Comparison of Conventional and Rapid Methods for *Salmonella* Detection in Artisanal Minas Cheese

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Abstract

Artisanal cheeses traditionally produced from raw milk have a diverse microbiota and, due the varied changes that occur in this type of food matrix during the maturation, pathogens detection's may be impaired. In this study, the conventional method established by ISO 6579:2005 to evaluate the presence of *Salmonella* was compared with two alternative rapid methods, PCR-BAX® (DuPont) and VIDAS®-SLM (BioMérieux), to analyze artisanal Minas cheese, a typical Brazilian product. *Salmonella* was not detected by conventional or PCR-BAX® in 63 artisanal Minas cheese samples analyzed. Although highly specific and accurate, the immunoassay (VIDAS®-SLM) presented 3.17% of false positives. Good manufactures practices were absent in some producers of Minas artisanal cheese and, the fact of *Salmonella* was not detected in analyzed samples should be related with presence of high and diverse endogenous microbiota, including approximately, 10^7 $CFU.g^{-1}$ of lactic bacteria, and a low pH and water activity, conditions that can minimize pathogens growth, provide cellular injury and hamper the recovery strategies.

Keywords: *Salmonella*, artisanal Minas cheese, PCR-BAX®, VIDAS®-SLM

1. Introduction

Artisanal cheeses are appreciated around the world and constitute a group of specialty cheese whose manufacture is characterized by small scale and limited volume production. These cheeses are manufactured in farmhouses following traditional techniques, without the deliberate addition of selected starter cultures (Randazzo, Caggia, & Neviani, 2009) and are generally made from raw milk. Besides their cultural, social and economical significance these cheeses are recognized to have great microbial biodiversity, which is generally found at both genus and species level, associated with varied changes that occur in this food matrix during the maturation. High microbial loading was expected in artisanal products that were manufacturing without the observation of good manufactured practices (GMP) and this diverse contamination could promote pathogens inhibition. However, in the wake of food scares in recent decades, raw-milk cheeses have often been categorized as ''risky'' food (West, 2008) and outbreaks involving pathogens as *Salmonella*, *Listeria monocytogens* and *Staphylococcus aureus* have been associated with the consumption of these cheeses (Hall & French, 2011).

Salmonella is a food-borne pathogen influencing on food safety and public health around the world. According to Scallan et al. (2011), *Salmonella* is the second major cause of foodborne disease acquired in the United States and leads episodes of hospitalization and death. Hall and French (2011) evaluated reports on case material dating from January 2000 up to December 2010, in which raw milk or raw milk cheeses were implicated in 772 clinical cases and two deaths due contamination by *Salmonella*.

The various safety management tools of the worldwide food industry, such as the Hazard Analysis and Critical Control Points (HACCP), use microbiological analyses to monitor emerging risks (Vanne, Karwoski, Karppinen, & Sjiiberg, 1996). In Brazil, pathogens such as *Salmonella* sp. must be absent in 25 grams of cheese (Brazil, 2001). In contrast, European legislation mandates a stricter criterion for *Salmonella* sp., which must be absent in one gram of the same type of product (Directive 92/46/EEC). As a result, a small amount of the pathogen must be detected quickly and securely to avoid damage and legal repercussions generated by contaminated food.

Conventional culture methods for *Salmonella* detection in foods consist of a series of steps that include nonselective enrichment, selective enrichment, and selective/differential plating and, finally, morphological,

biochemical and serological confirmation as described in ISO 6579:2005 method (International Standards Organization, 2005).This standardized classical culture method is rather sensitive and quite inexpensive, but it requires at least, three working days to produce a negative result and five to ten working days for a confirmed positive result. Nevertheless, it is still in use by many labs, especially by regulatory agencies, because it is harmonized method, looked at as the "gold standards" in food diagnostics (Jasson, Jacxsens, Luning, Rajkovic, & Uyttendaele, 2010). Although reliable and officially approved, the conventional methods for the detection of microorganisms in foods require a long period of time to obtain results. Moreover, due to environmental factors, variations in gene expression of microorganisms can occur and may affect the discriminatory power of biochemical tests. Furthermore, viable but nonculturable cells are not detected by the conventional methodology (Malorny et al., 2003).

Alternative and rapid methodologies for the detection of *Salmonella* in foods are attractive and allowing convenience, flexibility and potential for automation (Oslen, 2000; Koyuncu, Andersson, & Häggblom, 2010). Molecular methods designed for targeting *Salmonella* DNA by conventional and quantitative real-time PCR (RT-qPCR) technologies have the potential to reduce detection time in one to three days (Cheung & Kam, 2012). One example was the commercial automated BAX®-PCR system (Dupont Qualicon, USA), that reduces the enrichment steps, the risk of contamination by PCR amplicons and the demand for laboratory resources because is automated and eliminate the need for gel electrophoresis. The BAX® system combined all the essential PCR reagents, such as DNA polymerase, nucleotides, primers, internal positive control, and fluorescent dye (SybrGreen), into a single small lyophilized pellet in each PCR tube, to which the sample cultures could be added. The melting curve profiles showing *Salmonella*-specific temperature peaks could also eliminate the possibility of getting false positive results (Cheung & Kam, 2012). Despite the advantages of methods based on DNA amplification, such as high specificity and sensitivity, degradation of the target DNA sequence and the presence of food interference, including fat and calcium (Glynn et al., 2006), might impair the performance of these methods. Alternative methods also included those immunoassay-based techniques, such as, the VIDAS® *Salmonella* assay (bioMérieux, France) that can produce negative results in one to three days. Despite of require onerous commercial kits and sophisticated equipments, the alternative methods present high productivity and quality of results and the cost effective should be considered mainly by testing laboratories that analyze samples routinely for foodborne pathogens. Some of these rapid methods have already been validated and accepted by international authorities as standard methods and have become increasingly popular among testing laboratories (Cheung & Kam, 2012). Although rapid methods are efficient, with many containing protocols that have already been validated, these methods are still used as presumptive tests. Moreover, in cases where a pathogen is detected, the conventional method must be used for confirmation (Jasson et al., 2010). The aim of this study was to evaluate two rapid methods and compare them with the conventional method to detect *Salmonella* in artisanal Minas cheese samples.

2. Materials and Methods

2.1 Microbiological Analysis

A total of 63 samples of artisanal Minas cheese from different manufacturers and with different maturation times were collected in the region of Serro, Minas Gerais, Brazil. The BAX®-PCR method from Qualicon DuPont, Wilmington, USA (AOAC Official Method 2003.09) and the immunoassay VIDAS®-SLM method from bioMérieux, Marcyl'Etoile, France (AOAC Official Method 2004.03) were compared with the conventional ISO 6579 method (2005), that include pre-enrichment, enrichment, selective and differential plating, biochemical and serological identification. The lactic acid bacteria were count on DeMan, Rugosa and Sharp (MRS) and total and thermotolerant coliforms was determined by Most Probable Number (MPN) (Downes & Ito, 2001). Additional tests, such as Gram stain and catalase, were conducted to confirm the lactic acid bacteria count. All tests were performed on an analytical unit of 25 g.

2.2 Physical-chemical Evaluation

Water activity (aw) was measured on an automated analyzer (Decagon Aqualab CX-2, Washington, USA). Determination of salt, pH and acidity was according to Richardson (1985).

2.3 Statistical Analysis

We evaluated the performance attributes of rapid methods such as sensitivity, specificity, accuracy, false positives, and false negatives. The analyses were performed using the Epi-Info software (1993). The following definitions were used:

$$\text{Sensitivity} = \frac{\text{N}^{\circ}\text{of samples positive by conventional method} - \text{false negative}}{\text{Total of positive samples for the conventional method}} \times 100\%$$

$$\text{Specificity} = \frac{\text{N}^{\circ}\text{of samples negative by conventional method} - \text{false positive}}{\text{Total of negative samples for the conventional method}} \times 100\%$$

$$\text{Accuracy} = \frac{\text{N}^{\circ}\text{of sample positive by conventional method} - \text{false negative} + \text{N}^{\circ}\text{of sample negative by conventional method-false positive}}{\text{Total of samples}} \times 100\%$$

3. Results and Discussion

The contamination of artisanal cheeses by *Salmonella* is commonly reported and its occurrence varies (Kousta, Mataragas, Skandamis, & Drosinos, 2010; Hall & French, 2011). In the present survey, *Salmonella* was not detected by the ISO 6579 or PCR-BAX® methods in all 63 artisanal Minas cheese analyzed. However, VIDAS®-SLM immunoassay method indicated two samples contamined with *Salmonella* and this represent 3.17% of false positives (Table 1). Regardless this result, VIDAS®-SLM showed high values of specificity and accuracy (Table 1). Both alternative methods PCR-BAX® and VIDAS® significantly decreased the time to obtain results compared to the standard ISO method and this is an important factor in the selection of an analytical method.

Reports on the comparison of performance between the PCR-BAX® and other analytical methods, included the immunoassay-based and the standard culture methods, for *Salmonella* detection are available. The PCR-BAX® method was able to detect low contamination by *Salmonella* sp. in cheese, orange juice and fish samples with a sensitivity of 76%, 95% and 98%, respectively (AOAC, 2005). According to Cheung and Kam (2012), the comparison between the BAX® system, the immunoassay and the standard culture methods, for *Salmonella* detection showed that the BAX® system has very low false-positive and false-negative rate and generated results comparable with those of the standard culture methods. Other studies have also reported results regarding the efficiency of PCR-based methods (Tapchaisri et al., 1999; Von Rückert et al., 2008, Prendergast et al., 2012; Mcguinness, Barry, & O'Grady, 2012).

Table 1. Comparison of conventional *versus* rapid methods for detection of the *Salmonella* sp. in samples units of Minas Traditional cheese

Number of sample	VIDAS®-SLM	PCR-BAX®
Total	63	63
Positive by conventional method	0	0
Positive by rapid method	2	0
Negative by conventional method	63	63
Negative by rapid method	61	63
False positive	2	0
False negative	0	0
Sensitivity (%)	Nd[*]	Nd
Specificity (%)	96.83	100
Accuracy (%)	96.83	100

Nd[*] = Not determined

Sensitivity = Number of samples positive by conventional method - false negative/Total of positive samples for the conventional method x 100

Specificity = Number of samples negative by conventional method - false positive/Total of negative samples for the conventional method x 100

Accuracy = (Number of samples positive by conventional method - false negative) + (Number of samples negative by conventional method - false positive)/Total of samples x 100

The sensitivity and specificity values observed for the VIDAS®-SLM method differ from those found in literature for other foods with low natural contamination rates, such as milk powder, raw pork, chocolate milk, casein (AOAC, 2005), and chicken carcasses (Von Rückert et al., 2008). A good correlation (97.5%) between a VIDAS®-SLM assay test and conventional culture technique were obtained when bulk tank milk and in-line milk filters were analyzed with the advantages of taking less time to obtain a negative or presumptive positive result (Walker, Kinde, Anderson, & Brown, 2001). Analysis of food surface using VIDAS®-SLM revealed lower false positives rates of 1.56% (Yeh, Tsai, Chen, & Liao, 2002). The occurrence of false positive results may depend on the specificity of the antibody, which may present cross-reactions with correlated antigens. However, a recent report on the use of VIDAS® for screening raw meat and by-products from pork and beef showed that the number of positive samples detected was two-fold higher than that by culture method (Meyer, Thiel, Ullrich, & Stolle, 2010).

The presence of coliform bacteria and high number of lactic acid bacteria in cheeses made from raw milk (Table 2) can impair the recovery of pathogens and compromise results. High microbial loading was expected in artisanal products that were manufacturing without the observation of good manufactured practices (GMP) and among this diverse contamination, some could promote the pathogens inhibition. In fact, De Medici et al. (1998) have observed that the presence of competing microbiota (6.0×10^6 CFU mL^{-1} of *Citrobacter freundii*) and low amounts (ranging from 1 to 2 CFU mL^{-1}) of *Salmonella* sp. generated false negative results by VIDAS®-ICS. Low number of *Salmonella* Enteritidis, ca 2.5 to 3.0 log CFU mL^{-1}, was detected in raw milk only when counting of endogenous microbiota was below 5.5 log CFU mL^{-1} (Nero, Matos, Barros, Beloti, & Franco, 2009a). *In vitro* antagonistic activity of natural microbiota isolated from raw milk or from Minas artisanal cheese against *Salmonella* sp. was demonstrated (Alexandre, Silva, Souza, & Santos, 2002; Nero et al., 2009b).

Table 2. Lactic acid bacteria and coliforms in Minas Traditional cheese

Endogenous microbiota	Log CFU or MPN g^{-1} (Mean ± SD)
Lactic acid bacteria (log CFU.g^{-1})	7.3 ± 0.9
Total coliform (log MPN.g^{-1})	2.2 ± 1.6
Thermotolerant coliform (log MPN.g^{-1})	1.6 ± 1.3

In addition to the endogenous microbiota of lactic bacteria and coliforms, the physicochemical properties of artisanal cheeses such as water activity, low pH, acidity and high percentages of NaCl (Table 3) can minimize pathogens growth, provide cellular injury and hamper the recovery strategies.

Table 3. Physical-chemical characteristics of Minas Traditional cheese

Physical-chemical parameters	Mean ± SD
Water activity (Aw)	0.886 ± 0.037
pH	5.607 ± 0.503
Titratable acidity (% lactic acid)	1.056 ± 0.330
Salt (% NaCl)	0.806 ± 0.315

The results obtained in this study using different methods indicated absence of *Salmonella* in samples of artisanal Minas cheese made with raw milk, even though much of producers did not attempt to GMP. However, attention should be paid to an inappropriate interpretation of these results, once it is necessary to evaluate if these methods allow maximally resuscitation and recover low numbers of sublethally injured cells. On the other hand, our results may contribute to further detailed evaluations and validations of alternative techniques with different food matrices before they could be accepted as cost-effective and standard methods for *Salmonella* testing in the future.

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