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RESEARCH ARTICLE

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Evaluation the latex agglutination test for detection of the causative agents of bacterial mastitis in milk samples

Mohammad Khosravi[a], Mohammad Bafandeh Dehaghi[b], Saad Gooraninezhad[c], Darioush Gharibi[a], Masood Ghorbanpoor[a]

[a] Department of Pathobiology, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz , Iran. [b] Graduate Student of Veterinary Medicine, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Iran. [c] Department of Clinical Sciences, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Iran.

ABSTRACT

Mastitis is the main disease in dairy farms that causes serious losses. The early diagnosis and specific treatment can prevent the spread of the disease and the economic losses. The aim of this study was to evaluate the accuracy of the latex agglutination assay for detection of the main bacterial mastitis agents. The antiserum against *Staphylococcus aureus*, *Trueperella pyogenes*, *Streptococcus agalactiae* and *Escherichia coli* were prepared from immunized rabbits. The couplings of antibodies to latex particles were optimized and after that, the limit of detection (LOD) of latex agglutination test was evaluated for detection of the mentioned bacteria. The detection limit for the *Streptococcus agalactiae*, *E. coli*, *Staphylococcus aureus* and *T. pyogenes* were respectively 1.3×10^3, 2×10^7, 1.58×10^4 and 5.4×10^4 colony-forming unit per each milliliter of the bacterial suspensions. The prepared latex test has more sensitivity in the setting of phosphate buffered saline than in contaminated milk samples. This method can be used for the fast detection of the mentioned bacteria in bacterial cultures and milk samples. The latex agglutination test could be evaluated as a fast, cost benefit, and practical method in dairy farms.

Keywords

Mastitis, Agglutination, Latex

Abbreviations

LOD: limit of detection
PBS: phosphate buffered saline
SCC: somatic cell count
SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis
ELISA: enzyme-linked immunosorbent assay
PCR: polymerase chain reaction
CFU: colony forming unit
RPM: revolutions per minute
µl: microliter
mg: milligram
ml: milliliter

Corresponding author:
Mohammad Khosravi
Tell/Fax: 09376211901
Email: m.khosravi@scu.ac.ir

Introduction

Mastitis is inflammation of the mammary glands, and it is associated with physical and chemical changes of the milk and pathological changes in udder glandular tissue. The quality and quantity of milk production are dependent to the state of mammary tissue, the efficiency of secretary cells, and the availability of nutrients. Mastitis is associated with decreases of the milk secretion and changes of the milk components [1]. Mastitis is one of the worldwide most important diseases in dairy cattle herds. It is assumed to be the most important breeding failure. Mastitis could reduce the cow price, herd's income ,and profits. The economic impact of the mastitis can be categorized as the direct effects including medicinal treatment, veterinary expenses and mortality, and the indirect effects including decreased milk production, reduced protein concentration in the produced milk, increased reproductive failure rate, and reduce d survival rate [2, 3].

Mastitis is caused by various factors such as infectious agents, and mechanical, or chemical trauma. The infectious agents of mastitis include bacterial agents, viruses and fungi [2]. The main cause of the bacterial mastitis is invasion and toxin production of the bacteria in mammary glands; for this reason, the mastitis treatments are based on the antibiotics therapy [4]. The main bacterial mastitis cases are caused by *Streptococcus agalactiae, Streptococcus dysgalactiae, Staphylococcus aureus, Trueperella pyogenes, Mycoplasma bovis, Streptococcus uberis and Coliform bacteria* [5].

Detection of the causative agents of bacterial mastitis is necessary for accurate treatment, management, and appropriate antibiotic therapy. Conventionally, the bacteria are identified using general microbial culture methods. This is time-consuming assays and needs at least 24 to 48 hours for initial diagnosis.

[6]. However, rapid detection and immediate handling are critical issues in the management of the clinical mastitis. Delays would results in increase of the therapy period and extensive antibiotics utilization [7]. In addition, interpretation of the cultivated plates is associated with challenges; there are some differences on diagnostic results of the laboratories, although they use similar standardized methods [8].

Enzyme-linked immune-sorbent assay (ELISA) is another method for detection of the bacterial agents of mastitis; but despite the abundant advantage, it failed to detect the low concentration of some antigens [9]. Another routine method that is used for diagnosis of the causative agents of mastitis is polymerase chain reaction (PCR). The main weakness of the conventional PCR is utilization of the agarose gel electrophoresis with very weak clarity and precision [10]. The other difficulties in mastitis diagnosis by PCR and ELISA are the limited use of related equipment at farm, the high cost, and requirement expertise. The number of somatic cells does not always correlate with mastitis; the elevation of the somatic cell count (SCC) may occurr due to the problems other than mastitis such as lactation, the level of the milk production, the number of the milking, stress, seasons, and the animals breed [11]. Other disadvantages of the SCC method are the required high time, expensive equipment and cost [12]. The electrical conductivity of milk could be a useful diagnosing method for the detection of mastitis, but due to the large number of false positives, could not be a reliable assay [13]. The milk production industry and animal husbandry farms are searching for the alternative fast and accurate diagnostic methods for mastitis. At now, the farms are bigger than past and they produce more milk; these conditions need automatic milking machines, milk processing machine and a hygienic environment. An appropriate screening and detection test is an important point in early management of the infectious diseases. [14].

Latex particles have been employed in the immunoassay and related biomedical diagnostic methods. The appropriate ratio of the positive samples and coated latex particles in a mixture gives visible sediment in a few minutes. Latex particles uptake antigen/antibody and suspend it on the surface; so when acquainted with their specific antigen/antibody, the reaction is easily visible as physical accumulation [15].

The latex agglutination assays have suitable sensitivity and specificity, are affordable, and require little skills, tools and time. It could be a good alternative method for the detection of bacterial mastitis agents. This research evaluated the sensitivity and accuracy of the latex agglutination test for the detection of selected bacterial mastitis agents.

Results

The rabbits produced a proper antibody titer after three sets of immunization; however *S. agalactiae* and *S. aureus* produced a greater antibacterial antibody titer on micro-agglutination test (Table 1) than the others. The SDS-PAGE analysis showed appropriate purification of the antibody by using ion-exchange chromatography (Figure 1).

The antibacterial antibody titers of the purified IgG were analyzed using micro-agglutination test. According to the results (Table 1) using method of affinity purification, could not extract the total specific antibodies.

Coupling of antibodies to latex

The bacterial latex at optimum conditions of the antibody-latex coupling showed appropriate visible positive and negative agglutination after 30-180 sec-

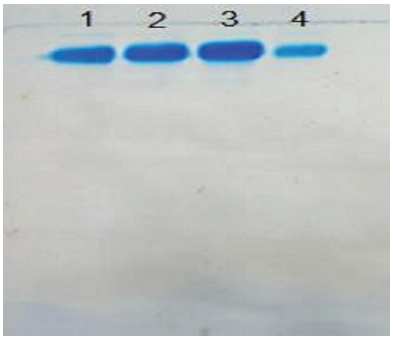

Figure 1
Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of the purified IgG by ion exchange chromatography. The four clearly bands depicted good efficacy of the methods. 1. T. pyogenes, 2. S. agalactiae, 3. S. aureus and 4. E. coli

Table 1
The antibacterial antibody titers of the purified IgG by Ion Exchange chromatography and affinity purification.

Bacteria	*E. coli*	*S. aureus*	*S. agalactiae*	*T. pyogenes*
Ion exchange	512	1024	1024	512
Affinity purification	256	256	128	512

onds. The stability test showed excellent results at least for six months (Figure 2).

The sensitivity latex agglutination test for diagnosis of bacterial mastitis

Ten 1/10 serial dilutions of the bacteria were prepared from the initial concentration adjusted to 2 MacFarland standard in normal saline and sterilized bovine milk; the 2 final dilutions were cultured on blood agar for accurate assessment of the bacterial count. The agglutinated reactions were graded +1 to +4 based on the time and severity of the reactions. As seen in Table 2, different sensitivities observed in 4 bacterial latex reactions; the sensitivity directly correlated to the antibacterial antibody titer of the corresponding hyper-immune sera (Table 1).

The detection limit of the bacterial number in milk and normal saline samples were calculated according to the culture method. The detection limit of the bacterial latex has been noticeably reduced in milk samples than normal saline (Table 2). Incubation of the polluted milk samples at 37°C for 30-120 minutes, enhanced the detection limit 10-100 times; however, incubation at 37°C more than 120 minutes led to the coagulation of the milk samples. Also, the essential bacterial number for induction of the positive reaction 10-fold decreased by using specific antibody instead of the total IgG.

The total IgG against different bacteria mixed at the same concentration and attached to the latex particles. The produced multi-bacterial latex could agglutinate four types of the corresponding bacteria at different detection limits. These results (Table 2) have direct correlation with the corresponding antibacterial antibody titer of the hyper-immune sera.

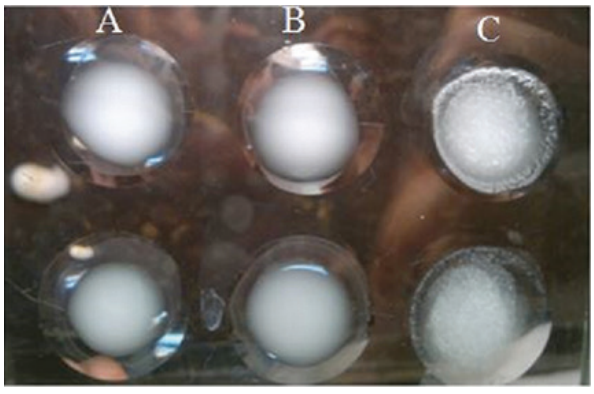

Figure 2
The stability of the prepared bacterial latex after six months. A and B) negative results of the control samples and C) positive results of the samples.

Discussion

In the current study the main bacterial agents of mastitis were successfully detected by latex agglutination test. The detection limits of these latex agglutination tests were dependent to the antibacterial antibodies titer in the corresponding serum samples. The detection limit was 1.3×10^3, 2×10^7, 1.58×10^4 and 5.4×10^4 colony forming unit per milliliter (CFU/ml) of the normal saline for *Streptococcus agalactiae, E. coli, Staphylococcus aureus* and *T. pyogenes,* respectively.

Hechemy et al. [17] coupled the anti *E. coli* IgG to the latex particles; the obtained sensitivity was 1.5-5.7×10^6 CFU/ml. Similarly, Hajra et al [18] reported the detection of *E. coli* at 5×10^6-5×10^7 CFU/ml by latex agglutination. The sensitivity of the latex test for the detection of the *Streptococcus* reported as 10^3 CFU/ml [19].

Sumithra et al [20] reported a detection limit of 5×10^4 for *Bacillus cereus* using polyclonal antibody. Based on the results of this study, detection limit of the latex test for bacteria tracking is directly dependent to the level of the specific antibody, affinity and avidity of the used antibody. In comparison with other reports, the detection limit of the produced latex for the detection of the bacteria in the polluted milks can be reported as appropriate.

Also, current study tried to develop a rapid and easy method for purification of the specific antibacterial polyclonal antibody. However, the antibacterial antibody titer of the purified antibody was 50-60% lower than the total IgG; the affinity purified antibody enhanced the limit detection of the latex tests up to

Table 2
The detection limit of the latex and multi- latex tests for the detection of bacteria in milk and normal saline samples.

Bacteria	*T. pyogenes*	*S. aureus*	*E. coli*	*S. agalactiae*
Normal saline	5.4×10^4	1.54×10^4	2×10^7	1.3×10^3
Milk	4.8×10^7	7.5×10^5	3.9×10^6	11.28×10^6
Multi-latex in normal saline	6.3×10^4	8×10^4	4×10^7	5.2×10^3

10-fold. Due to the poor efficiency of the current affinity method, the total IgG which purified by ion exchange chromatography were preferred in this study. Utilization of a cost benefit and appropriate method for purification of the specific antibodies suggested for commercialization of latex agglutination test for detection of the bacterial infections. The related research confirmed the priority of the specific antibodies than total IgG. The latex agglutination test for the detection of *S. aureus* had cross reaction with *Staphylococcus saprophyticus* [21]. Also, the *Staphylococcus aureus* and its enterotoxins' detection of in milk samples had failed results [22]. Moser et al [23] showed that latex agglutination test has detected the S. aureus with different genomic profile. The previous research often analyzed latex on pure bacteria obtained from culture but the current study successfully reported the sensitivity of the latex test for detection of the main causes of bacterial mastitis in the milk samples.

In the current study, the best detection limits were obtained by employment of the specific antibody, ion exchange purified IgG and ammonium sulfate precipitated antibody, respectively. The polyclonal IgG is often used for sensitization of the latex particles; however employment of the monoclonal antibody resulted in a higher specificity [24]. Due to the simple steps of purification, higher level of the affinity and specificity, the IgG isotype is often used for sensitization of the latex particles; despite the higher ability of IgM in induction of the agglutination reactions [25].

Conclusion

In conclusion, the results showed appropriate detection limit of the latex agglutination test for the detection of mentioned mastitis agents. Also, in agreement with the previous studies, the latex agglutination test in association with the bacterial culture and biochemical tests could help the correct and rapid detection of the bacterial mastitis agents. Finally the current study suggested the use of the high affinity IgG and coloured latex which can enhance the detection limit of the latex agglutination test in milk samples.

Materials and methods

Bacterial preparation

The native isolation of *Streptococcus agalactiae* (clinical isolate of bovine mastitis), *Trueperella pyogenes* (clinical isolate of bovine mastitis), *Staphylococcus aureus* (PTCC: 1431) and *Escherichia coli* (PTCC: 1399) were prepared from archive section of Microbiology department, Veterinary Faculty of Shahid Chamran University of Ahvaz. The bacteria were cultured in blood agar medium and incubated at 37°C for a period of 24-48 hours depending on the type of bacteria. The biochemical tests were used to confirm the identity of the purred bacteria. The obtained bacterial samples were killed by incubation in 95°C for 2 hours; in order to ensure the inactivation. The inactivated bacteria were cultured on blood agar medium.

Immunization

The eight male rabbits with average weight of 2 ± 0.2 Kg were divided into 4 groups and maintained according to the animal care division. The 0.5 ml of the inactivated bacteria, at a concentration adjusted to 4 McFarland standard, was mixed with equivalent amount of the complete freund's adjuvant. The prepared antigens were injected subcutaneously and intramuscularly to each rabbit. The booster antigens were prepared by mixing 0.5 ml of the inactivated bacteria at a concentration adjusted to 2 McFarland standard with an equal amount of the incomplete freund's adjuvant; three sets of booster were injected as before at 2-weeks intervals (26). The micro-agglutination test was used to assess the antibacterial antibody titer for each immunized rabbit. The hyper-immune sera were harvested from the immunized rabbits and stored at -20°C. The micro-agglutination test was used to assess the antibacterial titers of the prepared hyper immune sera.

Antibody purification

The IgG antibodies were purified by ion exchange chromatography on DEAE-C column (Sigma, Number: D3764) according to standard guidelines [16]. Purification of the specific antibodies for each bacterium has been done in accordance with the following steps: The 2 mg/ml of purified IgG was mixed with 0.5 ml of the corresponding bacteria at a concentration adjusted to 4 MacFarland standard and incubated at 37°C for one hour, along with shaking. The antigen-antibody complexes were precipitated by centrifugation for 5 minutes at 10,000 RPM; the resulting sediments were harvested and the supernatant was used for more purification as in the previous steps. The process repeated twice and the precipitated complexes were collected together. The precipitates were mixed with 1 ml phosphate buffered saline (PBS). The suspension pH was adjusted to 3.5 and shaked for 3 minutes. Then the same centrifuge conditions were used for bacterial sedimentation. The supernatant, which contained specific antibody was collected and the pH was adjusted to 7.2. The quantity, purity and titer of the purified antibody were checked by Bradford protein assay, SDS-PAGE and micro-agglutination test, respectively.

Coupling of the antibody to latex particles

Different buffers, pH and temperature conditions were tested for latex activation and antibody coupling to the latex particles. The optimum conditions were optimized as described in the following steps. The latex particle (prepared in our department) 100µl (5 mg) were mixed with 400µl of PBS (pH, 7.2) and 500µl Triton X-100 0.02%. The suspension was centrifugated for 8 min-

utes at 7000 rpm. The precipitated latex was mixed with an equal amount of PBS and Triton X-100 and washed twice as before. The washed latex was mixed with a solution containing Cyanogen bromide (200mg/5ml). The pH of the prepared suspension was fixed on 10.5 by adding NaOH 0.3%. The mixture left on electrical shaker for 30 minutes; after that the mixture centrifugated and the activated latex particles washed as before. The precipitated latex was mixed with 50 mM MES buffer (pH: 6.1). 750µl IgG (600 µg) was added to the latex suspension. The mixture was kept on electric shaker for 30 minutes and overnight in 4°C. The bovine serum albumin 250µl (4mg/ml) were added as blocker. The reagents shaked for 1 hour at room temperature. The suspension was centrifugated for 8 minutes at 6000 rpm. The precipitated latex was mixed with 350µl of MES buffer containing BSA (4mg/ml). The stability of the produced antibacterial latex was tested during 6 months. In addition, mixtures of the 4 prepared antibodies were attached to latex particles as mentioned above.

Latex Agglutination test

The ready to use bacterial latex (25µl) was poured on agglutination plate and mixed with an equal volume of the various concentrations (seven 1/10 serial dilutions of the bacteria prepared from the initial concentration equal to 1 MacFarland standard in normal saline and sterilized bovine milk) of the tested bacteria. The plate was shaked constantly and the reaction was observed for 4 minutes. Along with the test samples, negative sample of bacteria and latex was used as control. The crossing reactions were tested by combination of the bacterial latex with bacteria other than corresponding bacteria. The sensitivity of the bacterial latex was tested for the detection of various numbers of the tested bacteria in experimentally contaminated milk. Firstly, bacteria were mixed with 1ml PBS at a concentration adjusted to 2 McFarland standard. The bacterial suspensions were centrifugated and the precipitates were mixed with an equal volume of the sterilized milk. Seven 1/10 serial dilutions of each bacterium were prepared on sterilized normal milk samples; the 2 last dilutions were cultured on blood agar and the bacterial colony were counted (CFU/ml). The ready to use bacterial latex particles were diluted as 1/3 in MES buffer. The agglutination tests were done by mixing of the diluted latex (75µl) with dilutions of the experimentally contaminated milk (25µl). The negative samples were incubated in 37°C and were tested every 30 minutes.

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Author Contributions

Conceived and designed the experiments: M.Kh., M.Gh., D.Gh. S.G. Performed the experiments: M.Kh M.B.D., D.Gh Analyzed the data: S.G. Research space and equipment: M.Kh., M.Gh., D.Gh. S.G Contributed reagents/materials/analysis tools: M.Kh., M.Gh., D.Gh. S.G.

Conflict of Interest

Author declares that they have no conflict of interest.

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