Presence of drug resistance in intestinal lactobacilli of dairy and human origin in Turkey

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Received 7 January 2004; received in revised form 23 April 2004; accepted 10 May 2004

First published online 19 May 2004

Abstract

The prevalence of different resistance genes was investigated in lactobacilli of human and dairy origin by PCR. The presence of \textit{erm}, \textit{van}, \textit{tet}, and \textit{cat-TC} genes were determined in 16 raw milk, 15 cream, 10 yogurt, 50 hand-made cheese, and 20 industrially produced white-cheese samples of dairy origin and 16 mouth, 32 fecal, and 36 vaginal samples from different subjects of human origin. Lactobacilli of dairy and human origin were found to carry only \textit{erm}(B) and \textit{tet}(M) genes. The majority of the isolates, \textit{Lactobacillus crispatus} (61), \textit{Lactobacillus gasseri} (49), \textit{Lactobacillus plantarum} (80) studied were found to harbor either \textit{erm}(B) or \textit{tet}(M) gene or both. No resistant lactobacilli was found in raw-milk and cream samples. All the human fecal samples and the majority of vaginal (29 of 36) and mouth (10 of 14) samples were found to carry the resistance genes. While a third of the hand-made cheeses carried resistant lactobacilli only one industrially produced cheese was found to carry resistant lactobacilli. Furthermore, the genes were found in the non-starter species, \textit{Lactobacillus acidophilus} and \textit{Lb. plantarum}, indicating that industrially produced cheeses in this respect could be considered more favorable. These results indicate that drug resistance seems to be very common in Turkey. Even though the number of dairy samples harboring the resistance genes (17 of 111) is smaller in regards to human samples, 10% of them were still found to carry the resistance genes as well. The presence of the resistance genes in majority of the samples of human origin and in minority of the samples of dairy origin indicates that drug resistance may be acquired in the intestinal tract during passage and spread to dairy products by the hands of workers during production.

Keywords: \textit{erm}, \textit{tet}, \textit{van}, and \textit{cat-TC} gene resistance; Lactobacilli; Human; Dairy products

1. Introduction

Antibiotic resistance is an ever-increasing worldwide problem. It is actually the extensive use of antibiotics that creates the selective pressure resulting from mutation of normal cellular genes, acquisition of foreign resistance genes, or a combination of these two mechanisms and leading to the acquisition and spread of a variety of antimicrobial resistance determinants [1–3]. Intrinsic mechanisms of resistance to some antimicrobials or classes of antimicrobials, notably rifampicin or the fluoroquinolones, occurs primarily through point mutations and is not transferable [4,5]. Resistance genes are however often imported. In this case, bacteria use a complex array of mechanisms to share and spread resistance determinants. Mechanisms important for the evolution and dissemination of antibacterial resistance determinants include: conjugation, transformation, and transduction. The main mechanisms of horizontal transfer in bacteria in natural environments are conjugation and most probably bacteriophages [6]. Plasmids are another conjugative genetics elements with regard to antibiotic resistance transfer [6–9].

Lactobacilli are important microorganisms as being an important element of the commensals of the human and animal body (intestine, nasopharyngeal, and vaginal mucosa), and in environments (mainly plants) where
spontaneous fermentations of carbohydrate containing substrates occur. *Lactobacillus* species often harbor many plasmids of different sizes. The data on drug resistance of the industrially important lactobacilli are rare. There are however reports showing the successful transfer of plasmids to lactobacilli [10–12]. Recently, the acquisition and incorporation of the tet(M) gene into the plasmid, pMDS057, of *Lactobacillus plantarum* was reported (Danielsen M., 2002). *Lb. plantarum, Lactobacillus casei, Lactobacillus salivarius* carry intrinsic resistance towards vancomycin [13]. Vancomycin resistance is also reported to be spread by a transposon, Tn 1546, carrying the van(A) gene cluster [14,15]. The most frequently found macrolide resistance genes in bacterial isolates from animals and humans are the *erm* genes [16–18].

Dairy products are an important part of the food consumed daily in Turkey. *Lactobacillus delbrueckii* subsp. *bulgaricus* is one of the components of famous Turkish White-Cheese of industrial importance (similar to Bulgarian Feta Cheese but harder). It is a well-preserved starter culture strain by the industrial producers yet there is no investigation on dairy products whether the starter culture strains harbor resistance gene(s) or not. Additionally, this species is usually found in Turkish Yogurt as well. However, it is not used in the production of hand-made cheese (a kind of hard white-cheese). Therefore, an investigation on the drug resistance profiles of lactobacilli common to both humans and dairy samples seem to be necessary in order to understand the size of dissemination of resistance and resistant bacteria in both human and animals. In this respect, the presence of the tet(M), cat-TC, *erm*(ABC), and van(A) genes were investigated to answer the question of the prevalence of resistance in lactobacilli of both dairy and human intestinal origin.

### 2. Materials and methods

#### 2.1. Primer design

*tet, erm, and van* gene classes deposited in GenBank (Table 1) were utilized to design and use specific primers [19–25]. Chloramphenicol acetyltransferase (cat-TC) gene providing resistance to chloramphenicol of *Lactobacillus reuteri* G4 was taken as a basis in designing the primer for cat-TC gene [26] by comparing with other chloramphenicol acetyltransferase (CAT) genes [27–29].

A set of two primers was designed by our group from the 1746-bp sequence from *Lb. reuteri* G4 encoding chloramphenicol acetyltransferase (cat-TC). The designed primers were 18-bp and located between 657 and 1375 nts.

#### 2.2. Bacterial cultures and identification of isolates

A total of 195 samples were studied for the presence of the tet(M), cat-TC, *erm*(ABC), and van(A) gene classes. Among these 84 were of human origin and 111 were from dairy products (Table 2). All 84 human samples were collected in 2002–2003 in Turkey from non-hospitalized patients. Human samples were collected from patients of urban origin. The all had a history of antibiotic treatment for BV at least once in their previous life, but they were not under treatment for at least 6 months. Samples of human (mouth, fecal, and vaginal) and dairy (raw milk, cream, yogurt, and cheese) origin were directly inoculated in MRS broth as soon as they were received and incubated in an anaerobic chamber at 37 °C for 16–18 h. The isolates were identified to species level by picking single colonies from each agar plate, extraction of total DNA from each single colony, and application of PCR with species-specific primers (Jensen, 1999) (data not included) [20].

### Table 1

<table>
<thead>
<tr>
<th>Primer pair</th>
<th>Sequence (5′ to 3′)</th>
<th>5′ position*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>VANA</td>
<td>+ATGAATAAGATAAAAAGTGTGAATAC</td>
<td>1–25</td>
<td>[24]</td>
</tr>
<tr>
<td>VANA1</td>
<td>−CCCCCTTAACCGCTATACGAT</td>
<td>1009–1009</td>
<td></td>
</tr>
<tr>
<td>VanB</td>
<td>+ACCCGTCTTTTTGTAACCCGGGAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>−CCCCCCAAGATCAACACGCGAAGGCCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cat-TC</td>
<td>+5′-CAT ATC AAA TGA ACT TTA ATA-3′</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>−5′-CGT TTT GTG AAG TAG TAC ACT-3′</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>TetM-F</td>
<td>+5′-GAYACN CCN GGN CAY RTN GAY TT-3′</td>
<td></td>
<td>[25]</td>
</tr>
<tr>
<td>TetM-R</td>
<td>−5′-CAC CGA GCA GGG ATT TCT CCAC-3′</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tn554-2</td>
<td>+TCAAAGCCTGTCGGAATTTG</td>
<td>4634–4653</td>
<td>[20,37]</td>
</tr>
<tr>
<td>Tn544-1</td>
<td>−AAGCGGTAAACCCCTCTGAG</td>
<td>5074–5055</td>
<td></td>
</tr>
<tr>
<td>ErmB-1</td>
<td>+CATTAAACGAGCGAACTGGC</td>
<td>836–855</td>
<td>[20,37]</td>
</tr>
<tr>
<td>ErmB-2</td>
<td>−GGAACATCTGTTGATGGCGG</td>
<td>1260–1241</td>
<td></td>
</tr>
<tr>
<td>ErmC-1</td>
<td>+ATCTTTGGAATCGGTCAGG</td>
<td>2639–2620</td>
<td>[20,37]</td>
</tr>
<tr>
<td>ErmC-2</td>
<td>−CAAACCGTATTTCCAGATT</td>
<td>2354–2364</td>
<td></td>
</tr>
</tbody>
</table>

*5′ position in *E. faecalis vanA*; +, sense primer; −, antisense primer; N = A,C,G, and T; R = A and G; W = and T; Y = C and T.
2.3. DNA preparation and PCR detection of tet, erm, cat, and van genes

The presence of the tet(M), cat-TC, erm(ABC), and van(A) gene classes were investigated by PCR with specific primers towards these genes conferring drug resistance to verify the results obtained from each isolates. Total genomic DNA from each plates in which single colonies were grown, was extracted and purified by the method of Cataloluk [30]. Typing of Lactobacillus species was performed by species-specific PCR primers targeted to the 16S–23S rRNA regions of each species. The sequences of the primers, their related positions on selected to the 16S–23S rRNA regions of each species. The sequences of the primers, their related positions on selected genes, and their sources are listed in Table 2. The $T_m$ values for the individual primers were taken from references and three of the $T_m$ values were modified.

The PCR assay mix (total volume, 50 μl) contained 20 pmol of each primer (MWG Biotech, Germany) (Table 2), 10× PCR buffer (Invertas, Spain), each deoxynucleoside triphosphate at a concentration of 200 μM and 1 U Taq DNA polymerase (Invertas, Spain). A 50-ng portion of purified total DNA was used as a template. tet, erm, cat, and van genes were detected with primers specific for these genes.

All PCR amplifications were performed in an Eppendorf Master Cycler PCR system with the following temperature program: initial denaturation at 94 °C for 5 min; 30 cycles of 94 °C for 1 min, annealing temperature (Table 1) for 1 min, and 72 °C for 2 min; and a final extension step at 72 °C for 7 min.

PCR products (5 μl) were separated by electrophoresis on a 1% agarose gel and visualized by ethidium bromide staining.

3. Results

3.1. Classification of tet, erm, cat, and van genes

The results of PCR amplifications for selected gene classes in tested isolates were given in Table 2. Using PCR, the presence of erm(B) and/or tet(M) genes was found in 91 samples. Positive samples were mainly of human origin (254 of 489 isolates). erm(A), erm(C), van(A), and cat-TC genes were not found in any isolates. Of 195 samples, 91 were found to carry either erm(B) or tet(M) genes or both. Among the positives 31 were carrying erm(B) gene only and 17 were tet(M) gene only, and 43 were carrying both erm(B) and tet(M) genes.

3.2. Prevalence of selected erm and tet genes among bacterial isolates of human and animal origin

From the primary isolation plates for 195 samples, 637 single colonies were picked and grown further. Of these 489 were identified to species level with species-specific primers (Table 3). DNA from these bacteria was isolated and resistance genes were identified. Positive amplicons of erm(B) and tet(M) were obtained from isolates of both human and animal origins. Of the 195 samples, 91 were found to contain either erm(B) or tet(M) genes or both (Table 2). The number of Lactobacillus strains of dairy origin carrying the resistance genes was 49 whereas the number of Lactobacillus strains of human origin carrying the resistance genes was 254. Of the 489 single colony isolates, 95 were Lactobacillus crispatus, and 61 of these were found to carry the erm(B) and/or tet(M) genes (Table 3). Hundred and four were Lb. casei, and 80 were containing the erm(B) and/or tet(M) genes. The number of Lactobacillus gasseri isolates was 79 of which 49 were containing the erm(B) and/or tet(M) genes. Lactobacillus rhamnosus was purified from 63 samples and 43 of these were containing the erm(B) and/or tet(M) genes. Lactobacillus fermentum was isolated from 50 samples of which 20 were positive for the erm(B) and/or tet(M) genes. Lactobacillus acidophilus was isolated from 38 samples of which 18 were the erm(B) and/or tet(M) genes positive. Lactobacillus johnsonii was found in 32 samples of which 26 were the erm(B) and/or tet(M) genes positive. Lb. casei was found in 28 samples of which 6 were the erm(B) and/or tet(M) genes positive.
Thus, the majority of the isolates (303) were found to harbor either \textit{erm}(B) or \textit{tet}(M) gene or both (Table 4). \textit{Lb. delbrueckii}, \textit{Lactobacillus zeae}, \textit{Lactobacillus sharpae}, and \textit{Lb. reuteri} were not found in human isolates. One yogurt sample was found to contain a \textit{Lb. plantarum} species carrying both \textit{erm}(B) and \textit{tet}(M) genes. Of the hand-made cheese samples 16 were found to contain \textit{erm}(B) and/or \textit{tet}(M) genes. One industrially produced White-Cheese was found to contain a \textit{Lb. acidophilus} species carrying \textit{erm}(B) gene and a \textit{Lb. plantarum} species carrying both \textit{erm}(B) and \textit{tet}(M) genes. No raw-milk and cream samples were found to contain resistance factors at all. Most of the human fecal (all samples) vaginal (29 of 36), and mouth (10 of 14) samples were found to carry the resistance genes. No PCR product for \textit{van}(A) gene cluster was amplified from either lactobacilli of human or dairy origin suggesting that lactobacilli of human and dairy origin do not contain the \textit{van}(A) gene cluster. In addition to the DNA obtained from single \textit{Lactobacillus} strain colonies, PCR was also performed on DNA preparations from samples in broth (original culture vessel) and samples of confluent growth (primary isolation plates) on MRS agars. Obtaining of the same PCR products in all these three growth vessels proved that the number species present in each original culture medium were matched with the number of species found in the primary isolation plates and with the single colony plates. This also verified the optimization of PCR amplifications.

4. Discussion

The use of lactobacilli in both the food industry and clinical applications as probiotics has raised discussion of new safety aspects, one of them being the nature of acquiring and distribution antibiotic-resistance genes such as vancomycin, erythromycin, and chloramphenicol resistance.

Some species of lactic acid bacteria commonly used in the food industry, e.g., many species among \textit{Lactobacillus}-, \textit{Pediococcus}-, and \textit{Leuconostoc} genera are intrinsically resistant to vancomycin, which means that vancomycin susceptible strains of these species do not exist. Being vancomycin-resistant, however, these species are susceptible to many other antibiotics and they have not been reported to easily acquire antibiotic-resistance determinants like the \textit{enterococci} [31]. Even though we were not able to find relevant genes for chloramphenicol resistance, the presence of \textit{erm}(B) for the resistance to erythromycin could provide resistance to other antibiotics in the group.
Out of 637 single colony isolates 489 were Lactobacillus strains (76.8%). The number of Lactobacillus species carrying the resistance genes was 303 (61.96%). Out of 303 resistant isolates only 49 were of dairy origin (16.2% of the resistant isolates). However, the percentage of resistant lactobacilli of dairy origin with regards to the total number of lactobacilli identified to the species level was 10% (49 of 489 isolates). The results we had showed that the dairy products in which the drug resistance genes were found were mostly locally consumed hand-made cheeses. The presence of tet(M) and/or erm(B) genes in two industrially marketed cheese samples indicates that either the starter cultures are contaminated with the resistance genes or it is the result of contamination of the resistant species during the production of the cheese. However, the second option seems likely due to the reason that we were not able to find any resistant Lb. delbrueckii subsp. bulgaricus species, which is one of the key elements of Turkish-Cheese, in dairy products nor it was found in any samples of human origin. Thus, this needs a further study in detail. Additionally, Turkish farmers are reluctant in getting educated to establish a prolific farm and produce healthy dairy products. These farms are in a form of “small family farms” of weak economical importance. Many times these farmers bring their dairy products illegally to the market place while their animals are on medical treatment. This is because of the fact that the economical pressure of these farmers is heavily dependent upon selling the dairy products they produced whether or not they are healthy, an established and effective monitoring system is not available for the products they brought to the market places, and no sanitary precautions or sanctions are taken for them as well. This situation may produce a high risk for the consumers. On the other hand, consumers themselves are ignorant of the use of antibiotics and use them without consulting to practitioners as well. These together may also lead to the gaining of resistance by many of the Lactobacillus strains found in both dairy products and in the intestine.

Neither the lactobacilli of human nor dairy origin was found to harbor cat-TC, erm(AC), and van(A) gene classes. Thus, our results are in accordance with previous findings in Lactobacillus, Pediococcus, and Leuconostoc species, which have shown that the vancomycin resistance in these species is intrinsic, chromosomally coded, and non-transferable [31–33]. In general, 61.9% of the isolates carry either erm(B) or tet(M) genes or both. The number of resistant lactobacilli of human origin was 254 (51.9%) and its proportion to the total amount of resistant lactobacilli was 83.8%. However, only the 10% of dairy products were found to carry either erm(B) or tet(M) genes or both. Even though the number of animal samples harboring the resistance genes (17 of 111) is smaller with regards to human samples, 10% of the dairy isolates were still resistant.

This indicates that lactobacilli of dairy origin in Turkey harbor such drug resistance genes in a high number indeed. The presence of the resistance genes in the majority of the lactobacilli of intestinal origin suggests that transfer of such genes from an unknown origin during the passage from the intestinal tract is more likely. The presence of identical genes in different bacteria should be an indication of a common reservoir for resistance or evolution from the same ancestor [36,37]. Moreover, identical genes in lactobacilli should be a result of both clonal and/or horizontal gene transfer among closely related bacteria of the same genus. Further studies of the position and the mobility of different tet and erm genes are clearly needed to determine whether the horizontal or clonal transfer of genes is important in terms of distribution of resistance genes among the members of the same and/or related species among lactobacilli.

These results indicate that drug resistance is a common problem both for hand-made cheese production and for the human medicine in Turkey for the time being, however, as to the lack of resistance genes in industrially utilized Lactobacillus starter cultures such as Lb. delbrueckii subsp. bulgaricus it essential to claim that industrial strains of lactobacilli are still safe. It also indicates that drug resistance may be acquired in the intestinal tract by humans and spread to animals or the dairy products while processing them.

Acknowledgements

We thank to Dr. Morten Danielsen for his valuable critiques on the development of the data and for the patience in getting a flawless and coherent language ability. We also thank to Dr. Sung-Sik Yoon and Takahiro Matsuki for helpful comments in the preparation of the manuscript.

References


