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Monitoring of antibiotic residues in muscles, milk and eggs of food-producing animals in Umbria and Marche regions (Central Italy) during the period time 2012-2021

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Abstract

Introduction. The use of antibiotics in food-producing animals for infections treatment, metafilaxis and, although not allowed in Europe, as growth enhancer is responsible for the presence of antibiotic residues in animal derived foodstuffs. For this reason, it is very important to perform a monitoring.

Methods and results. Muscle samples from bovine, pig, poultry, turkey and fish, as well as bovine milk and hen's egg samples, deriving from 444 farms of both Umbria and Marche regions (Italy) were analyzed by well-established and validated analytical methods in order to evaluate the presence or not of antibiotic residues (penicillins, quinolones, tetracycline and sulphonamides). The samples were collected during 2012-2021 period of time. In total, 15/2,354 samples resulted positive to the analyses. The amount of antibiotics found in the 15 samples resulted below the maximum residue limit fixed by EU Regulation 37/2010 and for this reason considered compliant.

Conclusions. Despite irregular samples were not found, the presence of antibiotic residues in foodstuff represents a risk for public health as they are responsible for the selection of resistant strains contributing to antimicrobial resistance problem spread. In the present work, this aspect was evaluated in relation to the results obtained from the analyzed samples coming from Umbria and Marche regions.

Key words

- antibiotics residues
- muscles
- eggs
- milk
- antibiotic-resistance
- One Health

INTRODUCTION

Food safety and control of antimicrobial resistance (AMR) are among the aims of the One Health concept that considers the health of humans, animals and environment strictly interconnected [1, 2].

In a recent study, referring to 2019, is reported that 1.27 million people worldwide died because of infections associated to bacterial AMR [3]. Without appropriate strategies aimed at limiting this phenomenon these numbers would rise. It is in fact estimated that the number of death would reach 10 million/year by 2050 due to infections associated with multi-resistant micro-organisms [4].

The main factors responsible for the AMR are: i) antibiotics overuse and misuse in both humans and animals; ii) absorption of antibiotic residues deriving both from the environment (contaminated water, air, soil, or manure) and food; iii) direct animal-to-human contact on farms and slaughterhouses [5].

In food producing animals, antibiotics are used for therapeutic purposes, for disease prevention or as growth promoters [6], the last practice was banned in Europe starting from 1st January 2006.

The massive use of antibiotics in food producing animals represents a serious health care problem as the foodstuff is a vehicle for AMR transmission. Through foodstuff consumption, antibiotic residues could be transmitted to humans and, once internalized, they could promote the selection of AMR microorganisms. The latter could also develop in the animal continuously

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exposed to antibiotics so that the animal derived foodstuff could also represent a vehicle for the transmission of resistant bacteria or genes [6].

Considering the European scenario, according to the data collected from the European Medicines Agency (EMA), the use of antibiotics in the livestock's changes in the different countries [7]. In Nordic-Baltic nations for example, the antibiotics consumption is very low due to the combination of national strategies (surveillance program as well as good practice veterinary guidelines) aiming to limit the use of antibiotics and thus to control the AMR phenomenon [8, 9].

The largest users of antibiotics are: i) Poland, Italy and Spain where the amount used per livestock unit is 10-20 times higher than the lowest users (Nordic-Baltic countries); ii) France and Germany where the usage levels are about 5-10 times higher per livestock unit than the lowest users [7].

It is well demonstrated that the use of antibiotics in food producing animals contributes to AMR problem with consequent impact on the global health [10].

For this reason, over the years it was considered necessary to elaborate projects aiming to perform a deep surveillance and collaboration among the countries in order to better control and monitor the antibiotics consumption and thus AMR.

In 2005 the European Centre for Disease Prevention and Control (ECDC) was established. It is an agency of the European Union (EU) born with the aim to control the infectious diseases. ECDC performs a surveillance of both antibiotics' consumption in humans as well as AMR. ECDC, together to the European Food Safety Authority (EFSA) and EMA, elaborates periodically a report about antimicrobial agent consumption and occurrence of antimicrobial resistance in bacteria from humans and food-producing animals. The purpose of this is to furnish periodic reports useful to provide an integrated analysis of the relationships between the use of antibiotics both in human and animals and the incidence of AMR in bacteria from humans and food [10].

The European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) started in 2009. This is a project aimed at collecting information about the use of antimicrobial medicines in animals in the EU. These data are useful to create a database to correlate the consumption of antibiotics in veterinary field to AMR. The Decision 2013/652/EU has a very significant importance for the collection of data about AMR. This document reports the rules useful to perform the monitoring and reporting of antimicrobial resistance in zoonotic and commensal bacteria according to harmonized practices in all the EU member states.

On 30th June 2017 the European Commission adopted the "European One Health Action Plan against Antimicrobial Resistance (AMR)" aiming to limit the use of antimicrobials together to the improvement of the information about the problems related to AMR. The adoption of these measures has produced a positive impact as demonstrated in the thirteenth ESVAC report, which highlights that the sales of antibiotics in veterinary field (reported as milligrams per population correction unit mg/PCU) decreased of 53.0% from 2011 to 2022 [11]. In Italy Decision 2013/652/EU was adopted starting from 2014 and the Piano Nazionale di Contrasto dell'Antimicrobico-Resistenza (PNCAR) launched a monitoring program aimed at counteracting AMR through an integrated plan involving the human, veterinary, food, environmental and agricultural fields.

According to this plan in Italy the main pathogen species, representing the main risks of developing acquired antibiotic resistance, are *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Enterococcus faecalis*, *Enterococcus faecium*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Acinetobacter*.

This paper deals with the examination of the results obtained from the search of selected antibiotic residues (penicillins, quinolones, tetracyclines and sulphonamides) within a surveillance study conducted in Central Italy by Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche, under the auspices of the Italian Ministry of Health. Meat samples (bovine, pig, poultry, turkey and fish), bovine milk and hen's eggs were analyzed in the period 2012-2021 in Umbria and Marche regions.

MATERIALS AND METHODS

Sample collection

2,354 samples (bovine, pig, poultry, turkey, fish muscle, hen's eggs and bovine milk) were collected during a ten-year period (2012 to 2021) from 444 farms of both Umbria (217) and Marche (227) regions, within the framework of the official control and self-control plan of the Italian dairy industry. 287 samples were submitted to penicillins, 454 samples analyzed for tetracycline, 990 for sulphonamides and 623 for fluorofluoroquinolones detection. Sampling was performed according to Piano Nazionale Ricerca Residui (PNR) 2021 from the Italian Ministry of Health and the analyses were carried out by the Istituto Zooprofilattico Sperimentale of Umbria and Marche "Togo Rosati".

Standards and reagents

Milli-Q system Millipore (Bedford, MA, USA, 18.2 mΩ cm^{-1} resistivity) was used to obtain ultrapure water. SPE SCX (100 mg, 3 mL) cartridges were purchased from Phenomenex (Torrance, CA, USA). The standards of antibiotics sulfamerazine, sulfamonomethoxine, sulfadiazine, sulfathiazole, sulfamethoxazole, oxolinic acid, flumequine, marbofloxacin, chlortetracycline, doxycycline, benzylpenicillin, cloxacillin, dicloxacillin, nafcillin, oxacillin were purchased from Dr. Ehrenstorfer (Augsburg, Germany); sulfachloropyridazine, sulfametoxipiridazine, sulfadimethoxine, sulfachinoxaline, sulfamethazine, sulfapyridine, ciprofloxacin, danofloxacin, difloxacin, enrofloxacin, norfloxacin, sarafloxacin, tetracycline, amoxicillin, ampicillin, purity ≥95% (HPLC), were purchased from Sigma-Aldrich (St. Louis, MO, USA). All the other reagents and solvents used were of analytical grade and were supplied by Carlo Erba (Milan, Italy).

Analytical methods

The detection of penicillins (nafcillin, dicloxacillin, cloxacillin, oxacillin, ampicillin, amoxicillin, benzyl-

penicillin) in muscle samples (bovine, pig, poultry, turkey, fish) was performed by Premi®Test (DSM, DSM Food Specialities R&D, Delft, The Netherlands) while for bovine milk samples Delvotest® (DSM, DSM Food Specialities R&D, Delft, The Netherlands) was used. They are microbiological assays, in which the samples, are submitted to antibiogram analysis based on the evaluation of growth inhibition of the strain *Bacillus stearothermophillus*. The limit of antibiotic detection is ≥25 µg/kg for muscle samples while for milk samples concentrations ≥3 µg/L for ampicillin, amoxicillin, benzylpenicillin and ≥20 µg/L for nafcillin, dicloxacillin, cloxacillin, oxacillin are detectable.

Before the analysis, muscle samples were treated according to the following procedure: 4 g of minced muscle was placed in a 50 mL Falcon® tube and added by 10 mL of extraction solvent constituted by acetonitrile (ACN)-acetone 70:30 v/v. The sample was homogenized for 10 min and then centrifuged at 4,000 rpm for 10 min. The supernatant was placed in a 15 mL Falcon® tube and the solvent removed under nitrogen at 40-45 °C. The solid was then suspended in 500 µL di Lab Lemco broth (Thermo Scientific™, Roma, Italy) and vortexed. Premi®Test was performed for screening e post-screening using 100 µL (screening) and 230 µL (post-screening) of the extract (*Figure 1*).

Bovine milk samples were analyzed without preventive preparation procedures (*Figure 2*). The determinations were carried out following the kit manufacturer instructions [12, 13].

Tetracyclins (doxycycline, chlortetracycline, tetracycline, oxytetracycline) were detected by TetraSensor (Tissue) – KIT036 for both muscles (bovine, pig, poultry, turkey, fish) and hen's eggs and KIT014 for bovine milk (Unisensor, Seraing (Ougrée) – Belgium). The detection limit is ≥40 µg/kg for muscles and ≥25 µg/L for milk and ≥75 µg/kg for eggs. Before the analysis, the muscle samples were prepared as follows: the homogenized muscle (10 g) was put in a stomacher bag added by 30 mL of extraction buffer provided in the kit. The sample was then homogenized in stomacher for 2 min. One mL of extract was ultracentrifuged at 10,000 rpm for 3 min. Then 200 µL of the extract were seeded in the microplate well. The dipstick was put in the well and left for 10 min. Afterwards the dipstick was removed and performed the analysis by Readsensor reader (*Figure 1*). In the case of bovine milk samples, they were assayed without preliminary extraction procedures (*Figure 2*). Hen's egg samples were prepared as follows: the homogenized eggs (10 g) were put in a centrifuge tube (50 mL) then added by 30 mL of extraction buffer (prepared according to kit procedures). The sample was centrifuged (4,000 rpm, 20 min), then put in a centrifuge tube (15 mL), added by n-hexane (5 mL) vortexed, centrifuged (4,000 rpm, 10 min) and the hexane removed. The remaining aqueous phase (200 µL) was used for the assay (*Figure 1*). The determinations were carried out following the kit manufacturer instructions [14].

Fluoroquinolones (flumequine, difloxacin, ciprofloxacin, marbofloxacin, norfloxacin, sarafloxacin, danofloxacin, enrofloxacin, oxolinic acid) were detected by

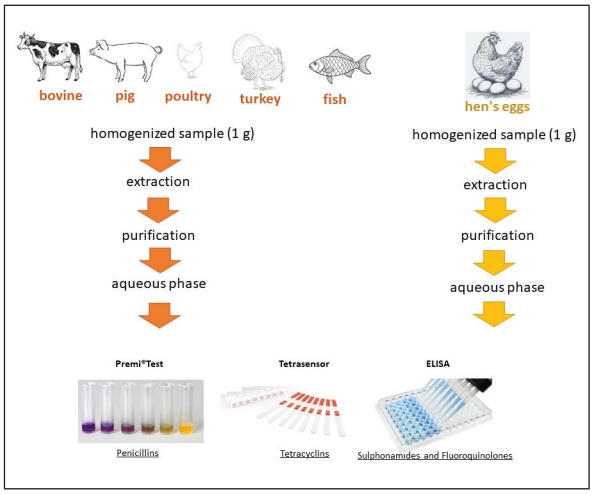

Figure 1
Scheme of the procedure followed for both muscles and eggs samples preparation before the analysis of the different antibiotics.

enzyme immunoassay using the immunoenzymatic kit chinolone ridascreen® cod. R3113 (r-Biopharm, Darmstadt, Germany). The detection limit is ≥25 µg/kg for both muscles and eggs and ≥15 µg/L for bovine milk. The samples (bovine, pig, poultry, turkey, fish) were prepared using the methods proposed by Scortichini *et al.* [15] starting from 1 g of homogenized muscle or eggs (*Figure 1*).

Extraction of fluoroquinolones was obtained by introducing 4 mL of extraction solution (m-phosphoric acid 0.45%/ACN 70/30 v/v). Then the tube was vortexed for 10 min. Afterward the tube was placed in a water-bath for 30 min at 45-50 °C in order to induce the precipitation of proteins. Then the sample was left to cool and centrifuged (4,500 rpm for 10 min), the supernatant was filtered in a 15 mL Falcon® tube by using a nylon syringe filter (30 mm, 0.45 µm). The obtained sample was resubmitted to another extraction cycle adding 4

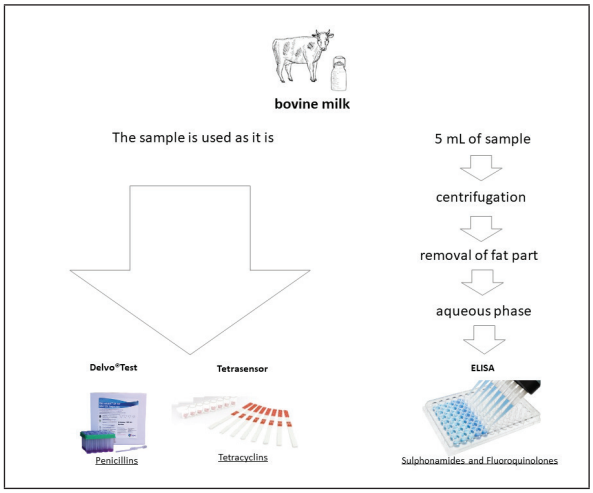

Figure 2
Scheme of the procedure followed for bovine milk samples preparation before the analysis of the different antibiotics.

mL of extraction solution (m-phosphoric acid 0.45%/ACN 70/30 v/v) and performing the steps described above. The extracts (~8 mL) were combined and an aliquot of 4 mL was evaporated under a nitrogen flux (40-50 °C) until the complete evaporation of ACN (until 2 mL). Then the concentrated extract was diluted with 4 mL of water. The extract purification was performed by loading on the OASIS HLB cartridge previously conditioned with 1 mL of MeOH and 1 mL of Milli-Q water. Subsequently, the cartridge was washed with 2 mL of phosphate buffer (0.025 M, pH 3)/MeOH 95:5 (v/v) and with 2 mL of water. The fluoroquinolones were eluted with 2 mL of MeOH/ammonia 95:5 (v/v). The solvent was removed under nitrogen (40-50 °C), just before application to the microtiter plates, the residue was dissolved in 2 mL of MeOH/water 35/65 (v/v). The determinations were carried out following the kit manufacturer instructions [16]. In case of the milk 5 mL of sample were centrifuged (4500×*g* for 10 min) in order to eliminate the fat fraction (*Figure 2*).

Sulfonamides (sulfamerazine, sulfamonomethoxine, sulfadiazine, sulfachloropyridazine, sulfametoxipiridazine, sulfadimethoxine, sulfachinoxaline, sulfathiazole, sulfamethazine, sulfamethoxazole, sulfapyridine) detection was performed by ELISA method using a sulphonamides ELISA KIT cod. SM390 (Tecna srl, Trieste, Italy), detection limit ≥20 µg/kg for muscle, egg and milk samples. The ELISA determinations were carried out following the manufacturer instructions. Muscle samples were prepared as described by Galarini *et al*. [17].

The samples (bovine, pig, poultry, turkey, fish) were prepared as follows: 1 g of homogenized muscle was placed in a 50 mL Falcon® tube. Then 5 mL of ethyl acetate were added and the sample was vortexed for 10 s and then stirred at 300 rpm for 15 min. Afterwards the sample was centrifuged for 10 min at 4,000 rpm. Three mL of supernatant (corresponding to 0.6 g of muscle) were taken and placed in a 15 mL Falcon® tube. The solvent was removed under nitrogen atmosphere at 50 °C. The obtained solid was then suspended in 0.6 mL of buffer provided in the kit and added by 1 mL of n-exane. The sample was vortexed for 30 s and centrifuged for 10 min at 4,000 rpm. The supernatant was removed and the aqueous phase submitted to the analysis. For the analysis 50 µL of sample were used for the seed in the microplate (*Figure 1*).

For bovine milk samples 5.0 g were centrifuged for 15 min at 4000 rpm and 4 °C in order to remove the fat fraction. Then the sample (2.5 g) was put in a centrifuge tube (50 mL), added by ethyl acetate (5 mL) and mixed for 1 min. The sample was then left in static conditions at room temperature for 10 min in order to obtain the phases separation. The supernatant (4 mL) was then dried under nitrogen at (50 °C). The obtained solid was then solubilized in 1 mL of buffer prepared according to kit procedures and 50 µL used for the assay (*Figure 2*).

For hen's egg samples 1.0 g of homogenized sample was added by ethyl acetate, vortexed for 10 min and put in a mechanical stirrer for 15 min at 300 rpm. The sample was then centrifuged, 10 min at 4,000 rpm. The supernatant (3 mL) was then dried under nitrogen at 50 °C and the obtained solid resuspended in 0.6 mL of buffer prepared according to kit procedures. The sample was then added by n-hexane (1 mL), vortexed for 30 sec, centrifuged for 5 min at 4,000 rpm. The supernatant was removed and the aqueous phase (50 µL) used for the analysis (*Figure 1*). The determinations were carried out following the kit manufacturer instructions [18].

Methods validation

The assays used for antibiotic residues identification are widely developed and implemented as routine laboratory tests for official analyses, due to the low costs and reduced working times allowing well-timed decisions. This is particularly important in the search of antibiotic residues in foodstuffs deriving from food-producing animals. The validation was performed according to the Commission Decision 2002/657/EC regulating the performances of analytical methods applied in EU official monitoring programs (*Table 1*). The main parameter considered in the validation is represented by the detection capability (CCβ) defined as "the smallest content of the substance that may be detected identified and/or quantified in a sample with an error probability of β". In the case of substances with an established permitted limit, the detection capability is the concentration at which the method is able to detect the allowed limit concentrations with a statistical certainty of 1 – β" (point 1.12 of the Annex to CD 2002/657/EC). β error represents the probability that the considered sample is truly non-compliant, even though a compliant measurement is obtained (false compliant decision). For screening tests the β error is fixed ≤5% [19].

RESULTS AND DISCUSSION

During 2012-2021 food samples (bovine, pig, poultry, turkey and fish muscles, as well as bovine milk and hen's eggs) deriving from farms of both Umbria and Marche regions were analyzed for the search of the following antibiotics residues: penicillins, tetracycline, sulphonamides and fluoroquinolones. The search was performed according to "Piano Nazionale Residui" (PNR) 2021 hat prescribes the search of antibiotics residues in the following samples: muscles (bovine, porcine, ovine, caprine, equine, poultry, turkey, fish, rabbits, farmed game), milk, eggs, honey [20]. In PNR the groups of chemical substances to be investigated in such samples, provided in the Annex I of the Legislative Decree 158/2006, are divided in category A (anabolic substances and non-authorised substances) and category B (veterinary medicinal products and contaminants). The latter category, is further divided in B1, B2 and B3 sub-category. B1 is the sub-category of interest in this study as it represents the antibacterial substances.

The results obtained from the analyses performed showed that non-compliant (irregular) samples were not detected (*Table 2*). No positive samples were detected in both hen's eggs and bovine milk while in the case of muscles some samples resulted positive for tetracycline, sulphonamides and fluoroquinolones. In 2012 one poultry muscle sample was positive to the fluoroquinolone flumequine (47.9 µg/kg). During 2013 one

Table 1
Methods used for antibiotics determination in the different food matrices considered: data obtained in validation *vs* corresponding requirements (Commission Decision 2002/657/EC)

Test	Detection method	Antibiotic class	Matrix	Parameters considered during the validation according to Decision 2002/657/EC	In-house validation
Premi®Test DSM	Microbiological technique	Penicillins	Muscles (bovine, pig, poultry, turkey, fish)	Detection capability (CCβ): is the smallest analyte content that can be detected or quantified in a sample with an error of β: the maximum error rate for authorized substances should not exceed 5%. The value of CCβ depends on the regulatory limit for each substance or class of them. Specificity: is the power of an analytical method to discriminate between the analyte and any closely related substance. Ruggedness: is the ability of an analytical method to withstand minor changes of experimental conditions.	Analyzing at least 20 fortified blanks for the concentration level chosen according to the MRL of each substance, the lack of any false negative result demonstrated method compliance (percentage of false compliant results or beta-error ≤5%). After fortifying of representative blank samples at a relevant concentration with substances that could be interferences, the lack of false identifications demonstrated the specificity of the analytical method. The ruggedness tests of the analytical method were conducted using the Youden approach.
Delvotest® DSM	Microbiological technique	Penicillins	Bovine milk		
TetraSensor (Tissue) – KIT036 Unisensor	Receptorial technique	Tetracyclins	Muscles (bovine, pig, poultry, turkey, fish) and hen's eggs		
TetraSensor (Milk) – KIT014 Unisensor	Receptorial technique	Tetracyclins	Bovine milk		
Immunoenzymatic Kit Chinolone Ridascreen®	ELISA	Quinolones	Muscles (bovine, pig, poultry, turkey, fish), bovine milk, hen's eggs		
Immunoenzymatic Kit Sulphonamides Tecna®	ELISA	Sulfonamides	Muscles (bovine, pig, poultry, turkey, fish), bovine milk, hen's eggs		

MRL: maximum residue limit.

poultry muscle sample was positive to oxytetracycline (54.9 µg/kg), one to the fluoroquinolone flumequine (25.4 µg/kg); one pig muscle sample resulted positive to h (74.0 µg/>kg).

During 2014, two poultry muscles were positive to doxycycline (an amount of 49.0 µg/kg and 94.3 µg/kg respectively) and one to tetracycline (amount measured 34.2 µg/kg) (*Table 2*). Moreover, one poultry muscle sample was positive to the fluoroquinolone flumequine (30.0 µg/kg).

In 2015 one pig muscle sample was found positive to doxycycline (12.0 µg/kg), two pig muscle samples resulted positive to sulfamonomethoxine (amount 49.0 µg/kg and 13 µg/kg respectively) while one sample of bovine muscle was positive to sulfamonomethoxine (38.0 µg/kg). One fish muscle sample was positive to flumequine (20.0 µg/kg) as well during 2016.

During 2017 sulfamerazine (11.0 µg/kg) residues were found in one pig muscle sample and enrofloxacin (22.0 µg/kg) residues were found in one poultry muscle sample (*Table 2*).

In all cases, the amount of antibiotic residues found was not considered problematic as the values resulted compliant to UE Regulation 37/2010 in which the admitted maximum residue limits (MRL) are fixed for the different antibiotics. MRL can be defined as the maximum allowed concentration of antibiotic residues in animal derived foodstuff, after a therapeutic treatment, established based on the calculated acceptable daily intake from preclinical data and residue depletion studies in target animal species [21]. These limits are set according to safety assessment, taking into account toxicological risks, environmental contamination, as well as the microbiological and pharmacological effects of residues as reported in the Regulation (EC) 470/2009. The MRL value for the antibiotics found in the muscle samples are: 100 µg/kg for oxytetracycline, doxycycline, tetracycline, sulfamonomethoxine, sulfamerazine, enrofloxacin and 400 µg/kg for flumequine.

The samples resulted positive to antibiotic search (*Table 2*) are compliant to UE Regulation 37/2010 as the concentrations found are below the MRL. However, some important considerations must be done. The first of them is the relative low number of samples analyzed and, even more, the low number of positive samples that makes difficult to obtain statistically significant trends.

Moreover, it should be considered that the samples were analyzed by *ad hoc* methods, optimized for each class of antibiotics considered. The use specific methods have two main limitations: i) each sample should be analyzed for one molecule or group of molecules (one sample for one method), ii) each method is specific for one class of antibiotic molecules thus not useful to detect antibiotics of other classes. Moreover, the sensitivity of the method (limit off detection – LOD) is limited in comparison to other more efficient techniques. For this reason, since 2023 the EU suggested to use multi-residue and more sensitive methods capable of detecting, through the analysis of a single sample, many classes of molecules. LC-MS/MS is one of this technique allowing to detect also levels lower than 70 ppb [22]. Based on these considerations, it could be plausible to hypothesize that antibiotic residues may have been present in samples which resulted negative, due to the limited sensitivity of the analytical methods used.

It is well known the connection between the anti-

Table 2
Resuming table of the food samples analyzed (bovine muscle, pig muscle, poultry muscle, turkey muscle, fish muscle, hen's eggs, bovine milk) in Umbria and Marche regions (Central Italy) in the period 2012-2021. For each year and for each class of antibiotics selected (penicillins, tetracyclines, sulphonamides and fluoroquinolones) are reported: the number of samples analyzed and the number of positive samples. For the positive samples the amount of antibiotic found and the maximum residue limit (MRL) of UE Regulation 37/2010 are reported

Penicillins

Year	2012		2013		2014		2015		2016		2017		2018		2019		2020		2021		
Sample	N tested	N positive	N tested	N positive	N tested	N positive	N tested	N positive	N tested	N positive	N tested	N positive	N tested	N positive	N tested	N positive	N tested	N positive	N tested	N positive	Pos/total samples
Bovine muscle	21	-	58	-	20	-	5	-	1	-	-	-	-	-	1	-	-	-	-	-	0/106
Pig muscle	24	-	11	-	10	-	18	-	11	-	6	-	4	-	6	-	3	-	6	-	0/99
Poultry muscle	12	-	16	-	17	-	1	-	3	-	5	-	1	-	-	-	-	-	1	-	0/56
Turkey muscle	-	-	1	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0/2
Fresh fish muscle	-	-		-	-	-	-	-	2	-	-	-	2	-	1	-	3	-	2	-	0/10
Hen's eggs	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Bovine milk	6	-	4	-	2	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	0/14
	63		**90**		**50**		**26**		**17**		**11**		**7**		**8**		**6**		**9**		**0/287**

N: number.

Tetracyclines

Year	2012		2013		2014		2015		2016		2017		2018		2019		2020		2021		
Sample	N tested	N positive	N tested	N positive	N tested	N positive	N tested	N positive	N tested	N positive	N tested	N positive	N tested	N positive	N tested	N positive	N tested	N positive	N tested	N positive	Pos/total samples
Bovine muscle	21	-	58	-	20	-	5	-	1	-	-	-	-	-	1	-	-	-	-	-	0/106
Pig muscle	39	-	24	-	17	-	27	1[a]	19	-	20	-	15	-	12	-	13	-	19	-	1/205
Poultry muscle	13	-	16	1[b]	17	3[c]	1	-	3	-	5	-	1	-	-	-	-	-	1	-	4/57
Turkey muscle	-	-	1	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0/2
Fresh fish muscle	5	-	2	-	1	-	1	-	4	-	2	-	4	-	4	-	5	-	3	-	0/31
Hen's eggs	4	-	3	-	5	-	5	-	4	-	3	-	3	-	5	-	5	-	2	-	0/39
Bovine milk	6	-	4	-	2	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	0/14
	88		**108**		**63**		**41**		**31**		**30**		**23**		**22**		**23**		**25**		**5/454**

[a]doxycycline 12.0 µg/kg; [b]oxytetracycline 54.9 µg/kg; [c]doxycycline 49.0 µg/kg, doxycycline 94.3 µg/kg, tetracycline 34.2 µg/kg; the maximum residue limit (MRL) reported in UE Regulation 37/2010 is 100 µg/kg. N: number.

Sulphonamides

Year	2012		2013		2014		2015		2016		2017		2018		2019		2020		2021		
Sample	N tested	N positive	N tested	N positive	N tested	N positive	N tested	N positive	N tested	N positive	N tested	N positive	N tested	N positive	N tested	N positive	N tested	N positive	N tested	N positive	Pos/total samples
Bovine muscle	14		28	-	27	-	28	1a	23	-	21	-	20	-	20	-	19	-	8	-	1/208
Pig muscle	48	-	58	1b	41	-	50	2c	55	-	49	1d	40	-	45	-	44	-	40	-	4/470
Poultry muscle	14	-	25	-	26	-	5	-	26	-	27	-	26	-	24	-	23	-	24	-	0/220
Turkey muscle	-	-	1	-	1	-	1	-	1	-	-	-	-	-	-	-	-	-	-	-	0/4
Fresh fish muscle	1	-	1	-	1	-	1	-	1	-	2	-	3	-	2	-	4	-	2	-	0/18
Hen's eggs	2	-	5	-	5	-	8	-	8	-	3	-	7	-	6	-	9	-	2	-	0/55
Bovine milk	2	-	2	-	2	-	2	-	2	-	1	-	1	-	1	-	1	-	1	-	0/15
	81		**120**		**103**		**95**		**116**		**103**		**97**		**98**		**100**		**77**		**5/990**

[a]sulfamonomethoxine 38 µg/kg; [b]sulfamonomethoxine 74 µg/kg; [c]sulfamonomethoxine 49 µg/kg, sulfamonomethoxine 13 µg/kg; [d]sulfamerazine 11 µg/kg; the maximum residue limit (MRL) reported in UE Regulation 37/2010 is 100 µg/kg. N: number.

Continues

Table 2
Continued

Fluoroquinolones																					
Year	**2012**		**2013**		**2014**		**2015**		**2016**		**2017**		**2018**		**2019**		**2020**		**2021**		
Sample	**N tested**	**N positive**	**N tested**	**N positive**	**N tested**	**N positive**	**N tested**	**N positive**	**N tested**	**N positive**	**N tested**	**N positive**	**N tested**	**N positive**	**N tested**	**N positive**	**N tested**	**N positive**	**N tested**	**N positive**	**Pos/total samples**
Bovine muscle	21	-	58	-	20	-	5	-	1	-	-	-	-	-	1	-	1	-	-	-	0/107
Pig muscle	37	-	23	-	19	-	28	-	20	-	17	-	14	-	11	-	14	-	16	-	0/199
Poultry muscle	32	1[a]	36	1[b]	37	1[c]	6	-	25	-	21	1[d]	20	-	18	-	16	-	18	-	4/229
Turkey muscle	-	-	2	-	2	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	0/5
Fresh fish muscle	5	-	2	-	1	-	1	-	3	1e	2	-	3	-	4	-	5	-	3	-	1/29
Hen's eggs	4	-	3	-	7	-	6	-	4	-	2	-	3	-	4	-	5	-	2	-	0/40
Bovine milk	6	-	4	-	2	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	0/14
	105		**128**		**88**		**49**		**53**		**42**		**40**		**38**		**41**		**39**		**5/623**

[a]flumequine 47.9 µg/kg; [b]flumequine 25.4 µg/kg; [c]flumequine 30.0 µg/kg; [d]enrofloxacin 22.0 µg/kg; [e]flumequine 20.0 µg/kg; the maximum residue limit (MRL) reported in UE Regulation 37/2010 is 100 µg/kg for enrofloxacin and 400 µg/kg for flumequine. N: number.

biotics consumption and AMR occurrence in bacteria, in both humans and food-producing animals, as confirmed also in the fourth joint report published by EFSA, ECDC and EMA on January 2024 [10].

Data furnished by the European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) about the sales or prescription of antimicrobial veterinary medicinal products for food-producing animals shows that during the 2020 (in 31 countries) the main classes of antibiotics used were penicillins (31.1%), tetracyclines (26.7%) and sulfonamides (9.9%). Despite in Italy from 2011 to 2021 the overall use of antibiotics in veterinary field decreased by 53%, unfortunately the consumption in livestock's is still high classifying Italy as third country in Europe for antibiotics use [23].

Data provided by Italian Medicines Agency (Agenzia Italiana del Farmaco, AIFA) about the human antibiotic consumption, in both Umbria and Marche regions [24], show that the use of penicillins and their combinations decreased from 2015 (1.6 and 1.4 DDD/1000 ab *die** for Umbria and Marche respectively) to 2021 (0.7 DDD/1000 ab *die** for both Umbria and Marche).

From the data reported in *Table 2* no positive samples were obtained from penicillins analysis. Based on these data, a decrease in AMR should be observed for this class of antibiotics over the same time period.

However, the regional results found in the national report, elaborated by the Istituto Superiore di Sanità (ISS) about the antibiotic-resistance surveillance referred to 2020, show the opposite. An increase of *Streptococcus pneumoniae* strains resistant to penicillin (13.6%) emerged as well as a high resistance of *Enterococcus faecium* toward ampicillin (90.2% of the isolated strains) and methicillin (33.5%). Moreover, *Escherichia coli* resulted particularly resistant towards ampicillin (64.5%) and amoxicillin-clavulanic acid (42.9%) as well as *Klebsiella Pneumoniae* (57.2%) [25].

According to AIFA report 2021, in Italy tetracycline are one of the most prescribed antibiotics in food producing animals [24]. In this study residues of such class of molecules were found in five muscle samples between 2013-2015. Thus, could be hypothesized that the large prescription of this class of antibiotics could contribute to AMR spread observed in veterinary field.

Recently Russo *et al.* [26] observed that multi-drug resistant (MDR) *Salmonella* strains are particularly resistant to tetracycline. This study, performed on samples deriving from the food chain in the Marche region (Central Italy), showed a wide dissemination of tetracycline resistance in *Salmonella* strains (80%). Indeed, the ISS report of 2020 showed that in Italy *Streptococcus pneumoniae* is the main tetracycline-resistant strain (16.8%) [11].

The EU One Health 2020 Zoonoses Report [27] food, animals and feed are provided and interpreted historically. Two events impacted 2020 MS data collection and related statistics: the Coronavirus Disease 2019 (COVID-19, an EFSA/ECDC document, shows that campylobacteriosis is the most common reported zoonosis in Europe, representing more than 60% of all the reported cases in 2020, followed by salmonellosis. A document drafted from EFSA states that *Salmonella* is the second pathogen responsible for foodborne diseases [14]. In the period 2016-2018, statistically significant associations between tetracycline consumption in food-producing animals and tetracycline resistance were identified in both *Salmonella spp.* and *Campylobacter jejuni* from humans. The latter is the consequence of the development of tetracycline resistance in *Campylobacter jejuni* from poultry [28].

Sulfonamides were mostly detected in pig muscle and the highest number of positive compliant samples was registered during the year 2015 (*Table 2*). EFSA report, referring to 2015, shows that in the EU *Salmonella spp.*

* Average number of drug doses consumed daily by 1,000 inhabitants (AIFA 2021).

was isolated from fattening pigs showing a high level of resistance to sulfamethoxazole (~52.6%). The same report describes that *Salmonella* strains, isolated in 2015 from humans, show a fair degree of resistance to sulfonamides/sulfamethoxazole (32.4%) [15, 29]. Data gathered of 2019 and 2020 showed a degree of resistance of 50.6% and 49.2% respectively [30]. It was found that high levels of *Salmonella spp*. detected in Italy are higher than in Europe.

In particular sulfamethoxazole results ineffective in 44.9% of the cases, followed by tetracycline (40.4%) and ampicillin (37.4%) [31]. These data support the verified correlation between antibiotic resistance of *Salmonella* in humans, associated to antimicrobials consumption in the pig farms for food chain [32]. A recent survey of Marche region underlined a high resistance degree of *Salmonella* strains toward sulfisoxazole [26]. These findings are very important as salmonellosis is one of the most frequent foodborne zoonosis, representing one of the major worldwide health concerns [30].

About fluoroquinolones, the positive compliant samples showed residues of the molecule flumequine which is used in human for the treatment of urinary tract infections [33]. This is a second-generation fluoroquinolone, antibiotic used in poultry in the treatment of systemic bacterial infection due to gram-negative bacteria including colibacillosis [34].

During 2019 AIFA [35] and EMA decided to remove this antibiotic (together to cinoxacin, nalidixic acid and pipemidic acid) from the trade of human medicines as responsible for many long-lasting and potentially permanent adverse reactions. Thus, it is still available only for the veterinary purpose. This poses a serious problem about the risks to which humans are exposed through the consumption of food containing residues of this antibiotic [36]. From AIFA report [11] resulted that human consumption of fluoroquinolones in Umbria and Marche regions decreased from 2015 (3.8 and 3.3 DDD/1000 ab *die*** for Umbria and Marche respectively) to 2021 (1.8 and 1.6 DDD/1000 ab *die*** for Umbria and Marche respectively) as assessed by the Italian antibiotics report [24]. However, the problem of resistance toward this class of molecules is still high. Indeed, an Italian report of 2020 showed how antibiotic resistance of *Escherichia coli* was above 30% toward fluoroquinolones, *Pseudomonas aeruginosa* was 29.4% towards levofloxacin and ciprofloxacin was about 18%.

Moreover, few studies are available in literature dealing with the contribution of low levels of flumequine in the induction of mutations and modifications responsible for antibiotic resistance. Such as Wood *et al*. observed some mutations on a virulent wild-type *Aeromonas salmonicida* induced by the exposure to low flumequine concentrations [35, 37].

The contribution to AMR of antibiotic residues in foodstuff is well documented by many scientific studies. It is well established that the presence of antibiotic residues below the MRL value promote the adaptation/selection of resistant strains that become less sensitive to antimicrobial agents that can pass to humans, by food consumption, with consequent AMR problem acceleration and spread. For this reason, despite the EU Regulation 37/2010 reports MRL of antibiotic molecules used in veterinary field, they must be considered the possible problems deriving from the use of UE Regulation 37/2010 compliant foods as that found in the present study (*Table 2*).

For many antibiotics the minimal selective concentration (MSC) has been defined. It represents the lowest antibiotic concentration that can lead in the enrichment of resistant bacteria in a strain population responsible for the selection of high-level resistant bacteria [21]. The antibiotics found in the positive compliant samples of the present study (*Table 2*) are: oxytetracycline, doxycycline, tetracycline, sulfamonomethoxine, sulfamonomethoxine, sulfamerazine, enrofloxacin, flumequine.

In a study performed on *E. coli* and *Salmonella* enterica strains, the growth of resistant bacteria was observed using tetracycline concentrations of 15 ng/ml (corresponding to 1/100 of the minimum inhibitory concentration MIC value) [38]. In a recent work, considering *E. coli* resistant strains, the MSC values were calculated for amoxicillin (0.08 mg/L - 0.8 mg/L), doxycycline (0.4 mg/L - 4 mg/L) and enrofloxacin (0.0125 mg/L - 0.125 mg/L) [39]. MSC identified for oxytetracycline was 0.1 mg/L in *E. coli* strain [40] while in a recent study it was demonstrated that flumequine is able to increase the resistance by inducing mutations in *E. coli* GyrA gene at concentrations of 2 mg/L [41].

Comparing these concentrations with the MRL values of the antibiotics detected in the samples analyzed (*Table 2*), the main concerns could raise for tetracycline, enrofloxacin, oxytetracycline which MSC found in literature are below the MRL values suggesting that the admitted concentrations represent a risk for AMR spreading both in animals and humans.

There is no global consensus on the best strategy to choose in order to alleviate the risks to human, animal and even environmental health [42] but many institutions are very committed to solve this problem. The institutions involved in the changes and management of the system in the veterinary and human sectors in Italy are the Italian Ministry of Health, Zooprophylactic Institutes (Istituti Zooprofilattici Sperimentali, IIZZSS), AIFA and ISS.

The European Commission, EMA, ECDC and EFSA support Member States to achieve the same goal. All draw inspiration from the World Organization for Animal Health (OIE) and the World Health Organization (WHO). During the US-EU summit in 2009, EU and United States (US) established the Transatlantic Task Force on Antimicrobial Resistance (TATFAR) in order to intensify the cooperation in the fight against AMR; EMA is a member of TATFAR.

The objective of the taskforce is to increase levels of communication, coordination and cooperation between the EU and the US on human and veterinary antimicrobials. In October 2015 a plan for the period up to 2020 was launched in New York and then extended

** Average number of drug doses consumed daily by 1,000 inhabitants (AIFA 2020).

to Canada and Norway. It requires global cooperation increasing knowledge and awareness of the AMR problem together to its effects on global health. The vastness of the problem also requires the involvement of different skills.

The One Health approach was adopted as part of a joint plan of action of WHO, Food and Agriculture Organization (FAO), World Organisation for Animal Health (WOAH) and United Nations Environment Programme (UNEP). In the scenario of AMR, it has the objective to "preserve antimicrobial efficacy and ensure sustainable and equitable access to antimicrobials for responsible and prudent use in human, animal and plant health".

The control of specific pathogens and AMR have been extensively funded under European research initiatives such as FP7, Horizon 2020 and Innovative Medicines Initiative (IMI). The surveillance of AMR is the result of the collaboration between EMA, EFSA and ECDC.

Based on the One Health approach, on 30th November 2022, the "Piano Nazionale di Contrasto all'Antibiotico-Resistenza (PNCAR) 2022-2025" was approved in Italy aiming to control AMR through the following points: i) surveillance and monitoring of both antibiotic consumption and AMR; ii) prevention of infectious diseases, zoonoses, healthcare-associated and community-acquired infections; iii) correct use of antibiotics both in human and veterinary field as well as correct disposal of antibiotic-contaminated wastes.

CONCLUSIONS

The data report referring to 2012-2021 presented in this paper, dealing with the search of antibiotic residues in muscles, milk and egg samples, showed that in Umbria and Marche regions no positive non-compliant (irregular) samples were detected.

Despite the obtained results are promising in the perspective of public health preservation however some concerns may arise about the positive samples even though these samples are compliant to the maximum residue limits reported in the UE Regulation 37/2010. The consumption of such food samples can contribute to the expansion of AMR in both humans and animals as the low concentrations of antibiotic residues could be responsible for resistant strains selection. What strategies could be adopted to do this? In the perspective of One Health concept, the European regulation EU 2019/6 about veterinary medicines has the objective to introduce restrictions to limit the use of antibiotics to 50% within 2030 in farmed and aquaculture animals. For example, it could be useful to consider a prolonged wash-out period, after a therapeutic treatment, in order to reach the complete elimination of antibiotics residues in the animal body. Undoubtedly the habitual use of antibiotics must be avoided and it is necessary to find suitable alternatives to conventional antimicrobial treatments, when applicable. Thanks to the advancements of biotechnology and genetic engineering it is possible to exploit new strategies both as prevention (e.g., probiotics) and as therapy (e.g., antimicrobial peptides). Also, natural sources can be a valuable tool in the search of new antimicrobial agents as well.

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