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Research Article

Culture-Independent Techniques Approach for Detection of Antimicrobial-Resistance Gene Patterns in Cow Raw Milk Samples

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Abstract

Raw milk may be a source of bacteria which are resistant to antimicrobials or possess genes encoding resistance to such antibiotics. The purpose of this study was to investigate the abundance and diversity of tetracycline resistance genes (*tet*) in 49 raw milk samples collected from 21 farms located in Piedmont and Liguria regions (Northwest Italy). Specific primer pairs for amplification of genes *tet*(B), *tet*(C), *tet*(D); (II) *tet*(A), *tet*(E), *tet*(G), *tet*(K), *tet*(L), *tet*(M), *tet*(O), *tet*(S), *tetA*(P), *tet*(Q) and *tet*(X) were used to screen tetracycline resistance determinants in the milk samples. Results showed that 69% (n = 34/49) of samples were positive for at least one *tet* gene type with many of them positive for *tet* G (24/34; 71%), A (21/34; 62%), *S* (10/34; 29%), M (6/34; 18%), O (5/34; 15%), L (4/34; 12%), B (3/34; 9%) and K (1/34; 3%). Of these samples, 79% (n = 27/34) presented different combinations of *tet* genes. A selection of samples positive (n = 20) for at least one *tet* gene was then subjected to Next-generation Sequencing analysis (NGS) using a shotgun metagenomics approach to detect the presence of resistance genes also to other antibiotic classes. This analysis showed the presence of additional genes that confer resistance to different classes of antimicrobials such as beta-lactams (28.44%), aminoglycosides (12.03%), as well as macrolides, sulfonamides, fluoroquinolones, phenicols, diamonopyrimidines, rifamycins, phosphomycin and nitroimidazoles (> 6.0%). This study indicates that raw milk can be a reservoir of transferable tetracycline resistance genes potentially harboured by different bacterial species transmitted through the food chain.

Keywords: *Tetracycline Resistance Genes; Raw Milk; Multiplex PCR; Next-Generation Sequencing*

Abbreviations

tet: Tetracycline Resistance Genes; mPCRs: Multiplex PCRs; NGS: Next-Generation Sequencing Analysis; AMR: Antimicrobial-Resistance; MRSA: Methicillin-Resistant *Staphylococcus aureus*; NRL-AR: National Reference Laboratory for Antimicrobial Resistance; MRLs: Maximum Residue Levels; ST: Sequence Type

Introduction

Extensive use and misuse of antibiotics in human medicine and conventional animal agriculture have led to the emergence of various antimicrobial-resistance (AMR) mechanisms and resistant bacterial species, which is thought to pose an ever-increasing threat to public

health [1]. In particular, the use and misuse of antibiotics to treat food-producing animals, as those of the dairy cow industry, can act a factor for the selection of resistant microorganisms in cows' milk, also potentially causing the exposure of consumers to antibiotic residues in milk and other dairy foods [2]. There is strong evidence of AMR genes transfer among different bacterial species, including foodborne pathogens potentially transmitted to humans through the consumption of milk [3]. The broad spectrum tetracyclines, active against both Gram-positive and Gram-negative bacteria, are commonly used for the prevention and control of bacterial infections in both veterinary and human medicine. Different AMR mechanisms can be associated to the acquisition of genes mediated by transposons or conjugative plasmids [4]. There are three main mechanisms by which tetracycline-resistance is conferred to bacteria: active efflux by tetracycline-specific pumps, ribosomal protection and enzymatic inactivation. In particular, different *tet* genes encoding I) efflux pump genes *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(G), *tet*(H), *tet*(J), *tet*(Y), *tet*(Z), *tet*(30), *tet*(K) and *tet*(L), II) ribosomal protection protein (RPP) *tet*(M), *tet*(O), *tet*B(P), *tet*(Q), *tet*(S), *tet*(W), *tet*(T), and *tet*(32), III) and one enzymatic modification gene (*tet*X), have been described [5,6]. Several techniques for the detection of AMR genes that require no culturing, as shotgun metagenomics approach based on NGS data, have recently been developed competing with conventional techniques in cost, speed and accuracy for AMR genes screening in complex biological matrices.

In this study, we apply culture-independent techniques based on "classical" multiplex PCRs (mPCRs) and NGS, to investigate the presence and distribution of *tet* genes in bovine raw milk also comparing the capabilities of both methodologies to screen and identify these AMR genes in complex matrices.

Materials and Methods

Totally, 49 bulk tank milk samples collected from farms located in Piedmont and Liguria regions (Northwest Italy), randomly selected, were screened. Initially, all milk samples were analysed by standard diffusion test Delvotest SP NT (DSM, Netherlands) for the detection of residues of antibacterial substances in milk. In particular, Delvotest SP NT detects the following molecules (the number in brackets refers to the limit of detection (LOD): penicillin G (2 µg/L), oxacillin (10 µg/L), ampicillin (2.5 µg/L), cloxacillin (20 µg/L), amoxicillin (3 µg/L), dicloxacillin (15 µg/L), cephalexin (50 µg/L), sulfamethazine (150 µg/L), sulfadiazine (100 µg/L), tetracycline (200 µg/L), oxytetracycline (200 µg/L), chlortetracycline (400 µg/L), erythromycin (200 µg/L), tylosin (25 µg/L), spiramycin (400 µg/L), neomycin (1000 µg/L), dihydrostreptomycin (3000 *µg/L), streptomycin (4000 µg/L), lincomycin streptomycin (150 µg/L) and trimethoprim (250 µg/L). Briefly, the screening test consists of plates of 96 wells each containing a solid sugar agar medium seeded with a* standardised number of spores of *Bacillus stearothermophilus* var. *calidolactis* together with required nutrients for growth purposes and an antifolate trimethoprim. The principle of the test is based on the diffusion into the agar of possible inhibitory substances that may be present in the milk sample. If present, these substances reduces growth and acid production by the test organism and delays or prevents the agar from changing colour from purple to yellow.

Afterwards, total DNA from the samples collected was extracted by using the commercial QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions except for the following modifications. 50 µl of each milk sample, were added to 180 µl of Buffer ATL and 20 µl of proteinase K. The samples were vortexed and incubated to 56°C for 3 hours. 100 µl ultrapure water (Sigma-Aldrich, St. Louis, MO) was added to the spin-column containing the sample and incubated at room temperature for 5 minutes before collecting the purified DNA. Concentrations of the obtained DNAs were determined using Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA) at 260 nm (A260) absorbances.

Specific primer pairs were employed for the PCR amplification of 14 tetracycline-resistant genes. In particular, combinations of primers were used in mPCRs to detect 4 specific groups of *tet* genes: (I) *tet*(B), *tet*(C), *tet*(D); (II) *tet*(A), *tet*(E), *tet*(G); (III) *tet*(K), *tet*(L), *tet*(M),

tet(O), *tet*(S); (IV) *tetA*(P), *tet*(Q), *tet*(X) [7] (Table 1). Genomic DNA extracted from field strains of *Escherichia coli Sequence Type* (ST)-1485, *Campylobacter jejuni*, and Methicillin-Resistant *Staphylococcus aureus* (MRSA) supplied by the National Reference Laboratory for Antimicrobial Resistance (NRL-AR), Istituto Zooprofilattico Sperimentale del Lazio e della Toscana "M. Aleandri", were used as positive controls for mPCRs. Multiplex PCRs to detect the presence of *tet* genes, were performed in 25 µl total volume containing 12,5 µl of HotStar-Taq Master Mix (Qiagen GmbH, Hilden, Germany), 10 pmol of each primer pair (Sigma-Aldrich, St. Louis, MO), and 3 µl of template DNA. The optimized PCR conditions consisted of an initial denaturation at 95°C for 3 minutes followed by 35 cycles of denaturation at 95°C for 30s, annealing at 55°C for 30s, extension at 72°C for 1 minute, and a final extension at 72°C for 10 minutes. The amplified products were resolved by electrophoresis on 2.5% agarose gel (Sigma-Aldrich, Poole, Dorset, United Kingdom) and visualized using an ultraviolet transilluminator (Gel-Doc, Bio-Rad, Richmond, CA). Molecular weight sizes were determined by comparison with a 100-base pair (bp) DNA ladder plus (Bio-Rad, Richmond, CA).

	Tetracycline resistance gene	Primer sequence (5'-3')	Annealing Temperature (°C)	Amplicon size (bp)
Multiplex PCRI				
	tet(B)	TTG GTT AGG GGC AAG TTT TG GTA ATG GGC CAA TAA CAC CG	55°C	659
	tet(C)	CTT GAG AGC CTT CAA CCC AG ATG GTC GTC ATC TAC CTG CC	55°C	418
	tet(D)	AAA CCA TTA CGG CAT TCT GC GAC CGG ATA CAC CAT CCA TC	55°C	787
Multiplex PCRII				
	tet(A)	GCT ACA TCC TGC TTG CCT TC CAT AGA TCG CCG TGA AGA GG	55°C	210
	tet(E)	AAA CCA CAT CCT CCA TAC GC AAA TAG GCC ACA ACC GTC AG	55°C	278
	tet(G)	GCT CGG TGG TAT CTC TGC TC AGC AAC AGA ATC GGG AAC AC	55°C	468
	tet(G)	CAG CTT TCG GAT TCT TAC GG GAT TGG TGA GGC TCG TTA GC	55°C	844
Multiplex PCRIII				
	tet(K)	TCG ATA GGA ACA GCA GTA CAG CAG ATC CTA CTC CTT	55°C	169
	tet(L)	TCG TTA GCG TGC TGT CAT TC GTA TCC CAC CAA TGT AGC CG	55°C	267
	tet(M)	GTG GAC AAA GGT ACA ACG AG CGG TAA AGT TCG TCA CAC AC	55°C	406
	tet(O)	AAC TTA GGC ATT CTG GCT CAC TCC CAC TGT TCC ATA TCG TCA	55°C	515
	tet(S)	CAT AGA CAA GCC GTT GAC C ATG TTT TTG GAA CGC CAG AG	55°C	667
Multiplex PCRIV				
	tetA(P)	CTT GGA TTG CGG AAG AAG AG ATA TGC CCA TTT AAC CAC GC	55°C	676
	tet(Q)	TTA TAC TTC CTC CGG CAT CG ATC GGT TCG AGA ATG TCC AC	55°C	904
	tet(X)	CAA TAA TTG GTG GTG GAC CC TTC TTA CCT TGG ACA TCC CG	55°C	468

Table 1: *Primers used in the multiplex PCRs for amplification of tetracycline resistant genes (tet).*

A selection of milk samples (n = 20) positive for at least one *tet* gene, was then processed for the NGS technique. In particular, the DNA quality was checked and quantified on a Qubit 2.0 fluorometer (Invitrogen, USA) according to the manufacturer's instructions. Sequence libraries were fragmented and tagged with sequencing adapters by using the Nextera XT library preparation kit (Illumina) according to the manufacturer's instructions. The libraries were quantified using a Qubit 2.0 fluorometer and the quality and the size distribution were determined by using High-sensitivity DNA chips and DNA reagents on a Bioanalyzer 2100 (Agilent, USA). Sequencing was performed in the MiSeq (Illumina) system for a 300 cycle paired-end run. Basecalling and Illumina barcode demultiplexing processes were performed by the MiSeq control software v2.3.0.3. Raw reads were evaluated using the AmrPlusPlus metagenomics pipeline [8] under a local instance of Galaxy [9] using the MEGARes database. The MEGARes database contains sequence data for approximately 8.000 hand-curated antimicrobial resistance genes accompanied by an annotation structure that is optimized for use with high throughput sequencing [8]. The workflow of AmrPlusPlus metagenomics pipeline includes Trimmomatic [10], for removal of low-quality bases and sequences, BWA [11] for mapping against host DNA and resistance genes, Samtools [12] for removal of host DNA, SNPFinder for detection of haplotypes, ResistomeAnalyzer for analysis of resistome.

Results and Discussion

In the EU, the maximum residue levels (MRLs) concerning the concentration of antibiotic residues that are acceptable in milk for sale, are monitored [13]. Our results indicated that 16% (8/49) of milk samples were positive for beta-lactams (N = 5) and sulphamides (N = 3) residues. The detection of residues of antibacterial substances observed in raw milk analyzed is low but probably enough to further select resistant strains and to induce resistance *de novo*. The presence of antibiotics in the environment provides a positive selection for resistant pathogens and commensal microbiota. In particular, the commensal microbiota in food could become a reservoir for AMR determinants that could then further be transmitted through the food chain [14]. Whereas that tetracycline resistance marker was almost always associated with genes located on mobile genetic elements [15], *tet* genes can spread rapidly in environments that contain moderate levels of tetracycline or other antimicrobials promoting co-selection mechanisms [16]. When setting multiplex PCRs, tetracycline was selected because resistance against this antibiotic has been extensively documented among food-borne bacteria. The concentrations of extracted DNA from raw milk samples ranged from 500 ng/µl to 1000 ng/µl. The presence of 14 tetracycline-resistant genes, commonly found in both Gram positive and Gram-negative zoonotic pathogens, such as MRSA and different *Salmonella* serovars, was investigated. By using mPCRs, DNA fragments of the predicted size were observed and no primers interfered with the amplification of other targets. Our results showed that different tetracycline-resistant genes commonly existed in cow raw milk, as most of the analyzed milk samples carried *tet* genes. A set of 8/14 tetracycline-resistant genes were observed in this study. In particular, multiplex PCRs show that 69% (34/49) of milk samples were positive for at least one *tet* gene, with *tet*(G) as the most common type (24/34; 71%). The second most common *tet* gene was *tet*(A) (21/34; 62%), followed by *tet*(S) (10/34; 29%), *tet*(M) (6/34; 18%), *tet(*O) (5/34; 15%), *tet*(L) (4/34; 12%), *tet*(B) (3/34; 9%) and *tet*(K) (1/34; 3%) types (Table 2). Furthermore, we found that the carriage of more than one tetracycline resistance gene was common and that 79% (27/34) of milk samples presented different combinations of resistance genes (Table 2). T*et*(C), *tet*(D), *tet*(E), *tet*A(P), *tet*(Q), and *tet*(X) genes were no detected. Most of the milk samples carried tetracycline resistance genes encoding various efflux pumps extruding tetracycline out of cells. The efflux genes are widely distributed in both Gram-positive and Gram-negative bacteria and are associated with plasmids, most of which are conjugative [17]. The common presence of *tet*(G) and *tet*(A) genes in this study is in agreement with the results previously reported [18,19] in *Salmonella* strains isolated from different animal and environmental samples. Efflux genes *tet*(K) and *tet*(L), that code for proteins which confer resistance to tetracycline and chlortetracycline, are found to be associated to small transmissible plasmids, that could be trasferred into the chromosome of staphylococci or the chromosome of *Bacillus subtilis* or into larger staphylococcal plasmids [20]. Milk samples analyzed in this study carried also tetracycline resistance genes *tet*(S) (29%), *tet*(M) (18%) and *tet*(O) (15%) encoding RPP. This mechanism of resistance is more common in Gram-positive bacteria, but it has also been described in Enterobacteriales, including *E. coli* [21]. In particular, t*et*(S) was first detected in *Listeria monocytogenes* BM4210, carried by self-transferable plasmids [22]. It has also been found in *Lactococcus lactis*, located on a conjugative plasmid and in *Enterococcus faecalis*,

where it has been shown to transfer from chromosome to chromosome by conjugative transposons [15]. Besides, some bacteria such as *Neisseria*, *Haemophilus* and *Streptococcus* spp. are naturally competent, which could help with the further dissemination of the tetracycline resistance genes [23]. Another most common resistance gene found in this study is *tet*(M), commonly contained within conjugative transposons, which have an extraordinarily broad host range [23]. In particular t*et*(S) and *tet*(M) genes were concomitantly detected in plasmids from lactic acid bacteria isolated from dairy products [24]. As for *tet*(O) gene, it has been frequently found in different Gram-positive species (*Lactobacillus*, *Enterococcus*, *Staphylococcus* and *Streptococcus* spp.) isolated from the oral and respiratory tract [25]. The shotgun metagenomic DNA sequencing approach based on NGS data allowed to highlight different resistance patterns characterized by the presence of additional AMR genes belonging to different antimicrobial classes other than tetracycline, as well as: beta-lactams, amphenicols and aminoglycosides. The analysis of raw milk samples data shows that AMR genes, from 2 to 14 different classes, were found in each samples analyzed with this NGS based approach. The most represented classes were beta-lactams (28.44%), aminoglycosides (12.03%) and tetracyclines (8.04%), moreover we have also identified genes encoding resistance to macrolides, sulfonamides, fluoroquinolones, phenols, diamon-pyrimidines, rifamycins, phosphomycin and nitroimidazoles (> 6.0%) (Figure 1). The comparison between the two methods regarding the detection of tetracyclines resistance genes, highlight that the results obtained by PCR were completely concordant to those obtained by NGS. These data confirm that the target-independent approach of NGS is complementary to the target-specific approach of PCR. Focusing on the NGS data analysis, the use of the AmrPlusPlus pipeline has made possible to analyze directly raw sequence data and therefore with less information loss. Furthermore, the use of a curated and annotated database as MEGARes, has allowed us to conduct an analysis that is not limited to the presence/absence of single genes but also related to the classes of antibiotics. The findings of our study indicate that raw milk can be a reservoir of *tet* resistance genes with potential for spreading through the food chain. Although most pathogens and their associated genes are destroyed by pasteurisation, consumption of raw milk and raw milk dairy products may represent a potential risk factor. The interest in drinking raw unpasteurised milk or consuming products made from such raw milk is increasing in the European Union [26] and in the United States, even though this practice poses a realistic microbiological hazard for the consumers' health or life. Moreover, in Italy there is a broad variety of traditional cheeses made from raw milk that may be contribute to the dissemination of resistance genes.

No. of tetracycline resistance genes	No. (%) of milk samples	Tetracycline resistance profiles								No. of milk samples
		tet(A)	*tet*(B)	*tet*(G)	*tet*(K)	*tet*(L)	*tet*(M)	*tet*(O)	*tet*(S)	
1	7 (21%)			X						3
							X			2
									X	2
2	19 (56%)					X		X		2
					X		X			1
								X	X	1
		X		X						15
3	3 (9%)					X		X	X	2
		X	X	X						1
4	5 (15%)	X	X	X					X	2
		X		X			X		X	3
Total	34	21 (62%)	3 (9%)	24 (71%)	1 (3%)	4 (12%)	6 (18%)	5 (15%)	10 (29%)	34

Table 2: *Distribution of tetracycline resistance genes (tet) in milk samples analyzes.*

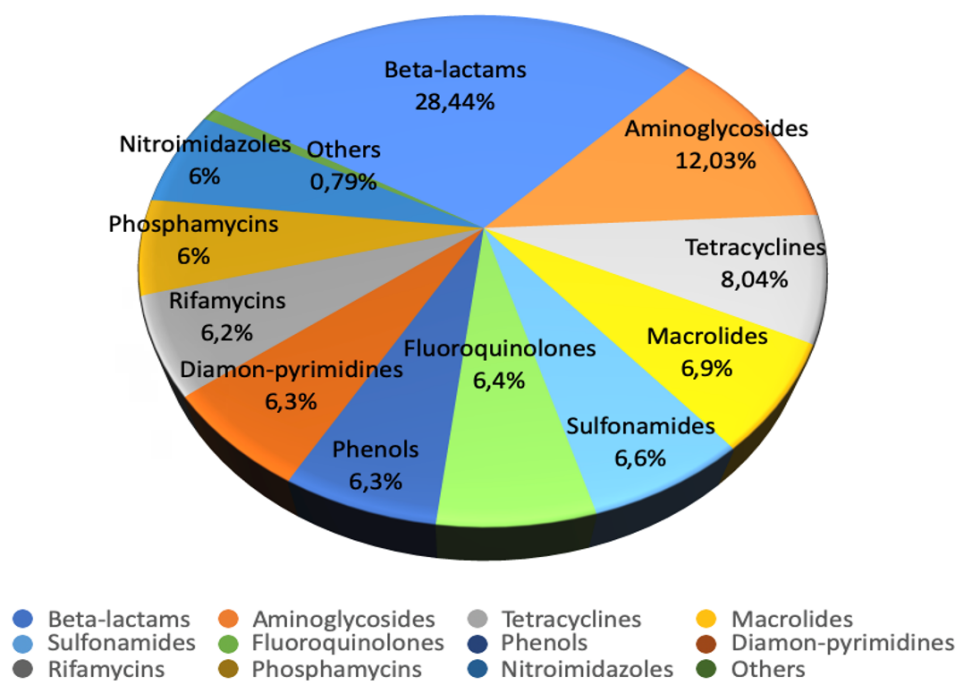

Figure 1: *Percentage of AMR genes for classes of antibiotics found in 20 tet positive samples analyzed by NGS using AmrPlusPlus with MEGARes database.*

Conclusion

The set of multiplex PCRs and the shotgun metagenomics approach based on NGS data used in this study to determine the presence of *tet* genes in milk, proved to be rapid and reliable screening methods. Furthermore, the results obtained highlight that the use of culture-independent methods is useful to directly map resistance genes from milk samples and to provide precise information about consumer risk level.

Conflict of Interest

Declare if any financial interest or any conflict of interest exists.

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