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Genetic heterogeneity of *Escherichia coli* strains isolated from raw milk, Minas Frescal cheese, and food handlers

[*Heterogeneidade genética de cepas de* Escherichia coli *isoladas de leite cru, queijo Minas Frescal e manipuladores*]

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ABSTRACT

From February 2004 to March 2005, 140 samples of food handlers – hands and nostrils – (92), raw milk (24), and minas frescal cheese (24) were analyzed for the presence of *Escherichia coli* in a dairy processing plant of Goiás State. Forty-seven *E. coli* strains were obtained and compared by DNA macrorestriction patterns obtained from pulsed-field gel electrophoresis following *Xba*I restriction in order to investigate the possible sources of cheese contaminations. Based on PFGE genotyping, one strain isolated from food the hands of a handler and five strains isolated from raw milk were identical or closely related to six strains from cheese suggesting, in these cases, the probable source of *E. coli* contamination in cheeses. No strain isolated from the nostrils was related to those found in cheeses or milk strains. The results showed high diversity among the strains, demonstrating a lack of predominance of an endemic clone in the dairy plant. This paper highlights the usefulness of PFGE as an epidemiological tool for determining the source of *E. coli* contamination in the food industry.

Keywords: *Escherichia coli*, pulsotypes, minas frescal cheese, raw milk, food handlers

RESUMO

Durante um ano, de fevereiro de 2004 a março de 2005, 140 amostras retiradas das mãos e das narinas de manipuladores de alimentos (92), do leite cru (24) e do queijo-de-minas frescal (24) foram analisadas para a presença de Escherichia coli, *em um laticínio do Estado de Goiás. As 47 cepas obtidas foram comparadas por macrorrestrição do DNA com enzima* Xba*I*, *seguida de eletroforese em gel em campo pulsado (PFGE), a fim de investigar as possíveis fontes de contaminação do queijo. Baseado na genotipagem pelo PFGE, uma cepa obtida do leite cru e cinco cepas obtidas dos manipuladores mostraram similaridade maior que 80% com seis cepas isoladas do queijo, denotando forte correlação genética entre elas e sugerindo, nestes casos, a fonte provável de contaminação do produto final. Nenhuma cepa isolada do nariz foi relacionada às isoladas do queijo ou do leite. Os resultados mostraram grande diversidade entre as cepas, demonstrando ausência de um clone endêmico no laticínio avaliado. Este estudo destaca a utilidade do PFGE como uma ferramenta importante em investigações epidemiológicas e na determinação de possíveis fontes de contaminação por* E. coli *na indústria de alimentos.*

Palavras-chave: Escherichia coli, *PFGE, queijo Minas Frescal, leite cru, manipuladores*

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INTRODUCTION

Many enteropathogenic microorganisms have been found in milk and dairy products such as cheese, which is usually stored under inadequate temperatures and consumed without any prior thermal treatment. They are frequently associated to outbreaks of foodborne diseases (Almeida Filho and Nader Filho, 2002).

Minas frescal cheese is widely consumed and has become part of the daily diet in many States of Brazil (Câmara et al., 2002; Cardoso and Araújo, 2004). This kind of cheese is produced from raw or pasteurized cow milk and because of several factors such as humidity content, its animal origin, and especially because it is hand manufactured, it can be easily contaminated and constitutes a favorable environment for bacterial survival and multiplication (Câmara et al., 2002).

The detection of coliform bacteria, especially *Escherichia coli,* in processed foods is an indication of fecal matter contamination. It also evidence the inadequate control of the raw material, the contamination of the handler involved in the production process, and the poor quality of the finished product. The fact that *E. coli* serogroups, whose pathogenicity is known by their toxigenic and infectious mechanisms (Câmara et al., 2002), can be isolated from cheese, also suggests that other enteropathogenic microorganisms could possibly be present in this food as well (Ritter et al., 2001).

Epidemiological studies have seek to establish the source of microorganisms in food by using bacterial typing techniques, characterizing strains from possible sources (animals, handlers, equipment, and others sources), and comparing them, in terms of genotype, to strains from food (Foxman and Riley, 2001). One of these methods is macrorestriction of DNA followed by pulsed-field gel electrophoresis (PFGE). This is a highly discriminatory and reproducible technique whose performance is the same or better than other techniques currently available and has been successfully applied for typing a large number of microorganisms. It is also the most effective technique for differentiating *E. coli* strains (Bidet et al., 2005).

The aims of this study were to characterize *E. coli* strains isolated from human nostrils and hands, raw milk, and cheese in a dairy processing plant by means of PFGE analysis and to investigate any relationship among the strains and the possible sources of cheese contamination.

MATERIAL AND METHODS

A total of 140 samples, 24 from raw milk, 24 from Minas Frescal cheese, 46 from anterior nostrils of food handlers, and 46 from their hands, were analyzed for the presence of *E. coli* in a small dairy plant in Goiás State, from March 2004 to February 2005. The industry was visited twice a month. The dairy plant had four workers handling milk and/or cheese. The study protocol was approved by the Ethical Committee at the Universidade Federal de Goiás and the participants gave informed consent. The hands and anterior nostrils samples were collected during each visit, from the handlers that were working on that day using the method described by Vandenbergh et al. (1999). Worker n^{o} 1 was sampled 16 times (M^1, N^1); n^{o} 2, 17 times (M^2, N^2); n^{o} 3 just once (M^3, N^3) and n^{o} 4 was sampled 12 times (M^4, N^4). Raw milk and cheeses were sampled following methodology described by Midura and Bryant (2001).

The samples were submitted to presumptive, confirmed, and completed tests for coliforms, fecal coliforms, and *E. coli* according to Feng et al. (2002). From each positive plate, five colonies were tested for *E. coli* confirmation. The strains were stored at -20^{o}C in tryptic soy broth[1] with 20.0% glycerol awaiting further analysis.

The genetic pattern of *E. coli* strains was carried out by pulsed-field gel electrophoresis (PFGE) of restricted DNA, using a CHEF DRII[2], following the method of Pfaller et al. (1992) with modifications. Briefly, a single isolated colony was grown overnight in TSB[1] at 37^{o}C. Cells were pelleted and resuspended in TEN buffer. The suspension was mixed with 1.2% SeaKem Gold agarose and plugs were made. They were placed in EC buffer with lysozime and incubated for 5h at 37^{o}C. The plugs were

[1]Oxoid Ltd. Cambridge, UK.
[2]Bio-Rad Laboratories, Hercules, CA, USA.

washed, placed in proteinase K solution and incubated at 50°C overnight. The plugs were washed five times with CHEF TE 1X buffer for 1h each at room temperature. DNA was restricted for 20h with 20U of *Xba*I enzyme at 37°C. DNA was separated for 19h by PFGE using 1.0% PFGE agarose in 0.5X TBE buffer under the following conditions: 14°C, 6 V/cm, 5-60 pulse times. A lambda ladder molecular weight marker[2] was included in each gel. Gels were stained with ethidium bromide (0.5mg/L) and then photographed under UV light.

Strains of *E. coli* were placed in groups of identical or related strains by comparing the banding patterns produced, using a combination of photographic visual inspection and computer analysis[3]. A pulsotype (PT) was defined as a unique electrophoretic banding pattern. Strains with identical restriction profiles were assigned as the same type and identified with a capital letter. Banding patterns presenting one to three differences among them were considered closely related and were assigned as subtypes (ST) indicated with a numeral suffix. Strains with more than three differences were considered to be different types (Tenover et al., 1995). The Dice formula was used to calculate the coefficients of pairwise similarity, and dendrograms were created by unweighted-pair group method (UPGMA) using arithmetic averages. The cluster cutoff was set at 80% similarity and all clusters were identified by arabic numerals.

RESULTS AND DISCUSSION

Sixty-nine *E. coli* strains were obtained from the 47 positive samples being 11 strains from the handlers, 33 from raw milk, and 25 from the minas frescal cheese. Out of the 92 samples collected from the handlers, *E. coli* was isolated in 11 (12.0%) of them, and from the 24 samples of raw milk and cheese, it was found in 19 (79.2%) and 17 (70.8%) of them, respectively (Table 1).

De Buyser et al. (2001) evaluated 60 outbreaks of milk and dairy-transmitted diseases, in France and other countries, from 1980 to 1997, and determined that pathogenic *E. coli* was responsible for around 20.0% of the notified episodes and that cheese made from raw milk or non-specified milk type was the most common food involved.

In Brazil, the presence of pathogenic microorganisms has been observed in minas frescal cheese, and several studies confirmed the presence of fecal coliforms in this product in Poços de Caldas, MG (Almeida Filho and Nader Filho, 2002), Mato Grosso do Sul (Câmara et al., 2002), Rio de Janeiro, RJ (Araújo et al., 2002), Distrito Federal (Cardoso and Araújo, 2004), Serro, MG (Brant et al. 2007), and região central region (Paneto et al., 2007).

E. coli was isolated in 75.0% of the handlers and more frequently from the hands than from the nostrils. This finding was expected since the anterior nostrils are not described as a normal habitat for *E. coli*. Monteiro et al. (2001) isolated *E. coli* in 55.0% from hands of handlers in the State of Ceará. According to Araújo et al. (2002), the presence of fecal coliforms is evidence of poor hygiene practices, since the finding of a high number of these microorganisms is evidence that hands are not properly cleaned and a clear indication of risk for food contamination by fecal particles.

E. coli was isolated in 79.2% of the raw milk samples. Smaller percentages were obtained by Chye et al. (2004). They isolated this bacterium in 64.0% of the raw milk samples from dairy farms in Malaysia. Enteropathogenic microorganisms have been found in milk consumed without thermal treatment. Contamination during milking, as well as inadequate temperatures during storage and transportation, can result in high levels of pathogenic bacteria, which can represent a health risk to consumers (Araújo et al., 2002).

E. coli was isolated in 70.8% of the minas frescal samples. Similar results were found by Câmara et al. (2002) in Mato Grosso do Sul. Rocha et al. (2006) found that except one trademark, all six others presented contamination levels above those recommended by the Brazilian legislation.

The presence of *E. coli* is a clear evidence that the controls of raw material manufacturing processes and the final product is inefficient.

[3]BioNumerics, v. 4.0; Applied Maths - Kortrijk, Belgium.

Table 1. *Escherichia coli* isolated from dairy staff, raw milk, and minas frescal cheese in a dairy plant

Source	Samples collected	Positive samples		Nº of strains
		Nº	(%)	
Dairy staff, nostrils	46	3	6.5	03
Dairy staff, hands	46	8	17.4	08
Raw milk	24	19	79.2	33
Cheese	24	17	70.8	25
Total	140	47	-	69

Out of the 69 strains, three of them (two from milk and one from cheese) could not be typed, even after four repetitions. The genetic analysis of the 66 strains resulted in 61 different banding patterns varying from 9 to 17 distinct bands in the range of 679kb to 48.5kb, showing a high level of genetic heterogeneity among the strains.

The dendrogram including all the genetic profiles was built on the basis of similarity levels (Fig. 1). The cluster cutoff was set at 80.0% similarity resulting in 57 clusters, numbered from 1 to 57. The 66 typed *E. coli* strains were grouped in 60 pulsotypes (PTs) and one subtype (ST).

Ten different electrophoretic profiles were obtained from 11 strains from handlers, indicating the absence of an endemic strain among them. The results revealed that the workers harbored more than one clone in their hands and nostrils, except in one occasion, in which M^2 presented the same clone in these sites (PT A) (Fig. 1).

Genetic typing of the 31 strains from the raw milk samples generated 30 different electrophoresis profiles, indicating the lack of an endemic strain. Only the PT T grouped two strains from a single milk sample (17la and 17lb) with identical band profiles, therefore, belonging to the same clone. The D pulsotype and the subtype D1 corresponded to two strains of the same raw milk sample (16la and 16lc), which were considered to be strongly related and are probably strains derived from the same clone (Fig. 1).

Picozzi et al. (2005), in Italy, used this technique to compare 12 *E. coli* strains isolated from cow milk and found different electrophoretic profiles, even when they used a 41.0% similarity level. This difference can be explained by the exposure of milk to several sources of contamination, such as the personnel involved in the milking process, utensils, and the farm environment, and the consequent poor hygiene/sanitation control of the milking activity in the farms that supply this product.

From the electrophoretic profiles obtained from the cheeses, there was no similarity since the 24 strains resulted in 24 different pulsotypes. Radu et al. (2002) found 28 different genotypic profiles in 28 strains of *E. coli* from samples of beef and poultry meat sold in Malaysia, using PFGE with a 90.0% similarity level.

After comparing the electrophoretic profiles and following the criteria established by Tenover et al. (1995), the 14qb (cheese) and 10m1 (hand) strains were considered identical. Therefore, it was possible to establish a contamination relationship of the final product by the handler (PT AA) (Fig. 1). This fact demonstrates that the handler represents a risk of bacterial transmission for the final product, especially when one considers that this small-scale dairy plant uses a manual process for producing minas frescal cheese.

Two pulsotypes contained identical strains isolated from milk and cheese: PT N (15q and 14l) and PT BF (10q and 11lc) (Fig. 1), according to criteria established by Tenover et al. (1995). Three cheeses strains were possibly related to three milk strains, thereby generating six distinct pulsotypes: PT J and PT K (13lb and 18q), PT V and PT W (14qa and 6l), and PT Y and PT Z (15lb and 17qc) (Fig. 1). These results suggest that the raw material is the probable source of contamination of the final product.

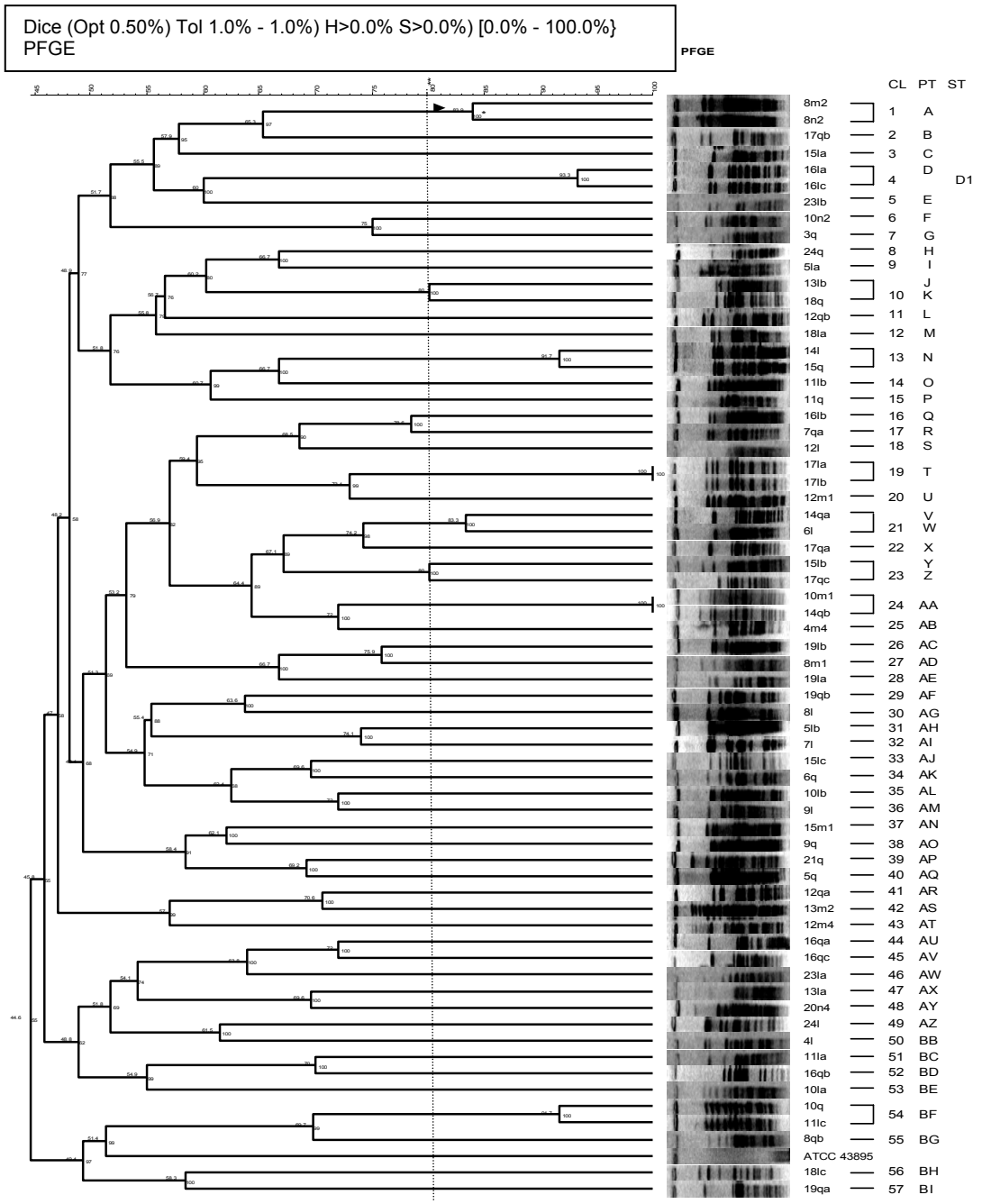

Figure 1. Clonal reletionships of 66 *Escherichia coli* strains established by *Xba*1 PFGE analysis. Clusters (CL) were labelled with arabic numbers, pulsotypes (PT), and subtypes (ST) with capital letters and letters and numbers, respectively.
► Similarity values, * Cophenetic correlations, ** Cutoff (80%).

According to Radu et al. (2002), the genetic diversity observed among the *E. coli* isolated by means of molecular typing techniques contributed to the investigation of potential epidemiological problems caused by this pathogen. Subtyping of strains helps in contamination studies in the food industry, because critical points can be identified and

appropriate measures implemented to guarantee product safety.

It was possible to observe that the use of a highly discriminatory technique, such as PFGE, made it possible to determine the genetic heterogeneity of *E. coli* strains, thus reflecting the complexity of this microorganism, especially in milk and cheese processing plants.

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