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Using of immunochromatographic analysis to determine antibiotics in milk

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Abstract

Background and Aim. Failure to comply with the rules for the use of antibiotics and/or the tim-ing of milk production from cows subjected to antibiotic therapy leads to the entry of residual amounts of drugs into the human body with dairy products, causing various pathologies. For practice, simple but sufficiently sensitive and specific rapid tests are needed to determine the safety of milk. Such tests can be developed based on immunochromatographic analysis (ICA).

Materials and Methods. Colloidal gold (CG) particles were prepared according to the method of Wang et.al. (2014) and examined using transmission electron microscopy and UV-visible spectroscopy. CG particles were used to label antibodies against streptomycin (STR), tetracycline (TC) and/or chloramphenicol (CAP) according to the method of Yakovleva et al. (2012). The ICA test was developed based on the principle of competition of antibiotics in milk with analogues immobilized on a nitrocellulose membrane.

Results. The multiplex ICA test showed sensitivity at the level of the best world analogues, detecting STR, TC and CAP in milk in concentrations equal to or exceeding the maximum residue limits (MRL) for these antibiotics: 200 ng/ml, 10 ng/ml, 0.3 ng/ml, respectively. The advantage of the ICA test is its affordability, since the proposed technologies for the production of antibiotic-specific anti-bodies and immunoassay test line reagents significantly reduce the cost of domestic products, making them more accessible to the country's consumers.

Conclusion. A domestic ICA test system has been designed for simultaneous rapid analysis of milk for the content of streptomycin, tetracycline and chloramphenicol.

Keywords: antibiotic; immunochromatographic test; milk; streptomycin; tetracycline; chloramphenicol.

Introduction

Milk is rich in nutrients such as high-quality proteins, fats, lactose, phospholipids, vitamins, enzymes, minerals, which especially plays an important role in the nutritious nutrition of human [1]. All over the world, including in the Republic of Kazakhstan (RK), the demand for milk is growing every year. According to the National Bureau of Statistics of the Republic of Kazakhstan, the gross production of commercial raw milk in the country in 2022 amounted to 3 million 975 thousand tons [2]. Various veterinary drugs, including antibiotics, are used to prevent diseases and treat dairy cows [3]. However, if the rules for the use of antibiotics and the holding period for obtaining milk from cows subjected to injection of antibiotics are not followed, the remains of medicinal substances can enter the human body through milk and dairy products [4-6]. Residual amounts of antibiotics can cause allergic reactions,

dysbacteriosis, nephropathy, carcinogenic and mutagenic effects, and also cause the development of antibiotic resistance - one of the modern global medical problems [7]. In May 2015, the 68th World Health Assembly adopted the Global Action Plan on Antimicrobial Resistance, which reflects the global consensus that antimicrobial resistance poses a serious threat to human health. The World Health Organization (WHO) has established MRL of antibiotics to control drug residues in products of animal origin [8]. The gold standard for monitoring antibiotic residues in meat and milk is the microbiological method. It is simple to perform, but the result of the study is achieved no earlier than 3-4 hours. Modern methods, such as real-time polymerase chain reaction, enzyme-linked immunosorbent assay, gas chromatography and/or high-performance liquid chromatography methods require expensive equipment and trained personnel and cannot be used for rapid analysis of milk for antibiotic content in food safety laboratories at dairy processing plants or food markets [9]. Thus, practice requires simple to perform, but sufficiently sensitive and specific tests that allow one to determine the presence or absence of antibiotics in animal products in a few minutes [10]. Such tests can be developed using the principles of ICA [11].

The goal of our work was to design a domestic test system based on ICA for simultaneous rapid analysis of milk for the content of three antibiotics: streptomycin, tetracycline and chloramphenicol.

Materials and methods

Reagents. The following antibiotics were used in the work: streptomycin sulfate (Sintez, Kurgan, Russia), oxytetracycline hydrochloride (BioPharmGarant, Vladimir, Russia), chloramphenicol (Panreac, Barcelona, Spain). Conjugates of antibiotics with protein carriers, such as ovalbumin and BSA (Jackson ImmunoResearch Inc, Pennsylvania, USA), as well as rabbit polyclonal antibodies (pAb) were obtained by us previously [12].

To construct an ICA test to determine the residual amount of the above antibiotics and test its sensitivity, goat anti-rabbit IgG (Jackson ImmunoResearch Inc., Pennsylvania, USA), tetrachloroauric acid (HAuCl4) (Fluka, Basel, Switzerland), nitrocellulose membrane CNPC 15 MDI Easypack kits (Advanced Microdevices; Ambala Cantonment, India), and a commercial ICA test kit (Beijing Meizheng Bio-Tech Co., Beijing, China).

Synthesis and characterization of colloidal gold. CG particles with a diameter of 20 nm were prepared according to the method described by Wang et.al. [13]. Briefly, an aqueous solution of chloroauric acid (100 mL of 0.01% (w/v) AuCl3•HCl•4H2O) was heated to boiling point, followed by the addition of 2 mL of 1.0% (w/v) sodium citrate solution. The reaction solution was simultaneously stirred and gently boiled for 5 minutes until the color of the solution changed from straw yellow to red. The resulting CG solution was stored at 4 °C for several months and used for conjugation with the puri-fied antibody. CG particles were examined using transmission electron microscope (TEM) (Jeol, Tokyo, Japan) and UV-visible spectroscopy (Biochrom, Cambridge, UK) in the wavelength range 400–800 nm.

Preparation of a conjugate of CG nanoparticles with antibodies. To 10 ml of solution CG with pH 7.0-7.5 was added dropwise with stirring to 1 ml of pAb solution with a concentration of 20 μg/ml, incubated with constant stirring for 30 minutes at room temperature. Then BSA was added to the re-sulting solution to a final concentration of 0.1%, sucrose to a final concentration of 10%, as well as 0.01% sodium azide. To remove unbound antibodies, the conjugate was centrifuged (30 min, 11000g, 4 °C). The supernatant was removed, and the sediment was redissolved in the required volume of phosphate-buffered saline (PBS) containing 0.1% BSA, 10% sucrose, and 0.01% sodium azide [14]. The mixture was stored at +4 °C until use. The resulting solution was applied to a pad of fiberglass membrane (manufacturer's name, city, country) and dried at room temperature for 8 hours.

Construction of an immunochromatographic composite. A solution of PBS with sucrose and BSA was used as a working buffer for the sorption of conjugates onto a nitrocellulose membrane. To form test and control zones on the membrane using an automatic dispenser Easy Printer Model LMP-0.2 (Advanced sensor systems private limited, Ambala cantt, India) a solution of antibiotic conjugates with OVA and a solution of anti-species antibodies were applied. The following concentrations of rea-gents were used: antibiotic conjugated with OVA – 0.5 mg/ml, anti-species antibodies labeled with CG – 0.250 mg/ml. The membrane with the applied reagents was dried at room temperature for 8 hours un-til completely dry or at 37 °C for 2 hours. The finished composites on the membrane were cut into strips using special equipment “SS - Programmable Strip Cutter” (Advanced sensor systems private limited,

Ambala cantt, India) for cutting. The strips were stored at room temperature in hermetically sealed containers.

Sample preparation of milk. Antibiotic-free milk samples were collected from a black-and-white cow that was healthy and not injected with any antibiotics. Milk samples were centrifuged for 10 minutes at 5000 rpm. The fat was then separated from the skim milk [15] and added a certain amount of antibiotics. Mix the sample thoroughly before testing, transfer 200 µl of the sample to the well of polystyrene plate for ELISA (Medpolimer, St. Petersburg, Russia) for further research.

Testing milk for antibiotic content. The ICA test strip sample pad was immersed in a well containing 200 µl of milk sample for 3 minutes. After 10 minutes, the test strip was removed from the well, placed on a dry surface, and the results of milk analysis for the presence of STR, TC and CAP were visually recorded. The results were recorded after 10 min. For statistical processing, all measurements were performed three times and in triplicate.

Statistical processing of results. Statistical analysis of test specificity were carried out according to the method described by Akinshina Yu.A. [16].

Results

CG particles were obtained by reducing chlorauric acid with sodium citrate. In order to obtain CG particles with a diameter of about 20 nm, a certain proportion of reagents were observed. The re-sulting CG had a wine-red color. After cooling the solution, it was poured into vials, the pH was meas-ured, and the CG particles were examined with a spectrophotometer and a transmission electron micro-scope. The concentration of hydrogen ions in the solution was 6.5. Spectrophotometric analysis of CG is a very important characteristic of its properties, which determine its suitability for use in immunochromatographic tests. The results of the study are shown in Figure 1.

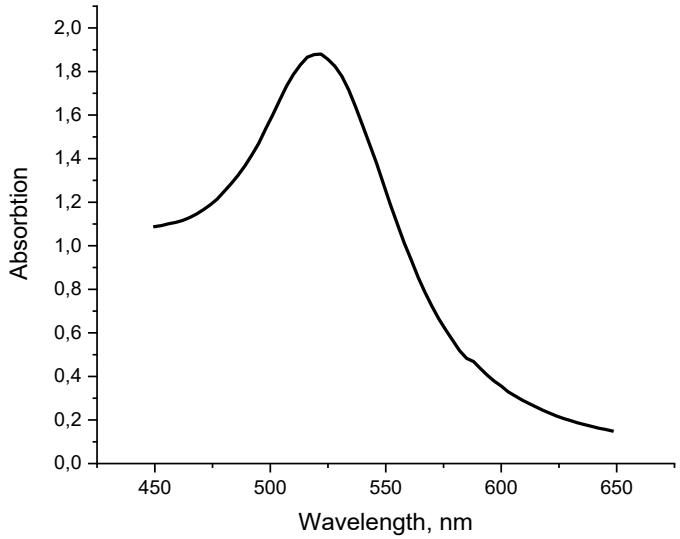

Figure 1 – Spectrophotometry of CG particles with a diameter of 20 nm

As can be seen from Figure 1, spectrophotometric analysis of CG particles showed the presence of one peak in the ultraviolet spectrum at OD520 with optical density = 1.8 (Figure 1).

The TEM image indicates that the CG particle is well dispersed (Figure 2)

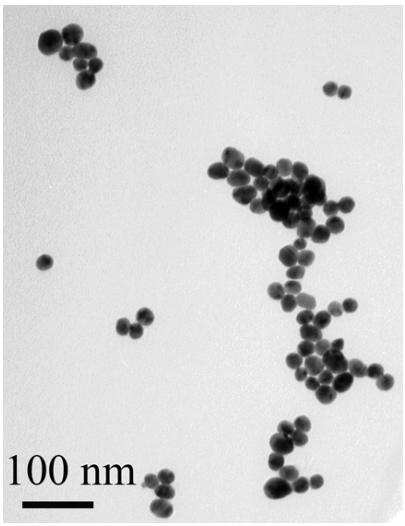

Figure 2 – Fragments of micrographs of CG particles

From Figure 2 it follows that the CG particles were almost the same diameter in the range 14 - 26 nm, and the average short circuit diameter was 19.47±2.5nm, which provided a good basis for using it as a label for pAb specific to the antibiotics used.

To simultaneously test milk samples for the presence of TC, STR and CAP, a multiplex competitive ICA test was prepared, the principle of which is shown in Figure 3.

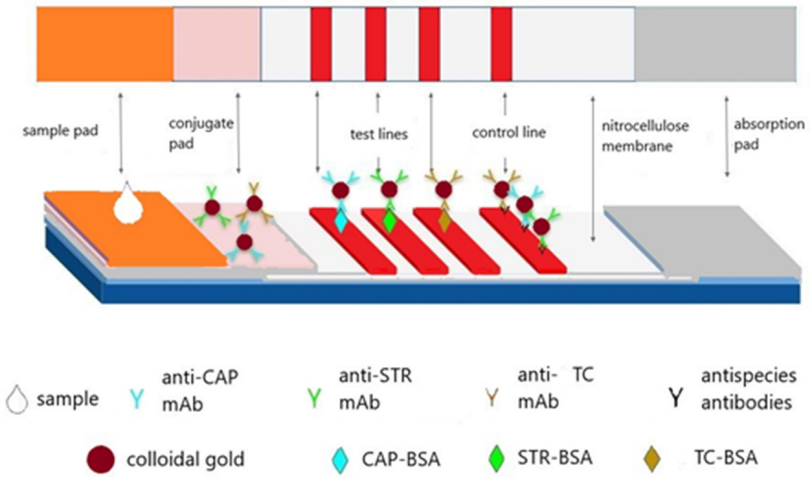

Figure 3 – Principle of competitive immunochromatographic test for determining TC, STR and CAP

The principle of the test is based on the competition of the analyte of the test sample and the antibiotic immobilized on the membrane with a carrier protein for binding to the antigen-binding site of pAb labeled with CG. Staining the control line confirms that there is a sufficient volume of the introduced sample and the correctness of the research methodology. If the ICA result is negative, i.e. if there is no antibiotic in the milk and/or its concentration does not exceed the MRL for STR, TC and CAP (200 ng/ml, 10 ng/ml, 0.3 ng/ml, respectively) [17,18], the control line and the test line corresponding to the antibiotic (TC, STR and/or CAP) are colored red. A positive reaction of the test system is characterized by the absence of staining of the test line of the corresponding antibiotic and the absence of staining of the control line. The manifestation of only the control line indicates that the content of all three antibiotics in the tested milk exceeds the MRL.

The specificity of the developed ICA test was determined on milk samples containing various concentrations of antibiotics STR, TC, and CAP (Figure 4).

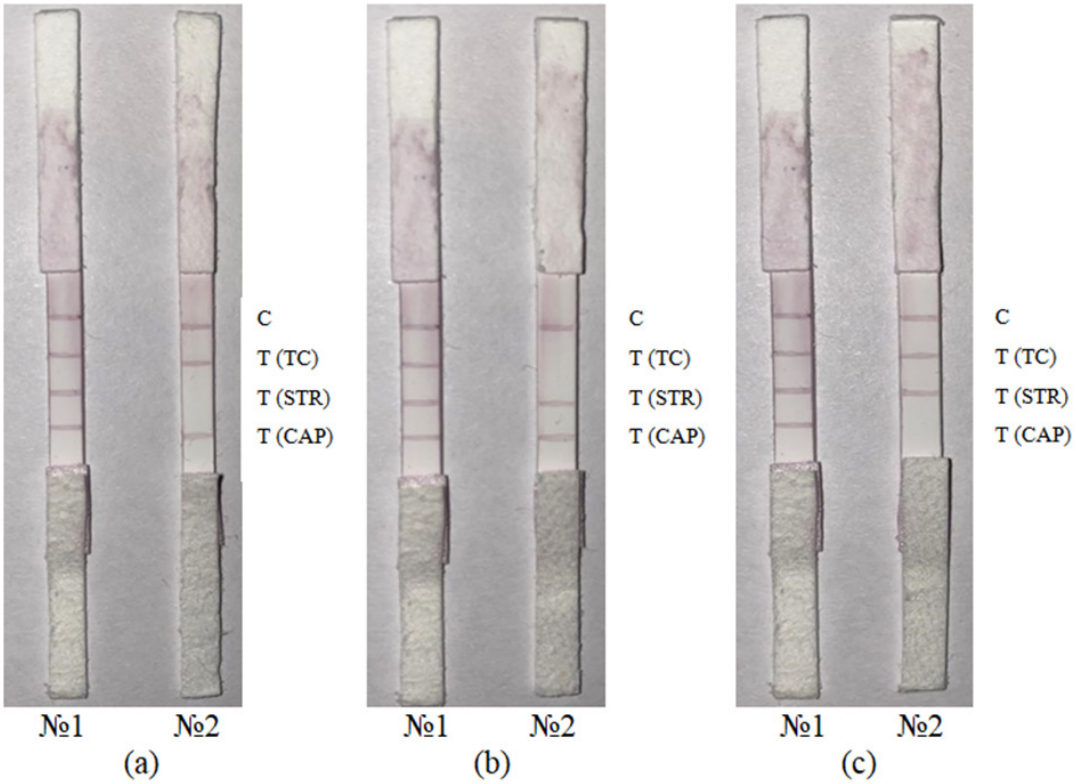

Figure 4 – Sensitivity of the developed ICA test
C- control line, T – test line; a, b, c, - №1: milk samples without antibiotics; a - №2: TC - 0 ng/ml, STR - 200 ng/ml, CAP -0 ng/ml; b - №2: TC - 10 ng/ml, STR - 0 ng/ml, CAP -0 ng/ml; c - №2: TC - 0 ng/ml, STR - 0 ng/ml, CAP - 0.3 ng/ml

From Figure 4 it can be seen that milk samples containing STR, TC and CAP within the MRL (200 ng/ml, 10 ng/ml, 0.3 ng/ml, respectively) give a positive reaction to the presence of the corresponding antibiotics. It should be noted that each of the prepared ICA test strips detected the presence of one antibiotic at a concentration equal to the MRL, but did not give positive results when examining milk samples containing other antibiotics.

The diagnostic value of the developed competitive ICA test was determined in comparison with a commercial analogue - the Pioneer Meizheng Bio-tech (China) express test kit, designed to determine residual amounts of four antibiotics, including STR, TC and CAP, in milk. For each analysis, milk samples containing one specific antibiotic at a specific concentration were used. The first sample was supplemented with the antibiotic TC at a concentration of 10 ng/ml, the second sample was supplemented with STR at a concentration of 200 ng/ml, and the third sample was supplemented with CAP at a concentration of 0.3 ng/ml. A commercial test kit was used according to the manufacturer's instructions provided.

The results of comparative studies of milk samples for antibiotics using a home ICA test and its foreign analogue are shown in Figure 5.

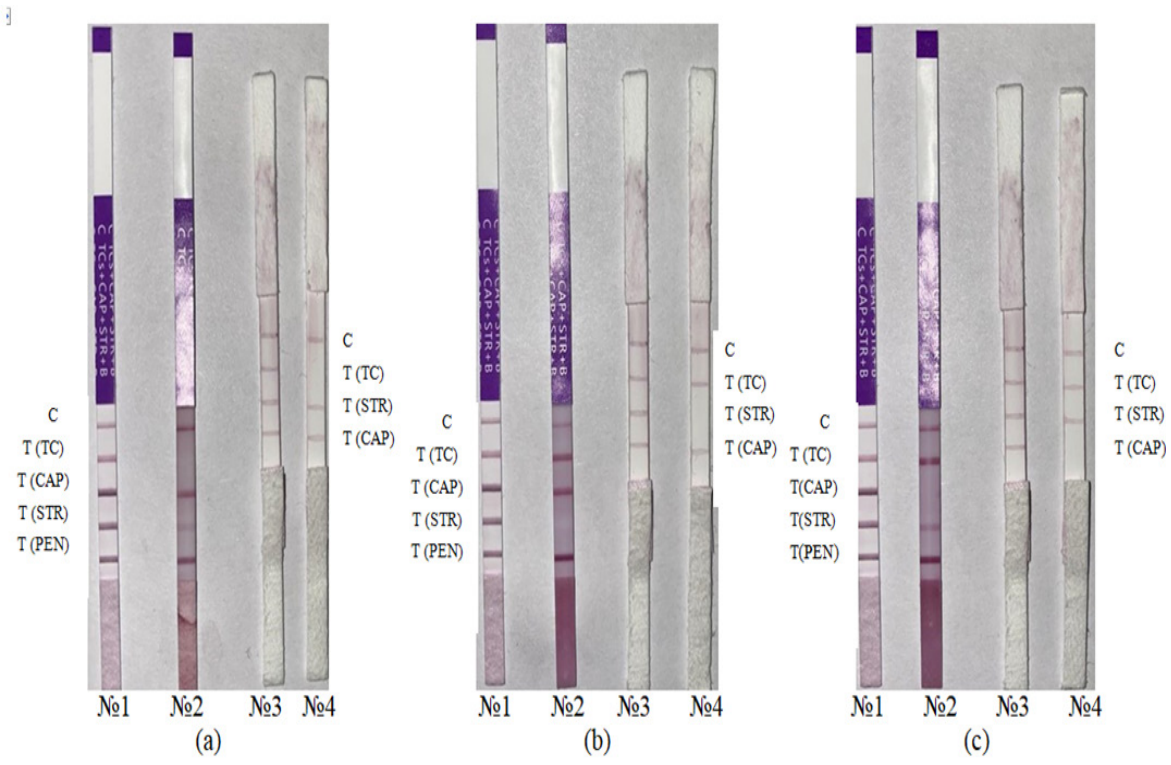

Figure 5 – Images of home ICA test strips and Pioneer Meizheng Bio-tech (China)
C- control line, T – test line; a, b, c, - №1 and № 3: milk samples without antibiotics; a - №2 and № 4: TC - 10 ng/ml, STR - 0 ng/ml, CAP -0 ng/ml; b - №2 and №4: TC - 0 ng/ml, STR - 200 ng/ml, CAP-0 ng/ml; c - № 2 and № 4: TC - 0 ng/ml, STR - 0 ng/ml, CAP -0.3 ng/ml

As the results of the comparative test showed, the absence of antibiotics in milk samples № 1 and № 3 was confirmed in both tests, while in samples № 2 and № 4 with antibiotic concentrations at the MRL, both home and commercial ICA tests gave positive results.

Milk samples from healthy cows (n=46), which were not administered antibiotics and/or other drugs, were examined for the presence of antibiotics STP, CAP, TC using domestic and commercial ICA tests. All samples were negative. The measured specificity was 100%.

Thus, the ICA test we developed in terms of its characteristics in detecting STR, TC and CAP in milk is not inferior to an imported commercial analogue and can be used at milk collection points, at milk processing plants and food markets for express determination of the safety of milk in terms of its content it contains the most widely used antibiotics.

Discussion

To reliably guarantee the quality of milk, it is necessary to control the content of residual amounts of medicinal drugs in it, primarily antibiotics, which are widely used in dairy farming for the prevention and treatment of diseases. Residual amounts of antibiotics in milk that exceed the MRL are not only harmful to human health, but also create a problem in the production of dairy products, inhibiting the growth and development of lactic acid bacteria. For rapid screening of food products for contamination with antibacterial drugs, enzyme-linked immunosorbent assay (ELISA) is becoming increasingly common. This test is recommended by European Union Directive (EC) 2002/657 for the determination of residues of veterinary drugs in animal products in the European Union [19]. However, this test is not used in practice, since the cost of milk analysis is very high. In addition, the equipment of domestic veterinary and sanitary laboratories in food markets leaves much to be desired. In this work, we have developed a more practical and cheaper test that can be used not only in poorly equipped laboratories, but also by the consumer himself. It is based on the use of a competitive ICA variant to detect the three most commonly

used antibiotics in milk - streptomycin, tetracycline and chloramphenicol in concentrations exceeding the MRL. In the post-Soviet space, GOST 32254-2013: Interstate standard "Milk" was developed to determine residual amounts of antibiotics in milk. The standard establishes the requirements for ICA tests for the rapid determination of penicillin, TC, CAP, STR in milk. In addition, there is GOST 32219-2013: Interstate standard "Milk and dairy products", developed taking into account the main regulations of the international standard ISO 18330:2003 "Milk and milk products – Guidelines for the standardized description of immunoassays or receptorassays for the detection of antimicrobial residues", which establishes high-quality immunological methods for the determination of antibiotics using ICA kits from manufacturers from foreign countries. However, imported diagnostics are still not used in food safety laboratories due to their high cost. For example, the average cost of one analysis using the ICA test PROQUITEST (Spain) is 1600 tenge [20]. Therefore, we need domestic express diagnostic kits that will be competitive in the market of veterinary drugs not only in sensitivity and specificity, but also in price offers. The developed domestic ICA test showed the same sensitivity as the imported analog Pioneer Meizheng Bio-tech (China), detecting STR, TC and CAP within the maximum MRL for these antibiotics: 200 ng/ml, 10 ng/ml, 0.3 ng/ml, respectively. The advantage of our diagnostic test is its affordability, since the manufacturing technologies we offer for ICA test components, namely pAb against STR, TC and CAP, as well as test line reagents, significantly reduce the cost of the domestic test system, making it more attractive to consumers in the country.

Conclusion

An ICA test has been developed for the simultaneous detection of STR, TC and CAP residues in milk, the content of which is equal to and/or higher than the MRL. The test is easy to use and is characterized by the rapidity of obtaining milk analysis results. The sensitivity of the test at the level of foreign analogues and proven technologies for obtaining pAb and manufacturing the antibiotic + carrier ICA test conjugate serve as the basis for its commercialization with the aim of introducing it into diagnostic practice in food safety laboratories of dairy processing enterprises and food markets. Our further research will be aimed at replacing pAb with monoclonal antibodies, which will standardize the ICA test and overcome the disadvantages inherent in polyclonal antibodies.

Authors' Contributions

ZhA and AJ: Conceptualized and designed the study, conducted a comprehensive literature search, analyzed the gathered data and drafted the manuscript. AB: Conducted the final revision and proofreading of the manuscript. All authors have read, reviewed, and approved the final manuscript.

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