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Sulfonamide resistance evaluation in five animal species and first report of *sul4* in companion animals

Angela Maria Catania [a], Maria Cristina Stella [a], Francesca Cimino [b], Simona Zoppi [b], Elena Grego [a,*]

[a] Department of Veterinary Sciences, University of Turin, Largo P. Braccini 2, Torino, Grugliasco 10095, Italy
[b] Istituto Zooprofilattico Sperimentale di Piemonte, Liguria e Valle d'Aosta, Via Bologna 148, Torino 10154, Italy

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ABSTRACT

Sulfonamides are one of the oldest groups of antibacterial agents with a broad-spectrum, used as first line treatment in bacterial infections. Their widespread use produced a selective pressure on bacteria, as observed by the high incidence of sulfonamides resistance mainly in Gram negative bacteria isolated from animals. In this research, the presence of sulfonamide resistance genes (*sul1*, *sul2*, *sul3*, and *sul4*) in phenotypically resistant *Escherichia coli* isolates has been studied. These genes were amplified in isolates recovered from five animal species, with different interactions to humans: cattle, swine, poultry as livestock, and dogs and cats as companion animals. Isolates were collected according to their phenotypic resistance, and the magnetic bead-based Luminex technology was applied to simultaneously detect *sul* target genes. The frequency of *sul* genes was highest in swine, among livestock isolates. The *sul1* and *sul2* were the most frequently sulfonamide resistance genes detected in all phenotypically resistant isolates. Notably, in companion animals, with a closest interaction with human, *sul4* gene was detected. To our knowledge, this is the first report of the presence of *sul4* gene in *E. coli* collected from animals, whereas previously the presence of this gene was reported in environmental, municipal wastewater and human clinical isolates. These results highlighted the importance of continuous antimicrobial resistant genes monitoring in animal species, with a special care to companion animals.

1. Introduction

Since their discovery, antibiotics represented a powerful weapon to tackle infectious diseases in human and veterinary medicine. However, their extensive use led to the rapid diffusion of antimicrobial resistant (AMR) bacteria, which represent a public-health hazard (Holmes et al., 2016).

Sulfonamides (SULs) are synthetic antibacterial drugs that competitively inhibit the enzyme dihydropteroate synthase (DHPS). DHPS is involved in the bacterial DNA and RNA production by the folate synthesis, consequently they hinder bacterial growth (Fernández-Villa et al., 2019). They are often used in combination with diaminopyrimidines (such as trimethoprim) to produce a synergistic effect by inhibiting the enzyme dihydrofolate reductase (DHFR) in the folic acid pathway (Fernández-Villa et al., 2019).

Sulfonamides are considered "high priority" drugs with a broad spectrum, widely used to treat, or prevent systemic or local infections (Papich and Riviere, 2009). Indeed, they are used in veterinary and human medicine to treat several types of infection such as urinary tract infection, meningitis, pneumonia, bronchitis, and diarrhea (Papich and Riviere, 2009). They are active against Gram-negative and Gram-positive bacteria unable to overcome the inhibition effects of DHPS (Nunes et al., 2020).

The main mechanism of sulfonamide resistance involves the acquisition of *sul* genes encoding alternative variants of the dihydropteroate synthase (DHPS) enzyme not inhibited by the drug (Fernández-Villa et al., 2019). The *sul* genes are both chromosomal and carried on plasmids and they are often associated with mobile genetic elements (MGEs) such as transposons and integrons which allow translocation of *sul* genes among chromosomes and plasmids (Wu et al., 2010). Moreover, plasmids carrying *sul* genes, can spread among bacteria of the same or different species or genera by conjugation or transformation, contributing to the wide spread of *sul* genes.

The extensive use of sulfonamides, as well as other antimicrobial

* Corresponding author.
E-mail address: elena.grego@unito.it (E. Grego).

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agents, in both humans and animals, applied a pressure for selection of resistant bacteria, thus facilitating the dissemination of AMR genes and bacteria in different ecosystems: agriculture (Economou and Gousia, 2015), environment (Fletcher, 2015), livestock (McKinney et al., 2010) and wildlife (Carroll et al., 2015).

In the past decade, high prevalence rates of sulfonamide resistance have been observed mainly in Gram-negative bacteria isolated from animals and humans all over the world (Wu et al., 2010). *Escherichia coli* is part of human and animal microbiota, and it is the most frequently isolated Gram-negative pathogen impacting health (Kaper et al., 2004). Among bacteria, *E. coli* is also recognized as an AMR barometer due to its ubiquity and genomic plasticity (Massella et al., 2021).

To date, four *sul* genes have been identified. The *sul1* and *sul2* genes were the first discovered in 1988 in Gram negative clinical isolates (Rådström and Swedberg, 1988; Sundström et al., 1988). In 2003, Perreten and Boerlin reported *sul3* gene in *E. coli* isolated from pigs in Switzerland (Perreten and Boerlin, 2003). After 14 years, a new plasmid-borne sulfonamide resistance gene, *sul4*, was described in environmental isolates (Razavi et al., 2017).

Previous research investigated the frequency of *sul1*, *sul2* and *sul3* in bacteria isolated from livestock (Wu et al., 2010). Recently, fecal resistome in slaughter pigs and broilers were studied in different European countries, highlighting a direct linkage between AMR levels, usage of antibiotics and fecal resistomes (Munk et al., 2018).

In recent years the relationship between companion animals and humans changed, with an increased number of pets in close contact with owners. This represents a public health concern because of the possible exchange of resistance genes between human and animal strains (Guardabassi et al., 2004). In a recent study, Belas et al., 2020 examined *β-lactamase* genes and other antimicrobial classes, including sulfonamides, in companion animals and their human households. Authors reported that *sul2* was the most frequently shared gene by pets and their owners (Belas et al., 2020).

The aim of the present research was to investigate the presence of the four sulfonamide resistance genes (*sul1*, *sul2*, *sul3*, *sul4*) in sulfamethoxazole phenotypically resistant/sensitive *E. coli* isolates, by Luminex xMAP technology. The isolates were collected from cattle, swine and poultry as food-producing animals, and cats and dogs as companion animals, due to their close relationship with humans.

2. Materials and methods

2.1. Bacterial isolates and identification

Escherichia coli isolates were obtained from clinical material of five animal species (cattle, swine, poultry, cats and dogs), arrived to the Istituto Zooprofilattico Sperimentale di Piemonte, Liguria e Valle d'Aosta (IZSPLV) for diagnostic procedures, in the period 2019–2023. The *E. coli* isolates included in this study originated from different farms and animals located in Piedmont. They are part of a broader collection maintained at the IZSPLV (Torino, Italy).

One hundred twenty-four *E. coli* isolates were included in this study: n=25 recovered from cattle, n=22 from swine, n=23 from poultry, n=28 from dogs and n=26 from cats. A detailed list of isolates included in this study is reported in Supplementary Table S1.

After isolation, *E. coli* strains were placed in Lysogeny Broth (LB) (Cryobank, Mast Diagnostic, Amiens, France) with 15 % [v/v] glycerol and stored at −80 °C, until used. To recover the isolates, LB (Cryobank, Mast Diagnostic, Amiens, France) cultures were prepared by overnight incubation at 37°C, in aerobic condition. Working cultures of each isolate were prepared by streaking the LB broth (Cryobank, Mast Diagnostic, Amiens, France) onto Sheep Blood agar plates (Liofilchem srl, Roseto degli Abruzzi (TE), Italy) and incubated aerobically at 37°C overnight.

The identification was performed by Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) analysis using Biotyper mass spectrometer (Bruker Daltonics, Bremen, Germany). Briefly, isolated colonies were spotted on the MALDI plate, overlayed with 1 µl of saturated α-cyano-4-hydroxycinnamic acid (HCCA) matrix solution and allowed to dry at room temperature. The mass spectra were analyzed by MALDI Biotyper software (version 3.1) and compared to a collection of spectra stored in MBT Compass database (version 4.1). Each isolate was tested in duplicate.

2.2. Antimicrobial susceptibility test (AST)

The selection of isolates was based on their phenotypic sulfonamide (sulfamethoxazole) resistance profiles. AST of *E. coli* was performed by broth-microdilution (minimum inhibitory concentration, MIC, µg/ml), using the Sensititre semi-automated susceptibility system (Thermo Fisher Scientific, Waltham, MA, USA) with a fixed Sensititre (Thermo Fisher Scientific, Waltham, MA, USA) panel of antibiotics for livestock and companion animals. Results were interpreted according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) VET01S (CLSI, 2020). Briefly, three distinct colonies were picked from the overnight culture plates and suspended into sterile physiological saline solution (Oxoid, Basingstoke, UK). Next, the bacterial suspension was adjusted to a 0.5 McFarland standard, after which 50 µl of suspension was mixed with 50 µl of Mueller-Hinton broth (Thermo Fisher Scientific, Waltham, MA, USA). Then, 100 µl of the suspension was inoculated to each well of a Sensititre™ plate and incubated at 37 °C for 24 h. Plates were read using the Sensititre™ ARIS automated system which interprets isolates based on the MIC as susceptible, intermediate, or resistant using the SWIN™ Software System according to CLSI VET01S guideline (CLSI, 2020). *E. coli* ATCC 25922 was used as quality control strains. The phenotypic sulfonamides resistance profiles of isolates are reported in Supplementary Table S1.

2.3. DNA extraction

For DNA extraction, isolated colonies were inoculated in LB (Cryobank, Mast Diagnostic, Amiens, France) and incubated overnight at 37° C. Subsequently, bacterial cultures were centrifugated, and nucleic acids were extracted from cell pellets using DNAzol™ Reagent (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. The concentration and purity of the DNA were measured by Nanodrop 2000 C spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.4. xTAG luminex assay

The detection of sulfonamides resistance genes was established by using the xTAG Luminex technology. The primers employed in the assay are reported in Table 1. The forward primers were modified by a "TAG" sequence separated from the primers' specific portion with an internal

Table 1
Primers and amplicon size for detection of sulfonamides genes by Luminex xMAP assay.

Primer	Sequences	Amplicon size (bp)	Reference
sul1	CTAAACATACAAATACACATTTCA-Spacer-CGCACCGGAAACATCGCTGCAC Bio-TGAAGTTCCGCCGCAAGGCTCG	163	[Xu et al. 2020]
sul2	TACTTAAACATACAAACTTACTCA-Spacer-TGCCAAACTCGTCGTTATGC Bio-CCCCCAGAGAAAACCCCA	182	
sul3	ATCTCAATTACAATAACACACAAA-Spacer-ACGAGATTTCACATCGGTTCC Bio-CGGGTATGGGCTTCTTTTTAG	158	
sul4	TACTACTTCTATAACTCACTTAAA-Spacer- CGCTTCATCGGGGTAAAAT Bio-CGGACCTATTAAGATGGGAAA	213	

spacer, and all reverse primers were biotinylated at the 5' terminus, according to manufacturing instruction (Table 1). PCR assays were performed using QIAGEN Multiplex PCR Plus Kit (Qiagen, Germantown, MD, USA) in a volume of 20 µL containing: 10 µL Multiplex Master Mix, 250 nM of each primer, 1 µL DNA (50–100 ng) and ddH2O to volume. The PCR product in the blank control tube was replaced by 1 µl of ddH2O. The thermal conditions were: 95 °C for 15 min; followed by 35 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s; and a final extension at 72 °C for 7 min. After amplification, 20 µL of appropriate MagPlex-TAG microspheres (about 2500 beads, Luminex Corporation, Austin, TX, USA) were combined with 1 µL of each PCR reaction and ddH2O to a volume of 25 µL. About 70–75 µL Streptavidin-R-phycoerythrin (SAPE) (Thermo Fisher Scientific, Waltham, MA, USA) were added and hybridized at 42°C for 30–45 min. After incubation, the mixture was transferred to a Bio-Plex 200 instrument (Luminex Corporation, Austin, TX, USA) for signal acquisition. A minimum of 100 events for bead set, were counted in each sample as recommended by the user manual (www.luminexcorp.com). The median fluorescence intensity (MFI) was calculated by the Bio-Plex Manager soft-ware v 6.2.

Blank controls, containing all reaction components except DNA, were used to define MFI background (MFIB) values. When the ratio of a sample's MFI (MFIS) and the MFIB was ≥ 3, the sample was considered positive; when MFIS/MFIB was <2 the result was negative; for values $2 \leq$ and < 3, the result was considered inconclusive, and sample needed to be reanalyzed.

To ensure the reproducibility of the results, three experiments were performed starting from independent bacterial cultures. The coefficient of variation (CV%) was calculated as CV% = (standard deviation (SD)/MFI) × 100 %.

To assess the sensitivity of the assay, a standard curve was generated by cloning specific PCR products in TOPO® XL PCR Cloning Kit (Thermo Fisher Scientific, Waltham, MA, USA) and preparing serial dilutions (2×10^6, 2×10^5, 2×10^4, 2×10^3, 2×10^2, 2×10^1) of the plasmid, which were used to define the limit of detection for each target.

2.5. Confirmation of sul genes by conventional PCR and Sanger Sequencing analysis

To confirm the presence of *sul* genes in the *E. coli* isolates, polymerase chain reaction (PCR) was performed, followed by Sanger sequencing analysis. The primers employed for *sul1* gene were: 5'-CGGCGTGGGCTACCTGAACG-3' and 5'-GCCGATCGCGTGAAGTTCCG-3' (Kozak et al., 2009); for *sul2* gene: 5'-GCGCTCAAGGCAGATGG-CAT-3' and 5'-GCGTTTGATACCGGCACCCGT-3' (Alves et al., 2014); for *sul3* were: 5'-CAGATAAGGCAATTGAGCATGCTCTGC-3' and 5'-AGAATGATTTCCGTGACACTGCAATCATT-3' (Xu et al., 2020); and for *sul4* gene were: 5'-CGCTTCATCGGGGTAAAAT-3' and 5'- CGGACC-TATTAAGATGGGAAA-3' (Xu et al., 2020). PCR reactions were performed in a volume of 25 µL containing 1x MyTaq Red Reaction Buffer, 400 nM each primer, 5 U MyTaq Red DNA Polymerase (Bioline-Meridian Bioscience Inc., Cincinnati, OH, USA) and the same DNA amount employed in Luminex assay (1 µL, 50–100 ng).

PCR conditions were: 95 °C for 1 min; followed by 35 cycles at 95 °C for 30 s, annealing at 60°C-56°C for 30 s, and 72 °C for 30 s; and a final extension at 72 °C for 7 min. PCR products were analyzed by electrophoresis on 2 % agarose gel stained with SYBR Safe (Thermo Fisher Scientific, Waltham, MA, USA). Moreover, the amplification products were purified with ExoSAP-IT™ kit (Thermo Fisher Scientific, Waltham, MA, USA) and then sequenced to confirm positive samples with SeqStudio Genetic Analyzer (Applied Biosystems, Waltham, MA, USA). Subsequently, sequences were analyzed in the web-based basic local alignment tool BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi/).

2.6. Statistical analysis

Fisher exact test was used to compare the frequency of *sul* resistance genes on different MIC groups (>8 and >16 µg/ml) by R software (https://www.r-project.org/). To assess the agreement of phenotypic (MIC) and molecular (Luminex) results, the Cohen's kappa statistic was calculated; Luminex sensitivity and specificity were also assessed using EpiTools (https://epitools.ausvet.com.au/).

3. Results

3.1. Luminex xMAP assay

The *E. coli* isolates were tested by Luminex technology. *Sul1* and *sul2* were the most prevalent genes in all tested isolates from the five animal species (Fig. 1). *Sul1* was detected in n=64/70 (91.4 %) of *E. coli* recovered from livestock and in n=42/54 (77.8 %) from companion animals, whereas *sul2* respectively in n=53/70 (75.7 %) and n=37/54 (68.5 %) of tested isolates (Fig. 1).

The *sul3* gene was detected in n=31/124 (25 %) isolates, mainly collected from livestock (n=24/124, 19.3 %), whereas the presence of *sul4* gene was highlighted in n=8/124 (6.4 %) *E. coli* isolates recovered from companion animals (n=6/124, 4.8 %) and in two isolates (n=2/124, 1.6 %) from livestock (Fig. 1).

To guarantee the reproducibility of the results, the data were confirmed in three independent experiments starting from different isolated colonies for each sample. Replications confirmed the positive results for each gene, with a coefficient of variation < 9 %. As example, the median fluorescence values (MFI) of ten isolates (two for each animal species) are reported in Supplementary Table S2.

The Luminex xMAP assay showed a limit of detection (LOD) of 2×10^1 copies/µL for *sul3*, 2×10^2 copies/µL for *sul1* and *sul4* and 2×10^3 copies/µL for *sul2* (Fig. 2).

3.2. Spread of sul genes in E. coli collected from different animal species

E. coli isolates included in the study were tested against sulfamethoxazole by broth-microdilution (MIC). One hundred and ten isolates showed resistance to sulfamethoxazole (twenty-two for each species), the remaining fourteen were classified as susceptible (Table 2 and Table 3).

In Table 2, the presence of *sul* genes in the food-producing animals is reported. In *E. coli* recovered from cattle, *sul1* and *sul2* genes were detected together in n=19/25 (76 %) isolates, while *sul3* and *sul4* in n=2/25 (8 %) and n=1/25 (4 %) isolates, respectively (Table 2).

Samples recovered from swine showed the greatest positivity for *sul* genes. Overall, more than 90 % of samples were positive for *sul1* (n=21/22) and *sul2* (n=20/22), and around 59 % (n=13/22) for *sul3* (Table 2). No positivity for *sul4* gene was detected (Table 2).

All phenotypically resistant isolates collected from poultry were positive for *sul1* genes, and n=12/23 (52.2 %) for *sul2,* while n=9/23 (39.1 %) were positive for *sul3* and n=1/23 (4.3 %) for *sul4* (Table 2).

In *E. coli* isolates recovered from companion animals, the frequency of *sul1* and *sul2* was also high. A positivity for *sul1* was found in n=20/28 (71.4 %) and n=22/26 (84.6 %) isolates recovered from dogs and cats,

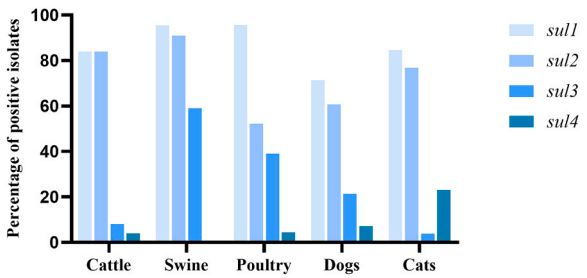

Fig. 1. Overview of *sul* genes detected by Luminex xMAP assay in *E. coli* recovered from five animal species.

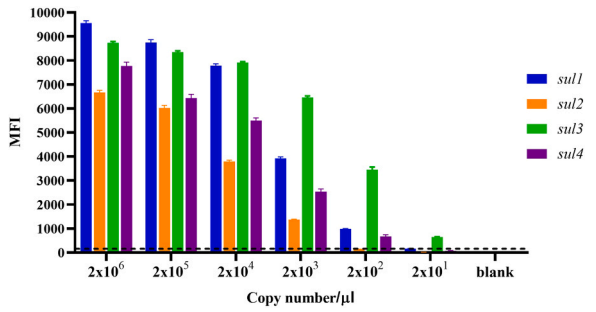

Fig. 2. Sensitivity of Luminex assay. Serial dilution of target genes cloned into recombinant plasmids are reported, blank refers to negative control (no template). The threshold defines signal background, and it is reported as dotted lines.

respectively (Table 3). A similar result was obtained for *sul2* gene in n=17/28 (60.7 %) *E. coli* isolates collected from dogs, and in n=20/26 (76.9 %) from cats (Table 3).

The *sul3* was detected in n=6/28 (21.4 %) isolates recovered from dogs (Table 3) and in n=1/26 (3.8 %) isolate from cats (Table 3). The gene *sul4* was detected in n=2/28 (7.1 %) *E. coli* isolates from dogs (Table 3) and surprisingly in n=6/26 (23.1 %) isolates from cats (Table 3).

The number of *sul* genes detected by Luminex, in each isolate, is not significantly different between phenotypic resistance values groups (MIC>8 or >16; Fisher exact test, $p > 0.05$). A perfect agreement between the phenotypic and molecular tests is observed, with a Kappa value=1.

The sensitivity and specificity of Luminex was 100 %, with 95 % confidence interval (CI), of 96.7–100 % for sensitivity and 76.8–100 % for specificity.

3.3. Standard PCR and sanger sequencing analysis

The positive samples were submitted to end-point PCR and Sanger sequencing analysis to confirm positive results. The nucleotide sequences showed a 100 % identity for *sul1* and *sul3* and a 99,9 % identity for *sul2* and *sul4* genes on Basic Local Alignment Search Tool (BLAST) (data not shown).

4. Discussion

The present research indagates the presence of sulfonamides resistance genes in *E. coli* isolates recovered from five animal species in Italy: three belonging to zootechnical animals and two companion animals. These two groups of animals were selected based on the different interaction with human.

The detection of *sul1, sul2, sul3, sul4* genes were evaluated by the highly sensitive bead-based multiplex assay using the Luminex technology.

In our study, *sul1* and *sul2* resulted the most abundant genes in the tested samples. Our results agreed with Zhuang et al. (2021) who included them among the most frequent antimicrobial resistance genes detected in livestock farms.

Sulfonamides are currently listed among class D compounds of Antimicrobial Advice Ad Hoc Expert Group (AMEG) classification, which is the first line therapy in veterinary field. This explains their intense use and the widespread presence of *sul* genes reported in animal species (Cheong et al., 2020) and in this research.

The frequency of *sul3* was higher in swine (59 %), compared to poultry (39 %) and cattle (8 %), an analogous result between animal species was previously reported by Guerra et al. (2003), although with lower percentages compared to our research.

Around 8 % of the samples (n=10/124) were positive for *sul4*,

Table 2
Detection of *sul* genes in *E. coli* isolates recovered from livestock (cattle, swine, poultry) samples by Luminex assay and phenotypic results (broth-microdilution, MIC, µg/ml). Results +/- indicate presence or absence of genes examined. R: sulphonamides resistant. S: sulphonamides susceptible.

Sample ID	Species	*sul1*	*sul2*	*sul3*	*sul4*	MIC	SMX µg/ml
37563/1	Cattle	+	+	-	-	R	> 8
18060		+	-	-	-	R	> 8
73350/A		+	+	-	-	R	> 8
88876/A1		+	+	-	-	R	> 16
10725/D1		+	+	-	-	R	> 16
61831/6/A5		+	+	-	-	R	> 8
68113		+	+	-	-	R	> 8
49375		-	+	+	+	R	> 8
61831/4/D5		+	+	-	-	R	> 16
46274/A/2		+	+	-	-	R	> 8
86555		-	-	-	-	S	1
88966		+	+	-	-	R	> 16
102102/D		+	+	-	-	R	> 16
59043/1		+	+	-	-	R	> 16
59043/2		+	+	-	-	R	> 16
87605/2C		-	-	-	-	S	1
30480/A		+	+	+	-	R	> 8
20026		+	+	-	-	R	> 16
10586/C1		+	+	-	-	R	> 8
10224/A1		+	+	-	-	R	> 16
89184/B1		+	+	-	-	R	> 16
94428/A1		+	+	-	-	R	> 16
107646		+	+	-	-	R	> 8
107639		+	+	-	-	R	> 16
27577		-	-	-	-	S	1
11947	Swine	+	+	-	-	R	> 8
76160		+	+	+	-	R	> 16
63115/A		+	+	-	-	R	> 16
71505/3/C		+	+	-	-	R	> 8
40512/2/A/B		+	-	+	-	R	> 8
77350/2/1		+	+	+	-	R	> 16
24838		+	+	+	-	R	> 8
37435/1		-	+	+	-	R	> 8
54446		+	+	-	-	R	> 8
54477/A		+	+	-	-	R	> 8
93441/2B		+	-	+	-	R	> 16
82632/C/1		+	+	+	-	R	> 16
77352		+	+	+	-	R	> 8
81844/A1		+	+	-	-	R	> 16
77777/B1/A		+	+	-	-	R	> 8
77777/B2/A		+	+	-	-	R	> 8
63806		+	+	+	-	R	> 16
63790		+	+	+	-	R	> 16
47583/A1		+	+	-	-	R	> 16
54874/A1		+	+	+	-	R	> 8
54890/A1		+	+	+	-	R	> 8
54890/A4		+	+	+	-	R	> 8
22180	Poultry	+	+	-	-	R	> 16
50812		+	-	-	-	R	> 16
72740/A1		+	+	-	-	R	> 8
69491/B		+	-	-	-	R	> 8
71505/3/C1		+	+	-	+	R	> 16
63115/A1		+	+	-	-	R	> 8
72677/C		+	-	+	-	R	> 8
73024		+	+	-	-	R	> 8
101261		+	-	+	-	R	> 16
97718		+	-	+	-	R	> 8
97428		+	+	+	-	R	> 8
71237/B/1		+	+	+	-	R	> 16
71234		+	+	-	-	R	> 8
68921		+	+	-	-	R	> 8
78911		+	-	-	-	R	> 8
65621/F		+	-	+	-	R	> 8
65621/6SV		+	-	+	-	R	> 16
65625/12SV		+	-	-	-	R	> 8
65628/14 S		-	-	-	-	S	1
67787/28SV		+	+	+	-	R	> 8
67783/2SV		+	-	+	-	R	> 16
76543		+	+	-	-	R	> 8
65433		+	+	-	-	R	> 8

Table 3
Detection of *sul* genes in *E. coli* isolates recovered from companion animals (dogs and cats) samples by Luminex assay and phenotypic results (broth-microdilution, MIC, μg/ml). Results +/- indicate presence or absence of genes examined. R: sulphonamides resistant, S: sulphonamides susceptible.

Sample ID	Species	*sul1*	*sul2*	*sul3*	*sul4*	MIC	SMX μg/ml
61081/1/A2	Dog	+	+	-	-	R	> 8
61081/1/A1		+	+	-	-	R	> 8
63164		+	+	-	-	R	> 8
63825/A2		+	+	-	-	R	> 8
58080/A*		+	+	-	-	R	> 8
58080/B*		+	+	-	+	R	> 8
61873		-	-	+	-	R	> 8
51932		+	+	-	-	R	> 8
51927/2		+	+	-	-	R	> 8
99555		-	-	-	-	S	1
51927/1		+	+	-	-	R	> 8
77875		+	+	-	-	R	> 8
87716		-	-	-	-	S	1
68234		-	-	-	-	S	1
105983/1		+	+	+	-	R	> 8
64424		+	+	-	-	R	> 8
62242		+	+	-	-	R	> 8
61878		-	-	+	-	R	> 8
62232		+	-	-	-	R	> 8
60559		+	+	-	-	R	> 16
57234		+	+	-	-	R	> 8
52921		+	-	-	-	R	> 8
52616		+	+	+	-	R	> 8
52617		+	-	+	+	R	> 8
60353		-	-	-	-	S	1
16779		-	-	-	-	S	1
66155		+	+	+	-	R	> 16
18483		-	-	-	-	S	1
60047/A2	Cat	+	-	-	-	R	> 8
63156		+	+	-	+	R	> 8
63137		+	+	-	-	R	> 8
63152/B2		+	+	-	+	R	> 8
63379/B1		+	+	-	+	R	> 8
63148/A2		+	+	-	+	R	> 8
20665/D1		+	+	-	+	R	> 8
60974/A2		+	-	-	-	R	> 8
50043		+	+	-	-	R	> 8
48244		+	+	-	+	R	> 8
76406/B		+	+	-	-	R	> 16
1104		-	-	-	-	S	1
61077		-	-	-	-	S	1
64776		+	+	-	-	R	> 8
53401		+	+	-	-	R	> 8
23441		-	-	-	-	S	1
45782		+	+	-	-	R	> 8
26350		+	+	-	-	R	> 8
9621		+	+	+	-	R	> 16
59194		-	-	-	-	S	1
66951		+	+	-	-	R	> 8
78841		+	+	-	-	R	> 8
86444		+	+	-	-	R	> 16
97412		+	+	-	-	R	> 8
83212		+	+	-	-	R	> 8
76543		+	+	-	-	R	> 8

specifically it was detected in n=1/25 from cattle, n=1/23 from poultry, n=2/28 from dogs and in n=6/26 *E. coli* isolated from cats. To our knowledge, this represents the first report of *sul4* in *E. coli* isolates recovered from animal samples.

Currently, a limited number of publications described the presence of *sul4* gene, which was reported in the environmental isolates (Razavi et al., 2017; Hutinel et al., 2022), in clinical human *E. coli* and *Salmonella* isolates (Xu et al., 2020; Peng et al., 2023) and recently also in marine bacteria (Shindoh et al., 2023). Interestingly, in this research the highest percentage of *sul4* positive samples was detected in companion animal group.

Furthermore, several co-presences of the *sul* genes were assessed. Overall, circa 80 % (n=99/124) of isolates were positive for more than one *sul* gene. The co-occurrence of *sul1* and *sul2* was the most frequent, observed in approximately half of tested isolates. These results were in accordance with a previous study which described *sul1* and *sul2* association as the most frequent combination, although with lower percentage compared to our research (Hammerum et al., 2006). In other studies, the simultaneous presence of *sul1*, *sul2* and *sul3* genes was investigated in sulphonamide resistant *E. coli* isolated from humans and swine, and co-occurrence was observed in ~20 % of the *E. coli* recovered from swine (Hammerum et al., 2006; Wu et al. 2010). In our study, the co-occurrence of *sul1*, *sul2* and *sul3* genes was assessed in *E. coli* isolates from livestock, in particular in almost half of *E. coli* recovered from swine. The presence of *sul4* gene was associated with *sul1* and *sul2* genes in 8 out of 10 *sul4* positive samples, with *sul2* and *sul3* genes in the positive sample isolated from cattle and with *sul1* and *sul3* in one of the two positive samples recovered from dogs. Interestingly, the presence of more than one *sul* genes in the same isolate is not associated with a greater phenotypic resistance (MIC>8 or >16).

The biological reason of resistance genes accumulation in the same microorganism is actually not clear. It could be hypothesized that the presence of multiple genes within the same isolate could be due to the individual transfer of each gene alone, in separate and successive transmission events or to the co-transmission of multiple genes mediated by mobile genetic elements such as integrons, transposons or plasmid (Racewicz et al., 2022). For example, the coexistence of the *sul1* and *sul2* genes in *E. coli* isolates may result from a possible acquisition of class 1 integron containing the *sul1* gene (Racewicz et al., 2022) as well as by a plasmid containing the *sul2* gene. Further studies should be performed to elucidate this issue. Anyway, the existence of more *sul* genes raises the possibility that *E. coli* develops a resistance to sulfonamides.

Sanger sequencing analysis was assessed to confirm positive samples, showing a percentage of identity over 99 % in the four *sul* genes tested (data not shown). This could mean that genes amplified in livestock and companion animals are genetically identical and they could be transmitted through bacterial populations of different animal species, remaining constant in their nucleotide sequence, without the occurrence of significant mutations.

Evaluation of the ARGs distribution is crucial for the control of AMR. The use of Luminex xMAP technology has advantages such as rapidity, multiplexing and an easy interpretation of the results.

According to our results, all phenotypical resistant isolates showed at least one positive gene detected by Luminex assay, and all phenotypical susceptible isolates were negative to Luminex (no genes were detected). Therefore, a perfect agreement between phenotypic and molecular test was achieved. Further experiments, with a higher number of MIC negative isolates, are necessary to confirm the specificity of the Luminex technology.

Among livestock isolates, *E. coli* collected from swine showed the highest incidence of *sul* genes, and the highest percentage (59 %) of *sul3* gene. High circulation of *sul* genes, particularly *sul3* was previously reported by Hammerum et al. (2006) and Wu et al. (2010), although with a lower incidence compared to our study, around 11 % and 9 %, respectively. Instead, in livestock isolates tested in the current study, *sul4* gene was detected in one cattle and one poultry *E. coli* isolate.

Lowest percentage of isolates positive for *sul* genes was detected in poultry, among livestock. This could be due to an increase of biosecurity joint to a reduction of antibiotic usage in Italian poultry farms, as reported by the last joint report from the European Centre for Disease Prevention and Control (ECDC), European Food Safety Authority (EFSA), and European Medicines Agency (EMA) (EFSA, 2021). However, the detection of sulfonamide genes in isolates recovered from poultry may be linked to the use of sulfonamides for the treatment of coccidiosis in poultry farming (Rumbeiha, 2023).

A high number of *sul* genes were identified in *E. coli* recovered from cats and dogs, suggesting a high circulation of these resistance determinants within these animal species. Our data agreed with a recent study by Joosten et al. (2020), who examined AMR in *E. coli* isolated

from companion animals in three European countries (Belgium, Italy, and The Netherlands). A high prevalence of sulfonamide resistance in pets from Italy, second only to ampicillin resistance was reported (Joosten et al., 2020). Moreover, resistance in isolates from dogs and cats from Italy was higher compared to the other two countries (Joosten et al., 2020).

In our research *sul4* gene was surprisingly found in two isolates from livestock samples and, in a greater frequency, in strains isolated from pets, with the highest prevalence in cats.

These data lead to various questions and hypotheses. Compared to livestock animals, pets can move more easily across the territory together with their owners. The finding of *sul4* gene in cats and dogs represents a novelty, and it is very interesting, since companion animals have a direct and close contact with humans, and the presence of AMR genes in their resistome may be a reservoir of antibiotic-resistant bacteria transmissible from animals to man or vice versa. In a previous study by Harada et al. (2012) companion animals (dogs) and the owners shared *E. coli* clones with the same antimicrobial resistance genes. A possible genes exchange animals-human was hypothesized by Zhao et al. (2022). Indeed, the gut microbiome, antibiotic resistance genes and mobile genetic elements was more similar between owned dogs and owners. than unrelated dogs (Zhao et al., 2022). The high prevalence of *sul4* gene, reported in this study, underlines the importance to monitor the presence of resistance determinants in companion animals for public health.

5. Conclusions

Our research highlighted a wide spread of *sul1*, *sul2* and *sul3* genes in phenotypically resistant *E. coli* isolates collected from food-producing and companion animals and the usefulness of Luminex xMAP technology for rapid identification and detection of ARGs.

Interestingly, we reported the first *sul4* gene detection in bacterial isolates recovered from animal species, with the highest frequency in feline isolates. Further studies are necessary to clarify the role of *sul4* gene to reduce the effect of sulfonamides.

The research underlines the importance of continuous monitoring of the AMRs genes diffusion in different animal species.

CRediT authorship contribution statement

Angela Maria Catania: Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft. **Francesca Cimino:** Methodology, Writing – review & editing. **Maria Cristina Stella:** Methodology, Writing – review & editing. **Elena Grego:** Conceptualization, Data curation, Funding acquisition, Supervision, Writing – review & editing. **Simona Zoppi:** Methodology, Writing – review & editing.

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.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetmic.2024.110170.

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