EXPERIENCE REPORT
https://doi.org/10.22239/2317-269x.01340

Evaluation of Violet Red Bile Glucose agar specificity for Enterobacteriaceae isolation in raw goat milk

Avaliação da especificidade do ágar Violeta Vermelho Bile Glicose para o isolamento de Enterobacteriaceae em leite de cabra cru

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Received: Jun 24, 2019
Approved: Dec 12, 2019

ABSTRACT

Introduction: Coliforms have been used over the years as indicator microorganisms for microbiological quality. However, several studies report the lack of direct correlation between their presence and that of pathogens in the evaluation of food safety, leading to the suggestion of new analysis proposals, such as the search for total enterobacteria, which includes all members of the Enterobacteriaceae family. **Objective:** This study aimed to determine the population of enterobacteria in raw goat milk samples based on the microbiological criteria suggested by the National Health Surveillance Agency - Ministry of Health (Anvisa - MS) - Public Consultation Nº 542 of July 17, 2018 for Pasteurized milk samples. **Method:** 21 samples from small producers from different regions of the state of Rio de Janeiro were used. Isolates were obtained from inoculation on VRBG agar according to ISO 21528-2 and identified by Matrix Associated Laser Desorption-Ionization – Time of Flight (MALDI/TOF) mass spectrometry. **Results:** Of the 222 isolates identified, only 26.6% belonged to the Enterobacteriaceae family. Another 61.3% belonged to the *Pseudomonas* genus, 4.5% to the *Acinetobacter* genus and 7.6% were *Stenotrophomonas maltophilia*. Although there are no microbiological parameters in Anvisa-MS RDC Nº 12 of January 2, 2001 for raw milk, the results of isolation on VBRG agar led to the investigation of other studies with similar findings regarding isolation of VRBG bacteria from goat and bovine milk samples. **Conclusions:** Detection in VRBG agar of non-Enterobacteriaceae-negative oxidase bacteria, such as those of the genus Acinetobacter, suggests the need for a debate on the methodology indicated by ISO 21528-2 for enumeration of enterobacteria, as the new regulation on standard microbiological data for food will no longer use the coliform group, but the Enterobacteriaceae/g or mL count, as an indicator.

KEYWORDS: Enterobacteriaceae; *Pseudomonas*; Milk; *Acinetobacter*; Legislation

RESUMO

Introdução: Bactérias do grupo dos coliformes têm sido empregadas ao longo dos anos como micro-organismos indicadores da qualidade microbiológica. No entanto, diversos estudos relatam a falta de correlação direta entre sua presença e a de patógenos na avaliação da segurança de alimentos, levando a sugestão de novas propostas de análise, como a pesquisa de enterobactérias totais, que compreende todos os membros da família Enterobacteriaceae. **Objetivo:** Determinar a população de enterobactérias em amostras de leite caprino cru com base nos critérios microbiológicos sugeridos pela Consulta Pública nº 542, de 17 de julho de 2018, da Agência Nacional de Vigilância Sanitária - Ministério da Saúde (Anvisa - MS), para amostras de leite pasteurizado. **Método:** Foram utilizadas 21 amostras de pequenos produtores de diversas regiões do estado do Rio de Janeiro. Os isolados foram obtidos a partir de inoculação em ágar VRBG, de acordo com a ISO 21528-2, e identificados por espectrometria de massas por *Matrix Associated Laser Desorption-Ionization* – Time of Flight (MALDI/TOF). **Resultados:** Dos 222 isolados identificados, apenas 26,6% pertenciam à família Enterobacteriaceae. Outros 61,3% pertenciam ao gênero *Pseudomonas*, 4,5% ao gênero *Acinetobacter* e 7,6% eram *Stenotrophomonas*

maltophilia. Apesar de não haver parâmetros microbiológicos na RDC nº 12, de 2 de janeiro de 2001, da Anvisa-MS, para leite cru, os resultados do isolamento em ágar VBRG levaram à investigação de outros estudos com achados semelhantes referentes ao isolamento de bactérias VRBG a partir de amostras de leite caprino e bovino. **Conclusões:** A detecção em ágar VRBG de bactérias oxidase negativas não pertencentes à família Enterobacteriaceae, como as do gênero *Acinetobacter*, sugere a necessidade de um debate acerca da metodologia indicada pela ISO 21528-2 para enumeração de enterobactérias, visto que o novo regulamento sobre padrões microbiológicos para alimentos não mais utilizará o grupo coliforme como indicador, e sim a contagem de Enterobacteriaceae/g ou mL.

PALAVRAS-CHAVE: Enterobacteriaceae; *Pseudomonas*; Leite; *Acinetobacter*; Legislação

INTRODUCTION

Hygiene and health conditions in the milk production chain are directly related to microbiological parameters and, therefore, to the quality of the final product[1].

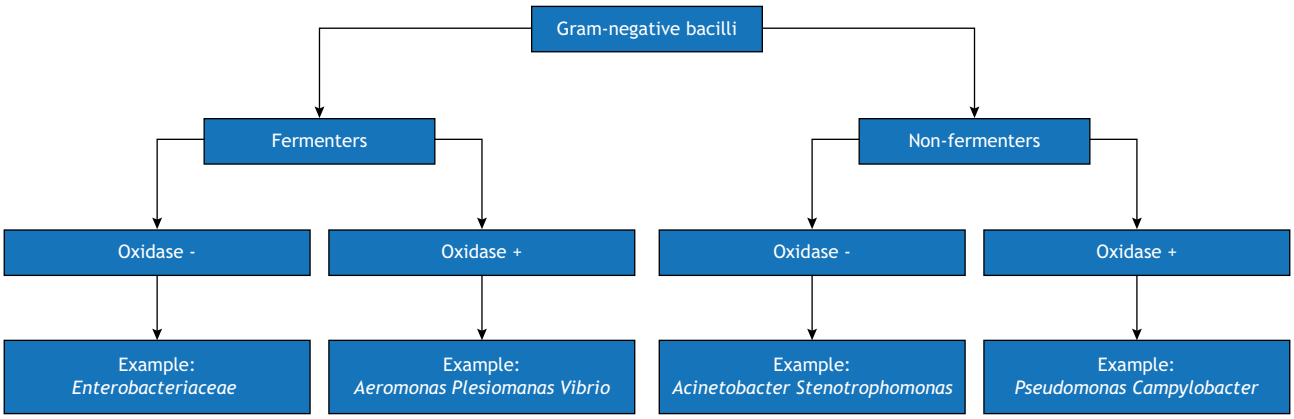
Resolution of the Collegiate Board (RDC) n. 12, of January 2, 2001[2], of the Brazilian National Health Surveillance Agency (Anvisa), approves the technical regulation on microbiological standards for food. In the food group called "milk and dairy products from bovines and other mammals", the microorganisms that should be researched are indicated, as well as their maximum tolerance values, so that at least the minimum quality standards of these products are ensured. For pasteurized fluid milk, the recommendation is to test for thermotolerant coliforms and *Salmonella* spp. As for cheese, the orientation varies according to the type and moisture of each product. In general, thermotolerant coliforms, coagulase positive staphylococci, *Listeria monocytogenes* and *Salmonella* spp. are to be tested. For raw milk, there is no established parameter, since its marketing has been banned[2].

Gram-negative bacilli are considered to be important causative agents of hospital-acquired infections. The most relevant of them are often related to food, with greater capacity for dissemination than Gram-positive cocci[3]. They are divided into two large groups, fermenters and non-fermenters, as shown in Figure 1.

The Enterobacteriaceae family is composed of 53 genera and more than 170 described species. Some pathogenic genera are of great clinical importance, such as *Enterobacter* and *Salmonella*, as well as the *Escherichia coli* and *Klebsiella pneumoniae* species[5].

Coliforms are a group of microorganisms used as indicators, with the purpose of verifying the general microbiological condition of a sample. The microorganisms in this group are aerobes or facultative and not sporulated anaerobes, which ferment lactose with production of acid and gas at 32ºC – 35ºC (total coliforms). Within this group there are also those that manage to ferment lactose up to a temperature of 45ºC, the so-called thermotolerant coliforms, where we can highlight the *E. coli* species, whose presence in food indicates fecal contamination due to its exclusively fecal origin. The members of the coliform group belong to the Enterobacteriaceae family, and the main genera are *Escherichia*, *Klebsiella*, *Citrobacter* and *Enterobacter*[6].

The initial concept of testing indicator bacteria was created to detect fecal contamination, which could serve as indirect evidence of the presence of pathogens[7]. However, over the years some studies have shown a lack of direct correlation between the presence of indicators and pathogens, which raises questions as to the usefulness of these microorganisms in the assessment



Source: Adapted from Koneman et al.[4]

Figure 1. Classification of Gram-negative bacilli and examples of their most relevant representatives.

of food safety[8]. In addition, the presence of certain coliforms in the environment also decreased their association as a group as indicators of fecal contamination[9,10]. There are also cases in which pathogens were detected in samples of raw milk that had low coliform count, which at first would suggest good microbiological quality[11].

Since the use of coliforms as indicators in dairy products has been discussed in the scientific community, new solutions have been proposed, like the use of the so-called EB Group or total Enterobacteria, which comprises all members of the Enterobacteriaceae family, including the coliform group. The use of the EB group as an indicator is advantageous because it detects possible contamination with greater sensitivity after pasteurization, in view of the greater scope of contaminating species. Another suggested group is that of total Gram-negative bacteria, which includes all enterobacteria and other non-fermenting Gram-negative bacteria, such as the *Pseudomonas* genus. This is the main genus associated with post-processing contamination in the dairy industry, and it may have biofilm-producing activity and production of deteriorating enzymes. Both groups, despite including pathogens, are considered hygiene-health indicators[6,12].

The present study aimed to determine the population of Enterobacteriaceae in samples of raw goat milk, based on the microbiological criteria suggested by Public Inquiry n. 542, of July 17, 2018, by Anvisa – Ministry of Health[13] for pasteurized milk samples.

METHOD

Twenty-one samples of raw goat milk from small producers from different locations in the Brazilian state of Rio de Janeiro were used. Seven from the Metropolitan area, four from the Serrana region (highlands) and ten from the northwest region of the state. The producers were selected after online searches about places where one could purchase raw goat milk, and also based on the authors' previous knowledge of some points of sale. The samples were collected directly in the containers where the product is sold to consumers, in two periods (March and August 2018). They were kept refrigerated until the moment of analysis, which occurred shortly afterward.

Twenty-five milliliters of each sample analyzed were homogenized with 225 mL of peptide water (BIOCEN, São Paulo) 0.1%. After serial dilutions of these suspensions, inoculations were made on Violet Red Bile Glucose agar (VRBG, Kasvi, São Paulo), prepared at most 3 h in advance, according to the methodology indicated by ISO 21528-2. The plates were incubated at 35°C for 24 h. For each new batch of VRBG produced, we did the quality control of the medium with the incubation of an unsown plate[14,15].

The typical colonies of pink and purple color, with the presence of precipitation halos (on average, 15 per sample) were inoculated in soybean tryptone agar (Casoy agar, BIOCEN, São Paulo) for mass gain, and later stored under freezing in cryotubes in Casoy broth (Merck, São Paulo) and 40% glycerol (Merck, São Paulo).

271 isolates were selected for identification by mass spectrometry by Matrix Associated Laser Desorption-Ionization - Time of Flight (MALDI-TOF; Microflex LT, Bruker, United States). For this, the bacterial cultures sown on Casoy agar were inserted in duplicate in the equipment's own metal plate, with the subsequent addition of 1 µl of 70% formic acid for cell lysis to occur. After the acid dried, the same volume of matrix was inserted, which was crystallized at the end of the process. The plate was then put into the equipment and, after the emission of laser beams, the sample was evaporated with the release of ionized proteins of different loads and masses. Subsequently a spectrum was generated as a function of the time elapsed until the detector. The equipment was previously calibrated with a control strain of *E. coli*, and the identification of each isolate was generated by Biotype 3.1 software[16].

RESULTS AND DISCUSSION

Result of the identification of the selected isolates

Of the 271 isolates, 222 were precisely identified using MALDI-TOF and only 26.6% corresponded to members of the Enterobacteriaceae family. Another 61.3% of the isolates were identified as species of the *Pseudomonas* genus and 4.5% as species of the *Acinetobacter* genus. In addition, 7.6% corresponded to the *Stenotrophomonas maltophilia* species, a species that was previously classified as belonging to the *Pseudomonas* genus. Figure 2 illustrates the general identification we obtained.

A Chinese study by Zhang et al.[17] assessed the bacterial diversity of raw goat milk and also detected the same groups found in this study, but in different proportions, with enterobacteria corresponding to about 25% of the total (together with some Gram-positive isolates), followed by *Pseudomonas* (13%), *Acinetobacter* (13%) and *Stenotrophomonas* (3%).

The *Pseudomonas* genus is extremely relevant in the deterioration of raw milk, especially because it is psychrotrophic and thus multiplies efficiently during cold storage, with proteolytic and lipolytic activity. *Pseudomonas* sp. have a great genetic diversity and metabolic variety, which enables their development in several environments, including surfaces and equipment used

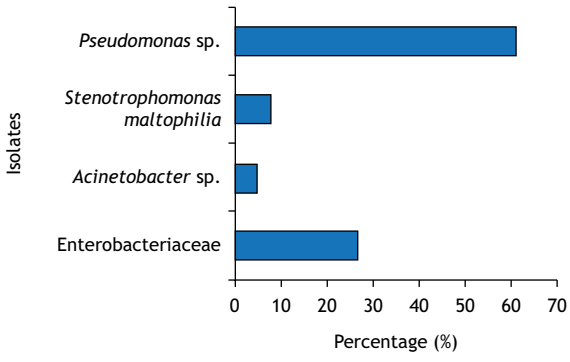


Figure 2. Distribution of the isolates identified in the analyzed raw goat milk samples.

in the dairy production chain. The *Pseudomonas fluorescens*, *P. aeruginosa* and *Pseudomonas putida* species are considered by the literature as the most relevant in milk[18].

Among the psychrotrophic microorganisms usually found in milk, species of the *Pseudomonas* genus have the shortest generation time at temperatures between 0ºC and 7ºC, which warrants their prevalence. *P. fluorescens* is the most isolated species in dairy products and the one that most produces deteriorating enzymes[19].

Although the microorganisms of this genus are inactivated by possible subsequent thermal treatments, the enzymes produced are thermoresistant and resist even ultra-high temperature (UHT) treatments. These enzymes can deteriorate the milk during the next stages of storage and transportation. The effects of the deterioration caused by *Pseudomonas* sp. vary significantly according to each strain and growing conditions[19,20].

The *Acinetobacter* genus is associated with high mortality rates in hospital-acquired infections and the expression of multidrug resistance, and its species are good competitors in the food matrix microbiota due to several factors such as biofilm formation capacity and long-term survival on surfaces[21].

Although little related to food, bacteria of the *Acinetobacter* genus have also been reported in milk. Asian studies have shown that hundreds of isolates, especially *A. baumannii*, from raw bovine milk, have shown multidrug resistance to a wide range of antibiotics[22,23].

In a recent study, multidrug-resistant strains (MDR) of the *Acinetobacter baumannii-calcoaceticus* complex, resistant even to carbapenems, were found in infant formulas and lactating utensils in a public hospital in Rio de Janeiro, which indicates a public health problem associated with this genus[24].

Recent studies highlight the occurrence of isolation of the *Pseudomonas* and *Acinetobacter* genera on VRBG agar, comparing the influence of this group on the detection of enterobacteria in food samples in different culture media. Depending on the type of sample, the temperature and the species present, different combinations of the microorganisms present can be obtained[25,26].

Association with Public Inquiry n. 542/2018

Initially, the study was focused on the detection of bacteria from the Enterobacteriaceae family and, therefore, the procedure with the VRBG agar was used. However, the largest proportion of isolates belonged to the *Pseudomonas* genus. VRBG agar is also associated with the recovery of microorganisms of the *Pseudomonas* genus, according to the international pharmacopeia of the World Health Organization (WHO). This medium is indicated for use with bile-tolerant Gram-negative bacteria and has the properties of growth promoter and indicator of the microorganisms in question[27].

In line with the debate of the global scientific community, Anvisa, through Public Inquiry n. 542/2018, proposed removing the coliforms group from the Technical Regulation on Microbiological Standards for Food and suggested replacing it by the research of Enterobacteriaceae and *Escherichia coli* nationwide[13]. However, the new legislation that will replace RDC n. 12/2001 has not been enacted yet.

In Brazil, Normative Instruction n. 62, of August 26, 2003[14], of the Ministry of Agriculture, Livestock Farming and Supply (MAPA), describes the recommended procedure for counting enterobacteria in food using VRBG agar[7]. The culture medium previously used to detect coliforms was *Violet Red Bile* agar (VRB), which had lactose in its composition. The coliform group is capable of fermenting both glucose and lactose, however, other species of the Enterobacteriaceae family ferment only glucose. Thus, VRB agar had lactose replaced by glucose in its composition, giving rise to VRBG agar and enabling the detection of enterobacteria. In the presence of these bacteria, the pH of the medium decreases due to the fermentation of glucose, causing the presence of pinkish colonies because of the presence of the neutral red indicator in the formulation. Halos also occur due to the precipitation of bile salts. Gram-positive microorganisms are inhibited by the presence of the violet crystal and bile salts[28].

In this study, colonies of all identified genera were very similar on the agar surface, with pink and halo coloration, and it was not even possible to differentiate glucose fermenters and non-fermenters in relation to the appearance of the isolated colony. Thus, members of the Enterobacteriaceae family and the *Pseudomonas* and *Acinetobacter* genera, although they have different behaviors, appeared in the same way when isolated directly from the sample.

Other studies also describe this fact. A study done in Japan has shown the results of a test made with VRBG agar in raw and pasteurized milk, in which the development potential of *Pseudomonas* against the enterobacteria was verified, with significant growth of both groups[20]. In Germany, a study using powdered milk used VRBD agar (another name for VRBG agar) with the specific objective of isolating *Acinetobacter* sp. It obtained different species of the genus together with members of the Enterobacteriaceae[26] family. Njage et al.[29] used VRBG agar with the strict objective of isolating camel milk enterobacteria, however, when identifying the isolated colonies, microorganisms of the *Pseudomonas* and *Acinetobacter* genera were found, as in our study.

Since more advanced identification techniques, such as mass spectrometry, are still extremely costly and difficult to access, IN n. 62/2003 from the MAPA and international references indicate that after plating on VRBG agar, colonies should be subjected to staining Gram test and oxidase test to confirm the presence of enterobacteria[14,15]. In the routine of a microbiological analysis laboratory that will routinely investigate enterobacteria in many food classes, countless isolates that are possibly not enterobacteria will be generated, as seen in this study, and these isolates will have to be tested, which will increase costs and the need for labor.

Gram staining would not generate any differentiation between enterobacteria, *Pseudomonas* and *Acinetobacter*, since these are all Gram-negative bacilli. The oxidase test would discard *Pseudomonas*, but the *Acinetobacter* genus would eventually be counted as enterobacteria, since it is also oxidase negative. Thus, in addition to the increased labor and cost, overestimated counts could be generated.

CONCLUSIONS

The diversity of the population of Gram-negative bacilli identified in this study, as well as the identification of potentially pathogenic groups, highlights the risk of consumption of raw goat milk, which occurs mainly in towns of the Brazilian countryside.

Although the Brazilian legislation is focused on pasteurized milk and not on raw milk, the results obtained in this study, corroborating those found by other authors, pointed to the need for a debate on the methodology indicated for counting enterobacteria at the national level. This is necessary because the new technical regulation on microbiological standards for food will no longer use the coliform group as an indicator, but the count of Enterobacteriaceae/g or mL, using the VRBG agar isolation as the official methodology.

From the point of view of the Brazilian legislation, the inclusion of the Enterobacteriaceae/g or mL of sample parameter sparked a debate about the recommended quantification method, which may lead to inaccurate results, causing the indication of a sample as inappropriate due to overestimated results.

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Acknowledgments

To the Laboratory of Research in Medical Microbiology, of the Institute of Microbiology Paulo de Góes, Federal University of Rio de Janeiro, for the availability of the mass spectrometer (MALDI-TOF).

Authors' Contributions

All authors participated in the conception, planning (study design), acquisition, analysis, interpretation of data and writing of the paper. All authors approved the final draft of the paper.

Conflict of Interest

Authors have no potential conflict of interest to declare, related to this study's political or financial peers and institutions.