Research Note: Detection of antibiotic-resistance genes in commercial poultry and turkey flocks from Italy

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ABSTRACT Antibiotics are routinely used in commercial poultry farms for the treatment of economically important bacterial diseases. Repeated use of antibiotics, usually administered in the feed or drinking water, may also result in the selection of resistant bacteria in animal feces, able to transfer their antimicrobial-resistance genes (**ARG**), residing on mobile elements, to other microorganisms, including human pathogens. In this study, single and multiplex PCR protocols were performed to detect tetracycline-, lincomycin-, chloramphenicol-, aminoglycoside-, colistin-, vancomycin-, and carbapenem-resistance genes, starting from 38 litter samples collected from 6 poultry and 2 turkey Italian flocks. The ARG were confirmed for all investigated classes of antimicrobials, except for colistin (*mcr-1, mcr-2, mcr-3,mcr-4 mcr-5*) and carbapenem (*IMP, OXA-48, NDM, KPC*), while the *vanB* gene was only detected for vancomycin. The highest positivity was obtained for tetracycline (*tet[L], tet[M], tet[K], tetA[P]*) and aminoglycoside (***aadA2***) ARG, confirming the predominant use of these antimicrobials in the veterinary practice and their potential to enhance the resistance patterns also in humans as a consequence of environmental contamination. On the contrary, the dissemination by poultry of ARG for critically important antimicrobials seems to be of minor concern, suggesting a negligible environmental dissemination by these genes in the Italian poultry industry. Finally, the molecular screening performed in this study using a noninvasive sampling method represents a simple and rapid tool for monitoring the ARG patterns at the farm level.

Key words: broiler, turkey, antimicrobial-resistance genes (ARG), PCR, environmental contamination

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INTRODUCTION

The antimicrobial-resistance genes (**ARG**) can be defined as a new type of biological pollutant, potentially able to have negative effects on the human and animal health (Duan et al., 2019). The horizontal transfer of mobile-resistance genes is considered an important spreading factor of antimicrobial resistance (**AMR**) that leads to the selection and maintenance of multiresistant bacteria in the environment (Heuer et al., 2011).

This mechanism can be influenced by the selective pressure exerted by the use of antibiotics for diseases treatments in humans and animals and, consequently, by the antibiotic residuals eliminated in water and soil through the sewage and animal manure, often used as fertilizers in agriculture. At the farm level, it has been estimated that up to 90% of used antimicrobials is released in the environment through animal excreta (urine and feces) (Heuer et al., 2011), with a long-time persistence and thus contributing to the development and selection of resistant bacteria. Once released in the environment, the microorganisms and their ARG can persist and eventually stabilize into the microbial community (Petrin et al., 2019).

In this regard, poultry litter produced by intensive flocks has been proven to be a prime reservoir of AMR and the relative genes for other microorganisms including human pathogens (Duan et al., 2019).

Current methods applied for monitoring of AMR are mainly based on culturing indicator bacteria followed by phenotypic AMR determination. This procedure targets a limited number of species and isolates present in the microbiota and, therefore, probably represents only a fraction of its resistome (the collective pool of ARG). On the contrary, the biomolecular approaches used in

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recent studies may represent an alternative tool to monitoring and verifying the presence of ARG in environments with high microbial density such as intensive farming.

In Italy, studies have reported the antibiotic-resistance profiles of *Enterobacteriaceae* isolates such as *Escherichia coli* and *Salmonella* Infantis, obtained from animals samples (Cavicchio et al., 2015; Carfora et al., 2018). More recently, Laconi et al. (2021) examined the ARG composition of soil and livestock manure in Northern Italy, including poultry farms also. Compared to the swine and cattle sectors, the poultry manure appeared moderately interested by the ARG distribution, with a more prevalent diffusion of β-lactamase–encoding and erythromycin ribosome methylation–encoding genes (*bla* and *erm* genes). These data highlighted the potential risk of the environmental distribution of ARG in poultry Italian flocks, and additional studies should be carried out, including other geographical areas or expanding the genetic target investigated, for the implementation of national strategies against antimicrobial resistance.

Therefore, the aim of this study was to evaluate the distribution of the ARG associated with the most common classes of antibiotics used in veterinary practice along with some antimicrobials considered critically important for human medicine in commercial broiler and turkey farms in Central Italy.

MATERIALS AND METHODS

Six broiler (B1–B6) and 2 turkey (T1 and T2) commercial flocks, located in Central Italy, were investigated. For environmental sampling, the litter specimens were collected by means of the boot socks method (Agritamp plus02; Biogenetics, Padua, Italy) and in accordance with the provisions of the National Plan for the control of Salmonellosis in poultry 2016/2018 (http://www.salute.gov.it/imgs/C_17_pubblicazioni_2453_appendix.pdf). Briefly, a 10-cm section of gauze material was applied over the foot, and then the operator walked through the entire area of the pens to expose the boot socks to the litter. After the sampling, the boot socks were stored at 2°C–8°C in sterile sample bags and promptly transferred to the laboratory.

For each flock, the pens were sampled twice, first at 7 d of age and then near to slaughtering (35–45 d of age for broiler, 100–110 d for turkeys), for a total of 38 samples. More in detail, 2 pens/farm in B1–B5 and 3 pens in B6 were sampled, respectively (n = 26). The sampling of turkey flocks included 3 pens/farm (n = 12). The size of flocks ranged from 6,000 turkeys/pen to 10,000 broilers/pen.

The boot socks were homogenized in 20 mL of sterile physiological solution, mixed properly by a Stomacher (VWR International PBI, Milan, Italy) and heated at 75°C for 20 min to inactivate bacterial vegetative cells and to avoid any additional proliferation. Then, 300 µl of each solution was used for DNA extraction using the Maxwell 16 Tissue DNA Purification Kit as per the manufacturer's instructions (Promega, Italy).

For the biomolecular screening, single and multiplex PCR protocols were used to identify the ARG specific for tetracyclines (*tet[A], tet[B], tet[C], tet[K], tet[L], tet[M], tetB[P], tetA[P]*), lincomycin (*lnu[A], lnu[B]*), chloramphenicol (*CatA1*), aminoglycosides (*aadA2, aadB, aac[3]IV*), colistin (*mcr-1, mcr- 2, mcr-3, mcr-4, mcr-5*), vancomycin (*vanD, vanM, vanC2, vanB, vanA, vanC1, vanN*), and carbapenems (*IMP, OXA-48, NDM, KPC*) using the previously published primers pairs, as reported in Table 1.

RESULTS AND DISCUSSION

All flocks were positive for 1 or more ARG, with slight differences for the class of antibiotics investigated and the species involved (Figure 1). In broiler flocks, 11 of 30 investigated ARG (*tet[A], tet[B], tet[K], tet[L], tet[M], tetA[P], CatA1, aadA2, vanB, lnuA,* and *lnuB*), belonging to all antimicrobial classes under study, were detected, except for the genes of colistin and carbapenem resistance. In turkey flocks, the chloramphenicol-, colistin-, carbapenem-, and vancomycin-resistance genes were not amplified, while for remaining classes, the specific fragments of *tet(K), tet(L), tet(M), tetA(P), aadA2, lnuA,* and *lnuB* genes were obtained.

The most common genes found in the litter are those against tetracycline and aminoglycosides, probably owing to a positive correlation between the use of these antibiotics in veterinary practice and the occurrence of ARG in farm manure. The *tet* genes are known to be present in a wide range of bacterial species, some of which are common in the gastrointestinal tract of healthy chicken, such as *Clostridium*, *Lactobacillus*, *Bacteroides,* and *Corynebacterium* (Wei et al., 2013). The highest number of positive samples resulted for *tet(L)* (24 of 38; 63.16% from all flocks, except for B4), as reported for poultry manure in Portugal, while other *tet* genes such as *tet(A)*, *tet(B),* and *tet(C)*, yet considered widely distributed in the animal and environmental isolates including livestock manure (Amador et al., 2019; Duan et al., 2019), resulted poorly or not at all detectable (*tet[C]*) in the flocks under study.

As far as the aminoglycosides are concerned, the *aadA2* gene appeared the most frequent ARG in all investigated flocks (35 of 38; 92.11%). The gene cassettes *aadA,* encoding aminoglycoside-adenylating enzymes, responsible for the resistance against streptomycin and spectinomycin, were frequently found in clinical and environmental bacterial isolates, including *E. coli* strains from Italian poultry, as described by Cavicchio et al. (2015). In addition, the European Medicines Agency reported that the resistance to streptomycin is very common in food-producing animals with the highest levels of resistance in *Campylobacter* spp., *E. coli*, *Enterococcus faecium,* and *Enterococcus faecalis* isolates from conventional broilers (www.ecdc.europa.eu). Based on these results, the *aadA2* gene could be used as an environmental indicator to monitor the presence of bacteria

Table 1. Details of single and multiplex PCR protocols applied during this study[1]

Multiplex PCR	Primer	Sequence 5'-3'	Size (bp)	Annealing
	Tet(K)F	TTATGGTGGTTGTAGCTAGAAA	382	50° C
	Tet(K)R	AAAGGGTTAGAAACTCTTGAAA		
1	Tet(L)F	ATAAATTGTTTCGGGTCGGTAAT	1077	
	Tet(L)R	AACCAGCCAACTAATGACAATGAT		
	TetA(P)F	CACAGATTGTATGGGGATTAGG	764	
	TetA(P)R	CATTTATAGAAAGCACAGTAGC		
2	TetB(P)F	AAAACTTATTATATTTATAAC	169	45° C
	TetB(P)R	TGGAGTATCAATAATATTCAC		
	lnu(B)F	CCTACCTATTGTTTGTGGAA	906	
	lnu(B)R	ATAACGTTACTCTCCTATTC		
3	Tet(M)F	ACAGAAAGCTTATTATATAAC	171	54° C
	Tet(M)R	TGGCGTGTCTATGATGTTCAC		
	lnu(A)F	GGTGGCTGGGGGGTAGATGTATTAACTGG	323	
	lnu(A)R	GCTTCTTTTGAAATACATGGTATTTTTCGATC		
4	CatA1F	GGCATTTCAGTCAGTTG	551	50° C
	CatA1R	CATTAAGCATTCTGCCG		
	TetCF	AACAATGCGCTCATCGT	1138	
	TetCR	GGAGGCAGACAAGGTAT		
5	Tet(A)F	GTAATTCTGAGCACTGT	954	45° C
	Tet(A)R	CCTGGACAACATTGCTT		
	Tet(B)F	ACGTTACTCGATGCCAT	1170	
	Tet(B)R	AGCACTTGTCTCCTGTT		
6	aadBF	GAGGAGTTGGACTATGGATT	208	54° C
	aadBR	CTTCATCGGCATAGTAAAAG		
	aadA2F	CGGTGACCATCGAAATTTCG	250	
	aadA2R	CTATAGCGCGGAGCGTCTCGC		
7	aac(3)IVF	TGCTGGTCCACAGCTCCTTC	653	63° C
	aac(3)IVR	CGGATGCAGGAAGATCAA		
8	Mcr-1F	AGTCCGTTTGTTCTTGTGGC	320	58° C
	Mcr-1R	AGATCCTTGGTCTCGGCTTG		
	Mcr-2F	CAAGTGTGTTGGTCGCAGTT	715	
	Mcr-2R	TCTAGCCCGACAAGCATACC		
	Mcr-3F	AAATAAAAATTGTTCCGCTTATG	929	
	Mcr-3R	AATGGAGATCCCCGTTTTT		
9	Mcr-4F	TCACTTTCATCACTGCGTTG	1116	56° C
	Mcr-4R	TTGGTCCATGACTACCAATG		
	Mcr-5F	ATGCGGTTGTCTGCATTTATC	1644	
	Mcr-5R	TCATTGTGGTTGTCCTTTTCTG		
10	VanDF1	TGGAATCACAAAATCCGGCG	311	58° C
	VanDR2	TWCCCGCATTTTTCACAACS		
	VanMF1	GGCAGAGATTGCCAACAACA	425	
	VanMR1	AGGTAAACGAATCTGCCGCT		
	VanC2F1	GCAAACGTTGGTACCTGATG	523	
	VanC2R4	GGTGATTTTGGCGCTGATCA		
	VanBF1	GATGTGTCGGTAAAATCCGC	640	
	VanBR1	CCACTTCGCCGACAATCAAA		
	VanAF1	GCAAGTCAGGTGAAGATGGA	721	
	VanAR1	GCTAATACGATCAAGCGGTC		
	VanC15F	GTATCAAGGAAACCTCGCGA	836	
	VanC16R	CGTAGGATAACCCGACTTCC		
	VanNF1	CCTCAAATCAGCAGCTAGTG	941	
	VanNR1	GCTCCTGATAAGTGATACCC		
11	IMPF	GGAATAGAGTGGCTTAAYTCTC	232	56° C
	IMPR	GGTTTAAYAAAACAACCACC		
	OXA48F	GCGTGGTTAAGGATGAACAC	438	
	OXA48R	CATCAAGTTCAACCCAACCG		
	NDMF	GGTTTGGCGATCTGGTTTTC	621	
	DMR	CGGAATGGCTCATCACGATC		
	KPCF	CGTCTAGTTCTGCTGTCTTG	798	
	KPCR	CTTGTCATCCTTGTTAGGCG		

[1]All references are available upon request.

resistant against the aminoglycosides, included those considered critically important for the human health, in food-producing animals.

The gene *CatA1* (chloramphenicol-resistance gene) was detected in 4 of 6 broiler flocks (B1–B4) in a total of 11 samples. The use of chloramphenicol in food-producing animals has been banned in the European Union (EU) since 1994, so the presence of *CatA1* may be related to other plasmid-mediated ARG. Indeed, a strong association between genes for chloramphenicol and streptomycin resistance was suggested (Esperón et al., 2018). Therefore, the presence of *CatA1* gene could be referred to the same plasmid carrying both *CatA1* and *aadA2* genes, even though the results of both fragments did not match completely.

The lincosamide-resistance genes *lnu(A)* and *lnu(B)* were detected in both broiler and turkey flocks (7 of 38 and 10 of 38, respectively), with *lnu(A)* being mainly

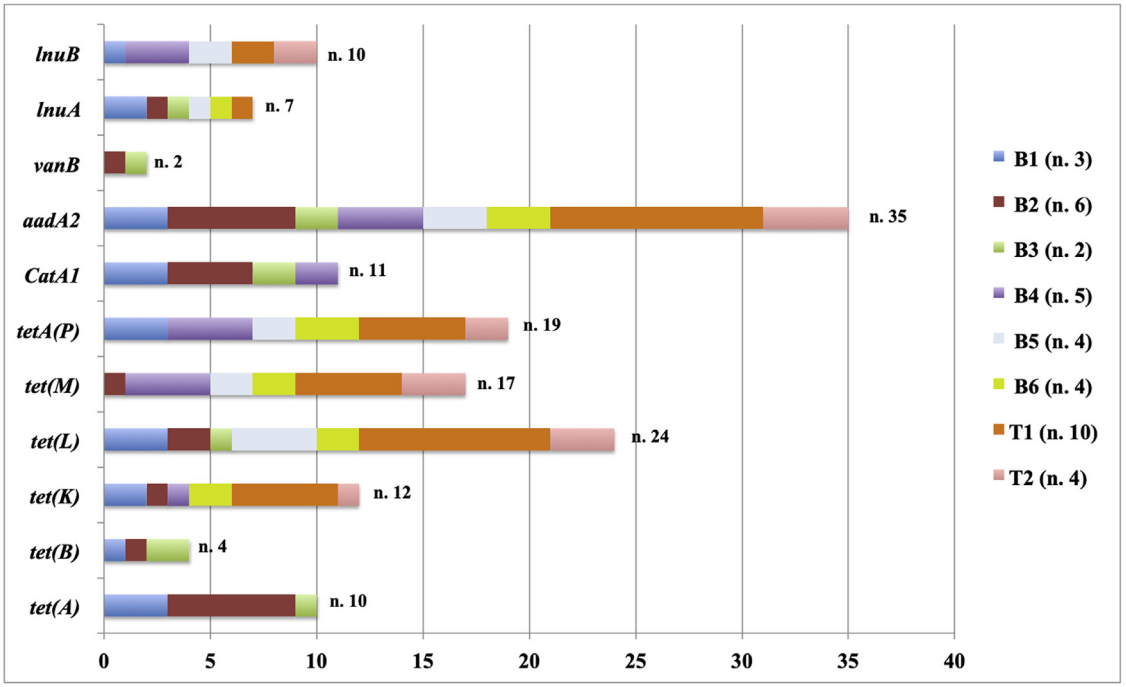

Figure 1. Number of samples resulted positive for antimicrobial-resistance genes (ARG) under study. For each flock, the total number of collected samples is reported in brackets. Abbreviations: B1–B6, broiler commercial flocks; T1 and T2, turkey commercial flocks.

detectable in broilers (6 of 28; 21.42%) compared with the turkey flocks (1 of 14; 7.14%). These results suggest a high abundance of these genes in the poultry litter. Consequently, the risk of transmission of antimicrobial-resistance determinants from farms to the environment, after the dispersion of poultry manure in soil, should be considered.

In this study, the *vanB* gene was detected in 2 fecal samples only, from broiler flocks B2 and B3, suggesting a moderate occurrence of the resistance for vancomycin. Despite the use of avoparcin been banned since 1997 by the European Union, the livestock is already considered a potential reservoir for vancomycin-resistance determinants worldwide. However, our results suggest a probable decline of this trend, as recently observed in Germany where only 1 vancomycin-resistant *E. faecium* isolate (carrying the *vanA* gene) was recovered from poultry slaughterhouses (Savin et al., 2020).

Finally, no evidence of carbapenem- and colistin-resistance genes was obtained from the investigated flocks, even if Laconi et al. (2021) reported both of them in poultry manure in Northern Italy. In European countries, the carbapenem resistance is not highly prevalent in livestock, suggesting limited public health relevance, while the colistin resistance is considered emerging because this antibiotic is one of the last treatment option for multidrug-resistance infection in humans. It is noteworthy that, in more recent year, a strong contraction of antibiotic use for diseases treatments was observed, especially in the poultry industry and in some countries such as Italy, Finland, Germany, Luxembourg, Norway, and Sweden (https://www.ecdc.europa.eu).

Probably, these trends could have influenced the negative results obtained for both carbapenemsand colistin resistance and the low detection level observed for the vancomycin too.

To our knowledge, only few data regarding the environmental ARG distribution in Italian commercial poultry flocks are available (Laconi et al., 2021), so this study can be considered an attempt to improve the knowledge about the occurrence of a wide range of resistance genes in the environment at the farm level, including the critically important antibiotics for human health.

Antimicrobial resistance is an important challenge threatening human and animal health, the economy, and the environment worldwide, which needs a multidisciplinary approach involving human activities, livestock farms, and wildlife. In this respect, the molecular culturing-independent screening performed in this study, based on a rapid and noninvasive sampling method, appears to be a relatively simple and at low-cost laboratory tool to monitor the AMR and ARG trends in the whole microbial community of the poultry farms, as previously observed in Spain (Esperón et al., 2018) and Portugal (Amador et al., 2019). Our study can be considered a preliminary step useful to address the further investigations to a more representative number of flocks, including also other kinds of farming methods as organic and antibiotic-free, and to study the effect of these alternatives systems on the composition of microbial resistome in poultry.

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DISCLOSURES

The authors declare no conflicts of interest.

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