High diversity of genes encoding tetracycline resistance in the microbiota of broiler chickens in Tunisia

Running title

Tet genes detection in broiler chickens in Tunisia

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Abstract

Tetracycline resistance is still considered one of the most abundant antibiotic resistances among pathogenic and commensal microorganisms. The aim of this study was to evaluate the prevalence of *tet* genes encoding tetracycline resistance in broiler chickens in Tunisia, by PCR. Individual cloacal swabs from 195 broiler chickens were collected at two slaughterhouses in the governorate of Ben Arous (Grand Tunis, Tunisia). Chickens were from 7 farms and belonged to 13 lots consisting of 15 animals randomly selected. Individual whole genomic DNA was extracted and tested for 14 *tet* genes. All the lots examined were positive for at least 9 *tet* genes, with an average number of 11 *tet* genes per lot. Of the 195 animals tested, 194 (99%) were positive for one or more *tet* genes. *Tet*(L), *tet*(M) and *tet*(O) genes were found in 98% of the samples, followed by *tet*(A) in 90.2%, *tet*(K) in 88.7% and *tet*(Q) in 80%. These results confirm the antimicrobial resistance impact in the Tunisian's poultry sector and suggest the urgent need to establish a robust national antimicrobial resistance monitoring plan.

KEYWORDS

Broiler chickens, microbiota, tetracycline resistance genes, PCR

1 | INTRODUCTION

Tetracyclines have been widely used in the disease prevention and therapy in food animals due to their broad spectrum of activity, relatively low cost, lack of side effects and availability. In addition to therapeutic purposes, tetracyclines have been often incorporated into livestock feed at subtherapeutic doses as growth promoters. Currently, among over 20 tetracycline molecules, tetracycline, chlortetracycline, oxytetracycline and doxycycline are the most common members used in veterinary medicine (Fritz and Zuo, 2007). In particular, tetracycline antibiotics are among the most commonly administered antibiotics in the commercial poultry sector worldwide (Chopra and Roberts, 2001). The extensive use of tetracyclines in clinical practice and livestock has subjected bacterial populations to selection pressure and increased the prevalence of tetracycline resistance, one of the most abundant antibiotic resistances among pathogenic and commensal microorganisms (Wang et al., 2017). Currently, it is well known that many antibiotics used in animal husbandery are poorly absorbed in the gut and consequently more than 75% of these molecules and their breakdown products are excreted into the environment. In this regard, it has been reported that about 25 % of oral dose of tetracycline is excreted in feces and 50 % to 60 % is excreted as an active metabolite in urine (Feinman and Matheson, 1978). From ecological point of view, these excreted metabolites that can persist in soil for long time may exert selective pressure for acquisition of resistance genes in indigenous bacteria or confer a selective advantage for indigenous bacteria carrying resistance genes. Taken together, livestock manure and waste represent a high risk of environmental spread of intestinal resistant bacteria as well as antibiotics that select resistant one in soil or in the aquatic environment where they are usually discharged.

Tetracycline resistance is generally caused by the acquisition of tetracycline resistance (*tet*) genes, often associated with either a mobile plasmid or a transposon. To date, at least 58 and eleven mosaic *tet* genes have been described (Nguuen et al., 2014; Roberts, 2020a). Three main resistance mechanisms are mediated by *tet* genes: pumping the drug out of the cell before it reaches its site of

action (active efflux pumps), protection of the ribosomal binding site which decreases drug binding, and enzymatic inactivation of the active compound. The first two mechanisms currently predominate in clinical settings (Roberts, 2005). In addition, it is worthy to note that tetracycline resistance determinants are mainly genetically associated with other genes encoding resistance to various antibiotics localized on plasmids, integrons or transposons (Le-Vo et al., 2019; Irrgang et al., 2020), leading to a multidrug resistant phenotype. Thus, assessment of genetic background of tetracycline resistance might not only indicate the *tet* genes pool in animal microbiota but also predict the occurrence of other antibiotic resistance phenotypes. In this regard, the aim of this study was to evaluate the presence of 14 tetracycline resistance genes in DNA samples from cloacal swabs of 195 broiler chickens sampled at two slaughterhouses in Tunisia.

2 | MATERIALS AND METHODS

2.1 | Samples

From February to March 2019, individual cloacal swabs from 195 broiler chickens were collected at two slaughterhouses in the governorate of Ben Arous (Grand Tunis, Tunisia). Chickens belonged to 13 lots from 7 farms (A-G), located in 5 governorates (Ben Arous, Bizerte, Béja, Zaghouan and Nabeul). Four farms were sampled repeatedly over time, while three only one (Table 1). Each lot consisted of 15 animals randomly selected. All the farms were industrial, except for one rural chicken farm (Farm E/Lot 7).

2.2 | Nucleic acid extraction

DNA was extracted from each cloacal swab using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) following the supplier's recommendations. One extraction control was also included consisting of kit reagents only.

2.3 | Detection of tetracycline resistance genes in broilers

DNA samples were investigated by PCRs to search 14 genes involved in the three tetracycline resistance mechanisms: the tetracycline efflux pumps [*tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(G), *tet*(K), *tet*(L), *tetA*(P)], the ribosomal protection [*tet*(M), *tet*(O), *tet*(Q), *tet*(S)], and the enzymatic inactivation [*tet*(X)], using specific primers as described by Ng et al. (2001). The DNA extracted from *Escherichia coli* field strains, containing tetracycline resistance plasmids, was used as a positive control. The extraction control and a distilled water negative control were also included.

2.4 | Sequencing

PCR amplicons were purified using a QIAquick PCR purification kit (Qiagen), and both DNA strands were sequenced (Bio-Fab Research, Rome, Italy). The sequences obtained were compared with the public sequences available using the BLAST server in GenBank (National Center for Biotechnology Information 2019).

3 | RESULTS

The results were reported in the Table 1. All the lots examined were positive for at least nine *tet* genes, with an average number of 11 *tet* genes per lot. The *tet*(A), *tet*(B), *tet*(K), *tet*(L), *tet*(M), *tet*(O), *tet*(Q), *tet*(S) and *tet*(X) genes were found in 100% of the lots. Of the 195 animals tested, 194 (99%) were positive for one or more *tet* genes. With respect to the *tet* gene frequencies, *tet*(L), *tet*(M) and *tet*(O) genes were found (each) in approximately 98% of the samples, followed by *tet*(A) *tet*(K), and *tet*(Q) genes which were found in 90.2%, 88.7%, and 80% of samples, respectively. *Tet*(C) (27.7%), *tet*(D) (18.4%), *tet*(P) (7.7%), *tet*(E) (2.5%), and *tet*(G) (0.5%) genes were detected at low frequencies. For each *tet* gene amplified, with the exception of the *tet*(E) gene, the identity of the amplicons was confirmed by the comparison between the sequence obtained and the corresponding sequences from antibiotic resistant Gram-positive or Gram-negative bacteria in the GenBank database, showing 99-100% nucleotide similarity. Sequencing failed for *tet*(E) amplicons,

probably due to the low signal. One sequence for each of the 13 *tet* genes successfully sequenced was deposited in the Gen-Bank database under accession numbers MW079481-MW079493.

4 | DISCUSSION

Public health implications of the antimicrobial resistance (AMR) are significant since the decreased effectiveness of antibiotics in treating common infections leads to an increase in the cost of health care in terms of days of hospitalization and requires intensive care. Multiple jurisdictions, especially in Europe, have adopted mandatory restrictions on antimicrobial use. The use of antibiotics as growth promoters in animal nutrition was banned in the EU by 1 January 2006 (European Commission 2005) and earlier in Scandinavian countries. Otherwise, in many extra-European countries the antimicrobial use in human and veterinary medicine is still unrestricted.

AMR is a current public health problem in Tunisia. In the last 15 years the country highlighted a strong increase of the antibiotic resistant bacteria, from human as well as from animal or foods of animal origin, in strict relation to overconsumption or incorrect use of antimicrobials (Abbassi et al., 2016). The Tunisian National Institute of Consumption reported that the use of antibiotics in Tunisia increased by 38% during the period between 2005 and 2013 (Mansour 2018). A recent study on the trend of antibiotic consumption from 2000 and 2015 in 76 countries (Klein et al., 2018), placed Tunisia in second place among the most consuming countries in 2015.

In the veterinary field, the AMR impact in the Tunisian's poultry sector is very strong, with highest resistance rates than those observed in the Tunisian's bovine and ovine husbandries (Abbassi et al., 2017). AMR occurring in poultry sector can spread to humans via food or water chain, environmental contamination by poultry waste or direct contacts with animals or biological substances. Both the transmission of zoonotic antibiotic resistant bacteria and of mobile genetic elements carrying genes encoding antibiotic resistance represent a public health concern,

considering that antibiotics used in poultry farming may be the same, or belong to the same class, as those used in human medicine (Ljubojevic et al., 2016).

The results of the present study showed high rates of tetracycline resistance genes in the chicken lots examined, 100% positive for at least 9 *tet* genes, in accordance to previous investigations performed in Tunisia (Jouini et al., 2009; Soufi et al., 2009, 2012; Kilani et al., 2015; Badi et al., 2017; Gharbi et al., 2018). Interestingly, genes involved in all the three mechanisms of tetracycline resistance were detected. In addition, a high gene diversity for antibiotic resistance was highlighted, *tet*(L), *tet*(M) and *tet*(O) genes exhibiting the highest rates of occurrence among *tet* genes in the chickens sampled. With respect to the *tet*(L) gene, Roberts (2005) reported in the last years a very large increase in the number of genera carrying *tet*(L) gene, up to the current 47 among Gram-positive and Gram-negative genera (Roberts, 2020b,c). The prevalence of *tet*(M) gene was consistent with other reports showing a wide distribution of this gene, probably because of its association with conjugative chromosomal elements (Roberts, 2005). Conjugative transposons appear to have less host specificity than do plasmids, which may explain the detection of *tet*(M) in 79 different genera including 39 Gram-positive and 40 Gram-negative genera (Roberts, 2020b,c). The *tet*(O) gene has been detected in 19 Gram-positive and 20 Gram-negative genera (Roberts, 2020b,c). This gene has been found on plasmids (Avrain et al., 2004) as well as in association with functional conjugative transposons (Brenciani et al., 2004). Interestingly, our results showed a high (72.3%) frequency of *tet*(X) gene, responsible for the enzymatic inactivation of the tetracycline molecule, which has long been considered as a rare resistance mechanism. Until now, *tet*(X) has been found in only Gram-negative genera (Roberts, 2020b,c). Little research has been conducted on *tet*(X) because this gene is not considered clinically relevant. However, recent studies suggested that *tet*(X) could be useful in the screening of various environmental contexts (Kyselková et al., 2015; Roberts and Schwarz, 2016). Furthermore, it is noteworthy the increasing reports of new *tet*(X) variants (especially *tet*(X3), *tet*(X4), *tet*(X5) and *tet*(X6)) conferring high-level tigecycline resistance, which is considered as a last-resort antibiotic for the treatment of multidrug resistant

Gram-negative bacteria, especially carbapenemase-producing *Enterobacteriaceae*. These transferable *tet*(X) variants are mainly vehiculed by different plasmids and could further spread and disseminate through associated mobile plasmids, raising public concern (Sun et al., 2019). Finally, the result obtained for *tet*(G), detected in only one sample, was not surprising, according to the low prevalence reported in literature (Pons et al., 2018).

The *tet* gene frequencies observed in the backyard chickens (Lot 7/Farm E) was comparable to that highlighted in the industrial poultry of the other lots, although the overuse of antibiotics is more common in industrial production. On the other hand, AMR is a complex topic attributable to many factors other than the medical administration of antimicrobials: i) most antimicrobial agents are produced by strains of fungi and bacteria that occur naturally in all environments, including soil (Martin and Liras, 1989) ii) bacteria may also acquire resistance determinants through horizontally mobile elements including conjugative plasmids, integrons and transposons (Radhouani et al., 2014) iii) the agricultural use of antimicrobial agents selects for antibiotic resistance, antibiotics persisting in soil and aquatic environment (Allen et al., 2010).

To the best of our knowledge, this is the first time a high number of *tet* genes was investigated in food animals in Tunisia. Previous surveys have mainly focused on *tet*(A), *tet*(B) or *tet*(C) genes in *E. coli* isolates of animal origins, *tet*(A) and *tet*(B) resulting predominant (Jouini et al., 2009; Soufi et al., 2009, 2012; Kilani et al., 2015; Badi et al., 2017). However, in some cases *tet* genes were not detected in tetracycline resistant bacterial isolates (Soufi et al., 2012; Badi et al., 2017), probably due to the few *tet* genes investigated by those studies. Otherwise, Klibi et al. (2013) tested by PCR *tet(*K), *tet*(L), *tet*(M), *tet*(O) and *tet*(S) genes in enterococcal isolates from poultry and beef/sheep meat, obtaining a higher gene frequency for *tet(*M) and an almost total correspondence between antibiotic susceptibility testing and *tet* gene molecular detection. According with Klibi et al. (2013), our results suggest testing a wide range of *tet* genes or at least including *tet*(M) gene, to avoid false negatives.

In this study, a culture-independent analysis to detect AMR genes was used, according to other studies investigating the presence of resistance genes in biological samples by molecular analysis (Blanco Pena et al., 2017: Di Francesco et., 2020). A limitation of culture independent methods is the inability to determine which bacterial species the *tet* genes originate from. On the other hand, these methods avoid a possible underestimation of the AMR occurrence due to a consistent nonculturable fraction of microorganisms. Singer et al. (2007) suggested that, due the capability of bacteria to transfer resistance genes, analysis of AMR emergence, dissemination and persistence might be better conducted at the gene level. Considering AMR genes as contamination markers, methods which allow searching for these genes directly rather than for the bacteria carrying them could help in epidemiological studies on the spread of AMR (Vittecoq et al., 2016).

In conclusion, our findings confirm AMR as a serious threat for human health and food production in Tunisia. Since the fight against antibiotic resistance represents a long battle, only a government commitment involving many actors including health care agencies, agricultural agencies and public communities, can have a chance of success. Tunisia has planned a national action plan for the five-year period 2019-2023, aligned with the WHO global action and integrating human health, animal health and environment. In the veterinary field the main problems consist in the scarcity of veterinary data on the procurement of antimicrobials and monitoring of their use, antibiotic therapy frequently conducted without the bacteriological examination and without determination of the antibiotic resistance profile of the pathogen, cost of analyses, lack of veterinary laboratories, usual self-medication on farms, purchase of antimicrobial through parallel markets. National surveillance of the antibiotic resistance of animal origin, awareness of the good practices of antibiotic therapy in veterinarians, breeders, pet owners and strict control of antibiotic trade appear essential to ensure a successful action plan.

ETHICAL APPROVAL

Ethical statement is not applicable since sample collection was obtained from dead animals.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in the GenBank database at https://www.ncbi.nlm.nih.gov/nucleotide / under accession numbers MW079481-MW079493.

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