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Whole genome-based antimicrobial resistance, virulence, and phylogenetic characteristics of *Trueperella pyogenes* clinical isolates from humans and animals

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ABSTRACT

Trueperella pyogenes is an opportunistic zoonotic bacterial pathogen, whose antimicrobial resistance, virulence, and genetic relatedness between strains from animals and humans are barely studied. These characteristics were therefore analyzed for clinical *T. pyogenes* strains from 31 animals of 11 different species and 8 humans determining their complete circular genome sequence and antimicrobial susceptibility. The MICs of 19 antimicrobials including 3 antiseptics correlated to the resistance genes identified in silico within the genomes revealing a predominance of resistance to streptomycin (*aadA9*), sulfamethoxazole (*sul1*), and tetracycline (*tet*(33), *tet*(W/N/W)) among strains from humans and cattle. Additional resistance genes (*erm*(X), *erm*(56), *cmx*, *drfA1*, *aadA1*, *aph(3'')-Ib* (*strA*), *aph(6)-Id* (*strB*), *aac(3)-IVa*, *aph(4)-Ia*) were found only sporadically. The resistance genes were localized on genetic elements integrated into the chromosome. A cgMLST-based phylogenetic analysis revealed two major clusters each containing genetically diverse strains. The human strains showed the closest relatedness to strains from cattle. Virulence genes coding for fimbriae (*fimA*, *fimC*), neuroamidase (*nanP*, *nanH*), pyolysin (*plo*), and collagen binding protein (*cbpA*) were identified in strains from different hosts, but no correlation was observed between virulence factors and strain origin. The existence of resistance genes typically found in Gram-negative bacteria within the Gram-positive *T. pyogenes* indicates a wider capacity to adapt to antimicrobial selective pressure. Moreover, the presence of similar antimicrobial resistance profiles found in cattle and human strains as well as their closest relatedness suggests common zoonotic features and cattle as the potential source for human infections.

1. Introduction

Trueperella pyogenes is a Gram-positive bacterium and a common inhabitant of skin and mucous membranes of the upper respiratory, gastrointestinal, and urogenital tracts of animals. It is also an opportunistic pathogen causing suppurative infections in numerous animals, including cattle, swine, and small ruminants, and thus, responsible for significant economic losses to animal husbandry (Jost et al., 2005; Rzewuska et al., 2019). Infections caused by *T. pyogenes* have also been reported in companion animals, such as dogs, cats and horses, and various wildlife species (Rzewuska et al., 2019). Although rare, infections in humans may also occur, especially in immunocompromised individuals and those who have contact with farm animals and their environment, highlighting the zoonotic potential of this microorganism (Rzewuska et al., 2019; Stuby et al., 2023).

The pathogenicity and opportunistic nature of *T. pyogenes* are related to several known and putative virulence factors required for adherence, colonization, and tissue damage. Among them, pyolysin (Plo), fimbriae (FimA, FimC, FimE, FimG), collagen binding protein (CbpA), and neuroamidases (NanH, NanP) have been characterized (Risseti et al., 2017).

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Previous studies have shown that differences in pathogenic potential among *T. pyogenes* strains may exist and may be dependent on their origin (Santos et al., 2010). Nevertheless, many aspects of the *T. pyogenes* pathogenicity remain poorly characterized and knowledge of virulence genotype is still limited, especially regarding human strains. In this context, investigations of virulence factors could contribute to the understanding of public health threats posed by the zoonotic transmission of this pathogen.

Infections caused by *T. pyogenes* are commonly treated with antimicrobials like beta-lactams and tetracyclines in both veterinary and human medicine (Rzewuska et al., 2019; Stuby et al., 2023). However, the non-prudent and extensive use of antibiotics is considered the main reason for the widespread emergence of antimicrobial-resistant bacteria, challenging both human and animal health in terms of morbidity, mortality, and healthcare costs (ECDC, 2023). Although the phenotypic antimicrobial susceptibility of *T. pyogenes* has been investigated in strains from livestock animals (Feßler et al., 2017), there is still limited information regarding data on strains from other animal species and humans, as well as on the genetic resistance mechanisms and their location within the genome through either gene mutations or acquisition of mobile genetic elements (MGE).

MGE such as plasmids, integrons (In), insertion sequences (IS) and transposons (Tn), have been suggested to play an important role in the acquisition and transmission of antimicrobial resistance genes in *T. pyogenes* (Jost et al., 2003; Jost et al., 2004; Billington et al., 2006; Liu et al., 2009; Zhao et al., 2011; Dong et al., 2017; Dong et al., 2020a; Dong et al., 2020b; Kwiecien et al., 2020). In this perspective, whole genome sequencing (WGS) analysis facilitates nowadays the determination of the genetic relatedness between strains and the clarification of the genetic basis of antimicrobial resistance, as well as the location of underlying antimicrobial resistance genes (ARGs) within the genome. WGS-based typing methods such as pangenome, core genome, or whole genome multi-locus sequencing typing (cgMLST, wgMLST) are also useful to fingerprint bacterial strains in a particular geographical area or population and determine their genomic relatedness.

Such a WGS-based method is essential to determine the degree of relatedness between strains of human and animal origin within a One Health approach and to identify potential niches of similar strains where exchange may have occurred. The detection of *T. pyogenes* in different animal species as well as in human infections in Switzerland prompted us to determine if specific strains are associated with the different hosts and settings and to identify virulence factors and antimicrobial resistance genetic elements. Such a WGS-based characterization may be useful to better understand the molecular epidemiology of *T. pyogenes* circulating in different settings and identify which animal strains are the most likely associated with human infections.

2. Materials and methods

2.1. Selection and culture of Trueperella pyogenes strains

A total of 43 *T. pyogenes* strains isolated between 1999 and 2022 were included in the study. Thirty-one animal and eight human *T. pyogenes* strains isolated within veterinary and human routine diagnostic procedures in Switzerland were obtained from the cryopreserved collections of the Centre for Zoonoses, Animal Bacterial Diseases and Antimicrobial Resistance of the Institute of Veterinary Bacteriology, University of Bern, and of the Institute for Infectious Diseases, University of Bern, Switzerland. Four additional Swiss strains, previously sequenced and deposited in the NCBI GenBank database (CP081508, CP096280, CP096279, CP097247; Marchionatti and Perreten, 2022) were also included. The strains were cultivated on trypticase soy agar containing 5% defibrinated sheep blood (TSA-S, Becton, Dickson) at 37°C for 24 hours under 5% CO_2 incubation. Identification of the species was confirmed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MALDI Biotyper, Bruker Daltonics GmbH, Bremen, Germany) using MALDI Biotyper MSP Identification Standard Method. Strains originated from infection sites of cattle (n=16), goat (n=4), sheep (n=2), swine (n=5), horse (n=1), dog (n=1), cat (n=1), wildlife (n=5), and human (n=8) (Fig. 1, Table S1).

2.2. Antimicrobial susceptibility testing

The minimal inhibitory concentration (MIC) of 16 antimicrobials was determined by broth microdilution method using Sensititre EUST2 plates (Thermo Fisher Scientific) and following the recommendations of the Clinical and Laboratory Standards Institute M45 (CLSI, 2016) and VET06 (CLSI, 2017). Briefly, colonies were suspended in 0.9% NaCl to a turbidity of 0.5 McFarland corresponding to 10^8 CFU/mL. In total, 10 µl of the suspension was mixed in 11 mL of cation-adjusted Muller-Hinton broth (CAMHB) containing 5% (v/v) lysed horse blood (LHB), and 50 µl of it was used to inoculate the microtiter plates that were then incubated at 37°C under 5% CO2 for 24 hours. Additionally, double serial dilutions (ranging from 0.25 to 512 µg/mL) of 3 antiseptics (chlorhexidine digluconate, octenidine dihydrochloride, benzalkonium chloride) were prepared in CAMHB in a 96 wells plate, inoculated and incubated as described above. The MIC was determined as the lowest concentration of antimicrobial that inhibited the visible growth of bacteria. MIC_{50} and MIC_{90} were also determined for each antimicrobial.

To have the same interpretation criteria for both animal and human strains, the CLSI M45 resistance breakpoints for Coryneform genera, which include *Trueperella*, were preferred as those from CLSI VET06 since CLSI VET06 only reports susceptibility breakpoints for penicillins, erythromycin, and trimethoprim-sulfamethoxazole. The following breakpoints were used: penicillin (≥4 µg/mL), gentamicin (≥16 µg/mL), erythromycin (≥2 µg/mL), ciprofloxacin (≥4 µg/mL), tetracycline (≥16 µg/mL), clindamycin (≥4 µg/mL), rifampicin (≥4 µg/mL), and quinupristin-dalfopristin (≥4 µg/mL). CLSI M45 reports susceptibility breakpoints for linezolid (≤2 µg/mL) and vancomycin (≤2 µg/mL), but no criteria for resistance determination are provided. The MIC for kanamycin (≥4 µg/mL), streptomycin (≥4 µg/mL), chloramphenicol (≥8 µg/mL), and sulfamethoxazole (≥512 µg/mL) were interpreted using resistance breakpoints from previously published data (Kwiecien et al., 2020; Dong et al., 2020a). No breakpoints were available for cefoxitin, trimethoprim, chlorhexidine digluconate, octenidine dihydrochloride, and benzalkonium chloride. Production of β-lactamase was tested by Nitrocefin assay (BD BBL™ Dryslide™ Nitrocefin) for strains that were non-susceptible to penicillin.

2.3. Whole genome sequencing (WGS)

Genomic DNA was extracted from a lawn of colonies grown for 24 hours on TSA-S at 37°C under 5% CO_2 using MasterPure TM Complete DNA and RNA Purification Kit (Lucigen, Middleton, WI). WGS libraries were prepared with the SMRTbell prep kit 3.0 following the manufacturer's instructions (Pacific Biosciences, Menlo Park, CA), and sequenced using a SMRT Cell 8 M on Sequel IIe system at the Next Generation Sequencing Platform, Institute of Genetics, Vetsuisse Faculty, University of Bern, Switzerland. PacBio HiFi reads were de novo assembled using Flye assembler v2.9.1 in PacBio HiFi mode using Geneious Prime® v2021.1.1 (Biomatters Ltd.). Genomes' annotation was performed with the NCBI Prokaryotic Genome Annotation Pipeline. The complete genomes of the 39 strains were deposited into the NCBI GenBank database under BioProject PRJNA954368 (accession numbers CP123391 to CP123429); the additional 4 strains were deposited under BioProjects PRNJNA755484, PRNJNA826253, PRNJNA834611 and have genome accession numbers CP081508, CP096280, CP096279, CP097247, respectively. The accession numbers of each strain are shown in Fig. 1.

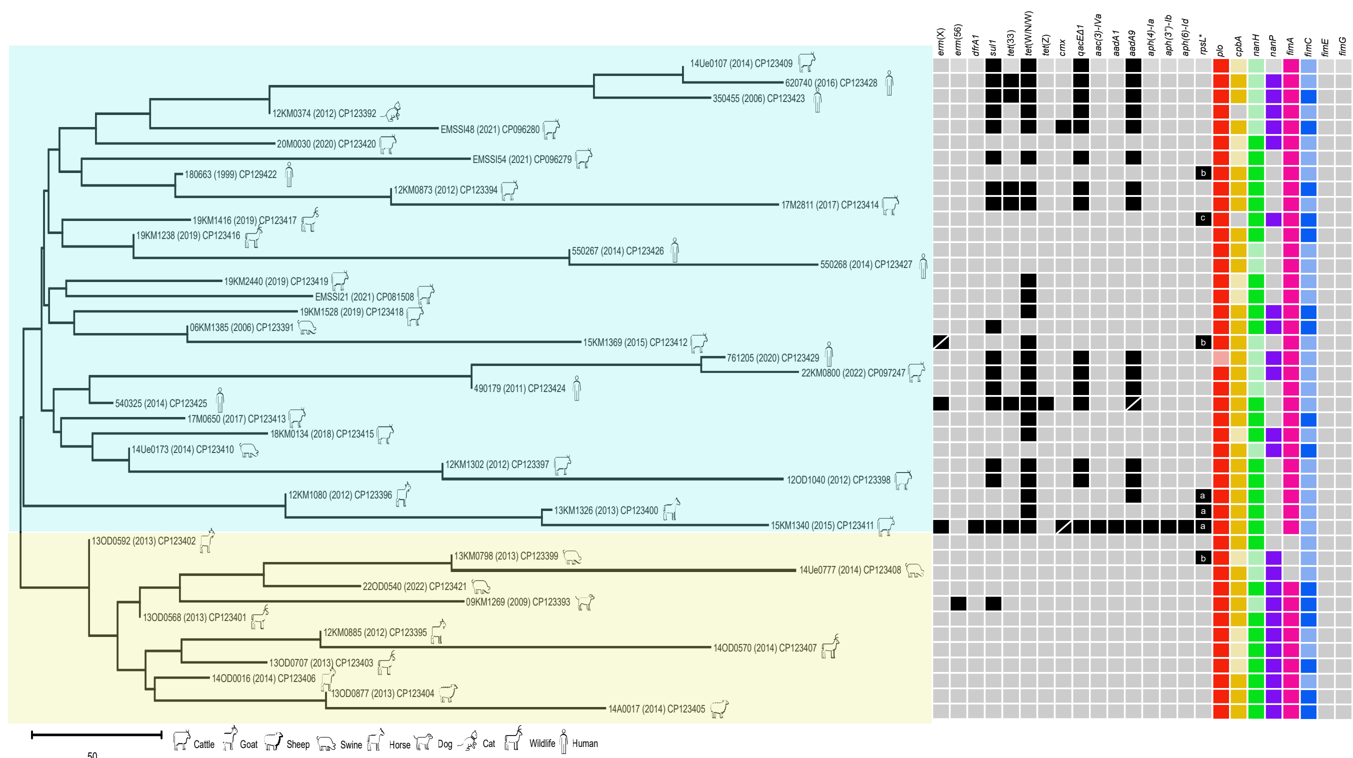

Fig. 1. cgMLST phylogeny, in silico antibiograms and virulence factor genes presence of the 43 *T. pyogenes* strains included in the study. Host, year of isolation, and GenBank accession number are indicated beside each strain name. The genomic features for antimicrobial resistance are represented by their presence (black square) or absence (gray square). Black squares with a white strike through represent the presence of resistance genes in the absence of phenotypic resistance. Letters within the black squares indicate *rpsL* mutations leading to a) Lys43Arg, b) Lys88Arg, and c) Lys43Thr amino acid substitutions. mutation indicate a) Lys43Arg, b) Lys88Arg, and c) Lys43Thr mutations respectively. The genomic features for virulence factor genes are represented by their presence (color square) or absence (gray square). The presence of homologues genes with different aa identity percentages are represented by different shades of color (*plo*: light red, 69%, dark red, 100%; *cpbA*: light yellow, ≤67%, dark yellow, ≥68%; *nanH*: light green, ≤76%, dark green ≥78%; *fimC*: light blue ≤70%, dark blue ≥98%). The figure was generated with MEGA11 using the Neighbour-Joining method (default parameters) and Adobe Illustrator.

2.4. WGS-based genetic characterization

Assemblies were screened in silico for ARGs using default parameters of Resfinder v4.1 (90% ID threshold, 60% minimum length) located at the Center for Genomic Epidemiology (https://www.genomicepidemiology.org) and CARD-RGI (perfect and strict hits only, exclude nudge) located at McMaster University (https://card.mcmaster.ca/analyze/rgi). The presence of virulence factor encoding genes *plo* (GenBank accession number AB027461), *cbpA* (GenBank accession number AY223543), *nanH* (GenBank accession number AF298154) and *nanP* (GenBank accession number AY045771) was evaluated by blastN search using sequence information available at the NCBI genome database, while a search of *fimA*, *fimC*, *fimE*, and *fimG* genes was performed using corresponding primer sequences published elsewhere (Silva et al., 2008) using Geneious Prime® v2021.1.1 (Biomatters Ltd.). cgMLST of the 43 *T. pyogenes* genomes was determined using chewBBACA v2.5.6. A phylogenetic tree was constructed based on cgMLST analysis with MEGA11 using the Neighbour-Joining method using default parameters.

3. Results

3.1. Antimicrobial susceptibility testing

The MIC distribution, as well as the MIC_{50} and MIC_{90} values for each antimicrobial and the susceptibility interpretation criteria, are shown in Table 1. MIC values above the applied breakpoints were frequent for streptomycin (n=20; 46.5%), sulfamethoxazole (n=17; 39.5%), and tetracycline (n=16; 37%); seven additional strains exhibited an MIC of 8 µg/mL for tetracycline, which placed them into the CLSI M45 interpretive category intermediate. However, these 7 stains contained all a tetracycline resistance gene (see below) and were therefore considered resistant due to the likelihood of therapeutic failure. One strain showed a MIC of 0.25 µg/mL for penicillin and also fell into the intermediate category. No known resistance mechanism was detected in this strain (see below). Three strains were resistant to erythromycin and clindamycin, one to trimethoprim, one to gentamicin, and one to chloramphenicol.

Eleven strains exhibited a MDR profile with three strains resistant to ≥ 4 and eight to 3 classes of antimicrobials; six strains were resistant to 2 antimicrobials and eleven to 1 antimicrobial. Fifteen strains were susceptible to all antimicrobials tested (Table S1). The MIC of each antiseptic tested, chlorhexidine digluconate, octenidine dihydrochloride, and benzalkonium chloride, were unimodally distributed indicating a wild-type phenotype and no acquired resistance (Table 1, Table S1). All the strains exhibiting increased MIC situated above the intermediate and resistance breakpoints contained resistance genes or point mutations explaining the resistance phenotype (Table 2), apart from the strain showing intermediate resistance to penicillin (see below).

3.2. WGS-based genetic characterization, detection of virulence genes, and ARGs

Genome sizes ranged from 2,234,126 bp to 2,390,155 bp and GC content from 59.3% to 59.8%. One genome could not be circularized. Four strains (12KM0873, 12KM1080, 490179, 761205) hosted plasmids of 4,864 bp, 2,435 bp, 4,799 bp, and 2,392 bp, respectively, which were related to the 2,439-bp *T. pyogenes* plasmid pAP1 of the rolling circle replication family (GenBank accession number U83788, Billington et al.,

Table 1
Minimal inhibitory concentration (MIC) distribution of 19 antimicrobial agents for 43 *T. pyogenes* isolates.

	Number of isolates classified by their MIC values (µg/mL)																		MIC$_{50}$	MIC$_{90}$
Antimicrobial	≤	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	>		
Kanamycin	-	-	-	-	-	-	-	-	-43-	∣				-	-	-	-	-	≤4	≤4
Rifampicin	-43-							-	-	-╫-	-	-	-	-	-	-	-	-	≤0.015	≤0.015
Erythromycin	-	-	-	-	-38-		2	‖	╫	1	1	-1-	-	-	-	-	-	-	≤0.25	0.5
Ciprofloxacin	-	-	-	-	-		1	31	9	2╫		-‖-	-	-	-	-	-	-	1	2
Tetracycline	-	-	-	-	-	20-				‖	7	6╫	-10-	-	-	-	-	-	8	>16
Streptomycin	-	-	-	-	-	-	-	-	-23-	∣	1	6	6	-7-	-	-	-	-	<4	>32
Quinupristin/dalfopristin	-	-	-	-	-	-43-				╫	-	-	-	-	-	-	-	-	≤0.5	≤0.5
Linezolid	-	-	-	-	-	-	-43-					-	-	-	-	-	-	-	≤1	≤1
Vancomycin	-	-	-	-	-	-	-43-					-	-	-	-	-	-	-	≤1	≤1
Clindamycin	-	-	-	-40-							-3-	-	-	-	-	-	-	-	≤0.12	≤0.12
Cefoxitin	-	-	-	-	-	-42-					1		-	-	-	-	-	-	≤0.5	1
Gentamicin	-	-	-	-	-	-31-		11	‖			∣╫	-1-	-	-	-	-	-	1	2
Sulfamethoxazole	-	-	-	-	-	-	-	-	-	-	-	-	-26-				‖	-17-	≤64	>512
Chloramphenicol	-	-	-	-	-	-	-	-	-42-		1‖				-	-	-	-	≤4	≤4
Penicillin	-	-	-42-			1			-	-╫-	-	-	-	-	-	-	-	-	≤0.06	≤0.06
Trimethoprim	-	-	-	-	-	-	-42-						-1-	-	-	-	-	-	≤1	≤1
Chlorhexidine digluconate	-	-	-	-	-				12	31								-	4	4
Octenidine dihydrochloride	-	-	-	-	-31-	12												-	≤0.25	0.25
Benzalkonium chloride	-	-	-	-	-			7	35	1								-	2	2

Dilution ranges in µg/mL of each antimicrobial tested are represented by the white area between the dashed (-) area.
Numbers between dasches (-n-): number of isolates with a MIC ≤ or > the lowest/highest concentration tested.
| MIC breakpoints from Kwiecien (2020) (Kwiecien et al., 2020).
‖ MIC breakpoints from Dong (2020a) (Dong et al.,2020a).
╫ MIC breakpoints for Coryneform genera including *Trueperella* species, CLSI M45, 3rd ed. 2016.

1998).

The distribution of the detected virulence genes is presented in Fig. 1. All 43 strains carried the pyolysin gene *plo* identified in *T. pyogenes* strain 42 (GenBank accession number AB027461, Ikegami et al., 2000); however, in strain 761205 from human, the pyolysin showed only 69% amino acid (aa) identity with that of the other strains suggesting the presence of putative new pyolysin homologs. For the CbpA, homologs were classified in two groups based on phylogenetic distance and percentage of aa identity being ≤67% (n=10) and ≥68% (n=31) (Fig. S1); strains 19KM1416 and 12KM0374 did not carry the virulence factor CbpA (Fig. 1). Twenty-three strains from diverse hosts carried the neuroamidase gene *nanP,* while the neuroamidase gene *nanH* was identified in all examined strains but separated in two groups exhibiting ≤76% and ≥78% aa identity in 15 and 28 strains respectively (Fig. S1). All the strains harbored at least one fimbriae gene with *fimC* present in all strains and *fimA* in 40 of them (Fig. 1). While the FimA fimbriae had ≥97% aa identity in all strains, the FimC showed two homologs' families with ≤70% (n=28) or ≥98% (n=15) aa identity (Fig. S1). The *fimE* and *fimG* genes were not detected in any of the examined strains (Fig. 1). No relationship could be observed between the presence/absence or homology group of the currently known virulence factors regarding the species of origin of the strains (Fig. 1, Fig. S1).

None of the plasmids contained resistance mechanisms; 15 ARGs and 3 point mutations associated with antimicrobial resistance were identified in the chromosome of 28 strains (Fig. 1, Table 2). They consisted of genes conferring resistance to tetracycline [*tet*(W/N/W) (n=23), *tet*(33) (n=6), *tet*(Z) (n=1)], sulfamethoxazole [*sul1* (n=17)], streptomycin [*aadA9* (n=15), *aadA1* (n=1), *aph(3'')-Ib* (*strA*) (n=1), *aph(6)-Id* (*strB*) (n=1)], erythromycin and clindamycin [*erm*(X) (n=3), *erm*(56) (n=1)], chloramphenicol [*cmx* (n=2)], gentamicin [*aac(3)-IVa* (n=1)], and trimethoprim [*dfrA1* (n=1)], as well as nonsynonymous point mutations in the *rpsL* gene (n=7) associated with streptomycin resistance (Fig. 1, Table 2). Six strains contained *rpsL* mutations leading to the Lys43Arg (strains 12KM1080, 13KM1326, and 15KM1340) and Lys88Arg (strains 180663, 15KM1369, and 13KM0798) substitutions, which are known to confer streptomycin resistance in *Mycobacterium tuberculosis* (Sreevatsan et al., 1996). Strain 19KM1416 harbored a Lys43Thr substitution. The Lys43Arg substitution was accompanied by an Arg86His substitution not previously described, while the Lys88Arg and Lys43Thr substitutions were alone (Fig. 1, Table 2). The quaternary ammonium compounds resistance gene *qacEΔ1* (n=15) was also detected, but its presence did not correlate with presumptive reduced antiseptic susceptibility (Fig. 1, Table 2). One strain contained the *aph(4)-Ia* hygromycin resistance gene, for which MIC was not determined. Otherwise, the correlation between phenotypic resistance and the presence of a corresponding resistance mechanism was in concordance for most of the strains exhibiting a MIC situated above the used resistance or intermediate breakpoints except for strain 17M0650 which showed decreased susceptibility to penicillin (Table 2). In this strain, the mechanism conferring decreased susceptibility to penicillin remained unknown; no alteration was detected in the PBPs as compared to the PBPs of the susceptible strains (06KM1385, 09KM1269, 12KM0374, 12KM0873, 12KM1080, EMSSI54) and the *blaP1* gene known to confer β-lactam resistance in *T. pyogenes* (Zhao et al., 2011) was not found. The BBL DrySlide Nitrocefin test did not reveal β-lactamase production.

The presence of a known resistance gene accompanied by low MIC was observed in a few strains (Fig. 1, Table 2): the *erm*(X) gene was found in the erythromycin (MIC ≤0.25 µg/mL) and clindamycin (MIC ≤0.12 µg/mL) susceptible strain 15KM1369, however genome analysis of the upstream region of *erm*(X) revealed missing putative −10 and −35 promoter sequences as compared to *erm*(X) from *T. pyogenes* strain 98–4277–2 (GenBank accession number AY255627, Jost et al. 2003). Strain 15KM1340 harbored the *cmx* gene but the MIC of chloramphenicol (≤2 µg/mL) was low and situated below the resistance breakpoint proposed by Dong (2020[a]); likewise, strain 540325 harbored the *aadA9* gene but the MIC of streptomycin (≤4 µg/mL) remained below the

Table 2
Correlation between phenotype and genotype in strains exhibiting antimicrobial resistance.

Strain	Host	Type of infection/ origin	MIC of antimicrobials (μg/mL) and corresponding antimicrobial resistance genes							
			TET	GEN	STR	SUL	CHL	TMP	ERY/CLI	CHX/OCT/ BZK
15KM1340	Cattle	Abscess	>16 [*tet*(33), *tet*(W/N/W)]	>16 [*aac(3)-IVa*]	>32 [*aadA1*, *aadA9*, *aph(3'')-Ib*, *aph(6)-Id*, *rspL* (Lys43Arg)]	>512 [*sul1*]	**≤4 [*cmx*]**	>16 [*dfrA1*]	>8/>4 [*erm*(X)]	4/<0.25/2 [*qacEΔ1*]
540325	Human	Biopsy	>16 [*tet*(33), *tet*(W/N/W), *tet*(Z)]	≤0.5	**≤4 [*aadA9*]**	>512 [*sul1*]	≤4	≤1	4/>4 [*erm*(X)]	4/0.25/2 [*qacEΔ1*]
12KM0873	Cattle	Metritis	16 [*tet*(33), *tet*(W/N/W)]	≤0.5	32 [*aadA9*]	>512 [*sul1*]	≤4	≤1	≤0.25/ ≤0.12	4/0.25/2 [*qacEΔ1*]
17M2811	Cattle	Mastitis	16 [*tet*(33), *tet*(W/N/W)]	≤0.5	32 [*aadA9*]	>512 [*sul1*]	≤4	≤1	≤0.25/ ≤0.12	2/0.25/2 [*qacEΔ1*]
EMSSI48	Cattle	Surgical site infection	>16 [*tet*(W/N/W)]	≤0.5	16 [*aadA9*]	>512 [*sul1*]	8 [*cmx*]	≤1	≤0.25/ ≤0.12	4/<0.25/2 [*qacEΔ1*]
350455	Human	Sputum	>16 [*tet*(33), *tet*(W/N/W)]	≤0.5	>32 [*aadA9*]	>512 [*sul1*]	≤4	≤1	≤0.25/ ≤0.12	2/<0.25/2 [*qacEΔ1*]
620740	Human	Blood	>16 [*tet*(33), *tet*(W/N/W)]	≤0.5	32 [*aadA9*]	>512 [*sul1*]	≤4	≤1	≤0.25/ ≤0.12	4/<0.25/2 [*qacEΔ1*]
12KM0374	Cat	Otitis	8 [*tet*(W/N/W)]	≤0.5	16 [*aadA9*]	>512 [*sul1*]	≤4	≤1	≤0.25/ ≤0.12	2/<0.25/2 [*qacEΔ1*]
12KM1302	Cattle	Metritis	8 [*tet*(W/N/W)]	≤0.5	32 [*aadA9*]	>512 [*sul1*]	≤4	≤1	≤0.25/ ≤0.12	4/0.25/2 [*qacEΔ1*]
12OD1040	Cattle	Metritis	8 [*tet*(W/N/W)]	≤0.5	16 [*aadA9*]	>512 [*sul1*]	≤4	≤1	≤0.25/ ≤0.12	4/0.25/2 [*qacEΔ1*]
14Ue0107	Cattle	Abscess	8 [*tet*(W/N/W)]	≤0.5	32 [*aadA9*]	>512 [*sul1*]	≤4	≤1	≤0.25/ ≤0.12	4/0.25/2 [*qacEΔ1*]
22KM0800	Cattle	SSI	>16 [*tet*(W/N/W)]	≤0.5	16 [*aadA9*]	>512 [*sul1*]	≤4	≤1	≤0.25/ ≤0.12	2/0.25/2 [*qacEΔ1*]
EMSSI54	Cattle	SSI	>16 [*tet*(W/N/W)]	≤0.5	32 [*aadA9*]	>512 [*sul1*]	≤4	≤1	≤0.25/ ≤0.12	4/<0.25/2 [*qacEΔ1*]
490179	Human	Biopsy	>16 [*tet*(W/N/W)]	≤0.5	8 [*aadA9*]	>512 [*sul1*]	≤4	≤1	≤0.25/ ≤0.12	4/<0.25/2 [*qacEΔ1*]
761205	Human	Biopsy	>16 [*tet*(W/N/W)]	≤0.5	16 [*aadA9*]	>512 [*sul1*]	≤4	≤1	≤0.25/ ≤0.12	4/<0.25/1 [*qacEΔ1*]
12KM1080	Goat	Abscess	8 [*tet*(W/N/W)]	1	>32 [*rspL*(Lys43Arg)]	≤64	≤4	≤1	≤0.25/ ≤0.12	4/<0.25/2
15KM1369	Cattle	Abscess	8 [*tet*(W/N/W)]	1	>32 [*rspL*(Lys88Arg)]	≤64	≤4	≤1	**≤0.25/ ≤0.12 [*erm*(X)]**	4/<0.25/2
09KM1269	Dog	Abscess	≤0.5	1	≤4	>512 [*sul1*]	≤4	≤1	8/>4 [*erm*(56)]	4/<0.25/2
13KM1326	Horse	Abscess	16 [*tet*(W/N/W)]	≤0.5	>32 [*rspL*(Lys43Arg)]	≤64	≤4	≤1	≤0.25/ ≤0.12	4/<0.25/2
06KM1385	Swine	Peritonitis	≤0.5	1	≤4	≤64	≤4	≤1	≤0.25/ ≤0.12	2/<0.25/2
13KM0798	Swine	Abscess	≤0.5	≤0.5	16 [*rspL*(Lys88Arg)]	≤64	≤4	≤1	≤0.25/ ≤0.12	2/0.25/1
17M0650	Cattle	Mastitis	8 [*tet*(W/N/W)]	≤0.5	≤4	≤64	≤4	≤1	≤0.25/ ≤0.12	4/<0.25/2
18KM0134	Cattle	Pneumonia	16 [*tet*(W/N/W)]	1	≤4	≤64	≤4	≤1	≤0.25/ ≤0.12	4/<0.25/2
19KM1416	Reindeer	Cystitis	≤0.5	≤0.5	>32 [*rspL*(Lys43Thr)]	≤64	≤4	≤1	≤0.25/ ≤0.12	4/<0.25/2
19KM1528	Cattle	Abscess	16 [*tet*(W/N/W)]	≤0.5	≤4	≤64	≤4	≤1	≤0.25/ ≤0.12	4/<0.25/2
19KM2440	Cattle	Abscess	16 [*tet*(W/N/W)]	1	≤4	≤64	≤4	≤1	≤0.25/ ≤0.12	4/<0.25/2
EMSSI21	Cattle	SSI	>16 [*tet*(W/N/W)]	1	≤4	≤64	≤4	≤1	≤0.25/ ≤0.12	2/<0.25/2
180663	Human	Biopsy	≤0.5	≤0.5	>32 [*rspL*(Lys88Arg)]	≤64	≤4	≤1	≤0.25/ ≤0.12	4/<0.25/2

TET: Tetracycline, GEN: Gentamicin, STR: Streptomycin, SMX: Sulfamethoxazole, CHL: Chloramphenicol, TMP: Trimethoprim, ERY: Erythromycin, CLI: Clindamycin, CHX: Chlorhexidine, OCT: Octenidine, BZK: Benzalkonium chloride. SSI: surgical site infection. Inconsistencies between phenotype and genotype are shown in bold.

breakpoint indicated by Kwiecien (2020). Unlike for *erm*(X), no alteration in the upstream region of the genes was identified, suggesting a silent gene phenomenon known in other bacterial species such as *E. coli* and *Salmonella* spp. (Stasiak et al., 2021). Fifteen strains exhibited low MICs for all the antimicrobials tested and did not harbor any known ARGs or point mutations in antimicrobial target genes (Fig. 1, Table S1).

Among the 43 investigated *T. pyogenes*, 15 of the 16 strains of cattle origin and 6 of the 8 strains of human origin contained at least one ARG or a point mutation associated with antimicrobial resistance. The strains from dog, cat, and horse, exhibited $\geq$ 2 ARGs or point mutation each. Strains from swine, small ruminants, and wildlife carried the lowest number of resistance mechanisms (4 of 16 strains were resistant). The highest numbers of ARGs and point mutations associated with resistance were found in *T. pyogenes* strains 15KM1340 (n=14) and 540325 (n=7) of cattle and human origin, respectively (Fig. 1).

3.3. Genetic elements associated with ARGs

The *tet*(W/N/W) gene displayed 100% identity to the sequence of the formerly named *tet*(W)-3 gene present on transposon ATE-1 from *T. pyogenes* isolated from bovine specimens (GenBank accession number AY049983; Billington et al., 2006). The genetic organization of the 23 elements carrying *tet*(W/N/W) was structurally related to ATE-1, mainly differing at the 3′ end of the elements. They were all integrated between two housekeeping genes, *guaA,* and *pcrA*, encoding for a GMP synthase and a DNA helicase respectively (Fig. 2). As previously described, a *repA* and a *mob* gene coding for a helicase and a plasmid recombination protein respectively, were present immediately upstream of *tet* (W/N/W). The elements contained a site-specific integrase gene of the phage integrase family (Pfam accession number PF00589) at the 5′ end; in 3 strains the integrase was followed by an IS*1249* (Fig. 2D). The 3′ end of two of the elements harbored genes related to a toxin-antitoxin system (Figs. 2C, D).

Strains 12KM0873, 15KM1340, 17M2811, 350455, 540325, and 620740 had an additional tetracycline resistance gene *tet*(33) located in another genetic element flanked by two identical copies of IS*6100* located in the same orientation and preceded by a TetR/AcrR family transcription regulator (Figs. 3D, E). This IS*6100*-*tet*(33)-IS*6100* element was located in a larger MGE (see below). Moreover, strain 540325 harbored an additional tetracycline resistance gene *tet*(Z) integrated into the chromosome, flanked by core genome methyltransferase and NAD (P)-binding protein.

Other resistance genes were located within mosaic structures of MGEs, between two identical copies of IS*6100* elements facing the same direction and flanked by 14-bp inverted repeats (IR-L, GGCTCTGTTGCAAA; IR-R, TTTGCAACAGAGCC) which therefore likely belonged to composite transposons (Roberts et al., 2008). Six different MGEs (MGE-A to MGE-F) were identified in 17 strains (Fig. 3). They were integrated in three different locations in the chromosome namely between *cpbp* and *lytR-C* genes encoding for a metalloprotease and a LytR/CpsA/Psr regulator respectively, in 2 strains (MGE-A, Fig. 3A; MGE-B, Fig. 3B); between *α/β-hdcp* and *mfs* genes encoding for a hydrolase and a MFS transporter respectively, in 14 strains (MGE-C, Fig. 3C; MGE-D, Fig. 3D; MGE-E, Fig. 3E); or between *dass* and *nudix* genes encoding for a DASS transporter and a hydrolase respectively, in 1 strain (MGE-F, Fig. 3F). These MGEs contained several different IS (IS*6100*, IS*110*, IS*3*, IS*481*, IS*6*, IS*26*, IS*1249*) flanking resistance genes, as well as class I integron gene *intI1* (Fig. 3). The gene combination of *aadA9*, *qacEΔ1* and *sul1* was present in 4 MGEs (MGE-C, MGE-D, MGE-E, MGE-F), and in one of them, MGE-F, it was associated with a class I integron gene *intI1*, while in MGE-C, MGE-D and MGE-E it was preceded by a TetR/AcrR family transcriptional regulator instead of *intI1*. MGE-F also contained *cmx* linked to IS*481*. MGE-D and MGE-E additionally contained *tet*(33) flanked by IS*6100* as mentioned above. MGE-E had additional resistance genes (*aph(4)-Ia*, *aac(3)-IVa*, *aph(3'')-Ib*, *aph (6)-Id*, *erm*(X), *cmx*) which were each associated with different IS elements (IS*6100*, IS*481*, IS*6*, IS*26*, IS*1249*), as well as a class I integron with *dfrA1*, *aadA1*, *qacEΔ1* and *sul1*. MGE-A and MGE-B both contained

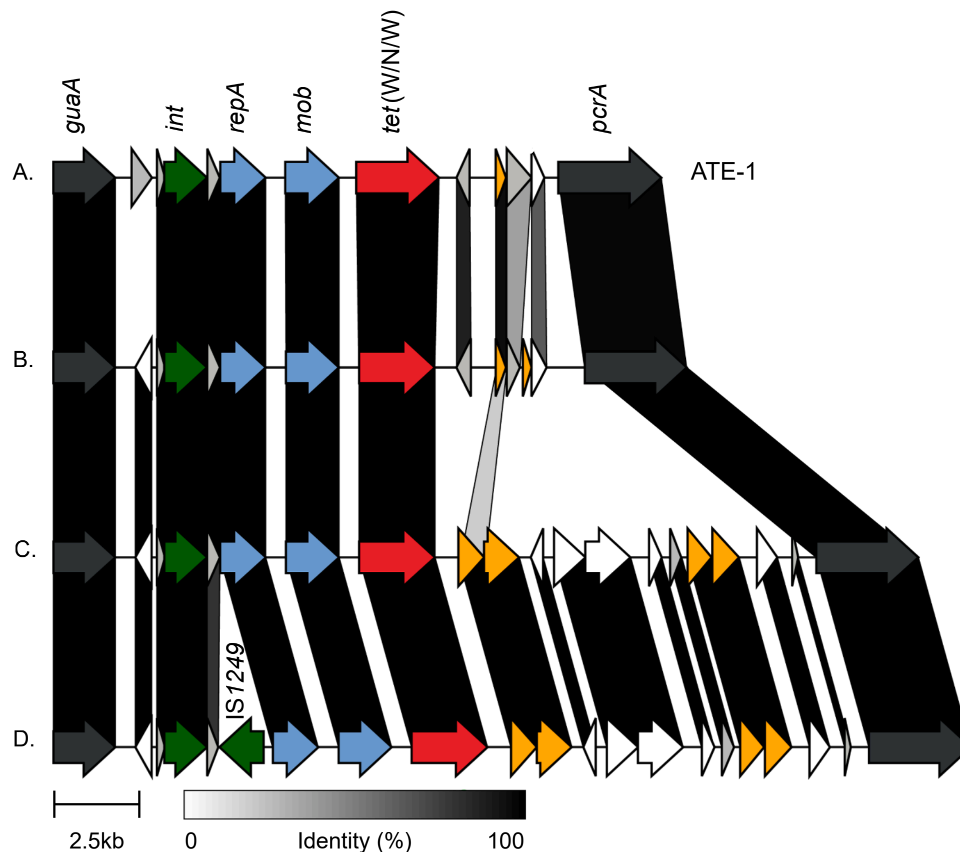

Fig. 2. Schematic gene map showing the *tet*(W/N/W)-containing element and flanking regions found in strains A.) ATE-1 transposon with GenBank accession number AY049983, B.) 12KM1080, 12KM0873, 13KM1326, 15KM1340, 15KM1369, 17KM0650, 17KM2811, 18KM0134, 19KM1528, 19KM2440, EMSSI21, EMSSI54, 540325, C.) 12KM1302, 12OD1040, 14Ue0107, 22KM0800, EMSSI48, 490179, 761205, and D.) 12KM0374, 350455, 620740. Black to gray areas represent regions showing similarity at the nucleotide level. Arrows represent open reading frames (ORFs). The ORF *tet*(W/N/W) is indicated by a red arrow. ORFs of integrase genes (site-specific integrase and IS*1249*) are indicated with green arrows. ORFs of plasmid protein genes (*repA* and *mob*) are indicated in blue. The ORFs of the toxin-antitoxin systems are indicated in orange. Other ORFs of hypothetical proteins are represented by light-gray arrows, while those of other genes by white arrows. Dark-gray ORFs at the left (*guaA*) and right (*pcrA*) ends of the sequence are housekeeping genes. The figure was generated using Clinker and Adobe Illustrator.

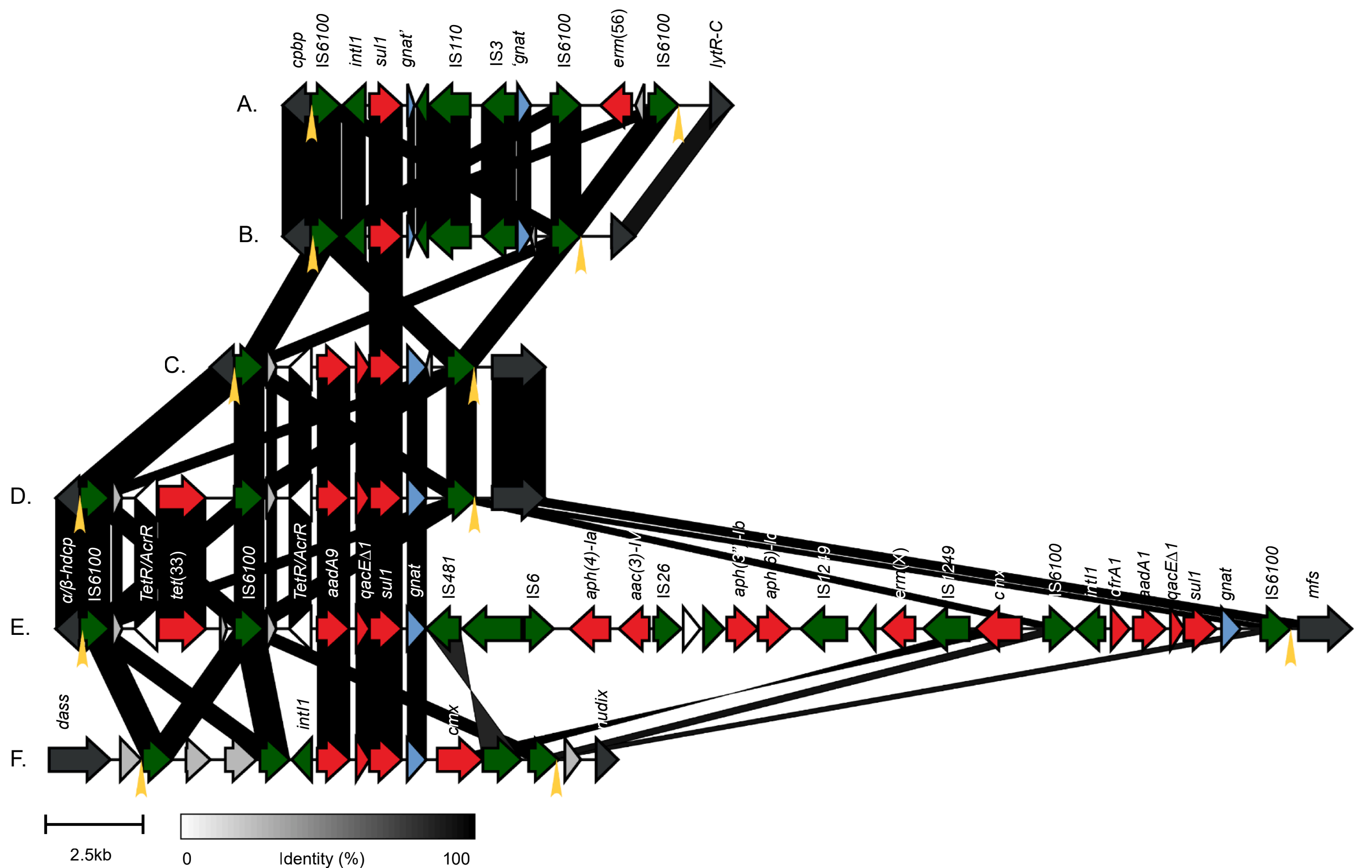

Fig. 3. Schematic gene map showing the MGEs and flanking regions. A.) MGE-A of strain 09KM1269, B.) MGE-B of 06KM1385, C.) MGE-C of 12KM0374, 12KM1302, 12OD1040, 14Ue0107, 22KM0800, EMSSI54, 490179, 761205, D.) MGE-D of 12KM0873, 17KM2811, 350455, 540325, 620740, E.) MGE-E of 15KM1340, and F.) MGE-F of EMSSI48. Black and dark-gray, areas represent regions showing ≥99% and 80% similarity at nucleotide level, respectively. Arrows represent open reading frames (ORFs). The ORF of resistance genes *tet*(33), *aadA9*, *aadA1*, *aac(3)-IVa*, *aph(3'')-Ib* (*strA*), *aph(4)-Ia*, *aph(6)-Id* (*strB*), *sul1*, *qacEΔ1*, *erm*(X), *erm*(56), *dfrA1*, and *cmx* are indicated by red arrows. ORFs of integrase (*intI1*), insertion sequences (IS), transposase, and recombinase are indicated with green arrows. The 3′-end truncated part (*gnat'*) and the 5′-end truncated part (*'gnat*) of the GNAT N-acetyltransferase gene, as well as the intact gene (*gnat*), are indicated in blue. ORFs of TetR/AcrR family transcriptional regulator are indicated in white. Other ORFs of hypothetical proteins are represented by light-gray arrows. Dark-gray ORFs at the left (*cpbp*, *α/β-hdcp*, *dass*) and right (*lytR-C*, *mfs*, *nudix*) ends of the sequences are housekeeping genes. Inverted repeats of the IS*6100* elements are indicated by yellow arrowheads (IR-L, GGCTCTGTTGCAAA; IR-R, TTTGCAACAGAGCC). The figure was generated using Clinker and Adobe Illustrator.

a class I integron gene *intI1* with *sul1*, and MGE-B had an additional *erm* (56) gene flanked by two IS*6100* (Fig. 3B).

3.4. cgMLST analysis

The cgMLST analysis based on 1610 core genes, revealed two major clusters each containing genetically diverse strains of diverse origins. However, human strains were all located in one cluster and were phylogenetically closest to the cattle strains. This cluster also contained most of the strains harboring antimicrobial resistance while the other cluster contained all susceptible strains, except one. This second cluster was formed by strains of swine, small ruminants, and wildlife origin. The phylogenetic tree based on cgMLST analysis is presented in Fig. 1.

4. Discussion

The development of bacterial antimicrobial resistance is a global and growing problem both in human and animal health. According to the World Health Organization, overuse of antimicrobials, incomplete length of treatment, and inappropriate choice of the antimicrobial agent, as well as transfer of ARGs among bacteria are considered the main reasons for the increase in bacterial antimicrobial resistance. *T. pyogenes* is the normal inhabitant of several different animal hosts that may play an important role as a reservoir of antimicrobial-resistant strains, and it may be transferred to other less adapted hosts including humans, posing a health risk in case of infections requiring antimicrobial treatment (Stuby et al., 2023). Within a One Health approach, the potential for zoonotic transmission of *T. pyogenes* as well as the potential ARGs transfer should not be underestimated (Rzewuska et al., 2019).

Few cases of human infections with *T. pyogenes* have been reported (Plamondon et al., 2007; Rzewuska et al., 2019; Stuby et al., 2023). To date, human colonization by *T. pyogenes* has not been demonstrated and human infections are estimated to be linked with occupational exposure to farm animals. Yet, there has been no previous assessment of the genetic relatedness between strains of humans and animals. Our cgMLST-based comparative genomic analysis generated two clusters with human and cattle strains clustering in only one of them. The presence of two such clusters of *T. pyogenes*, one containing exclusively strains from cattle origin and the other one with mostly strains from swine and small ruminant origin, has already been observed with analysis of *T. pyogenes* genomes from different countries, deposited in the GenBank database (Karthik et al., 2023; Thakur et al., 2023). Similar host-specific clustering was also observed in our study, further highlighting cattle-specific strains. Strains of wildlife and companion animals clustered in both branches, indicating that these animals can be affected by strains from both clusters. Of note, the human strains clustered together with cattle strains suggesting that these strains may have common zoonotic features or were acquired from animals. Nevertheless,

human colonization has been suspected in infected patients without clear exposure to animals or farm settings and warrants further investigation (Plamondon et al., 2007).

Resistance to streptomycin, sulfonamides, and tetracyclines were most common and mainly found in strains of human and cattle origin. In cattle, these antimicrobials are frequently used as first-line treatments and may have contributed to the selection of resistant strains, which were subsequently transferred to humans. Less phenotypic resistances and corresponding resistance genes were observed in strains from other animal species like swine, small ruminants, and wildlife. This may be due to the lower number of strains analyzed and, for wildlife, to a lower antimicrobial selective pressure in these animals.

Analysis of the virulence genes did not reveal that human and cattle strains display similar virulence factor patterns or that they were more abundant compared to strains from other animal species. Our analysis also revealed the presence of several homologs of pyolysin, neuroamidases, and fimbriae which may play a role in host adaptation and virulence. Considering this finding further investigations of the virulence mechanisms among *T. pyogenes* of different origins are warranted.

Different mechanisms, often related to MGEs, contribute to the dissemination of antimicrobial resistance among *T. pyogenes*. Several tetracycline resistance proteins have been described in *T. pyogenes*; the most common mechanisms include ribosomal protection proteins (RPPs) such as those encoded by the *tet*(W) gene family, and efflux pump proteins such as those encoded by *tet*(33) or *tet*(Z). In the present study, genetic tetracycline resistance determinants were found in all strains considered resistant or intermediate to tetracycline, indicating high accordance between phenotype and genotype. As previously reported, tetracycline resistance was mainly associated with the presence of *tet* (W/N/W) linked to the ATE-1 transposon (Kwicien et al., 2021). This gene has been identified in a wide range of anaerobic bacteria isolated from animal and human mucosal surfaces suggesting possible gene transfer (Billington et al., 2006; Kwiecien et al., 2021). Since *T. pyogenes* is frequently found in mixed infections alongside various bacterial species, especially Gram-negative anaerobes such as *Fusobacterium necrophorum* or *Bacteroides* spp. being particularly common partners, the likelihood of gene transfer is high (Rzewuska et al., 2019). The tetracycline resistance genes *tet*(33) and *tet*(Z) were also detected but in a lower proportion. As previously reported, the *tet*(33) gene was associated with insertion sequences IS*6100* located in the chromosomal DNA (Dong et al., 2020b, Karthik et al., 2022), while *tet*(Z), previously isolated on plasmid pAG1 of *Corynebacterium glutamicum* (Tauch et al., 2000) was integrated into the chromosomal DNA and was not linked to a MGE.

T. pyogenes is also considered an important reservoir of aminoglycoside resistance genes (Kwiecien et al., 2020). The predominant aminoglycoside resistance gene found in the present study consisted of *aadA9* conferring resistance to streptomycin and spectinomycin. This gene, when present, was always linked to *qacEΔ1* and *sul1* resistance genes as previously described by Kwiecien (2020). That study showed that a class 1 integron gene cassette carried the *aadA9* gene in 2 of 8 cases. Still, it was unable to elucidate, by PCR technique alone, the MGE structure carrying the *aadA9-qacEΔ1-sul1* gene combination in other strains. Our WGS analysis confirmed the association of this gene combination with a class 1 integron in only one cattle strain and identified the *aadA9-qacEΔ1-sul1* genes flanked by two identical IS*6100* within larger MGEs in the remaining 14 strains.

IS*6100* is part of the IS*6* family and can transfer resistance genes as part of a composite transposon, a region bounded by two IS copies that can move as a single unit (Roberts et al., 2008). The IS*6100*-associated transposons have been reported in a variety of Gram-negative and Gram-positive bacteria, including *Pseudomonas aeruginosa*, *Salmonella enterica*, *C. glutamicum*, *Streptomyces*, and *Mycobacterium fortuitum*, from where it was originally isolated (Martin et al., 1990). MGEs flanked by IS*6100* have already been reported associated with *erm*(X), *erm*(56), and *tet*(33) genes in *T. pyogenes* (Jost et al., 2003; Dong et al., 2020b; Karthik et al., 2022; Marchionatti & Perreten, 2023). Dong (2020[a]) reported IS*6100Δ1* sequences flanking the class 1 integron containing *qacEΔ1* and *sul1* in *T. pyogenes* strain TP1. The presence of IS*6100* flanking MGEs containing antimicrobial resistance genes in multiple MDR strains and in different locations as shown in the present study, suggests that these insertion sequences play an important role in the dissemination and integration of ARGs in *T. pyogenes*. Moreover, IS*6100* and class I integron seem to be able to capture and express diverse resistance genes in both Gram-positive and Gram-negative bacteria (Varani et al., 2021). The presence in *T. pyogenes* of genes usually present in Gram-negative bacteria is a specific feature of this Gram-positive bacterium, increasing its ability to rapidly adapt to antimicrobial selective pressure and different hosts.

The majority of the resistance genes detected were also associated with a corresponding resistance phenotype in most strains, further highlighting the functionality of Gram-positive and Gram-negative associated genes in *T. pyogenes*. However, 15 strains harbored the quaternary ammonium compounds resistance gene *qacEΔ1,* and the MIC of each antiseptic tested remained unimodally distributed indicating a wild-type phenotype and no association between *qacEΔ1* and increased MIC of antiseptics in *T. pyogenes*. The *qacEΔ1* gene, a disrupted form of the *qacE* gene, mediates resistance by a proton pump and confers bacterial reduced susceptibility to quaternary ammonium compounds such as benzalkonium chloride, and biguanide compounds such chlorhexidine (Jaglic & Cervinkova, 2012). The gene is widely spread in Gram-negative bacteria in association with class 1 integrons but may also occur in Gram-positive cocci (Kazama et al., 1998). However, multiple studies have reported no significant correlation between biocide susceptibility existed with the presence of *qacEΔ1* (Kücken et al., 2000; Romão et al., 2011). Our findings support the opinion that the *qacEΔ1* gene may not play an important role in reduced susceptibility to biocides in *T. pyogenes*. Nonetheless, as many efforts are being made to control the dissemination of MDR bacteria, and biocides have been widely used for this purpose, further investigations are warranted.

5. Conclusion

The presence of genetically related *T. pyogenes* strains in humans and animals, specifically, cattle, suggests that exchange may occur. This Gram-positive pathogen has also the ability to acquire genetic features typical of Gram-negative bacteria to survive antimicrobial treatment. Acquired resistance to several antimicrobials and the presence of known resistance genes accompanied by low MICs indicate that judicious use of antibiotics should be made based on antimicrobial susceptibility testing.

T. pyogenes is an important opportunistic pathogen in animals, and its presence in human infections deserves further attention regarding virulence and antimicrobial resistance as well as transmission routes.

CRediT authorship contribution statement

Emma Marchionatti: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Sonja Kittl:** Writing – review & editing, Resources, Methodology, Investigation, Formal analysis, Conceptualization. **Parham Sendi:** Writing – review & editing, Supervision, Resources, Conceptualization. **Vincent Perreten:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Funding acquisition, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetmic.2024.110102.

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