been other unreported signs. Our results indicate that when digestive disease or diarrhea are the reasons for consultation or the main clinical sign shown by the calves before dying, *S.* Typhimurium is the most likely serotype involved, especially in younger calves. Possibly, the adaptations of the digestive tract of neonates and the action of this serotype favor this presentation. The Dublin serotype was detected in 5 calves of 50 to 60 days of age from 4 outbreaks in which sudden death was the main reason for consultation. Recently, septicemic salmonellosis caused by *S.* Dublin in 1- to 3-month-old calves was described in Uruguay and Brazil [8, 32, 33]. On the other hand, in the pathogenesis of digestive disease caused by Typhimurium serovar, animals show an enteric inflammatory process that ultimately leads to diarrhea [34]. In this study, 13 cases of 9 outbreaks in which the Typhimurium serotype was identified presented diarrhea as the main clinical manifestation. However, septicemic salmonellosis was caused by both serotypes.

In the present study, the majority of macroscopic lesions observed at autopsy were highly suggestive of salmonellosis. In *S. enterica*-related outbreaks, macroscopic changes are observed mainly in the intestine, mesenteric lymph nodes, liver/gallbladder, spleen, and lungs, although lesions are not necessarily restricted to these organs [12]. In our study, the presence of enteritis grossly was associated with the Typhimurium serotype, and thus, finding this lesion upon autopsy should raise a suspicion of *S.* Typhimurium, especially in younger calves with diarrhea. In the present study, lesions were frequent in the mesenteric lymph nodes, liver, and lungs, while isolates were obtained from the same tissues and the intestinal contents. Sporadically, the kidneys, gall bladder/bile, spleen, cecum, ileum, and urinary bladder were also affected. By virtue of the main lesions found in our study and by Pecoraro et al. [17], we suggest that liver, mesenteric lymph node, ileum, cecum, bile, and lung samples are the most important for successful bacteriological culture. Similar samples were proposed by Holschbach and Peek [31]. In accordance with Guizelini et al. [32] and Ramos et al. [35], the main macroscopic lesions observed in our work were enteritis with or without fibrin, mesenteric lymphadenomegaly, and hepatomegaly. In contrast to what was reported by Pecoraro et al. [17], in our work, macroscopic lesions in the lungs and spleen were less frequent. Additionally, in all *S.* Dublin cases, macroscopic intestinal lesions were absent, and calves did not show diarrhea except for one case. Similar results were observed by Guizelini et al. [32] in Brazil. Assays performed with this serotype have shown that the aflagellated types isolated from the bloodstream can generate a lower intestinal inflammatory response and increased invasiveness [9].

Upon histologic examination, we observed enterocolitis, enterotyphlitis, typhlocolitis, or enterotyphlocolitis with necrotizing cryptitis as the most frequent lesion in calves infected with either serotype. Inflammatory activity and cytokine release at the intestinal level increase the process of cell injury, dehydration, and diarrhea that can, ultimately, lead to the death of the affected animals [31, 36]. These processes demonstrate the first contact of the bacteria with the tissues and the neutrophilic infiltrate, the response for preventing its spread. At a microscopic level, we confirmed the systemic process by detecting random necrosis in the liver and/or portal hepatitis, interstitial pneumonia, fibrinosuppurative splenitis and mesenteric lymphadenitis, and/or meningitis or inflammatory lesions in the rete mirabile. We also observed adrenocortical hemorrhage for both *S.* Dublin and *S.* Typhimurium serotypes, which is generally associated with acute infectious processes and circulatory imbalances and is often referred to as Waterhouse-Friderichsen syndrome [37]. The bacteria reach the adrenal gland, affecting the blood vessels with the consequent hemorrhage and failure of the gland [38]. We observed ulcerative abomasitis with abomasal edema. Abomasitis is a clinical syndrome that is present in lactating calves with the possible involvement of species of *Clostridium*, including the former *Sarcina* sp. and *S.* Typhimurium DT104 [39, 40], but in our work, none of our strains were DT104 (data not shown). However, to date, the implications of these agents remain unknown, and a multifactorial etiology has been postulated [41]. Hemorrhages in the urinary bladder, urocystitis, and tubulointerstitial nephritis have been described occasionally in calves that died from *S.* Dublin septicemia [8]. In our current work, *S.* Dublin was significantly associated with tubulointerstitial nephritis and fibrinosuppurative splenitis. These lesions, especially in older age with sudden death, should raise a suspicion for the Dublin serotype.

The molecular mechanisms by which *Salmonella* produces lesions have been studied [18, 42, 43]. Depending upon the stage of pathogenesis, different genetic mechanisms are involved in intestinal and systemic disease. Various environmental mechanisms activate and upregulate SPI [44] to achieve colonization, invasion, and spread into the host [45]. The pathogenicity islands SPI-1 and SPI-2 encode a type III secretion system (TTSS) that translocates effector proteins into host cells [46]. In general, SPI-1 genes are activated in the initial contact of the bacterium with host cells and are responsible for cell invasion, host immune and inflammatory responses, recruitment and apoptosis of immune cells, and the formation of biofilms [46, 47]. This study is the first to describe the frequency of a relatively large set of 17 virulence genes in *S.* Dublin and *S.* Typhimurium, isolated from fatal disease in cattle. These strains present vast virulence mechanisms to act in the different stages of infection. We detected a high frequency of the *inv*A, *prg*H, *org*A *spa*N, and *sip*B genes involved in the type III secretion complex, which are carried by most of the isolates studied from chickens, turkeys, and pigs [21, 48]. Most of these genes encode structural proteins that take part in channel formation and protein exportation [46, 49], which in the end allow the effector proteins of the secretion system to enter the cytosol [50].

On the other hand, as previously indicated, enteric lesions were one of the most frequent findings in our study. Many of the SPI-1-encoded proteins act on the host inflammatory response by inhibiting macrophage proinflammatory cytokine expression and leukocyte activity and inducing diarrhea [51]. The *sip*B gene was detected at high frequency. This gene is necessary to induce caspase-1-dependent macrophage pyroptosis and interleukin-18 release [52–54]. In addition, we detected a high frequency of *sop*B, a gene that promotes the secretion of fluids and chloride, induces inflammation and diarrhea, acts on polymorphonuclear cells [55–58], has antiapoptotic activity, and is related to intracellular replication [59] and damage to the epithelial barrier during intestinal invasion [60].

We also found the *tol*C gene in high frequency. This gene is part of an efflux system called AcrAB-TolC, which is necessary for the adhesion and invasion of epithelial cells. Furthermore, it confers innate resistance to a wide range of toxic substances, including tetracyclines [61] and fluoroquinolones, artificial and natural colorants, disinfectants, and detergents such as bile. When evaluating the antimicrobial resistance of the strains included in this work, we detected resistance to tetracycline and intermediate resistance to ciprofloxacin in 80% and 55% of the isolates, respectively [7]. In this context, in which efflux pumps participate in virulence through *Salmonella* adhesion and invasion, a more in-depth study is warranted to detect the implication of this mechanism in resistance to certain antibiotics.

The *lpf*C and *pef*A genes act in the first phase of contact and union of the bacterium with the intestine [49]. The *lpf*C gene, along with the bovine colonization factor (Bcf) necessary for colonization of bovine Peyer's patches, is required for long-term intestinal persistence of *S.* Typhimurium [62]. Accordingly, it would be appropriate to evaluate the presence of the *bcf* gene and determine if it could explain disease recurrence.

In the second stage of the infection process, the genes present in SPI-2 are expressed. The *sif*A gene stabilizes the *Salmonella*-containing vacuole (SCV) membrane, along with SPI-2 genes and persistent gene products in the cell cytosol derived from SPI-1, triggers intracellular events, and together contributes to establishing a niche of *Salmonella* in macrophages [49]. Associated with this mechanism, *pag*C and *msg*A are encoded in small islets of pathogenicity [63], detected together with *spi*A in 85% to 95% of our isolates. Additionally, the *spi*A gene encoded by SPI-2 regulates the secretion of proteins translocated under conditions that simulate the vacuolar environment and interferes with gallbladder trafficking, intracellular bacterial proliferation, and secretion [49].

Siderophores are bacterial molecules that carry iron and are important for bacterial growth in serum in the extracellular stage of systemic infection. *Salmonella* produces two siderophores, salmochelin and enterobactin. Salmochelin requires two genetic loci for a correct function, and the *fep* gene group and the iro operon comprise five genes. The *iro*N gene is one of the last to be expressed and encodes a membrane receptor. In this work, we found a high frequency of the *iro*N gene. Regardless of the presence of this gene, bacteria can acquire nutrients through various mechanisms. One of them is the *sit*ABCD operon, present in every isolate of our collection that is induced in iron-deficient conditions after invasion of the intestinal epithelium [64, 65]. The iron operon sit system might be the mechanism that allows *Salmonella* to overcome iron deficiency and establish an infection. The *spv*B gene, found in a very transmissible plasmid in *Salmonella*, was detected in almost every isolate of this work. Its presence is associated with the inhibition of autophagy, a mechanism of the immune system that is frequently used by the host to eliminate the pathogen [66]. The proteins they encode are translocated to the cell by the TTSS of SPI-2. The protein that encodes *spv*B prevents actin polymerization, destabilizes the cytoskeleton of infected cells, modulates the maturation and positioning of SCV, and induces apoptosis [67]. The plasmid carries four highly conserved genes (ABCDs) and correlates with greater virulence of the strains [68, 69].

The *stn* gene was detected in every isolate of the collection, as reported before. Widely distributed in *Salmonella*, it has been proposed as a target for diagnosis [70]. It acts in the early stages of pathogenicity of bacteria, causing diarrhea [71], and helps maintain the composition and integrity of the membrane [72]. Despite these facts, there is still no agreement on its role in virulence.

Overall, the strains of the two serotypes we studied have similar virulence genes that fulfill different structural or effector functions. In this sense, it could be inferred that the strains that affected the calves could belong to a common strain of *Salmonella* circulating in the study region, although further research is needed to elucidate this possibility. The use of new techniques such as single nucleotide polymorphisms (SNPs) has allowed the detection of genetic variants associated with diseases. Currently, their importance is widely recognized and justifies applying these molecular techniques to the study of virulence genes present in strains [73].

As previously described, in addition to observing a high frequency of diarrhea, we observed a significant association between *S.* Typhimurium with diarrhea and enteritis and the *pef*A gene and the Typhimurium serotype. This gene is located in plasmids [49]. The association between the Typhimurium serotype and the *pef*A gene could explain the high frequency of diarrhea in the presence of this serotype since this gene allows bacterial adhesion to intestinal epithelial cells. In this work, only 2/5 isolates of *S.* Dublin obtained from 5 calves had the *pef*A gene. This finding could be due to the presence of plasmids that carry the gene.

Both serotypes can cause systemic infection. In this work, we identified an association between mesenteric lymphadenitis and neurological signs and between gross enteritis and microscopic meningitis or fibrinous/neutrophilic perivasculitis in the rete mirabile, which indicates that *Salmonella* can spread from an intestinal location and alter the integrity of the blood–brain barrier, presumably through inflammatory mechanisms [74]. In this work, arthritis/serositis was associated with hepatitis and splenomegaly with fibrinosuppurative splenitis. Cytokine production in bacterial infections is rigorously balanced [75]. Th17 lymphocytes present in intestinal inflammation due to *Salmonella* in humans [76] play an important role

in the development of reactive arthritis due to the production of interleukin 17, which has receptors at the joint level [77–79]. On the other hand, the deficiency of this interleukin is associated with the production of granulomas in different organs due to intracellular pathogens such as *Salmonella*, and its downregulation was associated with the absence of sepsis and lesions [73]. These associations could explain the findings of this work. Immune reactions and their balance play an important role in *Salmonella* infection. Considering the breadth of its function, it would be appropriate to evaluate its behavior in a bovine model.

In our study, as age increased, calves were less likely to present diarrhea and be infected with *S.* Typhimurium, which could be related to the immunological development of calves, as well as the physiological development of the rumen, the establishment of microorganisms in the rumen and intestine and the reduction in abomasal pH [80]. In addition, as age increased, calves were more likely to be infected with *S.* Dublin. Segall and Lindberg (1991) [81] suggested that this finding could be linked to factors that develop in calves over time, but there are no studies to date that explain this finding, and more investigation is needed.

Conclusions

Salmonellosis represents an important cause of death in dairy calves in Uruguay. In 15 outbreaks evaluated due to *S.* Dublin and *S.* Typhimurium, the epidemiological data on morbidity, mortality, and lethality obtained far exceed acceptable production values. Diarrhea followed by respiratory and nervous signs were the main clinical manifestations. The virulence genes repertoire was similar in *S.* Dublin and *S.* Typhimurium, most genes, but *pef*A and *spv*B, were shared in both. *Salmonella* Typhimurium was observed to be related to the manifestation of diarrhea and associated with macroscopic enteritis and the *pef*A gene in calves less than 30 days old and *S.* Dublin with sudden death in calves at approximately 50 days, mostly without intestinal lesions. Microscopically, enterocolitis, enterotyphlitis, typhlocolitis, or enterotyphlocolitis with necrotizing cryptitis was the most frequent lesion in calves infected with any of the serotypes, and tubulointerstitial nephritis and fibrinosuppurative splenitis were significantly associated with *S.* Dublin. We describe for the first time a high frequency of 17 virulence genes in bovine *S. enterica* Typhimurium and Dublin strains. Characterization of the virulence genes of *Salmonella* in cattle and their association with lesions is still scarce. The inclusion of a greater number of isolates could shed light on the virulence battery of *Salmonella*, which could eventually allow us to make associations with clinicopathological manifestations and epidemiological data.

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Author contribution MLC, MF, and FG conceived the study. MLC, COS, RAC, MMR, RDC, CSS, VA, BDD, and FG performed the autopsy. MLC performed the bacteriological and molecular laboratory tests. COS, RAC, MMR, CSS, VA, BDD, and FG performed the microscopic analysis. WSN and FG analyzed data. MLC, MF, and FG wrote the first draft and final version of the manuscript. All authors read, edited, and approved the final version of the manuscript.

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Data availability The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Code availability Not applicable.

Declarations

Ethics approval Not applicable.

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Conflict of interest The authors declare no competing interests.

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CAPÍTULO II

Caracterización fenotípica y genotípica de una colección de aislamientos de *Salmonella enterica* de origen bovino

INTRODUCCIÓN

En el mundo, la resistencia a los antimicrobianos se ha incrementado en las últimas décadas de forma tal que se considera y se proyecta como una de las principales causas de muerte hacia el 2050 (Antimicrobial Resistance Collaborators, 2022). La aparición global de genes de resistencia en bacterias que anteriormente no se reportaban ha llevado a los expertos a tomar la decisión de clasificar los antibióticos en 3 categorías 1) de acceso, 2) precaución y 3) de último recurso, con el fin utilizar los mismos de forma adecuada y reservar los de último recurso para obtener efectividad en las infecciones multirresistentes (WHO, 2017).

La utilización y la resistencia a los antibióticos en animales varía de acuerdo con la especie animal que se evalúe. En producción animal la utilización de antibióticos presenta diferentes usos: la utilidad terapéutica para el tratamiento de enfermedades infecciosas, el uso profiláctico para la prevención de enfermedades, la metafiláxis utilizada en veterinaria para un grupo de animales cuando al menos uno presenta infección diagnosticada y el uso como promotores de crecimiento a dosis subterapéuticas (Simjee, & Ippolito, 2021).

Particularmente en bovinos, los reportes de la resistencia a los antibióticos varían de acuerdo con el área geográfica de estudio y el tipo de producción. Generalmente los informes que implican la resistencia de bacterias de origen bovino son escasos en países de mediano y bajo ingreso. Abundantes reportes existen sobre el perfil de susceptibilidad a los antibióticos en aislamientos bacterianos obtenidos a partir de muestras de granjas lecheras en el hemisferio norte. Específicamente los estudios sobre la resistencia observada en *Salmonella* son diversos y varían de acuerdo con el serotipo actuante. El serotipo Dublin, Typhimurium, Newport y Cerro lideran las afecciones en bovinos y presentan variaciones de predominio de acuerdo con el periodo de estudio (Holschbach & Peek, 2018).

El tratamiento antibiótico de elección para la salmonelosis bovina consiste en la utilización de ampicilina y sulfas (Constable et al. 2017). En *S.* Typhimurium la resistencia se observa en los antibióticos ampicilina, cloranfenicol, estreptomicina, sulfametoxazol y tetraciclina. Esta multirresistencia también esta descripta para el serotipo Dublin en múltiples granjas (Otto et al. 2018, Srednik et al. 2021).

De forma frecuente, la monitorización del incremento de bacterias resistentes lleva a la modificación en la utilidad de los antibióticos reservando aquellos considerados críticos. Dentro de estos se encuentran las quinolonas, cefalosporinas de 3° y 4° generación, macrólidos y aminoglucósidos, todos también utilizados en infecciones bacterianas en bovinos.

La transferencia de la resistencia desde bacterias de origen animal a otras bacterias que afectan al hombre o la infección de este a través del contacto directo o por medio del consumo de alimentos contaminados derivados de animales está por demás reportada (Ehuwa et al. 2021, CDC, 2019). A causa de esto, es necesario llevar adelante los estudios para determinar el estatus de la región de acuerdo con las infecciones en bovinos a causa de *Salmonella* y determinar el perfil de susceptibilidad a los antibióticos que poseen.

JUSTIFICACIÓN

Las afecciones por *Salmonella enterica* en rodeo bovino uruguayo son una de las principales causas de diarrea neonatal y es el único agente asociado a mortalidad de terneros en la región. Conocer las características fenotípicas y genotípicas de la RAM en este patógeno, no sólo brinda una herramienta para el tratamiento de las infecciones por *Salmonella*, permite obtener datos epidemiológicos y el estatus sanitario de los rodeos, sino que también permite brindar información para desarrollar potenciales herramientas del control de la salmonelosis bovina de Uruguay.

OBJETIVOS

Caracterizar una colección de 75 aislamientos de *Salmonella* representantes de los brotes de salmonelosis bovina de Uruguay, de acuerdo al perfil de susceptibilidad a los antibióticos y la colección de genes que confieren resistencia a los mismos, así como los elementos genéticos movilizables implicados en la resistencia.

RESULTADOS

El producto de este capítulo es el artículo **“Phenotypic and genotypic survey of antibiotic resistance in *Salmonella enterica* isolates from dairy farms in Uruguay”** el cual incluye los resultados fenotípicos y genómicos de la resistencia a los antimicrobianos de 75 aislamientos de *Salmonella* y detección de elementos genéticos movilizables asociados a la misma. El mismo fue publicado en la revista Frontiers in Veterinary Science. Se adjunta el original doi: https://doi.org/10.3389/fvets.2023.1055432

ARTÍCULO CIENTÍFICO CAPÍTULO II

RESUMEN EN ESPAÑOL

Salmonella enterica es un importante patógeno zoonótico que se identifica con frecuencia en los sistemas de producción lechera. El aumento de la resistencia a los antibióticos ha conducido a resultados inadecuados de los tratamientos, con repercusiones en la salud animal y humana. En este trabajo se evaluaron y compararon los patrones de susceptibilidad fenotípica y genotípica de aislados de *Salmonella* procedentes de ganado vacuno lechero y de entornos de explotaciones lecheras. Se evaluó una colección de 75 aislados de *S.* enterica y se determinó su susceptibilidad fenotípica. Para la caracterización genómica, se secuenciaron los genomas completos de los aislados y se determinaron los serotipos, los secuenciotipos (ST)) mediante el *core genome* (cgMLST) utilizando las herramientas de EnteroBase (Zhoue et al. 2021. https://enterobase.warwick.ac.uk/). Para caracterizar los genes de resistencia a los antibióticos y las mutaciones génicas, se utilizaron herramientas del *Center for Genomic Epidemiology* (Bortolaia et al. 2020, https://www.genomicepidemiology.org/*)*. Se incluyeron en la colección *Salmonella* Dublin (SDu), *S.* Typhimurium (STy), *S.* Anatum (SAn), *S.* Newport (SNe), *S.* Agona (Sag), *S.* Montevideo (SMo) y IIIb 61:i:z53. Se detectó un único tipo de secuencia por serovar utilizando el esquema de MLST de 7 genes. Los aislamientos no susceptibles fenotípicamente a la estreptomicina, tetraciclina y ciprofloxacina fueron muy frecuentes en la colección. Se observó resistencia a múltiples fármacos (MDR) en 42 aislados (56,0%), presentando SAn y STy mayor MDR que los otros serovares, mostrando no susceptibilidad a hasta 6 grupos de antibióticos. El análisis genómico reveló la presencia de 21 genes asociados a la resistencia a los antimicrobianos (RAM) en aislados de *Salmonella*. Más del 60% de los aislados portaban algún gen asociado a la resistencia a los aminoglucósidos y las tetraciclinas. Sólo se encontró un gen asociado a la resistencia a β-lactámicos, en siete aislados. Se identificaron dos mutaciones diferentes, parC_T57S y acrB_R717Q, que confieren resistencia a quinolonas y azitromicina, respectivamente. La precisión de la predicción de los fenotipos de resistencia a los antimicrobianos basada en los genotipos de AMR fue del 83,7%. El enfoque genómico no sustituye al ensayo fenotípico, pero ofrece información valiosa para el estudio de la resistencia antimicrobiana circulante. Este trabajo representa uno de los primeros estudios que evalúan la RAM fenotípica y genotípica en *Salmonella* del ganado lechero de Sudamérica.

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Phenotypic and genotypic survey of antibiotic resistance in *Salmonella enterica* isolates from dairy farms in Uruguay

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Salmonella enterica is an important zoonotic pathogen that is frequently identified in dairy farming systems. An increase in antibiotic resistance has led to inadequate results of treatments, with impacts on animal and human health. Here, the phenotypic and genotypic susceptibility patterns of *Salmonella* isolates from dairy cattle and dairy farm environments were evaluated and compared. A collection of 75 *S. enterica* isolates were evaluated, and their phenotypic susceptibility was determined. For genotypic characterization, the whole genomes of the isolates were sequenced, and geno-serotypes, sequence types (STs) and core-genome-sequence types were determined using the EnteroBase pipeline. To characterize antibiotic resistance genes and gene mutations, tools from the Center for Genomic Epidemiology were used. *Salmonella* Dublin (SDu), *S.* Typhimurium (STy), *S.* Anatum (SAn), *S.* Newport (SNe), *S.* Agona (Sag), *S.* Montevideo (SMo) and IIIb 61:i:z53 were included in the collection. A single sequence type was detected per serovar. Phenotypic non-susceptibility to streptomycin and tetracycline was very frequent in the collection, and high non-susceptibility to ciprofloxacin was also observed. Multidrug resistance (MDR) was observed in 42 isolates (56.0%), with SAn and STy presenting higher MDR than the other serovars, showing non-susceptibility to up to 6 groups of antibiotics. Genomic analysis revealed the presence of 21 genes associated with antimicrobial resistance (AMR) in *Salmonella* isolates. More than 60% of the isolates carried some gene associated with resistance to aminoglycosides and tetracyclines. Only one gene associated with beta-lactam resistance was found, in seven isolates. Two different mutations were identified, *parC_T57S* and acrB_R717Q, which confer resistance to quinolones and azithromycin, respectively. The accuracy of predicting antimicrobial resistance phenotypes based on AMR genotypes was 83.7%. The genomic approach does not replace the phenotypic assay but offers valuable information for the survey of circulating antimicrobial resistance. This work represents one of the first studies evaluating phenotypic and genotypic AMR in *Salmonella* from dairy cattle in South America.

KEYWORDS

bovine salmonellosis, antimicrobial resistance, calves, WGS, genotyping

1. Introduction

Salmonella enterica subspecies *enterica* (from now *S. enterica*) is one of the main pathogens affecting humans and animals and can cause various symptoms and illnesses, ranging from self-limiting acute gastroenteritis to septicemia and death (1, 2). Humans can be affected by typhoidal and non-typhoidal *Salmonella*. Typhoidal serotypes include invasive *Salmonella* Typhi and Paratyphi, which cause enteric fever (3). On the other hand, non-typhoidal *Salmonella* can cause enteric or invasive salmonellosis and is a major cause of morbidity and mortality globally (4). Many *Salmonella* infections in humans are due to the consumption of contaminated food, including meat of bovine origin, since this is a potential reservoir of the pathogen (5). *Salmonella* is now considered to cause more than 1 million infections annually in the US and is among the leading causes of foodborne illness (6). Animals are affected by non-typhoid serotypes such as Abortusovis, Dublin, and Gallinarum, which are adapted to sheep, cattle and poultry hosts, respectively, as well as other ubiquitous serotypes such as *S.* Typhimurium and *S.* Enteritidis (7).

In cattle, non-typhoid serotypes are capable of causing systemic disease similar to typhoid fever in humans (8). Salmonellosis in these animals is characterized by a more frequent occurrence in intensive farming systems. The disease varies according to different factors, such as the serotype involved, and the age, physiological and immune status of the host (9). *Salmonella* Dublin is the serotype adapted to cattle and can cause septicemia and abortions. *Salmonella* Typhimurium is frequently identified in calving systems and, in addition to enteric disease, can cause invasive diseases (10, 11). Both serotypes can rapidly spread in a herd, causing mortality and presenting a challenge in disease management (9).

Antibiotics are one of the main tools for treating bacterial diseases. However, in recent decades, the increase in AMR has led to inadequate treatment results, driving the search for new therapeutic options (12). When treating salmonellosis, antibiotics are indicated when the risk of systemic infection is increased, as in immunocompromised persons (13). Ampicillin and trimethoprim-sulfamethoxazole are used for the treatment of bovine salmonellosis, and fluoroquinolones and β-lactams are used for human treatment (14). However, the effectiveness of these antibiotics has decreased, mainly due to antibiotic resistance, which is a trend that is increasing in this bacterial genus and others (15, 16).

Plasmids are circular or linear extrachromosomal genetic elements present in various organisms (17). *Salmonella* serovars cause infections in animals and humans and may carry plasmids conferring virulence and AMR with particular biological properties that are subsequently expressed in the host (18). Within plasmids, antibiotic resistance genes are generally located in transposons. In addition, integrons are involved in the recruitment and expression of antimicrobial resistance genes (19).

In bacteria of animal origin, AMR varies among regions of the world. In the USA, *S. enterica* of bovine origin commonly shows resistance to cephalosporins and quinolones (20), while in Canada, the most common AMR pattern is resistance to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole and tetracycline (ACSSuT) (21). Additionally, in South America, the AMR *Salmonella* isolates obtained from calves differ among regions. In northeastern Brazil, the main type of resistance observed is to cefotaxime (22), whereas in southeastern Brazil, nalidixic acid and cefoxitin resistance is primarily observed to, with a high frequency of susceptibility among strains (23). In Uruguay, the main phenotypic resistance observed is to streptomycin, tetracyclines and ampicillin, with differences in susceptibility between serotypes, where Dublin is more susceptible to antibiotics than Typhimurium (24).

In recent years, the complete sequencing of microorganism genomes has made it possible to approach their study such that the information available about them from different approaches, such as clinical diagnosis, epidemiology and research, has increased considerably (25, 26). There are currently several bioinformatics tools for the analysis of complete genomes. For AMR investigation, there are specialized databases with information about resistance genes and mutations. These databases are generally free to access and are periodically updated (27). Although genome-wide analysis does not replace phenotypic methods of resistance analysis, there is a high degree of correlation between the two approaches, and genome-wide analysis is a useful complement for the identification of new mechanisms and relationships between isolates (28).

Studies characterizing bovine-derived *Salmonella* strains are scarce in our country. Hence, the objective of this work is to study the presence and distribution of resistance genes, plasmids and integrons using different bioinformatic evaluation systems in a diverse sample of *Salmonella* strains of bovine origin from Uruguay.

2. Materials and methods

2.1. Strain collection

The study was conducted with 75 strains of *S. enterica* obtained from 2016 to 2020 at the Plataforma de Investigación en Salud Animal—Instituto Nacional de Investigación Agropecuaria, La Estanzuela, Uruguay. The collection was represented by 75 isolates obtained from stool samples collected from 35 calves, 5 cows, and 1 heifer; 25 organ samples from autopsied animals; 5 samples from the dairy farm environment; 1 food sample; 1 udder swab sample from milking cows; 1 drinking water sample; and 1 bovine fetus autopsy sample. The fecal isolates came from 26 calves < 20 days old and 7 calves between 20 and 30 days old. There were also two isolates from calves aged 65 and 114 days. The cows from which *Salmonella* isolates were obtained from feces were between 2 and 4 years old, and the heifers were 1 year old. The calves had diarrhea in all cases, and the cows were pregnant. The calves from which samples of organs and fluids were obtained had ages of <10 days in 7 cases, between 11 and 20 days in 7 cases, between 21 and 35 days in 3 cases, between 45 and 60 days in 4 cases, and between 80 and 90 days in 3 cases (Supplementary Table 1).

2.2. *Salmonella* collection, maintenance and growth

For the isolation of *Salmonella* spp. from different samples collected, the procedures described by Casaux et al. (24) were used.

The strains were conserved in 20% glycerol and stored at −80°C. For reuse, they were cultivated in trypticase soy agar at 37°C for 18–24 h.

2.3. Antimicrobial susceptibility testing

The antibiotic susceptibility test was performed using the Kirby Bauer disc diffusion method (29). The antibiotics evaluated were ampicillin (AMP, 10 μg), amoxicillin-clavulanic acid (AMC 20/10 μg), cefotaxime (CTX 30 μg), sulfamethoxazole/trimethoprim (SXT 23.75/1.25 μg), ciprofloxacin (CIP 5 μg), enrofloxacin (ENR 5 μg), chloramphenicol (C 30 μg), streptomycin (S 10 μg), gentamicin (CN 10 μg), tetracycline (TE 30 μg), nitrofurantoin (NF 300 μg), and azithromycin (AZT 15 μg). *E. coli* ATCC 25,922 and *E. coli* ATCC 35,218 were used for quality control. The results were interpreted according to Clinical and Laboratory Standards Institute (31), and to facilitate the description of the results, both resistant and intermediate results were classified as "not susceptible." For enrofloxacin, the CLSI guidelines for bacteria isolated from animals were used (30) (Supplementary Table 1). In addition, multidrug-resistant isolates (MDR) were considered when the isolates showed non-susceptibility to 3 or more groups of antibiotics (32).

2.4. Whole-genome sequencing

The 75 isolates were sent to the sequencing facility of MicrobesNG (Birmingham, UK, http://www.microbesng.com) for whole-genome sequencing. Standard sequencing services were provided by the company. Genomic DNA libraries were prepared with the Nextera XT Library Prep Kit (Illumina, San Diego, USA) according to the manufacturer's protocol with the following modifications: input DNA was increased 2-fold, and the PCR elongation time was increased to 45 s. DNA quantification and library preparation were carried out on a Hamilton Microlab STAR automated liquid handling system (Hamilton Bonaduz AG, Switzerland). The pooled libraries were quantified using the Kapa Biosystems Library Quantification Kit for Illumina. Sequencing was performed with Illumina sequencers (HiSeq/NovaSeq) using a 250 bp paired-end protocol. Adapters were trimmed from the reads using Trimmomatic 0.30 with a sliding window quality cutoff of Q15 (33). *De novo* assembly was performed using SPAdes version 3.7 (34), and contigs were annotated using Prokka 1.11 (35).

2.5. Genoserotyping, MLST and core genome MLST

The genome sequences of the *Salmonella* collection were analyzed using the EnteroBase platform. The serotype was determined using the SISTR1 + SeqSero2 tools (36–38). The core-genome MLST V2 experimental scheme (cgMLST V2) was used to obtain the value of the sequence type (cgST), and the Achtman MLST scheme was used for the allelic profile and the sequence type (ST) (39) (Supplementary Table 1).

2.6. Antimicrobial resistance genes, mutations and plasmids

To identify AMR genes and to chromosomal point mutations, we used ResFinder and Point Finder 4.1 (40–42) from the Center for Genomic Epidemiology (CGE). For this analysis, we selected a threshold of 90% for the sequence ID and 80% for the minimum length coverage. Manual curation was performed in those cases where identification was not univocal and the gene sequence could contain a coding sequence disruption leading to a pseudogene. We also used the PlasmidFinder 2.1 (43) and IntFinder 1.0 (44) tools to identify plasmids and integrons. For PlasmidFinder, we used thresholds of 95 and 60% for identity and coverage, respectively, and for IntFinder, we used a threshold of 90% for both identity and coverage.

2.7. Correlation between phenotypic and genotypic resistance profiles

The phenotypic results of the antimicrobial susceptibility were used test as a reference and were correlated with the presence or absence of the resistance and/or mutation genes detected from the evaluation of the complete genome considering a resistant isolate. For each group of antibiotics, concordance was considered to exist for those results with a not susceptible phenotype for which at least one gene or mutation explained that finding, and concordance with phenotypic susceptibility was considered to exist when no corresponding gene or mutation was found. Sensitivity (Se), specificity (Sp), positive predictive values (PPVs), negative predictive values (NPVs), and accuracy were calculated according to Trevethan (45).

2.8. Phylogenetic analyses

The serovars SAn, SDu, Sty, and SNe were chosen to perform the phylogenetic analysis, as they were collected from more diverse sources and included more isolates for comparison. For each of these serovars, a phylogenetic tree was generated using different reference genomes: Anatum—USDA-ARS-USMARC-1728 (NCBI accession NZ_CP014664.1), Dublin—CT_02021853 (NCBI accession CP001144.1), Typhimurium—LT2 (NCBI accession NC_003197.2), and Newport 0007-33 (NCBI accession NZ_CP013685.1).

Snippy software (version 4.6.0) (Torsten Seemann. https://github.com/tseemann/snippy) was used to map the trimmed reads to the reference genome and obtain a whole-genome alignment. Snippy was run with the default parameters, and the snippy-core script was run to generate the whole-genome alignment file (.full.aln). This file was then used as the input for Gubbins software (version 3.1.6) to produce a new alignment, from which the regions of high polymorphism density (probable regions of recombination) were removed (46). Gubbins was used with the following options: –threads 8, –first_tree-biulder fasttree, –tree-builder raxmlng, and –first_model JC.

Whole-genome phylogenies were produced using RAxML-NG software (version 1.1.0) based on the filtered_polymorphic_sites files (obtained in the previous step). RAxML was used with the following options: –model GTR + G, –seed 3 and –bs-metric fbp (47). The branch support values were transferred to the best-scoring tree of each sample by the RAxML-NG pipeline. Bootstrapping converged after 50, 250, and 996 replicates for SDu, Sty, and SNe, respectively. For SAn, 1,000 replicates were performed, but convergence was not reached because of the very low divergence of the sequences.

Phylogenetic trees with annotations were generated using the online tool iTOL v5 (https://itol.embl.de/) (48). The final tree figures were edited with the software Inkscape 0.91 (https://inkscape.org/).

3. Results

3.1. Determination of serotypes by WGS

Seven serotypes were identified after analyzing the genomes of the 75 collected isolates. The greatest number of isolates (32) were classified as *S.* Typhimurium, 24 as *S.* Newport, 11 as *S.* Anatum, 6 as *S.* Dublin, 1 as *S.* Agona, 1 as *S.* Montevideo and 1 as IIIb 61:i:z53. Each serotype was represented by a single sequence type (ST). ST10, ST19, ST45, and ST64 were detected in all the isolates of *S.* Dublin, *S.* Typhimurium, *S.* Newport or *S.* Anatum, respectively. In addition, ST13 corresponded to *S.* Agona, ST138 corresponded to *S.* Montevideo and ST430 corresponded to the strain serotyped as IIIb 61:i:z53 were detected identified. Among a total of 31 *S.* Typhimurium genomes, 24 different cgMLSTs were distributed on 26 different farms. A farm with 24 isolates of *S.* Newport presented 9 different cgMLSTs. In *S.* Dublin, 6 different cgMLSTs were observed. In *S.* Anatum, 5 cgMLSTs were observed in 11 total genomes. cgMLSTs 261271, 260644, and 275391 were identified in IIIB 61:I:Z53, *S.* Agona and *S.* Montevideo, respectively (Supplementary Table 1).

3.2. Antimicrobial susceptibility testing

Most of the isolates showed non-susceptibility to quinolones, tetracyclines and aminoglycosides. Fifty-eight isolates were not susceptible to the second generation-quinolone ciprofloxacin (77.3%). In addition, in this group, 5 isolates (6.6%) were not susceptible to enrofloxacin. Fifty-eight isolates (67%) were not susceptible to tetracycline, 64 (85.3%) were not susceptible to streptomycin, and 7 were also not susceptible to gentamicin (9.3%).

Only nine isolates (12%) were not susceptible to beta-lactam ampicillin, and 4 (5.3%) were not susceptible to amoxicillin-clavulanic acid. Every isolate was susceptible only to cefotaxime, and only 3 (4%) of the isolates were not susceptible to trimethoprim/sulfamethoxazole. A similar situation was observed for chloramphenicol, to which only 1 (1.3%) of the strains was not susceptible. There were 15 (20%) isolates that were not susceptible to nitrofurantoin, and 7 (9.3%) were not susceptible to azithromycin.

Considering MDR, 42 isolates (56%) were MDR. Twenty-two isolates were not susceptible to three groups of antimicrobials (29.3%), 15 were not susceptible to 4 groups (20%), four were not susceptible to 5 groups (5.3%) and one was not susceptible to 6 groups of antimicrobials (1.3%). Three isolates of *S.* Anatum were MRD, as were 14 *S.* Newport isolates, 24 *S.* Typhimurium isolates, and the only IIIb 61:i:z53 isolate. The most frequent MDR phenotype was CIP-S-TE, observed in 15 isolates, followed by CIP-S-TE-NF, in 6 isolates, and S-TE-NF and CIP-S NF, in 2 isolates each (Supplementary Table 2).

3.3. Antibiotic resistance-related genes detected in *Salmonella* genomes

After using ResFinder, 21 genes that predict phenotypic resistance were detected. In addition to the presence of the *aac(6')-Iaa* gene in all 75 isolates, 50 (66.6%) presented the *aph(3")-Ib* and *aph(6)-Id* genes. The *aph(3")-Ia, aadA1*, *aadA2*, and a*adA17* genes, which also confer resistance to aminoglycosides, were detected at a low frequency. Only three genes that confer resistance to tetracyclines were identified. The most abundant of these genes was *tet*A, found in 48 (64%) isolates, followed by *tetB* and *tetM*. Sulfonamide resistance genes were observed in 29 (38.6%) isolates, all of which presented the *sul2* gene, and two of these isolates also presented the *sul1* gene. The only identified gene for beta-lactam resistance was the *blaTEM-1B* gene, detected in 7 isolates, and the only gene for quinolone resistance was the *qnrB19* gene, detected in 5 isolates. The *floR* and *cmlA1*, *fosA*, *qac*, *dfrA*, and *lnu* genes, which confer resistance to phenicols, fosfomycin, ammonium compounds, trimethoprim, and lincosamides, respectively, were detected in 4 or fewer isolates. According to serotype, the gene *tetA* was present in *S.* Typhimurium, *S.* Newport, *S.* Anatum and *S.* Agona. Only *S.* Typhimurium and *S.* Anatum presented the sul2 gene and the *bla-TEM1 gene*. Similarly, only *S.* Typhimurium and *S.* Dublin carried the *qnrB19* gene. Two mutations were detected using the PointFinder database. The *parC_T57S* chromosomal mutation, conferring resistance to quinolones, was detected in 38 (50.6%) isolates, including all strains of *S.* Anatum, *S.* Newport, *S.* Montevideo, *S.* Agona and the IIIB 61:I:Z53 isolate. This mutation was not present in the *S.* Typhimurium and *S.* Dublin isolates. The other detected mutation, *acrB_R717Q*, which confers resistance to azithromycin, was detected in one strain of *S.* Dublin (Supplementary Tables 1, 2).

3.4. Plasmid detection

Twelve different incompatibility (Inc) groups were detected in 70 strains. Forty-nine strains showed one Inc group, 17 strains showed two, and 4 strains presented three. Three strains, *S.* Newport, *S.* Agona and IIIB 61:I:Z53, did not present any of these groups.

All but five isolates carried at least one Inc group. The most frequent of these groups were IncFII(pHN7A8), IncFII(S), IncFIB(S), Col440I and IncI1. All *S.* Typhimurium isolates showed Inc groups, and the most frequent combination was Col440/IncFIB(S).

Salmonella Dublin strains presented either IncX1 or IncFII(S). In *S.* Anatum, the combination of Col440I/IncHI2A/IncQ1

TABLE 1 Incompatibility groups of plasmids distributed in different serovars.

	S. Typhimurium	*S.* Dublin	*S.* Anatum	*S.* Newport	*S.* Montevideo	Total
IncFII	ND	ND	ND	20	ND	20
Col440I	12	ND	1	ND	ND	13
IncFIB	2	ND	ND	ND	ND	2
IncFIB(S)	15	ND	ND	1	ND	16
IncFIC(FII)	2	ND	ND	ND	ND	2
IncFII(S)	15	3	ND	ND	ND	18
IncHI2A	ND	ND	1	ND	ND	1
IncI1	1	ND	10	ND	1	12
IncI2	ND	ND	1	ND	ND	1
IncI2(Delta)	1	ND	ND	ND	ND	1
IncX1	ND	3	ND	ND	ND	3
IncQ1	3	ND	3	ND	ND	6

ND, not detected.

TABLE 2 Resistance genes detected in plasmid incompatibility groups.

Inc. Group	Size (Kb)	Resistance genes	No
IncFII	85 kb	*tetA, aph (3")-Ib, aph (7)-Id*	21
Criptic plasmid	8,4	*tetA, aph (3")-Ib, aph (7)-Id, sul2,*	19
ColE1	2,8	*qnrB19*	4
IncFIB	51,8	*tetM, floR, intI-1, [dfrA12, aadA2, cmlA1, aadA1, and ΔqacL]*	2
IncFIB(S)/IncFII(S)/IncQ1	117/124	*tetB,aph (3")-Ib, aph (7)-Id, sul2, and blaTEM-1b*	2
incQ	11	*tetA, aph (3")-Ib, aph (6)-Id, and sul2*	2
IncQ	6,5	*aph(3")-1b, aph(6)-Id, sul2, and aph(3')-Ia*	1
IncQ	80,4	*tetA, aph (3")-Ib, aph (6)-Id, sul2, intI-1, [dfrA7, qacE1-1, sul-1]*	1
IncHI2A/IncQ	221	*tetA, aph (3")-Ib, aph (6)-Id, sul2, intI-1, [dfrA7, qacE1-1, sul-1]*	1
IncFIB(S)/IncFII(S)/IncQ1	113,6	*aph(3")-1b, aph(6)-Id, sul2, aph(3')-Ia, and blaTEM-1B*	1
incN	40	*tetA*	1
IncFIB(S)/IncFII (S)	97	*tetA, aph (3")-Ib, aph (6)-Id, sul2, aadA17, aph(3')-Ia, and lnu(F)*	1
IncI1-I(Alpha)	69,5	bla_{TEM-1B}	1

The genes that are between rect parenthesis are contained in Class I integrons. Genes grouped in brackets correspond to those included in class 1 integrons.

plasmids was present in 1 strain. Two strains presented 2 plasmids in the combinations IncI1/IncI2 and IncI1/IncQ1. Eight strains presented the IncI1 plasmid. A single strain of the *S.* Newport serotype presented the plasmids IncFII(pHN7A8) and IncFIB(S), and 20 strains presented the plasmid IncFII(pHN7A8). *Salmonella* serovar Montevideo had a single IncI1 plasmid (Table 1).

3.5. Plasmid localization of resistance genes and class 1 integrons

In 52/75 isolates, at least one plasmid incompatibility group and some antibiotic resistance genes were detected in the same contig (see the detailed results in Table 2).

The most frequently detected antibiotic resistance-related incompatibility group was IncFII, which was identified as an 85 kb contig in 21 isolates. This contig encoded the *tetA*, *aph(3")-Ib* and *aph(6)-Id* genes, which confer resistance to tetracyclines and streptomycin. These 85 kb fragments were 98% identical to two plasmids available in the NCBI databases (accessions CP042245 and CP025329) from veterinary isolates in China.

Second, a small 8.4 kb non-conjugative cryptic plasmid was detected in 19 isolates that encoded *sul2*, *aph(3")-Ib*, *aph(6)-Id*, and *tetA*, conferring resistance to sulfas, streptomycin, and tetracycline. This small plasmid without a defined incompatibility group is widely reported in databases such as CP023647 and CP049284.

A third group of plasmids that can be highlighted in the context of the accumulation of resistance genes was IncQ. Although only 8 isolates presented IncQ, these non-conjugative plasmids were highly heterogeneous in terms of the resistance genes involved and their sizes and mode of presentation. Thus, in four isolates, IncQ was found as the only incompatibility group of the contig, while in

another four, it was cointegrated with other incompatibility groups, such as IncHI2a, or even with the virulence plasmid of *Salmonella* spp. IncFIB(S)/IncFII(S). All detected gene arrangements can be seen in Figure 1.

Among the 75 isolates studied, only four carried class 1 integrons. Two highly related isolates of *S.* Typhimurium (39,218 and 39,235) presented the same integron on an IncFIB plasmid, with the arrangement intI-1, *dfrA12*, *aadA2*, *cmlA1*, *aadA1*, and *qacL*. Interestingly, isolate 39,235 but not 39,218 presented a second class 1 integron in the IncFIB(S)/IncFII(S) virulence plasmid encoding the aadA17 and *lnu(F)* genes.

On the other hand, two isolates of *S.* Anatum (39,215 and 39,255) carried the arrangement intI-1, *dfrA7*, *qacEΔ-1*, *sul1* in a contig associated with the cointegrated plasmid IncHI2A/IncQ1 or IncQ1, respectively.

3.6. Correlation between phenotypic and genotypic resistance

We recorded 900 phenotypic antibiotic susceptibility results and 1,575 genotypic data from CGE on 75 *S. enterica* isolates. The phenotype matched the genotype in 709 results. A total of 191 differences between phenotype and genotype were observed, 65 of which were due to the presence of phenotypic non-susceptibility with the absence of analyzed resistance genes/mutations, and 126 were due to phenotypic sensitivity but with the presence of resistance genes/mutations, with a general accuracy between the two tests of 83.6% (range 48–100%).

The highest correlation between phenotypic and genotypic resistance was found for gentamicin (100%), chloramphenicol (97.3%), trimethoprim-sulfamethoxazole (96%), macrolides (92%), tetracyclines (85.3%) and streptomycin for aminoglycosides (85.3%). The greatest number of discrepancies between the susceptibility profile and genes conferring resistance were observed in the of quinolone group (35 isolates), followed by the nitrofurantoin group (15 isolates). Among these three groups of antibiotics, the observed differences were due to the presence of non-susceptibility and the absence of genes and mutations in 40 isolates and 36 isolates with phenotypic susceptibility but also genes or mutations. According to the reference method, the overall sensitivity was 85.4% (14.3–100%), the overall specificity was 82.7% (38.8–100%), the PPV was 71.1% (11.6–100%), and the NVP was 90% (2–100%) (Table 3).

3.7. Phylogenetic analysis

Whole-genome phylogenetic trees were produced for strains of the serovars SAn, SDu, STy and SNe, as they were the most frequent serovars present on more than one farm and/or included more isolates for comparison. Independent phylogenetic trees were produced for each serovar, as this approach could resolve in more detail the intraspecific differences among isolates, and the results are shown in Figure 1.

In broad terms, while the observed genotypes were roughly the same among a given serotype, greater phenotypic diversity was observed for AMR. Strains from the same farm were clustered together in their respective trees.

The STy strains were the most genetically diverse group, in accordance with the broader range of sources and AMR observed for this serovar, as described above, whereas the SAn isolates were less diverse.

4. Discussion

Salmonella enterica is a pathogen present on dairy farms in Uruguay. In this study, 8 serotypes were identified, and each showed a single sequence type, suggesting the presence of a single lineage of strains.

As observed in Canada (21), *S.* Typhimurium ST19 was the most frequent sequence type isolated from calves with diarrhea, being identified 26 different times on 16 different dairy farms. This contrasts with findings in the USA, where the most frequent serotype is *S.* Dublin (49). ST19 has been identified in other countries and is associated with gastrointestinal disease due to showing a greater immune response to the flagellar antigens of these strains (50, 51). On one dairy farm, we identified 24 *S.* Newport ST45 isolates, most of which were susceptible to antibiotics. This ST affects both humans and animals, including cattle, and has been reported to show an MDR phenotype (52–54). In agreement with other reports, *S.* Dublin ST10 was isolated from 5 different farms, and *S.* Anatum ST64 was isolated from 2 farms (55–60).

Salmonella Agona is a pathogen frequently detected in food worldwide (61). In this work, the representative ST13 was isolated from a mesenteric lymph node. The isolate of *S.* Montevideo belonged to ST138, and this microorganism has also been reported as a foodborne pathogen (62). This sequence type of bovine origin is implicated in foodborne illness and comes from a different clade from those isolated from human outbreaks. Isolate IIIB 61:I:Z53 belongs to sequence type ST430, which has only been reported in association with human infections in the US due to contact with small turtles and their environment (63, 64). Here, it was isolated from the feces of a calf suffering from an outbreak of neonatal diarrhea and calf mortality, so its implications for farm animals should be evaluated.

We observed differences in the susceptibility profiles between the different serotypes. *Salmonella* Typhimurium was the serotype with the greatest differences in susceptibility. This could be explained by the diversity of geographic locations represented by the isolates since it was the most numerous serotype. In this study, the most frequent non-susceptibility pattern was found for aminoglycosides, tetracyclines and quinolones, identified in 11 (34%) isolates, 81% of which were MDR. A similar characteristic was observed in Canada by Otto et al. (21). Great differences are found among the reports of resistance in *S.* Dublin (21), and in our work, the isolates presented susceptibility to all antibiotics except aminoglycosides. The most frequent non-susceptibility pattern observed in *S.* Newport isolates, obtained from a single farm, was similar to that of *S.* Typhimurium, and this serovar is usually reported as MDR (65, 66), unlike *S.* Anatum, which was not susceptible to only aminoglycosides and quinolones.

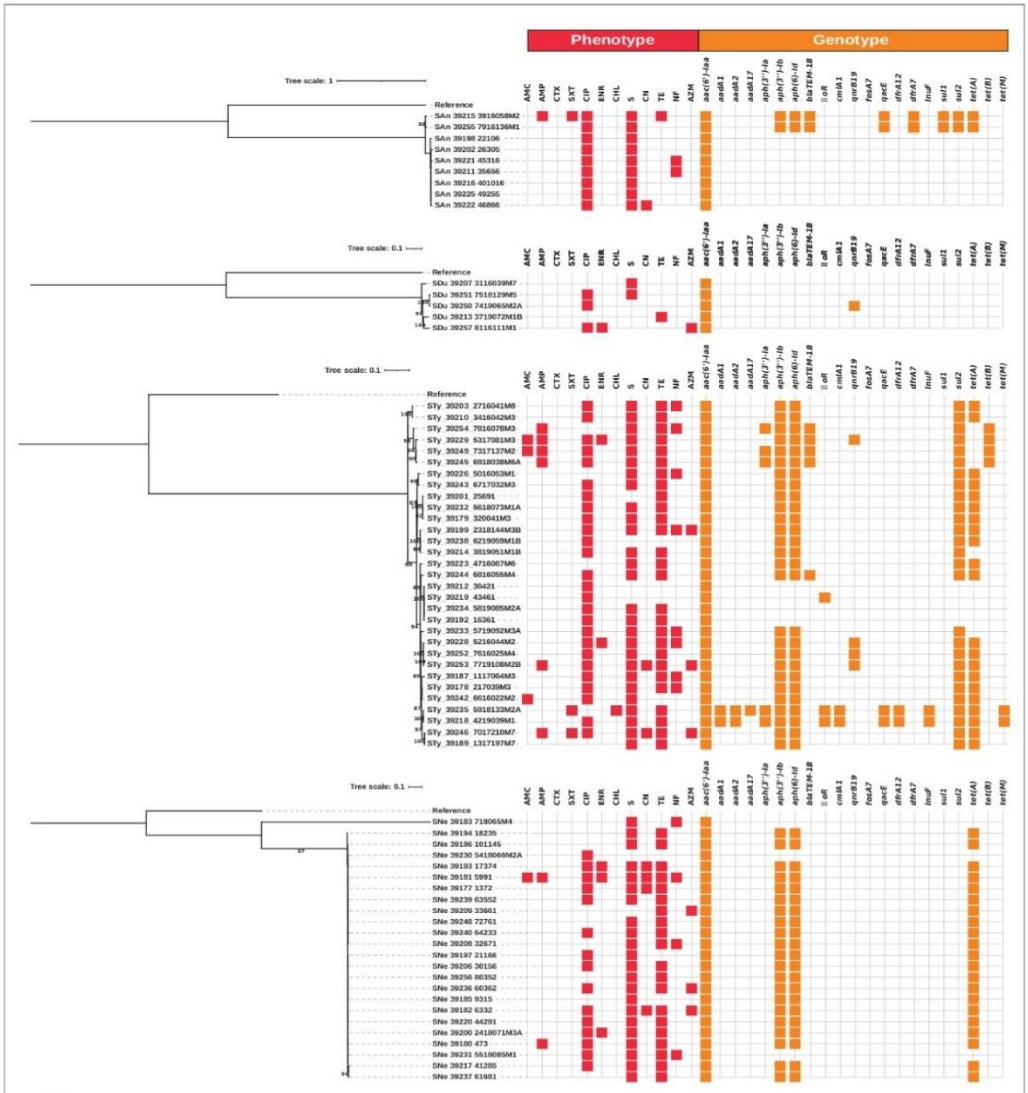

FIGURE 1
Whole-genome maximum likelihood phylogenetic trees (midpoint root) for the isolates from serovars Anatum (SAn), Dublin (SDu), Typhimurium (Sty) and Newport (SNe) in this study. Phenotypic antimicrobial resistance is indicated with red squares. Antibiotics: AMC, amoxicillin-clavulanic acid; AMP, ampicillin; CTX, cefotaxime; SXT, trimethoprim-sulfamethoxazole; CIP, ciprofloxacin; ENR, enrofloxacin; CHL, chloramphenicol; S, streptomycin; CN, gentamicin; TE, tetracycline; NF, nitrofurantoin; AZM, azithromycin. The presence of antimicrobial resistance genes is indicated with orange squares.

According to the evaluation of the obtained genomes, we observed that the most frequent resistance genes in all serotypes were those that confer resistance to aminoglycosides, tetracyclines and sulfonamides. Resistance to aminoglycosides mediated by the *aac (6')-Iaa* and *aacC (6')-Iy* genes is not considered by relevant platforms because studies indicate that they do not confer

TABLE 3 Sensitivity and specificity of genotype predictions of resistant antimicrobial phenotypes for 75 *Salmonella* isolates.

Antibiotics	Phenotype resistant/intermediate		Phenotype susceptible		Sensitivity % (a/(a + c)) × 100	Specificity % (d/(b + d)) × 100	PPV % (a/(a + b)) × 100	NPV % (d/(c + d)) × 100	Accuracy % ((a + d)/(a + b + c + d)) × 100
	Genotype resistant (a)	Genotype susceptible (c)	Genotype resistant (b)	Genotype susceptible (d)					
Aminoglycosides									
STR	64	0	11	0	100	ND	85,3	ND	85,3
CN	7	0	0	0	100	ND	100	ND	100
Tetracycline									
TET	44	7	4	20	86.2	83.3	91.6	74.0	85.3
Quinolones									
CIP	29	28	11	7	50.8	38.8	72.5	20.0	48.0
ENR	5	1	38	31	83.3	44.9	11.6	96.8	48.0
Penicillin									
AMP	5	4	2	64	55.5	97	71.4	94.1	92
Beta-lactam/beta-lactam inhibitor									
AMC	2	2	5	66	100	93	28.6	97	90.6
Cephems									
CTX	0	0	7	68	ND	90.6	ND	100	90.6
Trimetoprim-sulfametoxazol									
SXT	2	1	2	70	66.6	97.2	50	98.5	96
Macrolide									
AZM	1	6	0	68	14.3	100	100	91.9	92
Phenicol									
CHL	1	0	2	72	100	97.2	100	100	97.3
Nitrofuran									
NF	0	15	0	60	ND	100	ND	80	80
Total					85.4	84.2	71.1	90	83.7

STR, streptomycin; CN, gentamicin; TET, tetracycline; CIP, ciprofloxacin; ENR, enrofloxacin; AMP, ampicillin; AMC, amoxicillin-clavulanic acid; CTX, cefotaxime; AZM, azithromycin; CHL, chloramphenicol; NF, nitrofurantoin; ND, not determined.

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resistance and are ubiquitous in *Salmonella* (67). However, the aac(6')-Iaa gene was detected in 100% of the strains in our study. The *aac(6')-Iaa* gene has been previously detected in human *Salmonella* isolates from Uruguay (68). It confers resistance to tobramycin, amikacin and kanamycin, but this gene is less efficient in the acetylation of gentamicin, which may explain the low rate of detection of phenotypic resistance in our results (69). The *aph(6)-Id*, *aph(3)-Ib* and *aph(3)-Ia* genes were detected less frequently. These last three genes were recently reported in *S.* Dublin isolated from bovines in the USA (59).

The most frequent tetracycline resistance gene identified was the *tetA* gene, which was present in those strains that did not present the *tet*B gene; both of these genes expulsion pumps in gram-negative bacteria (70). In previous reports, it was observed that the *tet*A gene presented a greater dissemination capacity than the *tet*B gene on farms (71). This finding could be explained by the fact that these genes occur in mobile genetic elements that are frequently conjugative (72).

The *sul*2 gene was detected at the highest frequency, followed by *sul*1, both of which confer resistance to sulfonamides. This contrasts with findings in other animal sources, where *sul1* is more prevalent (72, 73). This result was not confirmed phenotypically because we assayed trimethoprim and sulfamethoxazole antibiotics together. According to this combination, only 4 isolates carried *sul1/sul2* and *dfr* genes that confer resistance to trimethoprim-sulfamethoxazole.

Plasmid-mediated quinolone resistance (PMQR) generates a low level of resistance to quinolones but promotes the selection of other resistance mechanisms, and their rapid spread and has been reported in different bacterial species of animal origin (74–77). In this study, the *qnrB19* gene was detected in only 4 *S.* Typhimurium and 1 *S.* Dublin isolates. Similar isolates harboring this gene have been observed in South America (78–80). These results contrast with the published data from the region, where the most frequent variants are indicated to be *qnrB2*, *qnrS1*, and *qnrE1* (80).

In this work, we only detected a mutation in *par*C at position *57*, causing a *Thr* to *Ser* change in the topoisomerase that confers resistance to quinolones (80). Enrofloxacin is a representative antibiotic used in the veterinary industry, and due to its wide range of activity, it is frequently used for treating various clinical presentation (81, 82). The *parCT57S* mutation has been reported as the most frequent mutation in environmental samples and animal feces isolates in Brazil (80), and a single mutation in a topoisomerase IV or DNA gyrase gene confers high-level resistance to this group of drugs (75). In Uruguay, the *parCT57S* mutation and the presence of *qnrB19* were detected in human *S.* Typhimurium isolates between 2011 and 2013 (68). Taken together, our findings suggest that these genes and mutations have existed for a long time.

In the USA, the detection of the *blaTEM* gene is very frequent (59, 83, 84); in contrast, only 7 strains carried this gene in this work, corresponding to <10% of the collection. Following the results obtained from Resfinder, the *blaTEM-1* enzyme was only identified in 5 strains of *S.* Typhimurium and 2 strains of *S.* Anatum. Among these 7 isolates, only five showed reduced sensitivity to ampicillin and two to amoxicillin-clavulanic acid. Most of these *blaTEM-1*-carrying strains were isolated from neonatal calves.

We detected the *aadA1* and *aadA2* genes in two MDR *S.* Typhimurium isolates, and one of them even carried the *aadA*17 variant, which is frequently reported in England (85) but has not previously been reported in *Salmonella* isolates in South America. This class of genes is located within integrons (86), together with genes such as *sul*, *qacE*Δ1, *drf*, and *cmlA* that confer resistance to sulfonamides, antiseptics and disinfectants, trimethoprim and phenicol, respectively (73, 87–89). In our work, *aadA17* was also found within a class 1 integron but was only accompanied by the *lnu(F)* gene encoded by the virulence plasmid of *Salmonella* spp. Inc FIB(S)/IncFII(S).

On the other hand, two isolates of *S.* Anatum with similar characteristics were detected on the same farm but came from two calves sampled months apart. These strains presented *blaTEM*, *sul2*, *tetA*, *dfrA7*, *qac*Δ*1*, and *sul1*, among which the last three genes are part of a class 1 integron.

The *fosA* gene is widely distributed among Enterobacteriaceae (90), and the f*osA7* variant has been detected in plasmids in *S. enterica* from birds and in pigs and cattle in China (91, 92), conferring a high level of resistance to fosfomycin. In our study, this gene was detected in *S.* Agona and *S.* Montevideo isolated from mesenteric lymph nodes and feces, respectively. This gene was previously detected in Uruguay, but within *E. coli* STEC strains (93).

Interestingly, a single strain of *S.* Dublin had the *acrB_R717Q* mutation, which confers resistance to azithromycin, and was recently identified in Bangladesh in *S.* Typhi (94). Additionally, this mutation was recently reported in a study involving *S.* Dublin isolates of bovine origin by García-Soto et al. (95) in Germany.

In general, most plasmids were exclusive to a certain serotype. The plasmids shared by different serotypes were Col440I in *S.* Typhimurium and *S.* Anatum; IncFIB(S) in *S.* Typhimurium and *S.* Newport; IncI1 in *S.* Typhimurium, *S.* Anatum and Montevideo; and IncQ1 in *S.* Typhimurium and *S.* Anatum. This agrees with findings reported by Feng et al. (96), who indicated that serotypes generally do not share the same plasmids due to their mostly vertical inheritance. Previous studies reported that the most frequent plasmids detected in *S.* Dublin were IncX1, IncA/C2, and IncFII (59, 97). Although the number of *S.* Dublin strains evaluated in our study was very limited, the detected plasmids were similar, except for IncA/C2. While *S.* Typhimurium was the sole serovar harboring the plasmids IncA/C, IncI, Col, IncFII, IncFIB, and IncFIA (similar to the plasmids detected in our strains), there was a difference in the Newport serotype (in which the only detected plasmids shared with this work were the IncFII and IncFIB) and in Anatum and Agona (84). It is important to deepen the study of the plasmids present in the strains since they are used for epidemiological studies and the interpretation of the virulence and resistance observed in different regions.

Since 1960, the most commonly reported MDR phenotype in *Salmonella* has been resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracyclines (98). Most of the isolates were MDR, and 35.7% of these isolates showed the CIP-S-TE profile. Genotypically, we also observed MDR in 40% of the isolates, which carried resistance genes from 3 to 7 groups

of antibiotics. Most of these MDR isolates were *S.* Anatum and *S.* Typhimurium.

Different publications have described notable correlations between resistance phenotypes and genotypes (83, 99). We detected a 100% correlation for gentamicin; above 90% for chloramphenicol, beta-lactams, trimethoprim-sulfamethoxazole and macrolides; and above 80% for tetracyclines, nitrofurans and streptomycin. Similar results were obtained by Campioni et al. (100) in *Salmonella* from different sources in Brazil.

In our work, although the estimated values of sensitivity, specificity, PPV and NPV were generally high, this was not observed in some specific drug groups (i.e., ciprofloxacin). With the strategy used in this work, we could not identify the presence of pseudogenes in the detected AMR genes that might account for part of this discrepancy. Other factors may be involved, such as low levels of expression or mutations in promoters or in Shine Dalgarno sequences. On the other hand, under the adopted strategy, we did not analyze the presence of efflux pumps since their effect depends on the level of expression. This aspect could explain certain levels of resistance that are not explained by our results.

Based on the above, phenotypic evaluation is still the most relevant approach when therapeutic decisions are being made.

This work represents one of the first studies in the region using the phenotypic resistance and WGS of *S. enterica* obtained from bovines as an analysis strategy. According to our results, *S.* Typhimurium was the serotype with the highest frequency and distribution, followed by *S.* Dublin, and all the serotypes detected were from a single sequence type. These serotypes were zoonotic, and we also found a high frequency of resistance to antibiotics used on farms and in human health. Taking these results into account, it is necessary to consider the implementation of strategies to limit the use of antibiotics that are considered critical and to mitigate the increase in resistance globally.

Data availability statement

The data presented in the study are deposited in the EnteroBase (https://enterobase.warwick.ac.uk) repository, accession numbers SAL_HB5975AA, SAL_HB5235AA, SAL_HB5236AA, SAL_HB5237AA, SAL_HB5238AA, SAL_HB5239AA, SAL_HB4194AA, SAL_HB5240AA, SAL_HB5241AA, SAL_HB5242AA, SAL_HB5243AA, SAL_HB5299AA, SAL_HB5300AA, SAL_HB5301AA, SAL_HB5302AA, SAL_IB4224AA, SAL_HB5471AA, SAL_HB5474AA, SAL_HB5476AA, SAL_HB5740AA, SAL_IB4223AA, SAL_HB5741AA, SAL_HB5742AA, SAL_HB5743AA, SAL_HB5744AA, SAL_HB5745AA, SAL_HB5746AA, SAL_HB5747AA, SAL_HB5748AA, SAL_HB5749AA, SAL_IB4193AA, SAL_HB5976AA, SAL_HB5977AA, SAL_HB5978AA, SAL_HB5979AA, SAL_HB5980AA, SAL_HB5981AA, SAL_HB5982AA, SAL_HB5983AA, SAL_HB5984AA, SAL_HB5985AA, SAL_HB5986AA, SAL_HB5987AA, SAL_HB5988AA, SAL_IB4194AA, SAL_HB5989AA, SAL_HB5990AA, SAL_HB5992AA, SAL_HB5993AA, SAL_HB5994AA, SAL_HB5995AA, SAL_HB5996AA, SAL_HB5997AA, SAL_HB6171AA, SAL_HB6172AA, SAL_HB6173AA, SAL_HB6179AA, SAL_HB6188AA, SAL_HB6194AA, SAL_HB6195AA, SAL_HB6201AA, SAL_HB6207AA, SAL_HB6208AA, SAL_HB6213AA, SAL_HB6217AA, SAL_HB6232AA, SAL_HB6235AA, SAL_HB6236AA, SAL_HB6238AA, SAL_HB6239AA, SAL_HB6240AA, SAL_HB6242AA, SAL_HB6244AA, SAL_HB6261AA, SAL_HB5975AA. Data has been available in the database since April 2021.

Author contributions

MC and MF conceptualized the study. MC performed the laboratory analysis and wrote the original draft. MC, BD'A, and RV performed data selection and data analysis. MC, BD'A, RV, and MF contributed to writing, proofreading, and editing. All authors contributed to the article and approved the submitted version.

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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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1055432/full#supplementary-material

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CAPÍTULO III

Evaluación genómica de factores de virulencia e islas de patogenicidad presentes en una colección diversa de aislamientos de *Salmonella enterica* de origen bovino

3.1 INTRODUCCIÓN

Las bacterias patógenas son aquellas capaces de causar daño en el hospedero merced al despliegue de un conjunto variable de factores de virulencia (Ribet & Cossart, 2015). En las diferentes etapas de interacción entre el patógeno y hospedador se activan un conjunto de genes que codifican la cápsula polisacárida, fimbrias, flagelos o toxinas entre otros que permite que el microorganismo sea capaz de adherirse, formar biopelículas, sobrevivir a nivel intracelular, captar nutrientes, evadir el sistema inmune e invadir entre otros (Liu et al. 2021).

Salmonella enterica presenta diversos mecanismos de virulencia que actúan en la patogénesis de la infección, entre los más importantes se encuentran los sistemas de secreción, flagelos, cápsulas, y otros determinantes como adhesinas, invasinas, fimbrias, hemaglutininas, exotoxinas y endotoxinas (Jajere et al. 2019).

Salmonella enterica presenta en su cromosoma o en plásmidos un grupo de genes que constituyen islas de patogenicidad (SPI) y codifican para distintos factores de virulencia (Wang et al. 2020). Dentro del género *Salmonella* se describen 24 SPI de las cuales las SPI 1 a SPI 5 se han detectado en todos los serotipos (Liu et al. 2023). Las SPI descriptas actúan en diferentes funciones que involucran la invasión de macrófagos y células epiteliales, resistencia al ambiente ácido, replicación intracelular, evasión del sistema inmune, entre otros (Wang et al. 2020). Las SPI 1 y 2 son las más estudiadas y presentan actividad diferenciada. SPI-1 codifica para genes que asisten en la invasión al intestino mientras que la SPI-2 codifica para determinantes que les permiten a las bacterias la sobrevida intracelular y su diseminación sistémica, ambas codifican para un sistema se secreción tipo 3 (SST3) a través del cual la bacteria transloca proteínas efectoras a la célula (Pico-Rodríguez et al. 2024).

Los sistemas de secreción proporcionan factores que promueven la colonización, la supervivencia y la superación de la resistencia del huésped. Dentro de los 11 sistemas de secreción descritos hasta el momento (SST1-11) se han descrito cinco sistemas de secreción en *Salmonella:* sistema de secreción tipo I (SST1), el sistema de secreción tipo III (SST3), el sistema de secreción tipo IV (SST4) el sistema de secreción tipo VI (SST6) y el sistema de secreción tipo X (SST-10) (Krone et al. 2023, Grossman et al. 2024). Estos sistemas intervienen de forma simultánea en funciones como: invasión, formación de biopelículas, inhibición de la producción de citoquinas proinflamatorias por parte de la célula hospedadora, formación de SCV, supervivencia de macrófagos, entre otras (Bao et al. 2020).

La adhesión es un paso inicial en la patogénesis de la salmonelosis y dentro de este mecanismo las fimbrias son uno de los factores más importantes. Las fimbrias son

estructuras proteicas que se unen a receptores complementarios de la célula huésped (Rehman et al. 2019). Las fimbrias pertenecen a cuatro categorías generales según su morfología, función o vía de ensamblaje: (1) pili F, (2) fimbrias tipo IV, (3) curli y (4) fimbrias de la vía chaperona-usher (Cheng & Wiedmann, 2021). La mayoría de las fimbrias pertenecen a la familia chaperona-usher (CU) (Cheng et al. 2022). La descripción de operones fimbriales aumentan a medida que se utilizan las herramientas de secuenciación (Cheng & Wiedmann, 2021), algunos de los más importantes en *S.* Typhimurium son fimbrias tipo 1 (*fim)*, fimbrias codificadas por plásmidos (*pef*), fimbrias polares largas (*lpf*), *S. enterica* serovar Typhimurium fimbriae (*stf*), factor de colonización bovino (*bcf*), *Salmonella* fimbriae (*saf*) y fimbrias agregativas (*agf*). (Humphries et al. 2001).

Las adhesinas no fimbriales están ampliamente distribuidas en las bacterias y participan en la unión a superficies abióticas, unión a células, en la interacción y agregación de células y en la arquitectura de biofilms. Se agrupan en dos categorías de acuerdo con si son secretadas por el SST-1 o el SST-5 (Berne et al. 2015). Las adhesinas más estudiadas secretadas por el SST-1 son de la familia de las proteínas *Bap* (*biofilm associated proteins*) y *SiiE* una proteína gigante cuyo sistema de secreción está codificado por la SPI-4 (Barlag et al. 2015, Azimi et al. 2020)). A partir del SST5 las proteínas mejor caracterizadas son *YadA de Yersinia adhesin A* y *BadA* de *Bartonella hensellae* ambas se unen a la fibronectina o al colágeno (Barlag et al. 2015).

Las adhesinas autotransportadas también forman parte del mecanismo de invasión de células epiteliales y persistencia intestinal al unirse a la fibronectina (Azimi et al. 2020). Dentro de ellas *MisL*, *ShdA* y *SadA* son proteínas autotransportadoras representadas por el SST4 distribuidas en bacterias Gram negativas. *ShdA* se une a fibronectina y colágeno tipo I y es necesaria para la colonización intestinal, *SadA* promueve la agregación celular, la formación de biopelículas y la adhesión a las células epiteliales intestinales mientras que la unión de *MisL* a la fibronectina da como resultado una mayor invasión de las células epiteliales (Wang et al. 2018).

Para las bacterias, el hierro es un nutriente esencial, pero se encuentra poco disponible. Es por eso que frente a su limitación se activan sideróforos para captar y transportar a su interior hierro y también pueden absorber sideróforos de otras bacterias, estos mecanismos constituyen factores que aumentan su virulencia (Chatterjee & O'Brian, 2018, Steunou et al. 2022). Otro componente esencial para la bacteria es el Mg^{+2}. En respuesta a la baja disponibilidad de Mg^{+2}, el pH ácido y péptidos antimicrobianos presentes en los fagosomas de macrófagos, se activa el regulador *PhoPQ,* que a través de la traducción de señales permite adaptarse al entorno, activando autotransportadores para la estabilidad del mangnesio (Iwadate et al. 2023).

En la interacción del patógeno con las células se activan genes que permiten la supervivencia dentro del ambiente ácido en macrófagos, la formación de biopelículas en la vesícula biliar y la resistencia a péptidos antimicrobianos. Frente a ambientes adversos, uno de los genes que se activa es el gen *mig*-14 y el mismo está regulado por *phoP*, que forma parte del sistema de regulación de 2 conponentes *phoP/phoQ*, el cual regula y controla la virulencia de *Salmonella* (Choi & Groisman, 2020; Jahan et al. 2022).

3.2 OBJETIVO

Diversos componentes relacionados a la virulencia son detectados en *Salmonella enterica*. El objetico principal de este capítulo es describir la distribución y detección de las islas de patogenicidad y genes de virulencia en el genoma de una colección de aislamientos de *Salmonella* de origen bovino.

3.3 MATERIALES Y MÉTODOS

3.3.1 Aislamientos, preparación, secuenciación

El origen de las muestras, la preparación y el proceso de secuenciación de los 75 aislamientos se detalla en el artículo “Phenotypic and genotypic survey of antibiotic resistance in *Salmonella enterica* isolates from dairy farms in Uruguay”, Capitulo II de este documento (Casaux et al. 2023).

3.3.2 Determinación de islas de patogenicidad

Se utilizó la base de datos de SPIfinder del “Center Genome of Epidemiology” (Yoon et al. 2014, https://cge.food.dtu.dk/services/SPIFinder/) para detectar la presencia de islas de patogenicidad presentes en los aislamientos. Se cargaron los archivos fasta y los resultados se registraron en planilla. Se seleccionaron los *threshold* con un umbral de 95% mínimo de identidad y un 60% mínimo de cobertura. Las funciones de cada

isla de patogenicidad detectada provistas por SPIfinder se listan en la Tabla 1 y Anexo 1.

3.3.3 Determinación de los genes de virulencia

Para determinar la presencia de genes de virulencia en cada aislamiento secuenciado se utilizó la base de datos de *Virulence Factor Database* (VFDB) (Liu et al. 2022, http://www.mgc.ac.cn/VFs/), para esto se cargaron los archivos fasta y se seleccionaron los *threshhold* para *Salmonella* 80% y 60%. Los datos arrojados se descargaron para posterior evaluación y descripción.

3.3.4 Análisis estadístico

Se realizó la asociación entre los genes de virulencia y las muestras de donde se obtuvieron los aislamientos y serotipos detectados, para determinar si existía una relación entre la presencia de genes en diferentes serovares de *Salmonella* y el origen de la muestra. Se excluyeron del análisis los hallazgos de baja frecuencia y variabilidad relacionados al origen y a los serotipos. Para esto, se realizó la prueba de Fisher para tablas r x c, también llamada prueba de Fisher-Freeman-Halton (Freeman & Halton, 1951), una extensión de las tablas r x c de la prueba exacta de Fisher, el cual define la probabilidad exacta de una distribución de números específica de ocurrencia en la tabla (cuando se conoce n y se establecen los totales marginales). Para lograr información más precisa sobre las correlaciones detectadas, es decir, entre qué niveles está la diferencia estadística, se determinaron comparaciones múltiples mediante comparaciones por pares con la corrección del p-valor de Benjamini-Hochberg. Los análisis fueron realizados en el software R versión 4.2.2 con el paquete rstatix.

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3.3.5 Análisis filogenético

Para evaluar la filogenia se utilizó la metodología descripta en el capítulo 2 (Casaux et al. 2023), La filogenia se construyó con todos los serovares detectados y los genes de virulencia en islas de patogenicidad. Se excluyeron del análisis los genes y SPI que se encontraban el 100% de los aislamientos. Como cepa de referencia se utilizó la cepa LT2 de *S.* Typhimurium.

3.4 RESULTADOS Y DISCUSIÓN

3.4.1 Detección de islas de patogenicidad

En la colección de aislamientos se identificaron 14 islas de patogenicidad diferentes de las cuales algunas fueron identificadas con más de una función. Todos los aislamientos presentaron las islas de patogenicidad SPI-1, SPI-2, SPI-3 y SPI-9 (Anexo 1 y Tabla 1). Estas SPI están asociadas a las funciones del sistema de secreción tipo 3, invasión de células intestinales, apoptosis, infección sistémica y patogenia intracelular, sobrevida en monocitos y captación de Mg^{+2}, entre otros (Tabla 1 y Figura 1). Las SPI-1 y SPI-2 son las más estudiadas y se encuentran distribuidas en distintos serotipos de *S. enterica* (Cheng et al. 2018, dos Santos et al. 2019). La isla de patogenicidad SPI-1 permite la invasión de enterocitos y su expresión se activa cuando el ambiente es ácido (Marcus et al. 2000). En esto proceso se activan proteínas efectoras como la proteína AvrA responsable de inhibición de la activación del factor de transcripción proinflamatorio NF-κB y la apoptosis. También se expresan: las proteínas Sip que intervienen en los sistemas de secreción translocando efectores, la tirosina fosfatasa (SptP) que produce la alteración del citoesqueleto y las proteínas Sop que intervienen en la quimiotaxis de leucocitos polimorfonucleares, la reorganización del citoesqueleto e invasión (Lou et al. 2019). Los productos de expresión de la SPI-2 actúan a nivel intestinal y son necesarios para la propagación de *S.* entérica de forma sistémica mediante la liberación de efectores en el medio intracelular a través de la SCV (Jennings et al. 2017).

En general, los efectores como SseF, SseG, PipB, SteA, SifA, SteD y PipB2, se clasifican en centrales, considerados presentes en todos los serotipos. Otros efectores son frecuentes en serovares que se encuentran a nivel intestinal, entre ellos *SseL*, *SifB*, *SopD2*, *SseJ*, *SteB*, *SteC*, *SlrP* y *SseK2.* En un tercer grupo están relacionados con elementos genéticos móviles y se denominan accesorios estos son *SspH2*, *SseK1*, *SrfJ*, *GtgA*, *GtgE*, *SseI*, *GogB*, *SteE, SseK3*, *SspH1*, *SpvB, SpvC* y *SpvD* (Jennings et al. 2017).

Por otro lado, las islas de patogenicidad SPI-3 y SPI-9 también fueron detectadas en todos los aislamientos, las mismas presentan genes diferentes de las SPI-1 y SPI-2 debido a características evolutivas diferentes (Blanc-Potard et al. 1999). La SPI-3 se relaciona con la infección crónica y la especificidad del huésped. Presenta los genes *mgtBC*, que constituyen un operón, y su expresión es necesaria para la supervivencia y la virulencia dentro del macrófago. La expresión de estos genes se activa frente a la escasez de Mg^{+2} y está regulada por *PhoPQ* (Marcus et al. 2000, Liu et al. 2023). La

SPI-9 presenta un operón cuya expresión aumenta con alta osmolaridad y bajo pH de una manera dependiente de *RpoS* (Velásquez et al. 2016).

Tabla 1. Islas de patogenicidad, función y serotipos detectados

SPI	Función	Genes asociados	Serotipo*	Frecuencia
C63	Sistema de captación de hierro	*sit*	SAn, SDu, SNe, STy, IIIB 61:I:Z53 SAg	98,6%
CS54	Colonización intestinal y determinantes de persistencia	*shdA, ratA, ratB, sivl, sivH*	SAn, SDu, SNe, STy	80%
Not Named (NN)	gen de proteína isla de patogenicidad putativa	*ssaD*	SAn, SDu, SNe, STy, SAg, SMo	98,6%
SPI-1	Sistema de secreción tipo III, invasión de células epiteliales, apoptosis.	Sistema de secreción Tipo, *invA*	SAn, SDu, SNe, STy, IIIB 61:I:Z53 SAg, SMo	100%
	proteína A de invasión no funcional debido a mutación			
SPI-2	Infección sistémica y la patogénesis intracelular al facilitar la replicación de bacterias intracelulares dentro de vacuolas que contienen *Salmonella* unidas a membranas	Sistema de secreción Tipo		100%
	proteína efectora	*spiC*	SAn, SDu, SNe, STy, SAg, SMo	98,6%
SPI-3	Invasión, supervivencia en monocitos, captación de Mg^{+2}	*MgtC, B, MarT, MisL*	SAn, SDu, SNe, STy, IIIB 61:I:Z53 SAg, SMo	100%
	Invasión, supervivencia en monocitos, captación de Mg^{+2}	*sugR, rhuMMgtC, B, MarT, MisL*	SAn, STy, SAg	17,33%
SPI-4	Sistema de secreción tipo I, supuesta secreción de toxina, apoptosis, necesario para la supervivencia intracelular en macrófagos.	-	SAn, SDu, SNe, STy, SAg, SMo	98,6%
SPI-5	Proteínas efectoras para SPI-1 y SPI-2	*SopB, SigD, PipB*	SAn, SDu, SNe, STy, SMo	96%
	Invasión, supervivencia en monocitos, captación de Mg^{+2}	*MgtC, B, MarT, MisL*	SAg	1,33%
	Proteínas efectoras	*sopB*	IIIB 61:I:Z53	
SPI-8	Exopolisacárido Vi, profago *SopE* y un operón pilus tipo IVB	-	SAg	1,33%
SPI-9	Aparato secretor tipo I, proteína similar a RTX	-	SAn, SDu, SNe, STy, IIIB 61:I:Z53 SAg, SMo	100%
SPI-10	Proteína similar a la fibrina	*sefD*	SDu	6,66%
SPI-12	Supervivencia intracelular	*msgA, narP*	SAn, SNe, STy	2,26%
SPI-13	Acetyl-coA dehidrolasa	*gacD*	SAn, SNe, STy, SDu, SMo IIIB 61:I:Z53	98,6%
	LysR regulación transcripcional	*gtrB*		
	Regulación transcripcional	*gta*	SAn, SNe, STy, SDu, IIIB 61:I:Z53	90,6%
SPI-14	Subunidad beta de la favoproteína de transferencia de electrones	*gpiA*	SAn, SNe, STy, SDu, SMo IIIB 61:I:Z53	97,3%
	Regulación transcripcional	*gpiB*		

*SAn: *S*. Anatum, SAg: *S*. Agona, SDu: *S*. Dublin, SNe: *S.* Newport, SMo: *S.* Montevideo, STy; *S.* Typhimurium.

La variabilidad de las SPI fue baja en *S.* Typhimurium. Todos los aislamientos de este serovar compartían las SPI C63PI, isla NM, SPI-1, SPI2, SPI-3 vinculada a invasión, supervivencia en monocitos y captación de Mg^{+2}; SPI-4, SPI-9, SPI-13 y SPI-14. Sólo un aislamiento no presentó CS54, pero fue el único aislamiento que presentó la SPI-3 relacionada con invasión, supervivencia en monocitos, captación de Mg^{+2} vinculada a los genes *sugR*, *rhuM*, *MgtC*B, *MarT*, *MisL*. Sólo 15 aislamientos de *S.* Typhimurium presentaron SPI-12 que se asocia con los genes msgA, y *narP* relacionados con supervivencia intracelular. De estos, 9 pertenecían a aislamientos obtenidos de órganos y secreciones y 5 a aislamientos obtenidos de heces. Ensayos realizados en ratones demuestran que SPI-12 contribuye a la supervivencia sistémica de *S.* Typhimurium (Haneda et al. 2009) y que, además pueden alterar genes de bacteriófagos adyacentes para aumentar la sobrevida en el hospedador (Tomljenovic-Berube et al. 2013).

Los seis aislamientos de *S.* Dublin portaban las SPI C63PI, CS54, isla NM, SPI-1, SPI-2, SPI-3. Estas SPI están vinculadas a la invasión, supervivencia en monocitos y captación de Mg^{+2}. Las SPI-4, SPI-5 se asocia a proteínas efectoras para SPI-1 y SPI-2, SPI-9, SPI-13 relacionadas con la enzima acetyl-coA dehidrolasa (gacD) y con un regulador transcripcional de la familia LysR y SPI-14. Además, cinco aislamientos de *S.* Dublin presentaban SPI-10, relacionada con las fimbrias *sef* (Townsend et al. 2001). En este trabajo la SPI-10 sólo se presentó en este serotipo. Si bien inicialmente se había propuesto que SPI-10 sólo se encontraba en serotipos específicos y se podía utilizar para predecirlos (Hensel, 2004), más tarde Saroj et al. 2008 concluyeron que estaba ampliamente distribuida en serotipos adaptados al huésped específico.

Salmonella Anatum presentó las SPI C63PI, isla NM, SPI-1, SPI-2, SPI-3 relacionada con invasión, supervivencia en monocitos, captación de Mg^{+2}, SPI-4, SPI-5 asociada a proteínas efectoras para SPI-1 y SPI-2, SPI-9, SPI-13 y SPI-14. La SPI-12 sólo se detectó en un aislamiento, representada por los genes *msgA* y *narP* asociados a sobrevida dentro de macrófagos. Por otro lado, esta isla porta el gen *shdA* de *S.* Typhimurium, el cual está implicado en la colonización y persistencia intestinal (Kingsley et al. 2003).

Los aislamientos de *S.* Newport presentaron las SPI C63PI, isla CS54, isla NM, SPI-1, SPI-2, SPI-3 relacionada con invasión, supervivencia en monocitos, captación de Mg^{+2}, SPI-4, SPI-5 como proteínas efectoras para SPI-1 y SPI-2, SPI-9, SPI-13 y SPI-14. La SPI-12 vinculada a sobrevida dentro de macrófagos se detectó en sólo 1 aislamiento.

Los serotipos *S.* Agona*, S.* Montevideo y IIIB 61:I:Z53 representados por un solo aislamiento cada uno en la colección, presentaron las SPI-1 relacionada con invasión de células epiteliales y apoptosis celular, SPI-2 relacionada con infección sistémica y la patogénesis intracelular, SPI-3 vinculada a las funciones de invasión, supervivencia en monocitos, captación de Mg^{+2} y SPI-9 relacionado al aparato de secreción RTX.

Excepto en el aislamiento *S.* Montevideo todos portaban la isla C63PI. De igual modo en todos los aislamientos se detectó una isla no nombrada (NM) asociada a una integrasa codificada por islas de patogenicidad salvo en IIIB 61:I:Z53.

La isla SPI-3 asociada a la invasión, sobrevida dentro de monocitos y captación de Mg^{+2}, se detectó en todos los aislamientos. Estas funciones están codificadas por los genes *mgtCB*, *marT*, *misL*; mientras que sólo se detectó en 13 aislamientos SPI-3 asociada a las mismas funciones codificada por los genes *sugR*, *rhuM*, *MgtC*B, *Mar*T y *MisL*. SPI-4 estuvo presente en todos los aislamientos excepto en IIIB 61:I:Z53. Esta SPI-4 contiene los genes de virulencia *sii*ABCDEF, que constituyen el sistema de secreción tipo 1 (SST-1), para actuar en la colonización y adhesión de células epiteliales (Liu et al. 2023).

SPI-5 se detectó en su función como proteína efectora de las SPI-1 y SPI-2 en 72 aislamientos (Amavisit et al. 2003); SPI-5 asociada a invasión y la función sobrevida dentro de monocitos y captación de Mg^{+2} sólo en el aislamiento de *S.* Agona, y SPI-5 relacionada con el gen *sopB* sólo se detectó en IIIB 61:I:Z53. Este último serotipo fue el único que presentó la SPI-8, esta isla se ha detectado en serotipos tifoideos y no tifoideos como Enteritidis y Typhimurium (Saroj et al. 2008, Espinoza et al. 2017).

La SPI-13, relacionada con la enzima acetyl-coA dehidrolasa (*gacD*) y con un regulador transcripcional de la familia LysR, se detectó en 74 aislamientos y estuvo ausente en *S.* Agona, mientras que para SPI-13 vinculada a un regulador transcripcional asociado al gen *gtA*, se detectó en 68 aislamientos y su ausencia estuvo mayormente representada en el serotipo Dublin. La SPI-13 describe en muchos serovares de *Salmonella* adaptados como en los no adaptados al huésped, pero se caracteriza por presentar una gran diversidad en su composición genética entre especies de *Salmonella* y entre serotipos de *S. enterica* (Elder et al. 2016, Elder et al. 2018). Espinosa y colaboradores, (Espinoza et al. 2017) han reportado que la acción de SPI-13 es necesaria para la internalización de *S.* Enteritidis en macrófagos y que se encuentra en baja frecuencia en serovares adaptados al hombre. Los macrófagos activados producen itaconato, el cual es un ácido que inhibe una enzima bacteriana cumpliendo funciones bactericidas. *Salmonella* ha adquirido un operón presente en la SPI-13 que codifica para las proteínas que degradan el itaconato (Hersch & Navarre, 2020). En este trabajo de tesis, se detectaron 3 componentes de la SPI que actúan inhibiendo el itaconato producido por los macrófagos: el gen *gacD* que codifica para una Acetyl-coA dehydrolasa, el gen *gtrB* el cual es un regulador transcripcional de la familia *LysR* y el gen *gtrA* también con función regulador transcripcional.

La SPI-14 con funciones de proteína de transferencia y regulación transcripcional se reporta presente sólo en *S*. Typhimurium (Sabbagh et al. 2010). En este trabajo estuvo presente para las funciones de regulación de la transcripción mediante el gen *gpiB* y el sistema de secreción tipo III, necesario para la infección sistémica y la patogénesis

intracelular, en todos los aislamientos excepto en los serovares *S.* Agona y IIIB 61:I:Z53.

3.4.2 Detección de genes de virulencia

Se identificaron múltiples funciones de virulencia las cuales fueron relacionadas a la presencia de 38 genes. Las funciones incluían adherencia fimbrial, adherencia no fimbrial, invasión, genes inducibles en macrófagos, resistencia sérica, adaptación al estrés, regulación, captación de magnesio, toxinas, sistemas de secreción, antifagocitosis, sistemas de glicosilación, formación de biofilms, entro muchos otros.

Los genes relacionados con adherencia fimbrial, sistema de secreción fueron los más representados, seguidos de adherencia no fimbrial, presencia de toxinas, genes inducibles en macrófagos y resistencia sérica.

De los 18 genes identificados de adherencia fimbrial los genes *Agf/Csg*, *Bcf*, *fim, stb, std* se encontraron en el 100% de los aislamientos. Los genes *sth*, *sti* y *saf*, *stf* se encontraban en 74 y 73 aislamientos respectivamente. En menor frecuencia se detectaron los genes *lpf* y *pef.* Estos genes están implicados en uno de los estadios más importantes en la patogénesis de *Salmonella* y se considera un factor clave en la patogénesis (Kolenda et al. 2018, Rehman et al. 2019). Se describen grupos principales de genes fimbriales que intervienen en la adhesión dentro de los cuales hay variaciones alélicas que representan una evolución patoadaptativa de adhesión a tejidos y hospederos (Yue et al. 2012). En un análisis realizado por Yue et al. (2012) se estableció que los genes *bcf, fim, stb, sth, std, saf y sti* estaban presentes en más del 80% de los serovares de *Salmonella* y que cumplían funciones esenciales. Estos resultados presentan similitudes y diferencias con este trabajo debido a que los serotipos evaluados no fueron los mismos, pero concordó en la frecuencia de los principales genes esenciales implicados en la adhesión (Yue et al. 2012). Estos datos, sirven de base para desarrollar estudios que implican la formulación de vacunas, así como el desarrollo de nuevos fármacos a partir de antígenos fimbriales bacterianos (Rehman et al. 2019).

De acuerdo con la detección de adhesinas no fimbriales, el gen *Mis-L* se detectó en todos los genomas y constituye un mecanismo de adherencia intestinal asociado a la matriz extracelular similar a *shdA* el cuál media la unión de proteínas de la matriz extracelular a través de un receptor en la célula huésped (Dorsey et al. 2005, Wang et al. 2018). *ShdA* es una proteína de membrana externa cuyo gen codificante se detectó en 73/75 aislamientos. En el modelo murino, esta proteína permite la persistencia de *Salmonella* Typhimurium en el intestino al unirse a la matriz extracelular, fibronectina

y colágeno tipo I (Kingsley et al. 2004). En menor medida también se encontró el gen *ratB* en 65/75 aislamientos involucrado en la persistencia intestinal (Branchu et al. 2018, Vila Nova et al. 2019. El gen *sinH* que se localiza en la isla CS54, es necesario para la colonización y persistencia intestinal en ensayos en ratones; el mismo fue detectado en el 100% de las muestras (Shah et al. 2012) y colabora en la adherencia intestinal.

Finalmente, se detectaron genes relacionados a adherencia, procedentes de otras especies bacterianas que en algunos casos presentan homología con proteínas detectadas en serovares de *Salmonella enterica*. Entre ellos, el gen que codifica para el pili Tipo IV de *Yersinia* spp. descripto en un plásmido de *E. coli*, presente en un plásmido en 14 aislamientos de *Salmonella enterica* de este trabajo; el gen que codifica para la fimbria K88 de *E. coli* detectado en 17 aislamientos, el cual tiene homología con una proteína cromosómica fimbrial de tipo 1 de *Salmonella* y el gen *Ehab* de *E. coli* detectado en 11 aislamientos y que codifica para una proteína autotransportadora presenta homología comuna proteína autotransportadora en *S*. Anatum.

Relacionados con los sistemas de secreción de *Salmonella* se identificaron 5 genes en total, entre ellos los codificados en la SPI-1, SPI-2, los efectores de traslocación generales y de translocación de SPI-1 y SPI-2 se detectaron en el 100% de los aislamientos.

Se detectaron dos genes que inducen su expresión en macrófagos. Por un lado, *mig-14* presente en todos los aislamientos que se induce cuando hay un bajo contenido de magnesio o pH ácido, y en 43 aislamientos el gen *mig-5*. Este último se detectó en al menos un representante de cada serotipo excepto en *S.* Montevideo. *Mig-14* es una proteína de membrana externa que promueve la resistencia bacteriana al péptido antimicrobiano relacionado con la catelina (CRAMP), la protamina y el péptido antimicrobiano de mamífero protegrina-1, la supervivencia dentro de los macrófagos activados y la infección persistente (Sheng et al. 2013, Brodsky et al. 2002). El gen *mig-5* se encuentra presente en plásmidos de virulencia de *Salmonella*, en este trabajo solo 9 aislamientos que presentaron *mig-5* no presentaban el operón *spvB* del plásmido de virulencia. Este gen se encuentra implicado también en la resistencia de *Salmonella* al sistema del complemento (Chacón et al. 2023).

El sistema de regulación PhoPQ estuvo presente en el 100% de los aislamientos. Este sistema de dos componentes detecta la acidificación del fagosoma y los péptidos antimicrobianos catiónicos para regular el contenido de proteínas y lípidos de la envoltura bacteriana. Las alteraciones que genera PhoPQ en las membranas bacterianas da como resultado una mayor resistencia bacteriana a los péptidos antimicrobianos catiónicos (CAMP) y una disminución de la detección por parte del sistema inmunitario innato (Dalebrux & Miller, 2014).

Rck se detectó sólo en 25 aislamientos de *S.* Typhimurium, 10 aislamientos de *S.* Newport y un aislamiento de *S.* Anatum. Es una proteína de membrana externa que actúa en la resistencia al complemento impidiendo la lisis bacteriana al bloquear el complejo de ataque de membrana y también se ha caracterizado por su capacidad para promover la invasión bacteriana in vitro de células de mamíferos (Koczerka et al. 2021).

El gen *sodCI* le brinda protección a *Salmonella* del superóxido fagocítico durante la replicación y el crecimiento en macrófagos (Krishnakumar et al. 2007). En la colección de aislamientos este gen se detectó en 40/75 en todos los serotipos excepto en *S*. Montevideo y *S*. Agona.

El gen *spvB* se detectó en 36/75 aislamientos de los cuales 24 fueron *S.* Typhimurium, 6 *S*. Newport, 4 *S.* Dublin y 1 *S.* Anatum y 1 IIIB 61:I:Z53. El gen *spvB* está localizado en un plásmido de virulencia de *Salmonella* e interviene en la autofagia, homeostasis del hierro y produce la disfunción de la barrera intestinal permitiendo la invasión bacteriana (Sun et al. 2019).

La mayor parte de los aislamientos (73/75) portaban el transportador MgtCB de Mg^{+2}. Las bacterias logran la homeostasis del Mg^{+2} a través de la activación de transportadores. El magnesio es crítico para la estabilidad de membranas bacterianas y ribosomas, neutralizan ácidos nucleicos y es cofactor de enzimas. (Groisman et al. 2012).

En ninguno de los aislamientos estudiados se detectaron genes que codifiquen para la cápsula.

3.4.3 Asociación de factores de virulencia

Se realizó una evaluación de los factores de virulencia detectados y su asociación con el origen de las muestras y los serotipos descriptos. Se excluyeron del análisis aquellos aislamientos obtenidos en baja frecuencia de alimento, agua y feto, y los serotipos *S.* Agona, *S.* Montevideo y IIIB 61:I:Z53 que se detectaron sólo en una oportunidad. Los mismos se presentan en la Tabla 2 y Tabla 3.

3.4.3.1 Asociación entre genes y serotipos específicos

Se observó una asociación entre genes y serotipos específicos. Como ya ha sido descrito, los genes *pef* y *stc* (adherencia fimbrial), *mig-5* (genes inducibles en macrófagos), *rck* (resistencia al complemento), *spvB* (toxina) y *Ehab* (autotransportador) se asociaron con el serotipo Typhimurium (Feng et al. 2012). Esta asociación puede explicarse dado que los genes *rck*, *pef* y *mig-5* se encuentran presentes en el plásmido de virulencia de *Salmonella* Typhimurium (Rychlik et al. 2006) por lo que se trasmiten en conjunto.

Los genes *peg* (p=<0,001*)* y *ste* (p=0,01) (adherencia fimbrial) se asociaron con el serotipo Newport. Estos operones fimbriales están descriptos y detectados en *S.* bongori y en todos los clados que constituyen *S. enterica* subsp *enterica* dentro de los cuales también se encuentra el serovar Newport, excepto en las subespecies diarizonae y houtenae (Worley et al. 2018, Cheng & Wiedmann, 2021). Los genes *lpf* (p= 0,018), *pef* (p=<0,001), *stc* (p=<0,001), *mig-5* (p=<0,001), *rck* (p=<0,001*)* y *spvB* (p=<0,001) vinculados a las primeras etapas de infección y a los procesos que permiten la diseminación y supervivencia de *Salmonella* se asociaron con *S.* Typhimurium. El gen *sodCI* (p=<0,001) (adaptación al estrés) se asoció con los serotipos Typhimurium y Dublin participando en la reducción del estrés oxidativo permitiendo la supervivencia de estos serotipos de *Salmonella* y también de otros patógenos (Sharma et al. 2023); un gen presente en el cromosoma de *Salmonella* homólogo del gen fimbrial K88 de *E. coli* involucrado en la adherencia se asoció con los serotipos Newport y Dublin.

De acuerdo con las islas de patogenicidad, también se observó una asociación entre serotipos y las SPI. *S.* Typhimurium estuvo asociado con las islas CS54 (p= 0,005) relacionada con colonización intestinal y determinantes de persistencia (*shdA, ratA, ratB, sivl, sivH*) (Sabbagh et al. 2010) y SPI-12 (p= 0,0003) vinculada a los genes *msgA*, narP la cual se ha demostrado ser necesaria para la infección sistémica en ratones infectados con *S.* Typhimurium (Sabbagh et al. 2010).

Tabla 2. Distribución y asociación genes de virulencia de acuerdo con los serotipos de los aislamientos

Genes de virulencia		Distribución de genes según serotipos					
Función	**ID**	***S.* Typhimurium (n=27)**	***S.* Dublin (n=6)**	***S.* Anatum (n=3)**	***S.* Newport (n=36)**	**Total**	**p: <0,05 ***
Adherencia Fimbrial	***Agf/Csg***	27	6	3	36	72 (100%)	-
	Bcf	27	**6**	3	36	72 (100%)	-
	Fim	27	**6**	3	36	72 (100%)	-
	Lpf	22	6	0	26	54(75%)	**0,018**
	Pef	27	2	0	2	50 (69%)	**<0,001**
	peg	5	4	3	29	41 (57%)	**<0,001**
	Saf	27	5	3	36	71(99%)	-
	sef	0	4	0	0	4 (5,6%)	-
	Sta	5	1	3	11	20 (28%)	**0,034**
	Stb	27	6	3	36	72 (100%)	-
	Stc	19	3	0	5	27 (37,5%)	**<0,001**
	std	27	6	3	36	72 (100%)	-
	ste	5	4	1	20	30 (42%)	**0,01**
	Stf	27	6	3	36	72 (100%)	-
	Stg	1	0	0	0	1 (1,4%)	-
	Sth	27	6	3	36	72 (100%)	-
	Sti	27	6	3	36	72 (100%)	-
Genes inducibles por macrófagos	*Mig-14*	27	6	3	36	72 (100%)	-
	Mig-5	26	6	2	9	43 (60%)	**<0,001**
Absorción de Mg^{+2}	transporte Mg^{+2}	27	6	3	36	72 (100%)	-
Adherencia no fimbrial	*MisL*	27	6	3	36	72 (100%)	-
	RatB	27	6	3	29	65 (90%)	0,23
	shdA	27	6	3	34	70 (97%)	-
	sinH	27	6	3	36	72 (100%)	-
Regulador	*PhoPQ*	27	6	3	36	72 (100%)	-
Sistemas de secreción	*SPI-1*	27	6	3	36	72 (100%)	-
	SPI-2	27	6	3	36	72 (100%)	-
Resistencia al suero	***rck***	25	0	1	10	36 (50%)	**<0,001**
Adaptación al estrés	***sodCI***	25	6	1	8	40 (55,5%)	**<0,001**
Toxinas	***spvB***	24	4	1	6	35 (49%)	**<0,001**
	Toxina tifoidea	0	0	1	1	2 (3%)	-

*p= <0,05 significativo. (-): asociación no determinada.

3.4.3.2 Asociación entre genes y origen específicos de los aislamientos

Hubo asociación entre el origen de los aislamientos y los genes *peg* (p= 0,002) y *stc* (p= 0,04) que se relacionan con la adhesión fimbrial al intestino. Los aislamientos de heces y ambiente presentaron mayor frecuencia de *peg* que los aislamientos de provenientes de de órganos (infecciones sistémicas). Por el contrario, hubo mayor frecuencia del gen *stc* en el grupo de aislamientos obtenidos de órganos.

Los genes *mig-5, spvB* y *sodCI* también mostarron asociación con el origen de los aislamientos (Tabla 3). La mayor frecuencia de estos genes se vio en los aislamientos de órganos y secreciones. Los genes *mig-5 y spvB* están codificados en plásmidos y se asocian a la inducción de genes en macrófagos. El gen *sodCI* está implicado en la sobrevida intramacrofágica y contibuye a la diseminación sistémica de *Salmonella* (Tidhar et al. 2015).

Los aislamientos de heces se encuentran asociados con el gen que codifica para el pili Tipo IV de *Yersinia* spp. (p= 0,001) y el gen *Ehab* de *E. coli (*p= 0,01). Estos genes están relacionados con la etapa de adhesión de *Salmonella* al epitelio intestinal, y el gen *Ehab* además promueve adhesión y la formación de biofilm (Wells et al. 2009). El gen fimbria K88 de *E. coli* (p= <0,001) se asoció con *S.* Newport y *S*. Dublin, *Ehab* (p= <0,001) y pili Tipo IV de *Yersinia* spp. (p= 0,04) con *S*. Typhimurium

Tabla 3. Distribución y asociación genes de virulencia de acuerdo con el origen de los aislamientos

Genes de virulencia		Lugar de obtención de los aislamientos			Total	p= <0,05*
Función	**ID**	**Heces (n=40)**	**Órganos y tejidos (n=25)**	**Ambiente (n=6)**		
Adherencia Fimbrial	***Agf/Csg***	40	25	6	71 (100%)	-
	Bcf	40	25	6	71 (100%)	-
	Fim	40	25	6	71 (100%)	-
	Lpf	29	21	4	48 (64%)	-
	Pef	27	21	2	54 (72%)	0,08
	peg	28	7	4	42 (56%)	**0,002**
	Saf	40	24	6	73 (97,3%)	-
	sef	1	3	0	4 (5,33%)	-
	Sta	14	6	1	22 (29,3%)	0,65
	Stb	40	25	6	71 (100%)	-
	Stc	12	17	1	30 (40%)	**0,004**
	std	40	25	6	71 (100%)	-
	ste	18	7	3	31 (41,3%)	0,31
	Stf	39	25	5	73 (97,3%)	-
	Stg	0	1	0	1 (1,33%)	-
	Sth	40	25	5	74 (98,6%)	-
	Sti	40	25	5	74 (98,6%)	-
	stj	39	20	5	68 (90,6%)	**-**
Genes inducibles en macrófagos	*Mig-14*	40	25	6	71 (100%)	-
	Mig-5	19	23	2	44 (62%)	**0,0002**
Absorción de Mg^{+2}	transporte Mg^{+2}	40	25	4	69 (97%)	-
Adherencia no fimbrial	*MisL*	40	25	6	71 (100%)	-
	RatB	34	24	5	63 (89%)	0,26
	shdA	40	25	4	69 (97%)	-
	sinH	40	25	6	71 (100%)	-
Regulador	*PhoPQ*	40	25	6	71 (100%)	-
Sistemas de secreción	*SPI-1*	40	25	6	71 (100%)	-
	SPI-2	40	25	6	71 (100%)	-
Resistencia séríca	*rck*	15	16	2	33 (46%)	0,09
Adaptación al estrés	***sodCI***	15	21	2	38 (54%)	**<0,001**
Toxinas	***spvB***	13	21	2	34 (48%)	**0,001**
	Toxina tifoidea	3	0	0	4 (6%)	-

*p= <0,05 significativo. (-): asociación no determinada.

3.4.4 Análisis filogenético

Se utilizaron todos los serovares para evaluar las similitudes y diferencias entre los genomas evaluados de acuerdo con la virulencia.

Los aislamientos se agruparon según los serotipos y se observan similitudes intraserotipo con la excepción del serotipo Typhimurium y Dublin los cuales muestran mayor diferencia en los genes de adherencia fimbrial (Figura 1). Esto podría relacionarse con al hecho de que las cepas de estos serotipos fueron aisladas de diferentes brotes de salmonelosis bovina en diferentes predios de producción los que representa mayor diversidad. Las diferencias observadas en la presencia de islas de patogenicidad podrían relacionarse con las metodologías de identificación, pero también al propio intercambio de material genético entre bacterias (Suez et al. 2013). Se observa en *S.* Dublin la presencia de la SPI-10 estando ausente en los demás serotipos de este trabajo, así como la presencia de la SPI-3 relacionada con invasión, supervivencia en monocitos, captación de Mg^{+2} en *S.* Anatum y la ausencia de la isla CS-54. De acuerdo con Zhao y colaboradores (2020), sería necesario realizar un estudio a mayor escala para determinar la distribución de las islas de patogenicidad que presentan los aislamientos de origen bovinos.

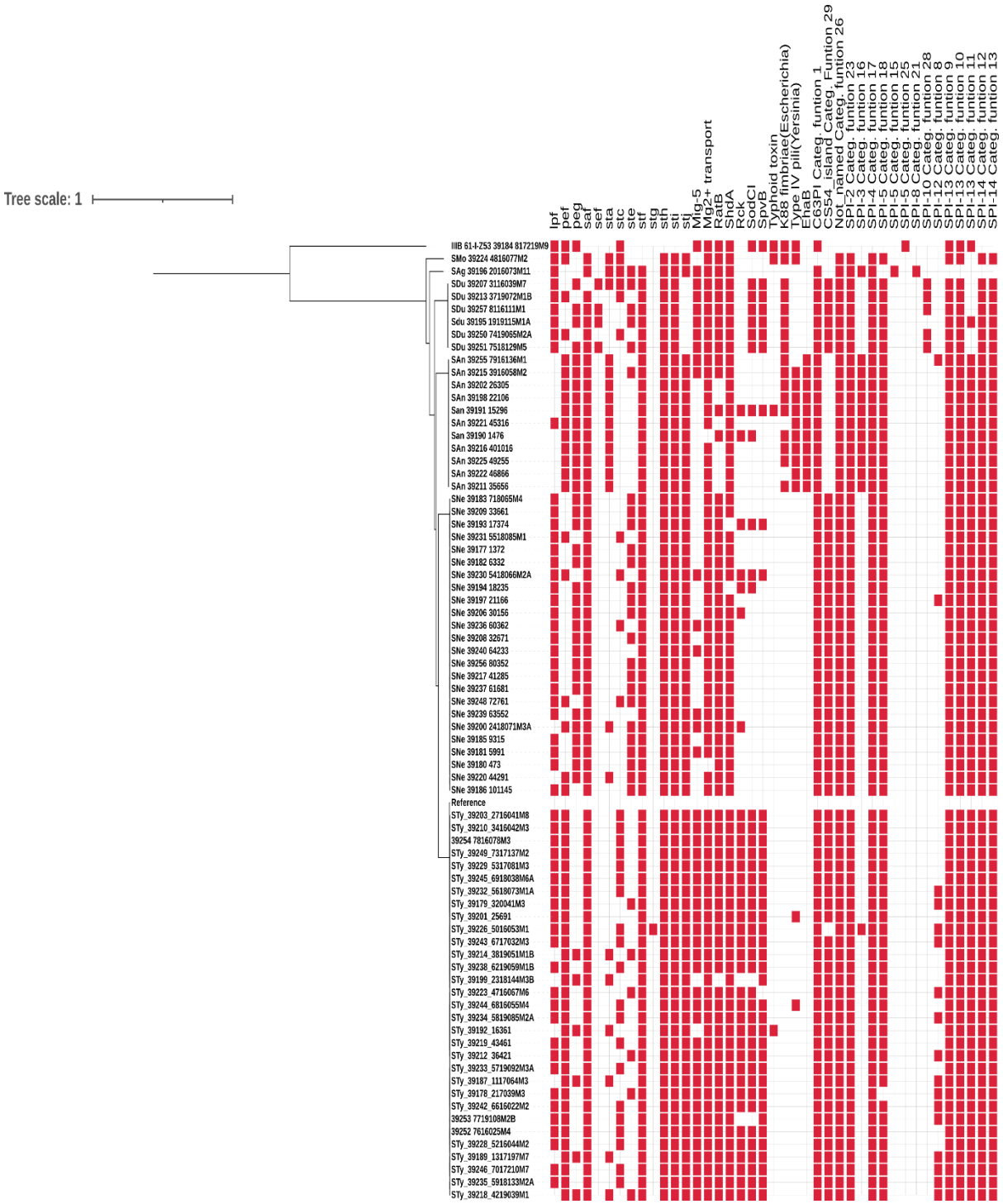

Figura 1. Relación filogenética entre los aislamientos de *S.* enterica y los factores de virulencia presentes. Se excluyeron los genes y SPI que estuvieron presentes en el 100% de los aislamientos.

3.5 CONCLUSIONES

Salmonella es un patógeno que afecta tanto a seres humanos como a animales, esta característica ha sido adquirida en su evolución por transferencia horizontal, presentando en el genoma bacteriano los genes necesarios para cumplir el ciclo infectivo. Estos genes suelen estar incluidos en islas de patogenicidad que son las responsables de la movilidad horizontal de dichos genes (Gal-Mor & Finlay 2003). En este trabajo detectamos 14 islas de patogenicidad diferentes con SPI-1, SPI-2, SPI-3 y SPI- 9 presentes en el 100% de los aislamientos. En una alta frecuencia se detectaron otras islas de patogenicidad con diversas funciones. Las mismas presentan los genes necesarios para cumplir con éxito las diferentes etapas de infección de *Salmonella*.

Por otro lado, los genes de virulencia identificados codifican para diversas funciones de adherencia, toxinas, componentes de sistemas de secreción, invasión, resistencia a péptidos antimicrobianos, captación de nutrientes como hierro y magnesio entre otros.

Se observó una asociación entre genes y serotipos. Principalmente el serotipo Typhimurium presentó asociación con genes de diferentes funciones esto puede ser debido a que es el serotipo más frecuentemente detectado. Los aislamientos obtenidos de heces se encuentras asociados en general a genes de adhesión fimbrial y afimbrial y los genes detectados en aislamientos obtenidos de órganos y secreciones presentan asociación con genes implicados en procesos sistémicos. En un proceso de vigilancia activo, esta asociación podría servir de señal de alerta al momento de ingresar nuevos clones a las instalaciones, de manera de poder anticiparse a la probabilidad de que se desarrollen brotes de infecciones extraintestinales.

Estos resultados representan la primera aproximación de los atributos generales de virulencia que presentan los aislamientos de *Salmonella* obtenidos a partir de bovinos lecheros de Uruguay utilizando el genoma completo de los aislamientos. Se han logrado identificar numerosos genes que son reconocidos en las diferentes etapas de infección. Por otro lado, los datos generados a partir de los genomas representan información útil para aplicar en futuras investigaciones y desarrollar herramientas para el control de la salmonelosis bovina.

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CONCLUSIONES GENERALES

En la ganadería lechera, la diarrea neonatal y la mortalidad de terneros son afecciones frecuentes que resultan en pérdidas económicas al sistema productivo. Dentro de los agentes infecciosos que pueden afectar a los terneros, *Salmonella* es una de las principales bacterias causantes de enfermedad. En estudios previos realizados en Uruguay se concluyó que es el único agente infeccioso asociado a mortalidad de terneros lecheros (Casaux et al. 2018, Caffarena et al. 2021). Este trabajo permitió determinar mecanismos involucrados en las características propias de *Salmonella* que le permite causar daño a los terneros, sobrevivir y persistir.

Se realizó una evaluación epidemiológica, patológica y genotípica de aislamientos involucrados en 15 brotes de salmonelosis bovina, concluyendo que los serovares que integran este trabajo, *S.* Typhimurium y S. Dublin, también son los serovares mundialmente reportados. Asimismo, las presentaciones clínicas de diarrea y mortalidad, lesiones entéricas, renales y esplénicas fueron las más frecuentes. Se observó que aquellos terneros que estaban infectados con *S.* Typhimurium tenían más probabilidad de presentar diarrea. Además, los terneros infectados con *S.* Typhimurium tenían menos de 30 días de vida. Esto puede deberse a las características de inmunosupresión que presentan los teneros en esta etapa de vida. En oposición, los terneros afectados por *S.* Dublin no presentaron diarrea y las presentaciones clínicas por este serotipo se daban en terneros de más de 50 días de vida. Sin embargo, ambos serotipos fueron capaces de generar lesiones y muerte. Justificando este tipo de hallazgos se determinaron por PCR en tiempo final 17 atributos de virulencia de aislamientos involucrados detectando que presentan una alta frecuencia en genes de virulencia implicados en diferentes funciones en ambos serotipos.

En línea con lo anterior, en una evaluación a nivel genómico de 75 aislamientos de *S. enterica* se detectaron numerosos genes de virulencia, donde los más numerosos fueron los relacionados con la adherencia fimbrial seguidos de adherencia no fimbrial. También se detectaron, en los serotipos más frecuentes, genes que cumplen funciones de invasión, componentes de sistemas de secreción, captación de nutrientes, sobrevida en macrófagos, evasión del sistema inmune, resistencia a péptidos antimicrobianos. Eso último pone de manifiesto la capacidad, que tiene este patógeno, de generar daño en el hospedero.

Se determinó que aquellos genes de virulencia que representan adhesinas fimbriales se encuentran asociadas con los aislamientos obtenidos de muestras de heces y que aquellos aislamientos de *Salmonella* obtenidos de órganos y secreciones estaban asociados a genes relacionados a diferentes etapas de la infección sistémica.

Por medio del análisis del genoma completo se detectó la presencia de genes asociados a la resistencia a antimicrobianos, mutaciones que confieren resistencia y

además de plásmidos e integrones. Se identificó que los aislamientos de *Salmonella* que circulan en los establecimientos ganaderos lecheros presentan genes que confieren resistencia principalmente a aminoglucósidos, tetraciclinas, sulfamidas y en menor media a quinolonas y β-lactámicos las cuales representan los grupos de antibióticos utilizados para tratamientos en los establecimientos ganaderos. Las drogas frente a las que se detectó resistencia son frecuentemente utilizadas para tratamientos en los establecimientos ganaderos. En contraste con la detección de genes, la frecuencia de resistencia a quinolonas debido a mutaciones fue relativamente alta, sólo se detectó la mutación *parC*_T57S en todos los serotipos excepto en *S.* Typhimurium y *S.* Dublin. En una comparación de la resistencia genotípica y fenotípica de los resultados se detectó una correlación de 85-90% entre ambos métodos, esto podría sugerir que los aislamientos presentan mecanismos por los cuales no expresan en su totalidad sus atributos de resistencia a antibióticos. Por otro lado, los datos obtenidos varían dependiendo de la plataforma de evaluación de genomas que se utilice. Es por este motivo que es necesario profundizar el análisis de los genomas con diferentes herramientas de forma que puedan explicar las discrepancias detectadas. En un contexto más amplio, generar información relacionada a los atributos de virulencia y resistencia que presentan las salmonelas aisladas de bovinos, permitirá abordar con datos concretos el desarrollo de diferentes herramientas para el control de los brotes de salmonelosis en Uruguay.

PERSPECTIVAS

A partir de este trabajo se logró generar información sobre las características de virulencia y resistencia antimicrobiana de aislamientos de *Salmonella* de origen bovino, así como datos epidemiológicos y patológicos de la salmonelosis bovina en Uruguay, logrando dos publicaciones en revistas internacionales indexadas y un tercer capítulo de tesis cuyos resultados van a ser publicado en un futuro próximo.

A partir de la caracterización de los aislamientos obtenidos en este trabajo se vienen desarrollando trabajos interinstitucionales para la creación de terapias alternativas para el control de la salmonelosis bovina. Por un lado, el desarrollo de nanopartículas de plata a partir de hongos específicos que sean efectivas frente a los principales serotipos involucrados en brotes; y por otro lado el desarrollo de nuevas vacunas a partir de la colección de *Salmonella* involucradas en este trabajo. Todas estas futuras aplicaciones llevarán ensayos en animales en un futuro próximo.

La secuenciación de los aislamientos y los datos generados permitieron generar información que es objetivo en nuevos estudios. Por un lado, evaluar en un futuro a partir de herramientas geográficas y características genómicas distribución de *Salmonella* en Uruguay y por otro lado profundizar en ensayos de virulencia seleccionando genes candidatos para lograr una mayor caracterización de las cepas. En un enfoque de salud global, sería interesante desarrollar una evaluación de la relación y características de *Salmonella* de origen humano y animal que permita abordar la relación que pueda existir entre ambos.

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Anexo

Anexo 1. Función de las diferentes islas de patogenicidad detectadas con su codificación en números funciones brindadas como salida del SPIfinder	
Tipo de función	**Función**
1	Operon *sit*, Sistema de captación de hierro
5	Sistema de secreción tipo III, invasión de células epiteliales, apoptosis.
8	*msgA, narP*
9	Acetyl-coA dehydrolase (*gacD*)
10	LysR family transcriptional regulation (*gtrB*)
11	Regulación transcripcional (*gtA*)
12	Subunidad beta de la favoproteína de transferencia de electrones (*gpiA*)
13	Regulación transcripcional (*gpiB*)
14	Sistema de secreción tipo III, necesario para la infección sistémica y la patogénesis intracelular al facilitar la replicación de bacterias intracelulares dentro de vacuolas que contienen *Salmonella* unidas a membranas.
15	Invasión, supervivencia en monocitos, captación de Mg^{+2} (*MgtC, B, MarT, MisL*)
16	Invasión, supervivencia en monocitos, captación de Mg^{+2} (*sugR, rhuMMgtC, B, MarT, MisL*), supuesta proteína fimbrial (*yadC/K./L/M*), probable chaperona de pilina (*ecpD1/D2*)
17	Sistema de secreción tipo I, supuesta secreción de toxina, apoptosis, necesario para la supervivencia intracelular en macrófagos.
18	Proteínas efectoras para SPI-1 y SPI-2 (*SopB, SigD, PipB*)
21	Exopolisacárido Vi, profago SopE y un operón pilus tipo IVB
22	Aparato secretor tipo I, proteína similar a RTX
23	*spiC*; proteína efectora secretada de la isla de patogenicidad 2
25	*sopB*
26	gen de proteína isla de patogenicidad putativa (*ssaD*)
27	invA; proteína A de invasión no funcional debido a mutación; ubicado en la isla de patogenicidad de *Salmonella* 1
28	Proteína similar a la fibrina (*sefD*)
29	Colonización intestinal y determinantes de persistencia (*shdA, ratA, ratB, sivI, sivH*)