A review on the most frequently used methods to detect antibiotic residues in bovine raw milk

Review

Methods to identify antibiotic residues in bovine raw milk

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Abstract

Bovine milk is a low cost and high nutritional value product, which is worldwide commercialized. To maintain high standard levels and to preserve consumer's health, tests could be used to identify antimicrobial drug residues, and control procedures must be planned and performed, according to legislative requirements. Ideally, a test should detect a large number of antibiotic molecules, should be rapid in the execution and not expensive. None of the actually available methods possesses the characteristics of the ideal test. Authors decided to write this review to resume the key features of immunoassays, high-performance liquid chromatography, liquid chromatography – tandem mass spectrometry, microbiological test, and biosensor assays, representative of the most used methods to detect antimicrobial residues in bovine raw milk. The final aim was to give essential information to veterinary practitioners and researchers who are engaged in on site screenings, official controls or in research.

Keywords: Analytical methods, Antimicrobials, Bovine milk, Residues, Screening test

1. Introduction

Bovine milk is one of the most consumed foods in the world and it is important for its high nutritional value and for its key role in the worldwide economy (FAO, 2019). Its composition comprehends proteins (3.0-3.9%), carbohydrates lactose and oligosaccharides (4.4-5.6%), fat (3.3-5.4%), and ash (0.7-0.8%) (Roy, Ye, Moughan, & Singh, 2020). Milk is an important dietary source of a variety of micronutrients, including calcium, phosphorus, magnesium, zinc, iodine, potassium, vitamin A, vitamin D, vitamin B12, and vitamin B2 (Dror & Allen, 2014). This variety of components is responsible for biochemical and nutritional features, and the derived energy is mainly due to the high presence of lactose fraction (Luiz, Bell, Rocha, Leal, & Anjos, 2018; Roy, et al., 2020).

One of the most important concerns about bovine milk is the contamination with veterinary medicine: antibiotics and anti-inflammatory drugs are administered to dairy cows to treat pathologies such as mastitis, endometritis, bronchopathies, pneumonia and lameness (EMA and EFSA, 2017; Han et al., 2015). The treatment of animals is mandatory to respect animal welfare but must be performed in a rational way: focusing on antibiotics, a targeted therapy with narrow spectrum molecules should be preferred, for the shortest time necessary to achieve a therapeutic outcome (Rajala-Schultz, Nødtvedt, Halasa, & Persson Waller, 2021). In the past, antibiotics were employed not only for the sole therapeutic purpose, but also to promote growth of food-producing animals and in metaphylactic protocols (Lees, Pelligand, Giraud, & Toutain, 2021). Without a stringent regulation, an overuse, or a misuse of antibiotics was diffused and, consequently, foodstuff contamination due to antibiotics and antibiotic residues was not unusual (Lees et al., 2021). The interest of the scientific community pushed several nations to change legislation to restrict antibiotic drug usage and preserve consumers' health (Lees et al., 2021; Luiz et al., 2018). European legislation (Regulation EU 2019/6) confirmed the ban of these substances as growth promoting agents and severely restricted prophylactic and metaphylactic uses. Recently, USA and China banned the use of antimicrobial drugs as growth promoting agents, as well (Lees et al., 2021).

Antibiotic residues might persist after the treatment and therefore, a withdrawal period is mandatory to ensure that the foodstuff derived from the treated animal do not contain levels of the drug that exceed the maximum residue limit (MRL) (Almashhadany, 2021; Jayalakshmi, Paramasivam, Sasikala, Tamilam, & Sumithra, 2017).

As defined by the European Union, a residue is a pharmacologically active substance, the parental drug, or its metabolites, which remains in foodstuff obtained from animals to which a medicinal product was administered (Regulation EC 470/2009; Regulation EU 37/2010). For each active substance, a MRL value has been established and is a precautionary value, which does not guarantee the absence of drug residues in milk or dairy products, thus adverse reactions in consumers are not excluded and might occur (Treiber & Beranek-Knauer, 2021). The MRLs established for antibiotics frequently used in dairy cows are summarized in Table 1.

Antibiotic residues from food can impair human health causing allergic reactions, mutations in cells, development of intestinal microbiome imbalances, and permitting to bacteria to get used to subtherapeutic concentrations of antibiotic drug and leading to the development and spread of AMR (Sachi, Ferdous, Sikder, & Azizul Karim Hussani, 2019; Treiber & Beranek-Knauer, 2021; Yazdanpanah et al., 2021). The direct consequences are complicated therapeutic outcomes, treatment failure, or the possibility to share and diffuse resistant pathogen microorganisms among animals and humans (Vercelli, Gambino, Amadori, & Re, 2022).

The control of milk to adhere to law requirements, the risks to share AMR through the worldwide consumption of milk and the preservation of consumers' health, are elements of a complicated network and they are all related each other (Ortelli, Cognard, Jan, & Edder, 2009). Ideally, a screening test should detect a large number of molecules in the same assay, should not be

time- and money–consuming and should be easy in the execution, even if performed by not specialized personnel. Nowadays several tests are commercially available or are under investigation to evaluate their possible use on site or in laboratory conditions, as confirmatory tests or for research purposes. Unfortunately, none of them possesses the characteristics of the ideal test. Thus, prior to use a test to detect the presence of antibiotic residues, advantages and disadvantages should be carefully evaluated to reach the most reliable result. Moreover, milk is a complex matrix, composed by several elements that may interfere with residues identification: purification, dilution and preparation of samples or specific storage conditions might be required (Serraino et al., 2013). Additionally, the purposes of the tests might be different: some tests are easy and fast in their execution and suitable for screening purposes but necessitate confirmation methods in case of positivity, leading to delay in obtaining result and to increased costs (Bilandžić et al., 2011; Burke & Adley, 2021). Regardless of the final use, test validation is mandatory accordingly to Commission Decision 2002/657/EC, concerning the performances of analytical methods and interpretation of results, thus establishing the criteria of linearity, sensitivity, specificity, intra- and inter-assay precision, and accuracy (2002/657/EC).

Considering all aforementioned factors, authors would like to summarize in the present review the most important methods that are currently used to identify antibiotic residues in bovine raw milk.

2. Current available methods to detect antibiotics residues in milk

2.1 Immunoassays

Enzyme-Linked Immunosorbent Assay (ELISA) technique was first described in 1971 by Engvall et al. (1971) and found multiple applications ranging from medical to biotechnological purposes. The method is based on an antigen-antibody linkage that produces a colorimetric reaction due to the presence of a chromophore linked to the antigen. The ELISA technique may be designed as qualitative (positive/negative result) or quantitative (result as measurement or concentration). Also,

a semiquantitative test exists, giving different levels of positivity and negativity that must always be compared to a reference scale (Belmar, Aly, Karle, & Pereira, 2019). Several ELISA-based kits are commercially available and used to rapidly detect a few families of antibiotics in bovine milk: due to the easy execution and the low cost, they can be performed as screening tests by veterinarians and dairy industry personnel (Belmar et al., 2019) (Table 2).

Anyway, in case of positivity and official controls, a confirmation test is necessary, using more specific and sensitive tests, such as high-performance liquid chromatography (HPLC) or Liquid Chromatography-Tandem Mass Spectrometry - LC-MS/MS) (Belmar et al., 2019). These two methods will be described in further paragraphs. In fact, the limit of detection (LOD – the lowest amount of analyte that can be identify in a sample) and the limit of quantitation (LOQ) (lowest amount of analyte in a sample that can be quantitatively determined) of immunoassay – based techniques are proven to be higher of that of analytical methods, such as HPLC or LC-MS/MS (Ahmed et al., 2020; Moudgil, Bedi, Aulakh, Gill, & Kumar, 2019).

The ELISA method was a milestone to develop new detection techniques such as a biochip-based test, which has been recently validated and seems to represent a significant improvement of the actual screening methods, considering the relative low-cost and rapid execution, even if it is able to detect only β-lactams (Yazdanpanah et al., 2021). Taking inspiration from antigen-antibody interaction, a new technique based on a magnetic immunoreaction has been developed to detect kanamycin and penicillin in dairy milk, demonstrating an extremely low LOD and a high sensitivity, but pretreatment and dilution steps of samples are necessary. This method is a proof-of-concept, not yet on the market (Pietschmann, Dittmann, Spiegel, Krause, & Schröper, 2020). A microarray assay ELISA- based has been recently validated: it can detect simultaneously norfloxacin, tetracycline, lincomycin and streptomycin in milk samples, reaching a remarkably high accuracy rate (ranging

132 from 77,6% to 116,4% of the different antibiotics), but having the disadvantage to require specialized
133 and trained personnel. So far, this method is suggested for control programs in the dairy industry (Du
134 et al., 2019).
135
136 A commercially available immunoassay method is Lateral Flow Immunoassay (LFIA), that
137 compared to ELISA is less time – consuming (easier samples preparation), with fast results
138 acquisition, permits to analyze a large quantity of samples, and it is less expensive (Ahmed et al.,
139 2020, Jiang et al., 2022). In the last few years, this method has been widely used to detect antibiotics
140 in milk, such as β-lactams, tetracyclines, streptomycin and chloramphenicol and it has been recently
141 implemented also for other matrices, such as honey, muscle, and liver (Ahmed et al., 2020).
142
143 Among immunoassays also radioimmunoassay (RIA), fluorescence immunoassay (FIA) and
144 colloidal gold immune-chromatographic assay (CGIA) must be listed (Ahmed et al., 2020).
145 Radioimmunoassays have been widely used in the past to detect antibiotic residues in foodstuff due
146 to its fast execution and low LOD (Yang & Carlson, 2004), but the short half-life of the radioisotopes
147 used to label the analyte, and the possible risks for the personnel and the environment connected to
148 the use of radioisotopes limited its application to a few clinical purposes (Ahmed et al., 2020).
149 Fluorescence immunoassay is based on the linkage of a fluorophore to the antigen, giving a precise
150 and well visualized detection, but background signal can interfere with the emission thus giving
151 ambiguous results (Ahmed et al., 2020). This specific immunoassay has been extensively used to
152 detect fluoroquinolones (Hu, Sheng, Zhang, Wu, & Wang, 2015), beta lactamases (Benito-Peña,
153 Moreno-Bondi, Orellana, Maquieira, & van Amerongen, 2005) and tetracyclines (Song et al., 2015)
154 in milk. Colloidal gold immune-chromatographic assay has been validated and used to detect
155 tetracycline, sulphonamides, and fluoroquinolones residues rapidly and simultaneously in milk
156 (Wang et al., 2017) but the scarce precision of the method leads in some cases to unclear results that
157 limited its application (Zhou et al., 2018).

158

159 *2.2 High-Performance Liquid Chromatography (HPLC)*

160 As previously explained, rapid tests are such as ELISA and other immunoassays are used as
161 first line, in-field screening methods and in case of positivity, the results must be confirmed using
162 more accurate methods such as HPLC or LC-MS/MS (Bilandžić et al., 2011).

163

164 The HPLC is a chromatographic technique that requires specific and expensive instruments,
165 time-consuming procedures and must be performed by trained personnel (Table 2). It is considered a
166 gold standard mehod to detect antibiotic residues due to the high sensitivity, specificity, and
167 quantification capability (Parmar, Chaubey, Gupta, & Bharath, 2021). The HPLC instrument is
168 composed of 5 major components: mobile phase, detector, pump, column, and sampler (manual or
169 automatic) (Fig. 1). Samples are injected and carried by the mobile phase flow through the column
170 where separation of compounds occurs according to their differential affinity for the mobile phase.
171 The pump generates optimal flow and pressure and pushes the mobile phase through the column that
172 reaches the detector: a signal is generated, and it is proportional to the amount of the compounds in
173 the sample (Parmar et al., 2021). Comparing the peaks resulting from the analysis to the calibration
174 curve (that is the reference), it is possible to identify the compound that has been isolated and to
175 calculate its concentration in the sample.

176

177 It has been validated the use of HPLC to detect tetracycline, sulfonamides and amphenicols
178 in milk (Darko, Borquaye, Acheampong, & Oppong, 2017) but it is sometimes very challenging to
179 adequately prepare and purify milk sample prior performing the procedure: fat, protein and sugars
180 present in milk can compromise the correct identification of residues (Luiz et al., 2018; Roy et al.,
181 2020; Yang, Guo, Liang, Zhou, & Zhu, 2022). The problems due to the matrix effect are reported in
182 the most recent literature related to innovative technique based on ultraviolet/visible spectroscopy
183 that has been validated to simultaneously identify multiple residues in milk (Parmar et al., 2021).

Moreover, results can vary according to pH and temperature, leading to alteration of some analytes that are not stable, such as tetracycline, leading to an incorrect or missing identification of antimicrobial residues in milk (Kurjogi et al., 2019).

A recent paper (Kumar, Panda, & Sharma, 2022) described the optimization and validation of HPLC-diode array detection (HPLC-DAD) method for the simultaneous determination of seven antimicrobial drugs: sulphadiazine, sulphamethoxazole, oxytetracycline, doxycycline, tetracycline, enrofloxacin and chloramphenicol residues in bovine milk. The final aim of this advances HPLC technique is to improve the features of the method, allowing multiple identifications and saving time. Samples preparation is a fundamental step and authors explained that solid-phase extraction (SPE) was performed to eliminate proteins from milk samples. This highlights once again the importance of a careful sample preparation prior the execution of analysis. This advanced technique was successfully validated in accordance with European Commission Decision 2002/657/EC (2002/657/EC) and applied for the analysis of antibiotic residues in 21 raw milk samples obtaining recovery values ranging from 83.3 to 111.8% (3.5-16.2% standard deviation). The LOQ values relative to all tested antibiotics, except for chloramphenicol, were below the MRLs, making the method dependable and susceptible for further implementation for routine application (Kumar et al., 2022).

2.3 Liquid Chromatography -Tandem Mass Spectrometry (LC-MS/MS)

Liquid Chromatography-Tandem Mass Spectrometry is an advanced analytical technique characterized by a high performance in identifying and quantifying antimicrobials, their metabolites or residues present in various foodstuff, also in milk, with remarkably high accuracy and precision (Parmar et al., 2021). Sample constituents eluted from the column are ionized and vaporized, subjected to fragmentation, and separated according to the mass-to-charge ratio (M/Z) in the mass

analyzer. The abundance or intensity of each ion with a different M/Z value is measured by the detector and it is proportional to the concentration of the analyte in the sample (Fig. 2).

When an MS/MS is used the "first MS" is set to select a parent ion (the M/Z value is most often the molecular ion of the substance), which then is passed into the "second MS" where it undergoes further fragmentation (Parmar et al., 2021). Ions are usually extremely specific to a given substance, and therefore their analysis provides an exceptionally reliable identification and quantification (Cronly et al., 2010; Zhao, Zulkoski, & Mastovska, 2017). This technique is extremely accurate (more than HPLC), and it is intended as high standard and confirmation tool. The main disadvantages of this technique are the high cost, the long execution, and the need to work in standardized experimental conditions (i.e., control ionization, pH, analyte stability) (Zhao et al., 2017). Also in this case, the complexity of milk composition may lead to misleading results and recent papers underlined that a reliable sample preparation is fundamental before LC-MS/MS analysis (Meklati et al., 2022; Zhao et al., 2017). The undoubted advantages are the simultaneous detection of multiple residues of different antibiotic classes and the very high analytical accuracy: this is ensured by the fact that structural information of the analyte is gained permitting the identification and quantification of very small amounts, having lower LOD and LOQ compared to HPLC (Parmar et al., 2021).

An advanced technique that has been successfully applied to detect antimicrobial residues is ultra-high performance liquid chromatography (UPLC), used alone (Castillo-Aguirre, Cañas, Honda, & Richter, 2021; Rahman, Hassan, & Chowdhury, 2021) or coupled with electrospray ionization and tandem mass spectrometry (UPLC–ESI–MS/MS) (Castillo-Aguirre et al., 2021; Luiz et al., 2018; Meklati et al., 2022). The method proposed by Igualada, Giraldo, Font, & Yusà (2022) aimed to identify 255 veterinary drug residues and contaminants in bovine raw milk. The procedure included two-step precipitation and ultra-performance liquid chromatography, operating both in positive and

235 negative multiple reaction mode (MRM). For most of the analytes, pretreatment process was a crucial
236 step to ensure a successful identification.
237
238 A study specifically focused to simultaneously detect 38 veterinary antibiotic residues in raw
239 milk was recently performed using UPLC–MS/MS (Han et al., 2015). The methodology differs from
240 others present in literature due to a purification of samples by Oasis HLB cartridge and a dilution
241 with water and acetonitrile (1mL sample; 0.5 mL water; 3 mL acetonitrile), before the injection into
242 the UPLC–MS/MS system. The results indicated variable recoveries of 68–118% for drugs belonging
243 to β-lactams groups, 79–118% for quinolones, 71–106% for sulfonamides, 76–116% for
244 tetracyclines, 78–106% for macrolides, and 88–103% for lincosamides, with coefficients of variation
245 less than 15% (Han et al., 2015).
246
247 *2.4 Microbiological tests*
248 Microbiological tests have been used for a long time as screening tests to detect residues in
249 milk: they are still used with significant improvements (Sachi et al., 2019). No sample preparation is
250 needed, and a milk sample is considered positive when an inhibition zone appears in the reference
251 bacterial cultures. The diameters of the inhibition zone must be measured to interpret the results
252 (Gaudin et al., 2004). This technique has a low specificity that might lead to incorrect results: high
253 somatic cells or pH variations in case of mastitis, may lead to false negative results (Wu et al., 2019).
254 Nevertheless, this kind of method continues to be the focus of several investigations to improve the
255 current method due to the brief time of execution (Table 2) and to the versability with various products
256 of animal origin, i.e., eggs and honey and raw, pasteurized, and bulk milk (Wu et al., 2019).
257
258 Advancement of this technique permitted to design the use of specific bacteria, sensitive to
259 particular antibiotics on agar gel including nutrients for bacterial growth and a pH indicator (Tumini,
260 Nagel, & Althaus, 2019). All elements are set up in a test tube where milk is added and then the tube

is incubated at the appropriate temperature to allow bacteria grow. Without antibiotic residues, the normal growth of bacteria is unaltered and visually detectable, appreciating a change of opacity of the medium and of the color due to acid pH. Otherwise, the growth of bacteria is inhibited, without observable changes (Nagel, Molina, & Althaus, 2013). Nowadays several kits based on this technique are commercially available. Anyway, few families of antibiotics can be identified using this kind of methods, and it is not possible to obtain a quantification of residues amount. Considering two of the most popular kits, Delvotest ST-NP can detect β-lactams, aminoglycosides, macrolides, sulphonamides, tetracyclines and diamino pyrimidine, while Charm Quad 1 test identifies only β-lactams, quinolones, sulfonamides and tetracyclines. Both tests claim in their datasheet the possibility to identify the presence of residues corresponding at least to MRL values, and both declare 95% of sensitivity (Charm QUAD1 datasheet; Delvotest datasheet;).

Low costs and simple execution are the main pros of this technique, while limitations could be the relative long incubation period (3-24 hours), the possibility of acquisition of antimicrobial resistance in the bacteria used for the detection, the subjective interpretation, the presence of inhibitors in abnormal milk (e.g., mastitis milk or colostrum) leading to misinterpretations (Tumini et al., 2019; Wu et al., 2019). The interest of the scientific community is to develop and validate new kits based on this method that can permit a qualitative identification of antibiotic residues even at concentrations lower than MRL (Gaudin et al., 2004; Tumini et al., 2019; Wu et al., 2019).

2.5 Other methods

A methodology based on Fourier transform near-infrared (FT-NIR) spectroscopy associated with principal component analysis (PCA) permitted to develop a portable prototype to detect ceftiofur hydrochloride traces in bovine milk in a fast and accurate way (Luiz et al., 2018). The method permitted to identify the parental molecule and the metabolites (Luiz et al., 2018).

Another promising technique that has been validated in the last years and that is continuously improved is the method based on the biosensors (Kivirand, Kagan, & Rinken, 2015). Several current methods of analysis are based on this technique, which does not require preliminary preparation of samples, enabling a selective detection for the on-site assessment of milk quality (Martins et al., 2019; Tumini et al., 2019). Biosensors are compact and usually portable devices, able to transfer the selective biochemical recognition into a measurable physical signal for real-time analysis, user-friendly, not requiring specific skills (Kivirand et al., 2015). Five distinct types of biosensor–based techniques are nowadays available: microbial biosensor, immunosensor, receptor and enzyme-based biosensors, aptasensors and molecularly imprinted polymer (MIP) sensors (Babington, Matas, Marco, & Galve, 2012; Beltrán, Berruga, Molina, Althaus, & Molina, 2015; Rebe Raz, Bremer, Haasnoot, & Norde, 2009). The most diffuse are immunosensors and aptasensors, that allow to perform the analysis without an extensive pretreatment of samples and in a brief time (30-40 minutes) (McGrath, Elliott, & Fodey, 2012; Reder-Christ & Bendas, 2011).

3. Discussion

In this review, authors aimed to summarize the current available methods to identify antibiotic residues in bovine raw milk, giving some simple explanations about the most notable features, a brief description of how they work, highlighting advantages and disadvantages. Authors are veterinarians having clear in mind the necessity to treat diseased animals, to apply reliable methods to correctly identify antimicrobial residues in milk, to preserve consumers' health, to limit AMR spread and to avoid alterations of dairy products (Burke & Adley, 2021; Kneebone, Tsang, & Townson, 2010).

The necessity to correctly identify antibiotic residues is only the end of a long story. Among the different pathologies that can affect dairy cows, mastitis are surely the most painful and debilitating conditions, impairing animal welfare and requiring immediate cure using anti-inflammatory drugs and antibiotics. The administration of a therapy is necessary to limit economic

losses due to the reduction in milk production and the discard of milk (Alves et al., 2020). Diagnosis of causative agent is often missing, and therapeutic protocols are applied in an empiric way leading to an increased risk to induce antibiotic resistance in bacteria (Vercelli et al., 2022). The presence of small amounts of antibiotic or residue in milk could play a significant role in the outbreak of allergic phenomena and in the dissemination of resistant bacteria or genes (Alves et al., 2020; Treiber & Beranek-Knauer, 2021).

The different methods differ each other for the expensiveness and the time-consuming procedure, the necessity to prepare or purify the samples, and the interference of matrix effect: all these parameters can affect specificity, sensitivity, and accuracy of each method (Alves et al., 2020; Kurjogi et al., 2019). Along the manuscript, authors highlighted that despite many advances on the detection techniques, sample preparation remains a problem influencing the determination of antibiotics in milk and interfering in analytical procedures (Aguilera-Luiz, Martínez Vidal, Romero-González, & Garrido Frenich, 2012). For the majority of the aformentioned techniques, the high protein and fat content in milk are the main responsible of this interference. The extraction methods are often based on long and tedious procedures, involving liquid–liquid extraction (García-Mayor, Garcinuño, Fernández-Hernando, & Durand-Alegría, 2006) or SPE (Darko et al., 2017) which also include a previous step to precipitate proteins.

In recent years, the method named QuEChERS (quick, easy, cheap, effective, rugged and safe) has been investigated and now it is commercially available (Zhang et al., 2019). The method is based on an acetonitrile extraction/partitioning of various compounds, while water and proteins are removed from the sample using sodium chloride and magnesium sulphate. Then, SPE clean uppermit the extraction of contaminats and antimicrobials residues that remain in the supernatant part after the centrifugation (Zhang et al., 2019). It is a rapid and reliable extraction system, flexible to different

situations and that can permit to achieve optimal results also in official controls for milk quality (Zhang et al., 2019).

Misleading results can occur with every methodology presented in the present review and this is the reason authors stressed the explanations about the rigorous preparation of the samples, the accuracy of the methods and the complexity of milk as matrix. There, the importance to use reliable tests and to obtain relevant results and to preserve consumers' health, especially fragile people, to maintain high standard levels in milk production and in dairy industry, limiting AMR spread.

4. Conclusions

Prior to write this review, Authors had to face the vast panorama of different methods designed to identify antimicrobial residues in bovine raw milk. After a careful consultation and selection of the most recent and relevant literature, they were able to summarize the advantages and disadvantages of the most important, dependable, and frequently used methods. It might be concluded that screening tests are mainly represented by microbiological, immuno- and biosensors assays. Among these three categories, several tools are available and significant differences of cost exist while all are characterized by a short time of execution. All of them require a confirmation test in case of positivity using a validated gold standard method (i.e., HPLC or LC-MS/MS).

Authors hope to have clearly exposed and successfully resumed such a complex topic and that this review will help other veterinarians and researchers dealing with antibiotic residues in bovine raw milk.

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