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Abstract. The presence of beta-lactam antibiotics in milk as raw material for other dairy products is prohibited by legal regulations. The research on the presence of beta-lactam antibiotics in milk was done during the six months (January – June) 2021, including 181 milk samples where 1 sample represented about 3000 liters of milk from an average of 60 farms per day. The analysis of the presence of beta-lactam antibiotics in the researched samples was carried out through the ROSA Pearl Reader Charm test. It turns out that the results of residues of beta-lactam antibiotics in fresh milk samples range from a low value of 0.014 µg/kg in May to a high value of 2.729 µg/kg in April and with the general average for all samples during the 6 months of research of 1.460 µg/kg. Since the European and Kosovo regulations refer to the maximum values of 4 µg/kg beta-lactam antibiotic residues, we can say that the results obtained from the 181 milk samples researched during the January-June period are below these values, which indicates that all values, even the highest of. 2.792 µg/kg, resulting negative in beta-lactam residues. As noted, the willingness of collection points and milk processing factories to carry out the test in milk for residues of beta-lactam antibiotic residues has led farmers to deliver milk without antibiotic residues, either because of their increased awareness of the damage that such waste brings to health, either because of the fear of punitive sanctions. Based on the results obtained during this research as well as on other potentiated factors, we can say that the milk which is accepted at the collection points and milk processing factories in Kosovo, is safe for wide consumption.

Keywords: Milk, beta- lactams, antibiotics, residues, results.

1. Introduction

If the milk is used for immediate consumption or as a raw material for the production of milk products, it must be organoleptically, physically, chemically, and microbiologically regular and not contain any harmful prohibited substances. [1],[2] Among the many prohibited substances in milk, antibiotic residues are the most common artificial inhibitory substances in cow's milk with a negative impact on human health, processing, and quality of the milk. [3] Antibiotic residues in milk can be a concern, as they may result from the use of antibiotics in dairy farming to treat and prevent diseases in cows. Farmers are required to adhere to withdrawal periods, which are specific waiting periods after administering antibiotics to dairy cows. During this time, the cow's milk is not collected for human consumption to allow the antibiotics to be metabolized and eliminated from the cow's system. This practice helps minimize the risk of antibiotic residues in milk. The presence of antibiotics in milk can pose several risks and challenges to the dairy industry. These risks can affect the industry's reputation, economic sustainability, and public health. The detection of antibiotic residues in milk also can lead to financial losses for dairy farmers and processors. When antibiotic-contaminated milk is identified, it may need to be discarded, resulting in economic losses for the producer. [4] The presence of antibiotic residues can damage the reputation of both dairy farms and the dairy industry as a whole. Consumers may lose trust in dairy products if they perceive that antibiotics are being used irresponsibly or if they believe that milk may contain antibiotic residues. [5] Antibiotic residues in milk can raise concerns about the potential health effects on consumers, including allergies and the development of antibiotic resistance. Public perception of the safety and quality of dairy products can be negatively impacted when antibiotic residues are detected. [6] Repeated instances of antibiotic residues in milk can erode consumer confidence in dairy products. This loss of trust can have long-term consequences for the industry's market share and profitability. [7]

The presence of antibiotics in milk can pose various risks to human health. When antibiotic residues are present in milk consumed by humans, it can have several adverse effects on health, including potential allergic reactions, because the consumption of milk containing antibiotic residues can trigger allergic reactions in sensitive individuals. [8] The presence of antibiotic residues in milk can contribute to the development of antibiotic resistance in bacteria, including those in the human gut. This can make it more challenging to treat bacterial infections with antibiotics in the future. [9], [10] In some cases, exposure to antibiotic residues in milk may lead to toxic effects or side effects in individuals, depending on the specific antibiotic and its concentration in the milk. [11]. Consumption of milk with antibiotic residues can disrupt the balance of the gut microbiome, potentially leading to digestive issues and other health concerns.[12]

It's important to note that rigorous monitoring and testing programs, along with strict regulations, are in place to minimize the risk of antibiotic residues in milk and to ensure that milk and dairy products on the market are safe for human consumption. Dairy producers are required to follow withdrawal periods and other best practices to prevent antibiotic residues from entering the milk supply.

Beta-lactam antibiotics are a class of antibiotics that contain a beta-lactam ring in their molecular structure. This class includes several important subclasses, such as Penicillins as Amoxicillin, Ampicillin, Penicillin G, Penicillin V, Oxacillin, Methicillin; [13] Cephalosporins as Cephalexin, Ceftriaxone, Cefuroxime, Ceftazidime, Cefixime. [14].; Carbapenems as Imipenem, Meropenem, Doripenem, Ertapenem, [15]. and Monobactams as Aztreonam [16].

The detection of antibiotics in milk is a critical aspect of ensuring food safety and compliance with regulatory standards. Various laboratory methods and techniques are used to detect the presence of antibiotics in milk. One of these methods of analysis with which we have worked during our research is also Enzyme-Linked Immunosorbent Assay (ELISA) which is a sensitive and specific immunological method used for the detection of antibiotics in milk. Antibodies specific to the target antibiotics are used to capture and detect the presence of antibiotics in milk samples.[17] Within this method, we can especially emphasize the Rosa Charm β Lactam test (Charm Sciences). The Charm Rosa Pearl Reader is a laboratory apparatus used for detecting the presence of antibiotics in milk and other dairy products. It utilizes a technology known as the Charm Rosa Pearl System, which is based on the principle of enzyme-linked immunosorbent assay (ELISA).

On the contrary, other methods are also used in practice, such as microbial inhibition assays, such as the Delvotest, which are widely used for screening antibiotic residues in milk. These tests rely on the ability of bacteria (usually Bacillus stearothermophilus) to grow in milk. The presence of antibiotics inhibits bacterial growth, leading to a visible change in the as say. [18]; High-Performance Liquid Chromatography (HPLC) is a widely used technique for the quantification of antibiotics in milk. It involves the separation of individual antibiotics based on their chemical properties and subsequent detection using ultraviolet (UV) or mass spectrometry (MS) detectors. [19].; Liquid Chromatography-Mass Spectrometry (LC-MS) is a highly sensitive and specific method for the identification and quantification of antibiotics in milk. It combines liquid chromatography separation with mass spectrometry detection to provide accurate results. [20].; Gas Chromatography (GC) is used for the detection and quantification of volatile and semi-volatile antibiotics in milk. It involves the separation of antibiotic compounds based on their vaporization properties and subsequent detection by a detector like flame ionization detection [21]. All these methods are commonly used by regulatory agencies, dairy processors, and testing laboratories to ensure the safety and compliance of milk and dairy products concerning antibiotic residues. The choice of method depends on factors such as the type of antibiotics being tested and the required sensitivity and specificity.

The maximum allowable values for antibiotics in milk and dairy products can vary depending on the specific antibiotic and the regulatory standards of the region or country. In the European Union, the regulatory levels or Maximum Residue Limits (EU-MRL) are defined by Regulation (EC) 470/2009 [22] and established by Commission Regulation (EU) 37/2010 [23]. For example, the MRL for Penicillin and Ampicillin in milk is set at 4 µg/kg.[24]. At the same time, the Kosovo Food and Veterinary Agency (KFVA) of the Republic of Kosovo after harmonizing the regulations with those of the EU, through Regulation no.12/2011 laying down specific rules on hygiene of food of animal origin and administrative instruction ma-no. 14/2006 on the determination of the maximum waste limit, also is set at 4 µg/kg for beta-lactams such as Benzilpenicilin, Amoxycilin, and Ampicilin.

2. Materials and methods

The research on the presence of beta-lactam antibiotic residues in milk was done during the six months (January – June) 2021, at the milk collection point "Jeta e Re" in the Municipality of Istog, Republic of Kosovo. The research included 181 milk samples where 1 sample represented about 3000 liters of milk from an average of 60 farms per day. Farmers have brought milk to the collection point with different canisters and in quantities from 20 to 200 liters of milk. Before accepting the milk, the milk sample was taken and the alcohol test was done by mixing in petri dish a 2 ml. of 85% alcohol and 2 ml. fresh milk. Only the samples that were negative in the alcohol test were taken and placed in 3 lactofreezes with a capacity of 1000 liters each. A sample for the analysis in the presence of beta-lactams was not taken from each lactofreeze from one sample, but a sample was taken from all three lactofreezes and this was done for economic reasons, to save the costs of the analysis.

Table 1. Number of milk samples throughout the months

Month	January	February	March	April	May	June	Total samples
No. of samples	31	28	31	30	31	30	181

The analysis of the presence of beta-lactam antibiotics in the researched samples was carried out through the Charm Rosa

Pearl System includes test kits for detecting beta-lactam antibiotics, such as penicillin and ampicillin, in milk. The Charm Rosa Pearl Reader is a laboratory apparatus used for detecting the presence of antibiotics in milk and other dairy products. It utilizes a technology known as the Charm Rosa Pearl System, which is based on the principle of enzyme-linked immunosorbent assay (ELISA). These methods, based on the use of specific receptors to detect antibiotics, were originally designed for the rapid detection of β-lactam antibiotics in cow milk. These methods, based on the use of specific receptors to detect antibiotics, were originally designed for the rapid detection of β-lactam antibiotics in cow milk.

Test Procedure

The Charm Rosa Pearl Reader test was employed following the manufacturer's instructions. For cows, 300 µL of milk sample was mixed with 300 µL of the dilution buffer (cow milk dilution buffer; Charm Sciences Inc.) and refrigerated for 10 min. Then, 300 µL of the mixture was placed in the sample compartment of the strip placed in the ROSA Incubator (Charm Sciences Inc.). The incubation time was set at 56°C for 8 min and results were interpreted visually and with the ROSA Pearl Reader, Charm Sciences Inc.). The Charm MRL BLTET test uses receptors that bind β-lactam drugs. As milk flows through the test strip, unreacted receptors bind at the BL (β-lactam) position and form a visible reddish test line. A weaker intensity BL line forms when β-lactam drugs are present in the milk sample. Visual interpretation of the results was carried out by comparing the BL lines with the control (C) line. If both lines are darker than or equal to the C line, the milk sample is negative (antibiotic-free). If either the BL line is lighter than the C line or the BL line does not form, the sample is positive (likely antibiotic presence). After the visual inspection of the strips with the milk samples investigated for the presence of beta-lactam antibiotic residues, all the strips are placed in the Charm Rosa Pearl Reader device, observing the four-digit results (values) and marking them in a list which will serve for statistical processing of the results.

3. Results and Discussions

Table 2. Beta-lactam residue values through Charm Rosa Pearl Reader (µg/kg)

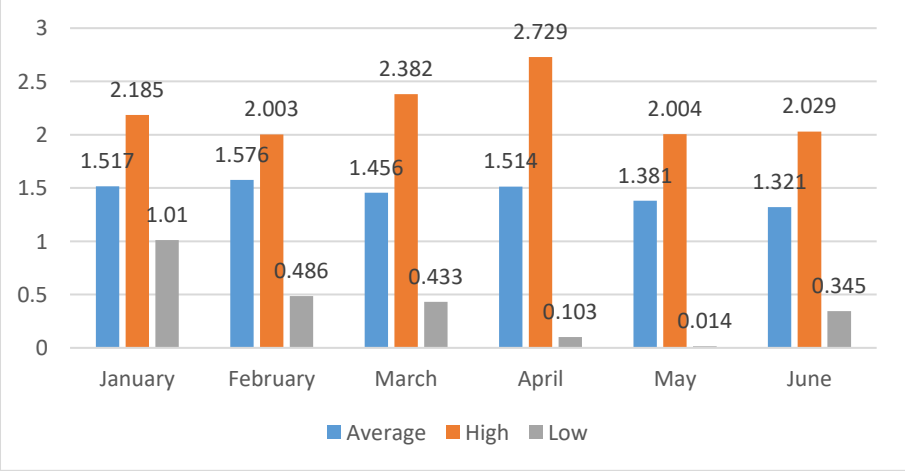
Date	January	February	March	April	May	June
1	1845	1965	1432	1862	2004	1845
2	1532	2003	1739	1432	1743	1463
3	2185	1643	1423	1050	1874	1352
4	1734	1324	1744	1011	1684	1141
5	1483	1593	1812	1259	1856	1061
6	1253	1521	1719	2729	873	1024
7	1784	1358	1761	103	1156	1243
8	1035	1611	1564	262	1063	1563
9	1714	1746	1495	2318	1748	1849
10	1242	1435	1322	1986	1849	1560
11	1854	1885	1270	1807	1754	345
12	1232	1939	1196	1649	1104	474
13	1645	486	1694	1886	14	481
14	1789	1358	1169	1720	1335	1325
15	1243	1342	1382	1880	1435	1463
16	1325	1542	2382	1657	1264	1244
17	1653	1432	1384	1677	1539	1352
18	1712	1722	1384	1368	1314	1241
19	1805	1845	1298	1246	1654	1631

20	1922	1744	1325	1617	1321	1345
21	1705	1840	1249	1125	1743	1232
22	1453	1643	854	1215	1231	1649
23	1305	1244	1734	1342	1456	1114
24	1024	1325	1442	1576	1273	1956
25	1142	1554	1347	1563	1849	1214
26	1125	1856	1325	1894	453	412
27	1654	1743	1644	1873	1349	2029
28	1010	1449	1732	1568	1855	1964
29	1233		433	1231	1439	1735
30	1634		1247	1542	1947	1325
31	1777		1644		432	

The values obtained through Charm Rosa Pearl Reader as seen in the table above are 4 digits. However, these values are in ppb (parts per million) or µg/kg. As an example, the value obtained from the sample dated January 1 is 1845, which indicates that the level of beta-lactam residues in this sample is 1.84 µg/kg or 1.845 ppb.

The reflection of the fluctuations of the minimum and maximum values of beta-lactam residues throughout the months are reflected in the following in graph no. 1

Graph no. 1. Reflection of fluctuations of minimum and maximum values of residues of beta-lactam antibiotics (µg/kg) in fresh milk throughout the months



Based on the results presented above, we note that the values of residues of beta-lactam antibiotics range from a low value of 0.014 µg/kg in May to a high value of 2.729 µg/kg in April and with the general average for all samples during the 6 months of research of 1.460 µg/kg. The European regulations (European Union, 2009), as well as those of Kosovo (Regulation no.12/2011 and Administrative Instruction ma-no. 14/2006) regarding the highest value of beta-lactam residues in cow's milk, refer to the maximum values of 4 µg/kg then we can say that the results obtained from the 181 milk samples of research during the January-June period are below these values, which indicates that all values, even the highest of. 2.792 µg/kg, based on the above-mentioned regulations, results negative in beta-lactam residues. The results obtained during the 6-month research for the detection of fresh milk for the presence of beta-lactam and antibiotic residues are below the maximum level allowed by regulations that regulate these issues and are considered negative results, respectively the milk delivered to the collection point by farmers do not contain antibiotic residues.

The obtained results indicate the reliability and sensitivity of the Charm Rosa Pearl reader in the detection of beta-lactam residues. This reliability is emphasized in the works of other authors as well. This statement can be justified by the works of many authors. So, M. C. Beltrán, et al. [25]. in their research, to evaluate the Charm MRL BLTET test for the detection of β-lactams and tetracyclines in the milk of small ruminants, an evaluation study was performed at Instituto de Ciencia y Tecnologia Animal of Universitat Politècnica de València (Spain). The test specificity and detection capability (CCβ) were studied following Commission Decision 2002/657/ EC. Specificity results obtained in this study were optimal for individual milk free of antimicrobials from uses (99.2% for β-lactams and 100% for tetracyclines) and goats (97.9% for β-lactams and 100% for tetracyclines). These results are similar to those obtained by Reybroeck et al. [26], using the Charm MRL-3 test to detect β-lactams in cow milk samples, the only exception being cloxacillin, which was also detected by those authors at a concentration below EU-MRL (14 μg/kg). Salter et al. also obtained appropriate sensitivity with the Charm 3 SL3 β-lactam test according to safe level/tolerance as stipulated by the FDA (2005). Also, Salter et al. [27]. indicate a specificity of 100% for raw commingled milk from cows for the Charm 3 SL3 β-Lactam test (Charm Sciences Inc).

The obtained results where we find the absence of detectable beta-lactam residues in all 181 milk samples indicate compliance with regulatory standards. This confirms the safety of the milk concerning beta-lactam residues.

Milk that the farmers deliver to the collection points which then either process this milk or as subcontractors deliver it to larger milk production factories, seems can be considered safe from the presence of residues of beta-lactam antibiotics. The reason for these results is that collection points and milk processing factories strictly and daily analyze the milk received from farmers for the presence of beta-lactam antibiotic residues. One of the reasons is to avoid damaging dairy products that cannot be processed if bacterial residues are present, such as fermented milk, since the presence of antibiotics would have inhibited normal fermentation processes during yogurt production. The second reason is that they maintain their reputation in tough competition to find the local market in which several competing companies operate.

Considering the reasons why collection points and milk processing factories necessarily carry out the analysis for antibiotic residues, it seems that farmers, knowing this, do not risk delivering milk that they know or suspect contains antibiotic content. This is because if their milk contains residues of beta-lactam antibiotics, then the farmer who has delivered such milk is obliged to pay all the economic damage caused in the event of the milk being discarded. Even in our case, we think that no farmer wants to be punished with the payment of the amount of milk of about 3000 liters.

4. Conclusions

The analysis of the presence of residues or beta–lactam antibiotics in the research from 181 fresh milk samples, results in a range from an average value of 1.460 μg/kg which is a lower value than the allowed value of 4.00 μg/kg which indicates that the values obtained are negative in the presence of antibiotic residues (beta-lactams) in milk.

The obtained results indicate the reliability and sensitivity of the Charm Rosa Pearl reader in the detection of beta-lactam residues.

The milk collection points that deliver this milk to the milk processing factories obligatorily and strictly analyze all milk samples for the presence of antibiotic residues (beta-lactam), following the legal regulations that regulate these issues.

Milk that is used as a raw material in the dairy industry in Kosovo and that is collected at collection points can be considered free of antibiotic residues and safe to use for general consumption.

It seems that farmers respect the withdrawal of antibiotics in milk as a result of their increased awareness of the damage that such waste brings to health, either because of the fear of punitive sanctions.

It is necessary to research the presence of antibiotic beta-lactam residues in fresh milk from the farms of farmers who do not deliver milk to collection points or milk processing factories but who sell that milk privately on the market or from that milk they produce milk products that they sell on the market.

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