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Evaluation of a rapid coliform detection kit from clinical mastitis milk using colloidal gold nanoparticle–based immunochromatographic strips

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ABSTRACT. The accurate identification of mastitis-causing bacteria assists in effective management by both dairy farmers and veterinarians and can be used to implement the selective use of antimicrobials for treatment. The purpose of this study was to evaluate the ability of our developed anti–ribosomal protein-L7/L12 antibody–coated immunochromatographic strip (ICS) test to detect coliforms in milk by comparing the results with the bacteriological culture method. We investigated the performance of the ICS test as compared with the bacteriological culture method using 308 milk samples from clinical bovine mastitis. First, to determine the optimal ICS test cutoff point for detecting coliform mastitis, we developed a receiver-operating characteristic curve. The result showed that the cutoff point was at 0.5 of our index. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value of the ICS test were 81.3%, 84.8%, 69.2%, and 91.54%, respectively. As the clinical signs increased in severity, the F-measure, a weighted harmonic mean of the sensitivity and overall PPV performance, increased. Because it is especially important to treat clinical mastitis appropriately in the early stages of detection, the ICS test, which can be used by both dairy farmers and veterinarians on dairy farms, is considered to be a useful tool for detecting coliform mastitis, which often presents with severe signs.

KEY WORDS: coliform mastitis, immunochromatographic strip, milk, rapid detection

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Mastitis is the most prevalent and costly disease affecting production in the dairy industry, and it commonly develops in response to an intramammary bacterial infection [7]. Although there are numerous contagious and environmental mastitis pathogens, coliform bacteria such as *Escherichia coli* and *Klebsiella* spp. are prevalent in the bovine environment and are among the most common mastitis-causing pathogens responsible for eliciting obvious clinical symptoms in cows [4, 21, 22]. Most coliforms infections are cleared from the gland with few or only mild clinical signs. However, when bacterial concentrations in milk increase enough to stimulate a marked immune response, severe mastitis (systemic signs) occurs. With severe systemic disease, it is essential to make an early diagnosis and initiate treatment immediately to increase cow survival rates. In particular, because the clinical signs of mastitis caused by *E. coli* or *Klebsiella pneumoniae* tend to be severe, it is important to identify these bacteria as early as possible. However, the use of the culture process for bacterial identification is complex and time-consuming. Furthermore, even if colonies are obtained from culture, skilled technicians are required for identification. Thus, to reduce the damage to cows, there is a need to develop a rapid and efficient tool to identify bacterial species in coliform-induced bovine mastitis [14].

Among currently available technologies, bacteriologic culturing or polymerase chain reaction (PCR) assays of the milk can be used to determine if mastitis is caused by coliform bacteria. However, in severe clinical cases, the results will not be known in time

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to have an effect on treatment. On the other hand, immunochromatographic strip (ICS) tests are rapid tests that can reduce the time spent waiting for test results from hours to minutes using classical immunochromatographic assays. These tests do not require any special equipment or technical training for operators, which makes them suitable for on-site testing [16].

Previous studies have reported the rapid diagnostic usefulness of ribosomal protein (RP)-L7/L12 as a target for the diagnosis of *Streptococcus pneumoniae* and *Mycoplasma pneumoniae* infection using ICS tests [19, 20]. Because RP-L7/L12 belongs to the 50S ribosome, which is richly expressed in many microbes, and because RP-L7/L12 contains specific sequences for individual bacterial species, ICS tests targeting bacterial RP-L7/L12 could be useful for the rapid diagnosis of various infectious diseases in humans and animals [1, 8]. In fact, our previous reports have shown the effectiveness of ICS using RP-L7/L12 for the detection of the causative organism of *Staphylococcus aureus* mastitis [15].

The appropriate treatment of clinical mastitis based on microbiological evidence is an important factor in improving the effectiveness of mastitis prevention programs to control infectious pathogens. In this study, we evaluated the ability of our developed anti–RP-L7/L12 monoclonal antibody–coated ICS tests to detect coliforms, targeting *E. coli*, *K. pneumoniae,* and other bacteria in the milk samples collected from cows with clinical mastitis. Because the provision of appropriate treatment in the early stages of clinical mastitis is especially important, this study focused on the rapid diagnosis of pathogenic micro-organisms of mastitis from milk at the initial visit.

MATERIALS AND METHODS

Milk sampling and preservation

We collected quarter milk samples from 302 Holstein dairy cows that showed signs of clinical mastitis from dairy farms in Ishikari district, Hokkaido, Japan. In each case in which clinical mastitis was noticed, we aseptically collected a quarter milk sample before the administration of antibiotic treatment and stored the sample frozen at −20°C until ICS testing, somatic cell counting, and using the culture method. A previous study already confirmed that freezing and thawing of milk samples does not affect the ICS test score [15].

Grading system for mastitis

Veterinarians diagnosed one-quarter of the cows as having clinical mastitis and classified them as grade 1–3 based on clinical signs. A simple system for grading the severity of mastitis was used [25]. The features of each grade are as follows. Grade-1 mastitis was defined as a mild case of mastitis characterized by only changes in the appearance of the milk. These changes could include flakes or clots seen only in foremilk. Grade-2 mastitis is a moderate case of mastitis characterized by changes in the milk and signs of inflammation in the affected quarter such as swelling, increased temperature and sensitivity, and redness. Grade-3 mastitis is considered to be a severe case of mastitis characterized by changes in the milk, signs of inflammation in the gland, and systemic signs that the cow is sick.

Somatic cell count

We conducted somatic cell counting using a DeLaval cell counter (DeLaval International AB, Tumba, Sweden) based on the method of Kawai *et al*. [5].

Identification of coliforms in milk using the bacteriological culture method and measurement of coliform counts

In our previous reports, we showed in detail the process of identification and coliform measurement in milk samples [14]. We plated each sample on a 5% sheep blood agar plate (Nissui Plate Sheep Blood Agar, Nissui Pharmaceutical Co., Tokyo, Japan) and cultured the samples aerobically at 37°C for 24 hr. We identified the coliforms based on the shape and other characteristics of the colonies that grew on each plate. In addition, we measured the coliform bacterial count by spreading a 1-ml aliquot of each milk sample on 3M™ Petrifilm™ Coliform Count Plates (3M, Minneapolis, MN, USA), which is known to be suitable for identifying mastitis pathogens [3, 11, 13], then incubated the plates at 37°C for 24 hr, followed by colony counting. We used the counts to calculate the colony-forming units (CFU)/ml. In addition, we considered culture-positive milk to be milk in which coliforms were detected by this method. Furthermore, to confirm that the bacteria on the plates were coliforms, colonies on blood agar plates were applied to the direct PCR amplification kit (RR180A, Takara, Kusatsu, Japan). We sequenced all PCR products at the authorized inspection agency. Obtained sequences were blasted with the GenBank database (http://www.ncbi.nlm.nih.gov/Blast; 16S ribosomal RNA gene sequences [Bacteria and Archaea]) for species or genus assignment.

Evaluation of the ICS test

As previously reported in our ICS test for *S. aureus*, we developed the ICS to detect RP-L7/L12 in *E. coli* and *Klebsiella* spp. etc., using colloidal gold-labeled antibodies, which are widely used for the construction of ICS tests [15]. Development of the ICS test was carried out based on the patent US 20190317093A1. The recombinant RP-L7/L12 protein used in the ICS test was constructed using the information present under GenBank accession no. QSZ64083.1. Using the ICS test, we attempted to detect coliforms in the milk collected from cows with clinical mastitis.

To lyse coliforms in milk, 300 µl aliquots of milk samples were incubated with 500 µl extraction buffer containing 1% (final concentration) of Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA), 2.2 mg/ml of lysozyme (Fujifilm Wako Pure Chemical, Osaka, Japan), 0.1 M MOPSO, pH 7.5 (Sigma-Aldrich), and 0.01% sodium dodecyl sulfate (Sigma-Aldrich) for 30 min at room temperature.

Next, the test strips were dipped into the milk samples to allow the bacterial antigen RP-L7/L12 and the gold-labeled clone antibody of the target bacteria to react. The appearance of a red line on the membrane of the test strips determined the presence of the target antigen in the sample. Since the liquid of the sample migrates through the membrane very rapidly, it was possible to detect the presence or absence of the antigen in about 5–15 min. In this study, the presence or absence of a red test line was determined by the naked eye after 30 min of the reaction to make a reliable judgment. The intensity of the red test line (level 0.5–8) was scored based on the index shown in Fig. 1.

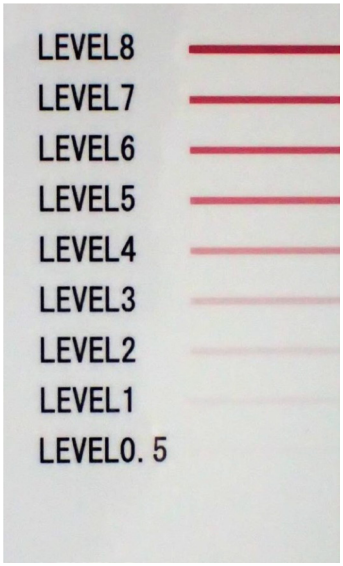

Fig. 1. Color chart for judging the immunochromatographic strip test results.

Statistical analysis

We conducted the statistical analysis using JMP 8 software (SAS Institute Japan Ltd., Tokyo, Japan) and Ekuseru-Toukei 2016 (Social Survey Research Information Co., Ltd., Tokyo, Japan). Before performing the statistical analysis, we transformed the coliform count data and somatic cell count to $\log_{10}$ to obtain a normal distribution. We performed statistical analyses using one-way analysis of variance followed by Scheffe's test to evaluate statistical differences between the groups for somatic cell count, coliform bacterial count, and ICS test score, respectively. Coliform count, somatic cell count, and ICS test scores are presented as mean ± standard error.

We used five measures to determine the prediction accuracy of the ICS test: sensitivity (i.e., the proportion of positive results found among infected animals), specificity (i.e., the proportion of negative results among noninfected animals), positive and negative predictive values (NPVs), the F-measure of the ICS test, and the corresponding 95% confidence intervals (CIs), using the bacteriological culture results as the gold standard. The F-measure was the weighted harmonic mean of the sensitivity and positive predictive value (PPV) representing the overall performance [6, 18]. This value is equal to the arithmetic mean when the sensitivity and PPV are equal. However, the F-measure becomes smaller than the arithmetic mean when one of the numbers approaches 0 (while the other is fixed). Possible values for these five measures range between 0 and 1: the closer to 1, the better the prediction. The F-measure is calculated by the following formula.

F-measure=2 × (sensitivity × PPV) / (sensitivity + PPV)

We also created receiver-operating characteristic (ROC) curves to determine the optimal ICS test cut point for the diagnosis of coliform mastitis by maximizing the area under the curve (AUC) using the method of Ekuseru-Toukei (2016). The AUC value generated from the ROC curve is used to measure the diagnostic test performance, classified as excellent (0.9–1), good (0.8–0.9), fair (0.7–0.8), poor (0.6–0.7), or fail (0.5–0.6) [24]. We also evaluated test performance using Youden's index (J=Se + Sp −1), selecting a cutoff point at which the index is maximized [17].

RESULTS

We obtained a total of 308 milk samples from 302 dairy cows that showed signs of clinical mastitis. We obtained 50, 70, and 188 milk samples from dairy cows with clinical mastitis score grade 1, grade 2, and grade 3, respectively. Somatic cell count ($\log_{10}$ cells/ml) was 2.81 ± 0.09 for grade 1, 2.78 ± 0.09 for grade 2, and 2.69 ± 0.06 for grade 3, and there was no significant difference between the grades (Table 1).

Of the 308 quarter samples, 266 samples of milk (86.4%) cultured positive. After performing bacterial cultures on 3M Petrifilm Coliform Count Plates, 91 milk samples (29.5%) yielded colonies of coliform bacteria. Subsequently, we confirmed those coliforms by PCR to be *E. coli* in 79 samples (25.6%) and *K. pneumoniae* in 12 samples (3.9%). However, no species belonging to other coliforms, such as *K. oxytoca*, *Proteus mirabilis*, or *Serratia marcescens*, were detected in the present samples. Of these milk samples in which *E. coli* and *K. pneumoniae* coliforms were detected, 5, 12, and 73 samples were obtained from dairy cows with clinical scores of grade 1, grade 2, and grade 3, respectively. The somatic cell count ($\log_{10}$ cells/ml) in their milk was 3.02 ± 0.15 for grade 1, 3.09 ± 0.15 for grade 2, and 2.71 ± 0.08 for grade 3, with no significant difference observed between the grades in the

Table 1. Comparison of somatic cell count with clinical score in all milk samples

	Clinical score		
	Grade 1	Grade 2	Grade 3
n	50	70	188
SCC ($\log_{10}$/ml)	2.81 ± 0.66	2.78 ± 0.79	2.69 ± 0.77

On the basis of clinical scores, we divided 308 milk samples into three tertiles: grade 1 (n=50), grade 2 (n=70), and grade 3 (n=188) clinical score groups. Each data point represents one-quarter milk sample and is expressed as the mean ± standard error of the mean. SCC: somatic cell count.

milk of coliform mastitis. The coliform bacterial counts and ICS test scores in their milk were 2.87 ± 0.14, 4.51 ± 0.62, and 6.42 ± 0.26 $\log_{10}$ CFU/ml and levels 0.10 ± 0.10, 2.50 ± 0.98, and 5.35 ± 0.37, respectively, and they were higher depending on the severity of mastitis (Table 2). We noted significant differences in coliform bacterial counts and ICS test scores between clinical scores of grades 1 and 3 (coliform bacterial counts: $P<0.01$, ICS test: $P<0.01$) and between grades 2 and 3 (coliform bacterial counts: $P<0.05$, ICS test: $P<0.05$), respectively. The experiments required for the ICS test in this study were completed within one hour.

To compare the predictive level of the ICS test for discriminating between milks from cows with coliform mastitis and cows with other mastitis, we performed an ROC curve analysis. The optimum cutoff point of the ICS test for coliform mastitis diagnosis was level 0.5 (Fig. 2). The corresponding sensitivity, specificity, PPV, NPVs, and F-measure values for this cutoff point were 81.3% (95% CI: 74.1–87.1%), 84.8% (81.7–87.2%), 69.2% (63.0–74.1%), 91.54% (88.3–94.2%), and 74.8% (95% CI: 68.1–80.1%), respectively (AUC: 0.8755, 95% CI: 0.829–0.922).

Furthermore, we re-created the confusion matrix based on the clinical score and calculated their indexes of ICS test and culture for milk samples. The result of 308 samples with clinical scores of grade 1 or higher has already been shown. Table 3, which includes the results for grade 1, shows the results of 258 samples with clinical scores of grade 2 or higher and 188 samples with grade 3 or higher. Regardless of the grade of clinical score, the sensitivity and specificity of the ICS test were 80% or higher. The sensitivity was 81.3% (95% CI: 74.1–87.1%) for grade 1, 85.9% (78.8–91.1%) for grade 2, and 90.4% (83.4–95.0%) for grade 3, and the specificity was 84.8% (81.7–87.2%), 83.8% (80.4–86.4%), and 80.9% (76.4–83.8%), respectively. The F-measure was 0.747 (95% CI: 0.681–0.801) for grade 1, 0.785 (0.720–0.833) for grade 2, and 0.820 (0.756–0.861) for grade 3, with higher values observed as the clinical sign became more severe.

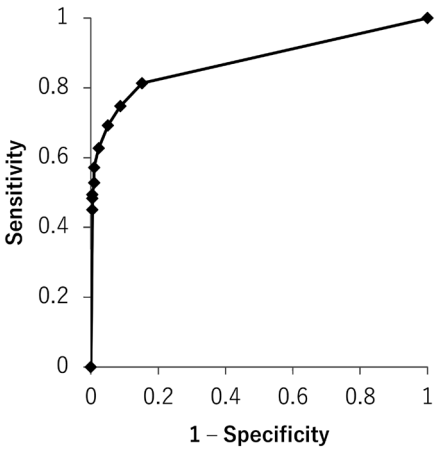

Fig. 2. Receiver-operating characteristic curve representing the diagnostic performance of the immunochromatographic strip test score as an indicator of coliform mastitis.

Table 2. Comparison of somatic cell counts, coliform counts, and immunochromatographic strip test scores among clinical scores in milk samples from coliform mastitis

	Clinical score		
	Grade 1	Grade 2	Grade 3
n	5	12	73
SCC ($\log_{10}$/ml)	3.02 ± 0.15	3.09 ± 0.15	2.71 ± 0.08
Coliform count ($\log_{10}$/ml)	2.87 ± 0.14 ^{A}	4.51 ± 0.62 ^{a}	6.42 ± 0.26 ^{Bb}
ICS test	0.10 ± 0.10 ^{A}	2.50 ± 0.98 ^{a}	5.35 ± 0.37 ^{Bb}

On the basis of clinical scores, we divided 90 milk samples into three tertiles: grade 1 (n=5), grade 2 (n=12), and grade 3 (n=73) clinical score groups. Each data point represents one-quarter milk sample and is expressed as the mean ± standard error of the mean. Different uppercase letters (A, B) correspond to statistically significant differences between grades ($P<0.01$). Different lowercase letters (a, b) correspond to statistically significant differences between grades ($P<0.05$). SCC: somatic cell count; ICS: immunochromatographic strip.

Table 3. Sensitivity, specificity, positive predictive value, negative predictive value, and F-measure of the immunochromatographic strip test for coliforms as compared with the bacterial culture method after grouping based on clinical score

Clinical score	Total	Culture		ICS test		Confusion matrix			
		Positive	Negative	Positive	Negative	TP	FN	TN	FP
Grade 1–3	308	91	217	107	201	74	17	184	33
Grade 2–3	258	85	173	101	157	73	12	145	28
Grade 3	188	73	115	88	100	66	7	93	22

Clinical score	Sensitivity (%)	(95% CI)	Specificity (%)	(95% CI)	PPV (%)	(95% CI)	NPV (%)	(95% CI)	F-measure	(95% CI)
Grade 1–3	81.3	(74.1–87.1)	84.8	(81.7–87.2)	69.2	(63.0–74.1)	91.5	(88.3–94.2)	0.747	(0.681–0.801)
Grade 2–3	85.9	(78.8–91.1)	83.8	(80.4–86.4)	72.3	(66.3–76.7)	92.4	(88.5–95.2)	0.785	(0.720–0.833)
Grade 3	90.4	(83.4–95.0)	80.9	(76.4–83.8)	75.0	(69.2–78.8)	93.0	(87.9–96.3)	0.820	(0.756–0.861)

Based on clinical scores, we evaluated the accuracy indicators of 308 samples obtained in grades 1–3, 258 samples obtained in grades 2–3, and 188 samples obtained in grade 3. Sensitivity, specificity, positive and negative predictive values, and F-measure of ICS test and their 95% confidence intervals were calculated based on bacteriological culture results. ICS: immunochromatographic strip; TP: true-positive; FN: false-negative; TN: true-negative; FP: false-positive; PPV: positive predictive value; NPV: negative predictive value; CI: confidence interval.

DISCUSSION

In the current study, we evaluated an ICS test for the rapid and accurate determination of bovine mastitis caused by coliforms based on anti–RP-L7/L12 mAbs and anti–RP-L7/L12 mAb-coated ICSs. Initially, to investigate the efficacy of the anti–RP-L7/L12 mAb-coated ICS test for use in the diagnosis of coliform infection in bovine mastitis, we examined the cutoff value of the ICS test by comparing it with the bacterial culture method using milk from cows with clinical mastitis. The results showed that the most appropriate cutoff value was at the 0.5 level of our index. Thus, we consider it necessary to suspect intramammary infection by coliform bacteria when even a very thin red line is confirmed by the ICS test in dairy farms. Next, we compared the efficacy of the ICS test with existing culture methods using milk samples of cows with clinical mastitis, and we found that both the sensitivity and specificity of the ICS test for coliform detection were greater than 80%, respectively. These results indicate that the anti–RP-L7/L12 mAb-coated ICS test is highly capable of detecting coliform bacteria in milk. In dairy cows, coliform mastitis caused by *E. coli* or *Klebsiella* spp. is generally characterized by an acute or peracute clinical course; thus, prompt action is required when intramammary infection by coliforms is suspected [4, 21]. Because the ICS test can be used by dairy farmers, it can lead them to take measures against coliform mastitis, such as frequent milking and cooling of the udder while waiting for the veterinarian to arrive [23]. This could lead to a reduction in losses due to coliform mastitis.

We have already reported that although the clinical score for coliform mastitis is unrelated to the somatic cell count, it is related to coliform bacterial counts [14]. The results of this study were similar to those of previous reports and also showed an association between clinical scores and ICS test scores. These results indicate that in order to determine the severity of coliform mastitis, it is desirable to measure coliform bacterial counts rather than somatic cell counts, suggesting the usefulness of ICS tests that can be determined on the farm. To identify the causative micro-organism of mastitis, the traditional gold standard microbial culturing and the PCR assay, which has become popular in recent years, can be used, but both methods require time and/or skill to determine the bacterial species [2, 12, 13]. On the other hand, the ICS test is effective only for the identification of a specific bacterial species, but it is considered to be useful when quickly determining the causative bacterial species is necessary [15]. Coliform bacterial load in milk is significantly associated with the clinical severity of coliform mastitis in cows and is considered a useful indicator for its optimal management [14]. In the ICS test, the time for result determination was one hour, which included the time for bacteriolysis and reaction; however, we were able to obtain the results as quickly as 30–40 min for milk samples from severe cases with high coliform counts. Since the reactivity of this ICS test depends on the target bacterial count, milk samples from severe cases with high bacterial counts can be analyzed more rapidly. However, coliform mastitis can cause severe damage due to endotoxins released from the causative bacteria. In such cases, it may take longer to determine the test results because the amount of target bacteria may be low. However, the ICS test is considered to have the advantage of obtaining results more rapidly than other bacteriological methods because the results are available within one hour of measurement.

In the present study, we obtained a few false negative and false positive results. After investigating the cause for each sample, we found that the majority of false negatives occurred when the bacterial count in the sample was below the detection limit of the ICS test, whereas false positives occurred when dead bacteria were present in the milk and were detected by the test. Other factors, such as the viscosity of the milk and the components in the milk, were also found to affect the ICS test results. Additionally, no coliforms other than *E. coli* and *K. pneumoniae* were detected in the present milk samples. However, this test is designed to detect *E. coli* and *K. pneumoniae* as well as coliforms in general, including *K. oxytoca*, *Proteus mirabilis*, and *Serratia marcescens*. In the future, with an increased use of the ICS test, the knowledge of its ability to detect other coliforms will accumulate.

In recent years, the emergence and spread of antimicrobial resistance has become an urgent matter of particular public interest; consequently, the use of antimicrobials in livestock production is a critically discussed subject [9]. In dairy cows, antibiotics are administered primarily to control udder inflammation; thus, dairy practitioners must use them appropriately and prudently in mastitis control [10]. In contrast, the diagnosis of causative agents of mastitis is often not performed, with treatment protocols applied according to veterinary-predefined protocols. The most frequent approach to treatment is the use of systemic or intramammary antibiotic as soon as possible after detection. These issues suggest that ICS tests, which can be tested easily on dairy farms, may contribute to solving the problem of antimicrobial resistance. In addition to the convenience available to dairy farmers, the ICS test used in this study has the potential to be a useful tool for veterinarians to select antibiotics appropriately. In particular, because the elevated clinical score increased the F-measure, this ICS test was indicated as being useful tool for the detection of coliform mastitis, which often presents with serious signs.

Identifying the causative agent of the intramammary infection of dairy cattle is important to improve control of this disease. Therefore, the development of a rapid and efficient bacterial species identification tool is necessary. In this study, we demonstrated as compared with the bacteria culture method, that anti–RP-L7/L12 mAb-coated ICS tests showed high sensitivity and specificity for the detection of coliforms in the milk of cows with clinical mastitis. In addition, this ICS test is simple and easy to use during routine diagnosis, because of its features that allow farmers or practitioners to perform it directly on the dairy farm. Therefore, we believe that this new method of detection using the ICS test for the diagnosis of bovine mastitis may be useful as a highly sensitive method of coliform testing.

CONFLICT OF INTEREST. The authors declare that this study received funding from Asahi Kasei Corporation. The funder had the following involvement with the study: collection and analysis. KM is an employee of Asahi Kasei Corporation and co-inventor of a patent application describing the method for detecting specific substances in milk (U.S. Patent No. 20190317093A1) but does not have stocks or options. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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