INTRODUCTION

Brazil is one of the largest milk producers, and ranks fifth in the world, with a production around 32,000 ton of milk per year (1).

The quality of milk is a subject of worldwide importance because it is a food of high nutritional value and is consumed by all populations, from newborns to the elderly. Thus milk and milk products intended for human consumption must be safe, without microbiological, physical or chemical contaminants (2).

Among the main contaminants of milk are toxic metals, mycotoxins, radionuclides, pesticide and veterinary drug residues (3,4).

Antibiotic contamination in milk occurs due to the misuse and/or fraudulent use of veterinary drugs in dairy cows. The milk obtained from animals still undergoing treatment must be discarded, and under no circumstances can it be used for consumption before the withdrawal period specific for each drug is reached (5).

Strict sanitary controls, including adequate feeding and good farming and production practices must be carried out on dairy farms, in order to reduce or even dispense with the use of these drugs (6). However, when the use is necessary, it is important to comply with the guidelines from the national supervisory bodies regarding their use for animals in lactation (7).

Several countries have stipulated Maximum Residue Limits (MRL) for the presence of different classes of antibiotics in milk, such as Beta-lactams, tetracyclines, sulfonamides, quinolones, aminoglycosides and chloramphenicol (8).

According to Brazilian legislation, antimicrobials should be used only when needed and in accordance with the Good Farming Practices. However, recent studies have reported AR contamination in milk marketed in Brazil (9-11).

This fact represents a serious public health problem because the presence of these contaminants above the MRL may trigger allergic processes, vomit, diarrhea, kidney problems, anemia and antimicrobial resistance (AMR), effects that are exacerbated in pregnant women, children and the elderly (12).

AMR refers to the capacity of microorganisms to resist antimicrobial treatments. The inappropriate use of antibiotics has been linked to the emergence and spread of microorganisms which are resistant to these antibiotics, rendering treatment of human diseases ineffective and posing a serious risk to public health (12, 13).

In the European Union, about 25,000 deaths per year...
are caused by resistant bacteria. Incorrect use of antibiotics in animals is considered one of the major causes of this resistance (14).

Another significant problem caused by the presence of antimicrobial residues in milk is undesirable effects on the production of dairy products. These residues can influence the quality of products by inhibiting fermentation by lactic acid bacteria in the production of yogurt, cheese and butter, causing serious economic problems for the dairy industry (15).

This work aimed to review studies published over the last 10 years about the occurrence of AR in milk marketed and consumed in Brazil. The "Maximum Residue Limit" prevailing in the country, the most commonly used methods for identification and quantification in milk and the results of published studies by authors and government agencies in recent years are discussed.

HAZARDS OF MILK CONTAMINATED WITH ANTIBIOTIC RESIDUES FOR HUMAN HEALTH AND IMPLICATIONS ON THE DAIRY INDUSTRY

Milk means the product from complete and uninterrupted hygienic milking of healthy, well fed and rested cows (16).

Milk and milk products are a rich and convenient source of nutrients for people in many countries and there is a significant international trade of milk-based commodities. It is an important component of a balanced diet, and is considered one of the world’s most complete foods and a rich source of proteins, vitamins and minerals, such as calcium, magnesium, phosphorus, potassium and zinc (17).

Normally when some disease is detected in dairy cows, particularly mastitis, regular doses of antimicrobials are given during the treatment. Antimicrobials, such as antibiotics, are substances used to kill micro-organisms or to stop them from growing and multiplying. They are commonly used in veterinary medicine to treat a wide variety of infectious diseases (14).

The Codex Alimentarius recommends that when dairy cows are treated with antibiotics, their milk should be discarded until the withdrawal period specified for each veterinary drug being used is reached; this can be days or even weeks (17).

The use of contaminated milk with antimicrobial residues is illegal and when intended of dairy products may interfere in the fermentation processes and, consequently, on the technological properties of the final products, causing economic losses to the industry.

When destined for human consumption it can cause severe allergic reactions, toxic and carcinogenic effects and induce bacterial resistance. A well known example of an bacterium that infects animals and humans and has acquired resistance to multiple antibiotics is Methicillin-resistant Staphylococcus aureus – MRSA (12, 13).

MRSA is considered to be a global public health problem that involves medical health care, veterinary medicine, agriculture and environment. Several recent studies have reported on the problems caused by consumption of contaminated milk and AMR in humans (12, 13).

The dairy farmer’s lack of knowledge concerning the negative effects of these drugs on human health contributes to aggravate such problems (18, 19).

However, it should be noted that not all antibiotics or other veterinary drugs used for animals confer risk to human health. What must be considered is the “Maximum Residue Limit”, which is defined, by the authorities, as the maximum concentration (in µg.L-1) of antibiotic residues in milk that bears no sanitary hazard for the consumer and has no negative effect on production processes. It is usually based on the Acceptable Daily Intake (ADI) for the consumer that is calculated from the NOEL (No Observable Effect Level) and a safety factor considering the toxicity, teratogenicity, mutagenicity, carcinogenicity and sensitizing potential for allergic reactions (immunotoxicity) of the substance (20).

The authorities consider there is no health hazard for the consumer and no effect on production processes below the MRL. The MRL for antibiotic residues in Brazilian milk is established by the Ministry of Health, using the Mercosul MRL as reference (21).

Table 1 shows the MRL for the main veterinary drugs permitted for the treatment of diseases in dairy cattle in Brazil. These limits were established in 2002 by the Ministry of Agriculture, Livestock and Food Supply (MAPA). When the substance registered for the specie in question has no MRL

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MRL (µg.L⁻¹)</th>
<th>Antibiotic</th>
<th>MRL (µg.L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlortetracycline</td>
<td></td>
<td>Flumequine</td>
<td>50</td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td></td>
<td>Cefotaxime</td>
<td>100</td>
</tr>
<tr>
<td>Tetracycline</td>
<td></td>
<td>Cloxacillin</td>
<td>30</td>
</tr>
<tr>
<td>Doxycycline</td>
<td></td>
<td>Dicloxacillin</td>
<td>30</td>
</tr>
<tr>
<td>Sulphathiazole</td>
<td></td>
<td>Ampicillin</td>
<td>4</td>
</tr>
<tr>
<td>Sulfamethazine</td>
<td>100</td>
<td>Amoxicillin</td>
<td>4</td>
</tr>
<tr>
<td>Sulfafoximethone</td>
<td></td>
<td>Oxacin</td>
<td>30</td>
</tr>
<tr>
<td>Sulfaclidine</td>
<td></td>
<td>Penicillin G</td>
<td>4</td>
</tr>
<tr>
<td>Sulfoxazole</td>
<td></td>
<td>Penicillin V</td>
<td>4</td>
</tr>
<tr>
<td>Sulfaclidine</td>
<td></td>
<td>Ciprofloxacine</td>
<td>100</td>
</tr>
<tr>
<td>Sulfaclidine</td>
<td></td>
<td>Enrofloxacine</td>
<td>100</td>
</tr>
<tr>
<td>Sulphathiazole</td>
<td></td>
<td>Chloramphenicol</td>
<td>0.30</td>
</tr>
</tbody>
</table>

MRL - Maximum Residue Limit. (22)
established, this value is equal 10 μg.kg-1 ou 10 μg.L-1 (22). The limits established by Brazilian legislation are similar to those set by the European Commission and can be considered adequate for public protection against AR contamination in milk (20).

As can be seen in table 1, the MRL for antibiotics in milk has concentrations of parts per billion (ppb or μg L-1), thus to determine the presence and quantifying the levels of these AR highly sensitive analytical methods are needed which must also be quick and convenient, are necessary.

METHODS OF IDENTIFICATION AND QUANTIFICATION

In Brazil, the control of residues and contaminants in milk is carried out by the Ministry of Agriculture, Livestock, and Supply - MAPA, through “The National Plan for Control of Residues and Contaminants” - PNCRC. This program aims to continuously assess samples of food products in general sold in the country.

The official methods of identification and quantification of AR used by the national reference laboratories are High Performance Liquid Chromatography with fluorescence detection (HPLC-FLD), HPLC coupled to mass spectrometry (HPLC-MS or HPLC-MS/MS) and the screening techniques based on immunoenzymatic reactions and microbial inhibition (8), as described below.

Methods based on microbial inhibition

Methods based on microbial inhibition are most commonly used for the qualitative evaluation of the AR in milk because they are fast and the results are obtained in hours; practical and a large numbers of samples can be analyzed simultaneously; and they are sensitive to different classes of antibiotics (23-25).

Among these methods, the most common are based on the inhibition of Bacillus stearothermophilus, in the presence of a pH indicator. If there is no AR in milk, the bacteria grows and increases the acidity of the medium, changing its color.

Other microorganisms can also be used for this purpose. Biacchi et al (26) used the inhibition method with Escherichia coli and Staphylococcus spp, and obtained results after 18 hours of incubation.

The sensitivity of these methods must be below the MRL established by national legislation so that any contaminated sample near these levels can be detected (5).

Positive samples can be confirmed using more specific methods. The methods described here are used in industry, when large amounts of milk need to be evaluated in a short time.

Immunoenzymatic tests

Immunoenzymatic assays as well as methods of inhibiting antimicrobials are also widely used by the dairy industry. They are sensitive to different groups of antimicrobials and the results are obtained in a few hours (5).

The principle of this method is based on the binding of antibiotics in milk with proteins conjugated to specific receptors fixed to a plastic unit, indicated by a color change in the reaction medium. It is a qualitative method that is considered more sensitive, faster and more specific than the microbial inhibition tests (9, 10).

To apply this method, as in microbial inhibition, the analyst must have a basic training and it does not require expensive equipment or specific installations in the industry.

Araujo (27) and Gomes (28) evaluated the effectiveness of several immunoenzymatic kits for the detection of different groups of AR in milk. They concluded that all the kits evaluated were suitable for this purpose and can be used as screening methods for monitoring substances in milk. However, the use of a positive and negative control and the exact temperature during the entire analysis are important points to obtain reliable results.

Gomes (28) observed that the acidity of the milk is also a critical point in these analyses because it can interfere in the results indicating false-positives. Therefore, it is very important to collect and store the samples under refrigeration until the moment of the test.

Folly et al (29) evaluated the efficiency of methodologies based on microbial inhibition, immunoenzymatic reaction and HPLC with a diode array detector for cloxacillin detection in milk. All methods identified the presence of the antibiotic; however, the advantage of using a chromatographic method is the possibility of identifying and quantifying the exact value of the antibiotic in the milk.

Chromatographic methods

In contrast to the previous methods mentioned above, the determination of AR in milk by chromatography is able to identify and quantify each antibiotic separately. These methods are more sensitive, precise and accurate, and therefore more reliable.

Simple techniques based on Thin Layer Chromatography (TLC) can be used for this purpose, with the advantage of being simple and having direct detection by the chromatographic profile obtained on the plate. This technique can be used for screening tests or direct quantification.

However, HPLC is considered more suitable to quantify with precision, sensitivity and accuracy the levels of AR in milk. Currently, several methodologies have been optimized and validated with this objective.

HPLC with reverse phase column, especially the C18 and Ultra Violet (HPLC-UV), diode array (HPLC-DAD) and fluorescence detector (HPLC-FLD) have been used to determine of a large number of antibiotics in milk samples, such as tetracycline, penicillin, ampicillin, sulfonamides and others (30-35).

HPLC can be coupled to a mass spectrometry system (HPLC-MS or HPLC-MS/MS), with excellent results to determine AR. After chromatographic separation the MS detects the antibiotic through its molecular weight thereby eliminating the necessity of a clean-up of the samples, and thus reducing the overall time of analysis.

The techniques based on HPLC-MS/MS fragment the molecule after their identification, generating a specific spectrum for each substance, which acts as a fingerprint for the AR, consequently, it is even more specific and reliable.

Thus, the main advantages of using HPLC methodologies are the absence of false-positive results and higher specificity of the analysis. However, to implement this methodology expensive equipment, specialized technicians, adequate facilities, equipment maintenance and frequent use of large volumes of solvents are needed.

Methods based on QuEChERS

The QuEchERS method (Quick, Easy, Cheap, Effective, Rugged and Safe) is considered very promising to determine AR in milk and has been studied by various researchers. QuEchERS was initially developed to examine pesticides in vegetables, proposed by Anastassiades et al (36). It is a
quick and easy method of extraction, economical because it uses a reduced amount of low toxic solvents and produces reliable results.

Since the advent of QuEChERS, many methods to determine residues and contaminants in food have been validated, including mycotoxins in food (37), pesticides in fruits (38), and veterinary drug residues in meat (39), among others.

Quantification after chromatographic separation is usually performed by FLD detection, UV, DAD or MS. HPLC or GC with MS/MS is the most common technique.

Recent research has been carried out in Brazil in order to validate simple techniques based on QuEChERS to determine AR in milk, demonstrating good analytical results and several advantages over conventional methods (40, 41).

When using these analytical methods to quantify AR in milk, mainly the techniques based on HPLC, GC, and MS, continuous validation is extremely important in order to have good analytical quality.

Thus, the main validation parameters considered are repeatability and reproducibility, recovery, limits of detection and quantification and specificity. The reference values for these parameters are determined by research bodies and their studies evaluate the suitability of the method for the purpose for which it was designed (42, 43).

OCCURRENCE OF AR IN MILK CONSUMED IN BRAZIL

The prevalence of subclinical mastitis in Brazilian bovine cattle is high and is frequently associated with the presence of the bacteria Staphylococcus spp and other groups like Streptococcus, Micrococcus, Bacillus and Enterobacter spp (44-46).

Many of these bacteria isolated from animals with mastitis have shown resistance to common antibiotics, which reinforces the concern about the possibility of resistant pathogens and AR being transmitted from animals to humans (13).

Over the past three years, the results of PNCRd to determine AR in Brazilian milk have demonstrated conformity of almost 100% of the evaluated samples in relation to the MRL set by the Ministry of Health. The results of the 521 samples collected in the years 2010, 2011 and 2012, showed disagreement in only one sample (0.2%), which was oxytetracycline detected in raw milk at a level corresponding to 981 µg.kg⁻¹ (22, 47,48).

Several authors have analyzed marketed milk for the presence of AR but found no positive results in various regions of the country (49-53).

On the other hand, other researchers have recently reported levels of AR contamination in samples of different types of milk. Oxytetracycline and penicillin G were the most

<table>
<thead>
<tr>
<th>Sample</th>
<th>State</th>
<th>Method of detection</th>
<th>Sampling n</th>
<th>Positive samples</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pasteurized milk</td>
<td>Paraná</td>
<td>Immunoenzymatic</td>
<td>79</td>
<td>19% (15)</td>
<td>(9)</td>
</tr>
<tr>
<td>Pasteurized milk</td>
<td>Bahia</td>
<td>HPLC-DAD</td>
<td>240</td>
<td>7.9% (19)</td>
<td>(54)</td>
</tr>
<tr>
<td>Pasteurized milk</td>
<td>Paraná</td>
<td>Immunoenzymatic</td>
<td>260</td>
<td>31% (80)</td>
<td>(10)</td>
</tr>
<tr>
<td>Pasteurized milk</td>
<td>Rio de Janeiro</td>
<td>Immunoenzymatic</td>
<td>57</td>
<td>65% (37)</td>
<td>(11)</td>
</tr>
<tr>
<td>UHT milk</td>
<td>Various</td>
<td>Microbial inhibition (Streptococcus thermophilus and Lactobacillus delbrueckii)</td>
<td>100</td>
<td>4% (4)</td>
<td>(23)</td>
</tr>
<tr>
<td>Different types</td>
<td>Rio de Janeiro</td>
<td>Microbial inhibition (B. stearothermophilus)</td>
<td>175</td>
<td>1.1% (2)</td>
<td>(24)</td>
</tr>
<tr>
<td>Organic raw milk</td>
<td>São Paulo</td>
<td>Microbial inhibition (B. stearothermophilus)</td>
<td>148</td>
<td>2.7% (4)</td>
<td>(25)</td>
</tr>
<tr>
<td>Pasteurized milk</td>
<td>Paraná</td>
<td>Immunoenzymatic</td>
<td>151</td>
<td>2.6% (4)</td>
<td>(55)</td>
</tr>
<tr>
<td>Raw milk</td>
<td>Various</td>
<td>Microbial inhibition (B. stearothermophilus)</td>
<td>210</td>
<td>11.4% (24)</td>
<td>(56)</td>
</tr>
<tr>
<td>Different types</td>
<td>São Paulo</td>
<td>Microbial inhibition (B. stearothermophilus)</td>
<td>1500</td>
<td>0.66% (10)</td>
<td>(57)</td>
</tr>
<tr>
<td>Different types</td>
<td>São Paulo</td>
<td>Microbial inhibition (E. coli e S.aureus)</td>
<td>100</td>
<td>45% (45)</td>
<td>(26)</td>
</tr>
<tr>
<td>Raw milk</td>
<td>Various</td>
<td>Microbial inhibition (B. stearothermophilus)</td>
<td>209</td>
<td>11.5% (24)</td>
<td>(58)</td>
</tr>
<tr>
<td>Pasteurized milk</td>
<td>RJ</td>
<td>HPLC-DAD</td>
<td>43</td>
<td>37.21% (16)</td>
<td>(59)</td>
</tr>
<tr>
<td>Raw milk</td>
<td>MG</td>
<td>Immunoenzymatic</td>
<td>158</td>
<td>1.89% (3)</td>
<td>(60)</td>
</tr>
<tr>
<td>Raw milk</td>
<td>Pará</td>
<td>Immunoenzymatic and microbial inhibition</td>
<td>103</td>
<td>10.68% (11)</td>
<td>(61)</td>
</tr>
</tbody>
</table>
frequent, and these are commonly used for the treatment of bovine mastitis (9, 19). Table 2 shows the results of the main studies concerning AR in milk that were published in specialized journals over the last 10 years.

Considering all samples from the studies evaluated in this review (n= 3,723 samples) including those without any positive samples, it is estimated that the occurrence of AR contamination in Brazilian milk is approximately 8%. This value indicates that the withdrawal period established after the application of the antibiotics is not being respected.

Korb et al (19) reported that the use of antibiotics to treat diseases of dairy cattle without a prescription from a veterinary professional at small farms is still common. This fact, together with the ease of obtaining these drugs in veterinary stores contributes to aggravate this situation and represents a risk to the consumers.

Another worrying fact is the sale of milk not inspected by federal, state or local bodies, which is still present in Brazilian municipalities. According to Martin (21), the Brazilian surveillance system still lacks efficiency and this author suggested that further discussions on these subjects should be conducted in an attempt to create conditions so that all milk production respects the current regulations in the country.

Concomitant with the trend of increasing awareness of Brazilian dairy farmers about the correct use of antibiotics in dairy cattle, there is a gradual decrease of MRL, requiring continuous implementation of quality management programs, especially those intended for milk production.

Also the development of analytical methods that are capable of identifying and quantifying the levels of each antimicrobial in milk are needed. These methodologies, mainly based on chromatographic and spectrometric techniques have been little explored in the current studies, with a predominance of techniques only based on “positivity or absence” of such substances through rapid detection kits. However, these kits do not provide sufficient information for a complete understanding of AR contamination.

Currently in Brazil, there is a tendency to increase the consumption of organic milk, which is free of AR because these drugs can not be used to treat the animals (21). In 2010 the national production of organic milk was about 5.5 million liters, which represents a small portion (<1%) of the total sale of Brazilian milk (16). Even so, this milk must be constantly evaluated by the supervisory bodies and researchers for the presence of AR, since this contamination has also been reported in a recent study (25).

CONCLUSION

The incidence of AR contamination in milk consumed and marketed in Brazil is high. The studies published in the last decade indicate that about 8% of the milk marketed in the country presents levels higher than that permitted by current legislation. Although the Brazilian regulatory limit is considered adequate for the protection of consumers more stringency in audits and the implementation of quality management programs, principally for the awareness and technical training of the dairy producer about the problems resulting from the inappropriate use of antibiotics, are needed. The free sale and distribution of these drugs without a veterinarian prescription should also be discussed by the competent authorities in order to implement better control of these antibiotics. In terms of the various methods to determine AR, the use of rapid detection kits based on qualitative results still predominates in the research works carried out in the country. The use of such techniques is important for screening, however, more studies are needed for the development and validation of methodologies based on quantitative techniques, particularly chromatographic and spectrometric, allowing accurate identification and quantification of each antibiotic. Research institutions and universities must be encouraged to produce more studies involving this theme, since only a few reports have been published concerning this theme in scientific journals in recent years.

RESUMEN

Los antibióticos se utilizan ampliamente para el tratamiento de las vacas lecheras y los residuos de estos medicamentos pueden permanecer en la leche y los productos lácteos, los que pueden ser una amenaza potencial para la salud humana. La exposición a bajos niveles de antibióticos se considera un problema de salud pública ya que pueden resultar en el desarrollo de cepas resistentes de bacterias humanos. De acuerdo con la legislación brasileña, los antimicrobianos deben ser utilizados de acuerdo con las Buenas Prácticas Agrícolas. Sin embargo, estudios recientes han informado de la contaminación de la leche comercializada en el país. Este trabajo tuvo como objetivo revisar los estudios publicados en los últimos 10 años, que describen residuos de antimicrobianos en la leche comercializada en Brasil. Se discuten los límites máximos de residuos, los métodos para la cuantificación y los resultados de los estudios publicados por autores y agencias gubernamentales.

Palabras clave: Antimicrobianos, límite máximo para residuos, seguridad alimentaria. calidad de la leche.

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