Journal of Microbiology, Biotechnology and Food Sciences

Skočková et al. 2012 : 1 (February Special issue) 777-784

REGULAR ARTICLE

DETECTION OF TETRACYCLINE RESISTANCE GENES IN *ESCHERICHIA COLI* FROM RAW COW'S MILK

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ABSTRACT

The occurrence of tetracycline resistance determinants in *Escherichia coli* recovered from raw cow's milk from the same farm sampled in two different time periods five years apart was investigated in this study. Detection of *E. coli* was performed and evaluated according to ISO 16649-2 and antibiotic resistance was screened by disk diffusion method. The polymerase chain reaction (PCR) was used to detect selected genes encoding resistance to tetracycline – *tet*A, *tet*B, *tet*C, *tet*D, and *tet*G. Of 89 samples of raw cow's milk, 84 (94.4%) were positive for *E. coli*. In total, 102 isolates were obtained. Fifty (49.0%) of these isolates were resistant to tetracycline. The most common gene detected in tetracycline-resistant isolates from 2010-2011was *tet*A (81.3 %), while *tet*B was most often (86.5%) found in isolates from 2005-2006. Neither the *tet*C, *tet*D, and *tet*G genes nor the co-occurrence of *tet*A and *tet*B genes were detected. None of the monitored genes was detected in two tetracycline-resistant isolates. Results of our study showed a significant shift in the distribution pattern of *tet*A and *tet*B genes in *E. coli* isolates from raw cow's milk five years apart.

Keywords: Resistance, tetracycline, tet-gene, *Escherichia coli*

INTRODUCTION

In the microbiological analysis, *Escherichia coli* is used to assess the hygienic quality of food and food ingredients. In addition, the level of antibiotic resistance in *E. coli* is considered to be a good indicator of the selection pressure exerted by antibiotic use (**Lei et al., 2010**). Raw cow's milk can be contaminated by *E. coli* either directly through animal feces or indirectly during milk collection through farm employees or the milking equipment (**Desmarchelier and Fegan, 2003**). The occurrence of bacteria resistant to antimicrobial agents has been increasingly reported in recent years. Growing prevalence of antibiotic resistant bacteria is a cause of concern for human and animal health. The responsibility for the prevalence of resistant bacteria is most often attributed to the overuse and misuse of antibiotics (**Scaria et al., 2010**). Tetracycline belongs to a family of broad-spectrum antibiotics. Its efficacy, low cost, and the lack of side effects make it the most popularly used antibiotic in livestock farming, including aquaculture. Its widespread and imprudent use caused a high prevalence of tetracycline-resistant bacteria nowadays. The main mechanisms conferring resistance to tetracycline to bacteria are an active efflux system, ribosomal protection and enzyme inactivation (**Koo and Woo, 2011**). The most common in gram-negative bacteria is the energy-dependent efflux pump system which is encoded by the genes *tet*A, *tet*B, *tet*C, *tet*D, and *tet*G.

The aims of this study were to monitor the presence of tetracycline-resistance genes in *E. coli* isolates from raw cow's milk and to detect shifts, if any, in the distribution of the *tet* genes over the five-year period.

MATERIAL AND METHODS

Sample collection

A total of 89 raw cow milk samples were collected in May 2005 - July 2006 (n = 55) and in May 2010 - February 2011 (n = 34). All samples were collected at regular intervals from the same dairy farm in the South Moravian Region. In 2005 – 2006, pooled samples of raw cow's milk were collected directly on the farm. In 2010 and 2011, samples were obtained from vending machines. All samples were collected into sterile sampling bottles and transported in an insulated sampling case to the laboratory for examination.

Isolation and identification of *E. coli*

The enumeration of *E. coli* was carried out according to ISO 16649-2. The detection of *E. coli* was performed after multiplication in buffered peptone water (BPW, Oxoid, UK) at 37°C for 24 hours followed by aerobic culture on TBX agar at 44° C for 24 hours. From each positive sample, one to two suspected *E. coli* isolates were involved in the study for confirmation and determination of resistance to antimicrobials. The confirmation of suspected isolates consisted of the detection of oxidase (OXItest, Pliva-Lachema, CZ) and production of indole (COLItest, Pliva-Lachema, CZ).

Antimicrobial susceptibility testing

Resistance to tetracycline was tested by the disk diffusion method according to the **CLSI protocol (2006a)**. Antibiotic disks were obtained from the Oxoid Company (UK). *E. coli* isolates were evaluated based on the size of the zones of inhibition and classified as susceptible (S), intermediate resistant (I) or resistant (R) according to the **CLSI criteria (2006b)**.

Detection of tetracycline resistance genes

In *E. coli* isolates found to be resistant to tetracycline, polymerase chain reaction (PCR) was used to detect selected genes encoding this resistance – *tet*A, *tet*B, *tet*C, *tet*D, and *tet*G. Bacterial DNA was isolated from a 24-hour culture on blood agar (BioRad, France), isolation was performed by lysis of bacterial cell suspension at 95.5°C for 10 minutes with the addition of 20% Chelex 100 (BioRad, France) followed by centrifugation. The supernatant was used as template DNA. For the detection of the *tet* gene, primers reported by **Ng et al. (1999)** in 25 ml of reaction mixture containing Taq-Purple DNA polymerase and MgCl2 (Top-Bio, CZ) were used. PCR products were analyzed by gel electrophoresis in 1.5% agarose (Serva, Germany) followed by visualization on a transilluminator after staining in ethidium bromide.

RESULTS AND DISCUSSION

E. coli was detected in 84 (94.4%) out of 89 raw milk samples collected in 2005-2006 and 2010-2011. Sixty-eight *E. coli* isolates were recovered in 2005-2006 and 34 *E. coli* isolates in 2010-2011. The results are summarized in Table 1.

Table 1 *Escherichia coli* detected in raw cow's milk

Time period	Samples analyzed	*E. coli* -positive samples		Counts of *E. coli*	*E. coli* Isolates	Tetracycline-resistant isolates	
	No.	No.	%	CFU/ml	No.	No.	%
2005-2006	55	50	90.9	<5 to >$7.5.10^3$	68	37	54.4
2010-2011	34	34	100	< 5 to $3.1.10^2$	34	16	47.1

The majority of *E. coli* isolates (54.4%) recovered in 2005-2006 were resistant to tetracycline and so were 47.1% of *E. coli* isolates from 2010-2011. These data suggest that the rates of tetracycline-resistant isolates differ between the two periods, showing a 7.4% downward trend. This drop is probably due to switching to β-lactams that are currently used as first-line antibiotics on the farm.

Among 37 tetracycline-resistant isolates from 2005-2006, *tet*B was the most common gene to be detected (86.5%) while *tet*A was revealed in 3 isolates (8.1%). None of the genes monitored was detected in two isolates. Out of 16 tetracycline-resistant isolates from 2010-2011, 13 (81.3%) were positive for the *tet*A gene and 3 (18.7%) for the *tet*B gene. Any other of the genes monitored or the co-occurrence of the *tet*A and *tet*B genes was not detected. The detection of tetracycline resistance genes in *Escherichia coli* isolates from raw cow's milk is summarized in Table 2.

Table 2 Detection of tetracycline resistance genes in *Escherichia coli* from raw cow's milk

Time period	Tetracycline resistance genes									
	*tet*A		*tet*B		*tet*C		*tet*D		*tet*G	
	No.	%	No.	%	No.	%	No.	%	No.	%
2005-2006	3	8.1	32	86.5	0	0	0	0	0	0
2010-2011	13	81.3	3	18.8	0	0	0	0	0	0

The obtained data show that over the last five years, there has been a shift in the distribution pattern of the genes responsible for resistance to tetracycline. While in 2005-2006, the most often detected gene was *tet*B (86.5%), *tet*A has become dominant on the farm (81.3%) five years later. Both of these genes encode an energy-dependent efflux pump system, one of the most frequently used mechanisms of tetracycline resistance in bacteria of the family *Enterobacteriaceae* (**Lewis et al., 2002, Tao et al. 2010**). The shift in the distribution pattern of the *tet*A and *tet*B genes is shown in Figure 1.

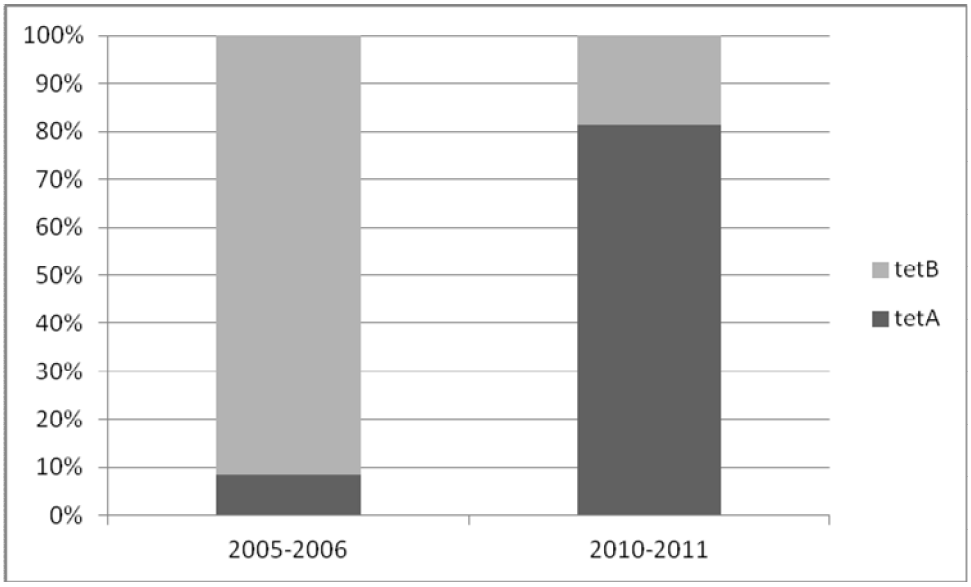

Figure 1 Distribution patterns of the *tet*A and *tet*B genes in *Escherichia coli* from raw cow's milk five years apart

Both *tet*A and *tet*B have been reported by many authors as the most common genes responsible for resistance to tetracycline. The genes detected in *E. coli* isolates from 2005-2006 in this study showed a similar distribution pattern as reported by **Sawant et al. (2007)** who found *tet*B and *tet*A to account for 93% and 7% of the tetracycline resistance genes in *E. coli* isolates from cattle feces. Bryan et al. (2004) have also pointed out the predominance of the *tet*B (63.0%) and *tet*A (35.0%) genes in animal *E. coli* isolates. On the other hand, data from recent studies are in line with our results from 2010-2011. The Korean scientists **Koo and Woo (2011)** have identified as the most frequent gene in tetracycline-resistant *E. coli* isolates from meat and meat products *tet*A (52.4%) followed by *tet*B (41.3%). Other genes occurred in their isolates only rarely (*tet*C in 1.7%) or, similarly to our study, were not detected at all. They have also found *tet*A to be more common among animal *E. coli* isolates while *tet*B is more often seen among clinical isolates of human origin.

Koo and Woo (2011) have also focused on the transmission of the *tet* genes. They have observed that 98.3% of meat-borne *E. coli* containing at least one of the *tet*A to *tet*D genes are able to transfer tetracycline resistance to a tetracycline-susceptible recipient strain of *E. coli*. Interestingly, two isolates carried both *tet*A and *tet*B, but only *tet*A was transferred to the recipient strain. It can be assumed that the *tet*A gene can be spread more easily in the environment than *tet*B. It is consistent with our results from 2010-2011 when the majority of tetracycline-resistant isolates carried the *tet*A gene. **Tuckman et al. (2007)** and **Koo and Woo (2011)** have also noticed that the presence of *tet*B in animal isolates is associated with higher minimum inhibitory concentrations (MIC) for tetracycline. Many authors have previously confirmed that the overuse and misuse of antibiotics in veterinary medicine plays an important role in the development of bacterial resistance, so we can assume that in an environment under higher selection pressure of tetracycline antibiotics, the *tet*B gene will predominate. The study of the isolates from 2005-2006 showed an 86.49% prevalence of *tet*B which indicates more frequent use of tetracycline drugs on the farm in that period. This conclusion is supported by the fact that the EU banned the use of growth regulators and other additives of this type, often based on tetracycline, as of January 1, 2006.

Another mechanism of resistance to tetracycline is ribosomal protection encoded by the genes *tet*M, *tet*O, and *tet*Q and *tet*S. This mechanism of resistance is more common in gram-positive bacteria, but it has also been described in *E. coli* (**Aminov et al., 2001**). It can be assumed that in two isolates negative for any of the *tet* genes, tetracycline resistance might be encoded by other genes than those monitored in this study.

CONCLUSION

Our study showed that the most frequently detected genes in the *E. coli* isolates from raw cow's milk on the monitored farm in the South Moravian Region are *tet*A and *tet*B. A significant shift in the distribution pattern of the *tet* genes was observed over a five-year period. Based on the data obtained, we can assume that the *tet*A gene might easily spread in an environment under low selective pressure of tetracycline antibiotics while the *tet*B gene could serve as an indicator of high selection pressure of tetracycline antibiotics.

Acknowledgments: This study was financially supported by research project MSM6215712402.

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