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Research Article

Diagnosis of *Staphylococcus aureus* Infection in Bovine Mastitis using its Affinity Purified Fraction

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Abstract | Affinity purified fraction from *Staphylococcus aureus* (*S. aureus*) was extracted, characterized and evaluated for its potency in diagnosis of *S. aureus* mastitis through indirect Enzyme Linked Immunosorbent Assay (ELISA). For purification of fraction, bacterial fresh cultures were centrifuged at 3000 rpm / 20 min and were homogenate in phosphate buffer saline and centrifuged at 15000 rpm / 20 min that known as the crude antigen. It entered to affinity column chromatography [Cyanogen Bromide-Sepharose 4B (CNBr-Sepharose 4B)] (Sigma Chemical Co.), obtain the fraction. In brief, IgG of cows *S. aureus* positive sera were purified by precipitation with 50% ammonium sulphate followed by purification with Protein A Sepharose gel. The separated IgG was dialyzed for 3 days against a 0.1 M NaHCO3 buffer pH 8.3 with 0.5 M sodium chloride and 0.02 percent NaN3 before being linked to CNBr-Sepharose 4B swollen beads. Bound fraction was eluted with mixture of glycine (0.1 M) and sodium chloride (0.5 M) at pH (2.3). Purified antigens and unbound were inspected of protein concentration. In the crude antigen, there were two bands with molecular weights of 73 and 64 KDa, compared to ten bands with molecular weights ranging from 209 to 24 KDa. The isolated fraction showed diagnostic properties of *S. aureus* mastitis using indirect ELISA with 100% sensitivity and 95 % specificity. The fraction validity lasted for more than one year of storage at -20 °C. The current study introduces effective way for *S. aureus* mastitis diagnosis through the using of purified fraction instead of the classical way.

Keywords | *Staphylococcus aureus*, Affinity column chromatography, Antigen, SDS-PAGE, Indirect ELISA

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INTRODUCTION

Mastitis is the mammary glands inflammation of dairy cattle which high prevalence worldwide (Abrahmsén et al., 2014). The disease causes economic losses due to alterations in milk quality, reduction in milk yield and increased costs of treatment which results in negative impact on the dairy industry (Kiku et al., 2017). Some scientists consider mastitis is the most challenging disease after foot and mouth disease in dairy animals (Sharma et al., 2007). The disease is caused by several bacterial species included *Staphylococcus aureus* which enters teat canal, colonizes, proliferates and secretes toxins that damage mammary gland cells (Bhosale et al., 2014; Rainard et al., 2020). Despite the importance of type 3 hypersensitivity, infection resulted in the production of several inter-leukeins (IL-17A, IL-17F, and IL-22) in milk and mammary glands (Rainard et al., 2021). The disease causes increase in somatic cell count, inflammatory cytokines levels, antibody production and bacterial count. Diagnosis of mastitis based mainly on counting milk inflammatory cells as macrophages, lymphocytes, neutrophils and eosinophils. California mastitis test is successfully used in diagnosis of the disease (Kandeel et

al., 2018). The diagnosis also depends on measurement of milk electrical conductivity, enzyme activity as lactate dehydrogenase (LDH), N acetyl b D glucosaminidase (NAG-ase), determination of inflammatory cytokines concentrations and antibody titer, bacterial culturing, Polymerase chain reaction for pathogen detection, immune response and proteomics based detection (Lakshmi, 2015). Multiplex PCR is suitable in diagnosis of mastitis where it identifies several pathogens in the same time. But its main disadvantage is competition of PCR substances which resulted in little sensitivity (Amin et al., 2011). Mass spectrometry and two dimensional electrophoresis identify host proteins expressed in infected animals (Lippolis and Reinhardt, 2005; Smolenski et al., 2007). Detection of immune responses depends on specific antigen(s) and sensitive assay(s) for accurate diagnosis.

Staphylococcus aureus has many virulence factors as surface proteins that responsible for cell wall adhesion and secreted proteins obvious during infection (Foster et al., 2014). These exposed proteins are fundamental for survival and proliferation of *S. aureus*. Isolation and identification of these antigens are essential for its utilization in the diagnosis of mastitis. Among these antigens, microbial surface component recognizing adhesive matrix molecules (MSCRAMMs) is one of four classes of *S. aureus* surface proteins and are the largest class. These proteins were identified by structural and functional analyses (Foster et al., 2014). During *S. aureus* bovine mastitis, iron regulated surface determinant A (IsdA) had very important role for *S. aureus* such as: surface adhesion, iron sequestration, and expressed highly, conserved and immunogenic proprietary (Misra et al., 2017). *S. aureus* possess unique proteins which were identified as a number of thirty eight. Some of these proteins (8) were portended to be surface proteins and involved in virulence of *S. aureus*. Iron-regulated surface determinant protein C (IsdC) and ESAT-6 extracellular protein (EsxA) were considered as two surface proteins and their immune reactivity was confirmed by ELISA (Misra et al., 2018). Interestingly, both proteins are conserved in 37 bovine *S. aureus* isolates as proved by PCR and amino acid symmetry discovered that sequences of IsdC and EsxA are highly memorized (Misra et al., 2018).

On the other hand, in acute or chronic mastitis, there are various immunoglobulins (IgG and IgM) secreted in blood and milk. The indirect ELISA used these secreted antibodies that are produced during infection for identification of the *S. aureus* antigens (Boerhout et al., 2016; Misra et al., 2018). Moreover, milk IgG1 antibodies are necessary for controlling and minimizing colony forming units so that these antibodies are produced in high concentrations. In experimental infections, it has been noticed that the existence of IgG1 isn't related to *S. aureus* infection only but also associated with different types of mastitis (Boerhout et al., 2016). *S. aureus* enterotoxins had been detected in food samples such as cheese, milk and cake using sandwich ELISA was also another tool for diagnosis of mastitis (Sundararaj et al., 2019).

Economic losses due to mastitis are attributed to late and improper diagnosis of etiological pathogens. Early and accurate diagnosis is essential where the disease developed very quickly and delay even in terms of few hours may lead to complete loss of teat and udder. Despite of multiple research and knowledge about diagnostics, effective diagnostic assays are still developing. Consequently, the current research aims to isolate and identify *S. aureus* immunogenic antigen to be coming out well applied for mastitis diagnosis.

MATERIALS AND METHODS

Ethical approval

No approval from the Institutional Animal Ethical Committee was required to conduct this investigation, because no intrusive procedure was employed.

Samples collection

S. aureus positive blood and milk samples, which were previously identified by PCR (Hussein et al., 2017) were used in the current study.

Antigen preparation

S. aureus was cultured onto medium (mannitol salt agar as a selective media for *S. aureus*), keeping at 37 °C for 24 hrs. Pure colonies were isolated and inoculated in Brain Heart Infusion (BHI) broth medium. It was incubated in shaker incubator (30cycles/min) at 37°C for 24 hrs. The grown bacteria were centrifuged (3000 rpm / 20 min). The supernatant was discarded and the precipitate was washed 3 times with distilled water. The bacterial suspension adjusted to a concentration of (4×10^6cfu / ml) in distilled water then boiled for 1 hour in water bath. The antigen was prepared according to (Al-Mayah and Saeed, 2013) by bacterial homogenization in 0.1M PH 7.2 phosphate buffer saline. The mixture was centrifuged in cooling centrifugation at (15000 xg / 20 min). After centrifugation, the supernatant and antigen were separated and freezed until use.

Ammonium sulfate precipitation of Immunoglobulins (IgS) of positive cow sera

Positive cow IgS were precipitated using a (50 %) saturated ammonium sulphate solution (SAS). For 3 days at 4°C, ammonium sulphate was extracted by dialysis against 15 mM phosphate-buffered saline (PBS). Lyophilization was used to concentrate the IgS. The precipitation process was according to (Abdel-Rahman et al., 2017).

Affinity chromatography purification of IgG s by protein A

Purification of IgG was done on protein A Sepharose gel with 0.1 M glycine as the eluting buffer. The procedure of purification was as follows (Abdel-Rahman et al., 2017).

Immuno-affinity column chromatography for antigen purification

The antigen of *S. aureus* was purified using Sepharose 4B affinity column chromatography. In a nutshell, immunoglobulins from positive cow serum were dialyzed for 3 days against NaHCO3 buffer (0.1 M-pH 8.3) containing Nacl (0.5 M) and NaN3 (0.02 %) before being linked to swollen beads of CNBr-Sepharose 4B (Sigma-Aldrich, USA) according to the manufacturer's instructions. 50 mM glycine, pH 2.3, containing 0.5 M Nacl pH 2.3 was used to elute the bound fraction (Hjelm et al., 1972).

Estimation of protein content

Protein content of both crude and purified fraction was assayed colorimetrically using folin reagent and the standard bovine serum albumin according to the method of Lowry et al. (1951).

SDS-page

Ten percent reducing polyacrylamide gel was used and 2-mercaptoethanol sample buffer separately mixed to the crude antigen and the purified fraction before loading in gel. After electrophoresis, proteins were stained with silver nitrate (Merck, Germany) and protein marker was used to calculate the bands molecular weights that get from Sigma-Aldrich (Laemmli, 1970; Shaapan et al., 2021). Bio Rad Gel Doc XR+ Apparatus was used to determine the molecular weights.

ELISA

Indirect ELISA was used to assess the diagnostic properties of *S. aureus* fraction in mastitis. It was also used to evaluate the purified fraction sensitivity and specificity and its validity. Antigens concentration, antibodies dilution and anti-bovine horse radish peroxidase (Sigma) dilution were determined according to checker board titration. This assay had been performed according to Paul and Akira (1974). In brief, microtitration plate was coated with 100 µl of each antigen separately and incubated overnight at 4˚C. After washing the plate was blocked with 05% Bovine Serum Albumin in phosphate-buffered saline for 1h at room temperature. 100 µl of each serum samples were added to each well after washing. 1.5h of incubation at 37˚C, the plate was washed and 100 µl of diluted peroxidase conjugated anti-bovine antibodies was added to each well and the plate was incubated for 1h at 37˚C. Ortho-Phenlene diamine substrate buffer containing H_2O_2 was added and the plate was read spectrophotometrically at 450 nm in Microplate reader ELx 800 USA.

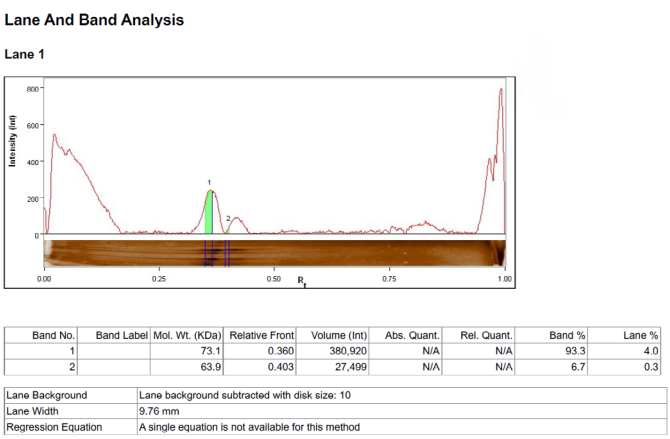
Specificity and sensitivity

Specificity, sensitivity, positive and negative predictive values were calculated according to Tabouret et al. (2001) and Parikh et al. (2008). Sensitivity (%) = [True positive/ (True positive + False negative)] ×100; Specificity (%) = [True negative/ (True negative + False positive)] ×100; PPV % = [True positive / (True positive + False positive)] ×100; NPV % = [True negative / (False negative + True negative)] ×100; Diagnostic Efficacy (%) = [(True negative + True positive) / (True positive +False positive + true negative + false negative x 100; Sensitivity, tp x 100/(tp + fn); specificity, tn x 100/(tn + fp); Diagnostic efficiency, (tn + tp) X 100/(tp + fp + tn + fn) (Gonzaleza et al., 2000).

RESULTS AND DISCUSSION

SDS-PAGE profile of crude antigen and purified fraction

S. aureus crude antigen and isolated fraction profiles obtained by SDS–PAGE showing in Figures 1 and 2. The crude antigen showed ten bands at molecular weights 208.9, 159.8, 131.6, 94.2, 74.7, 63.9, 49.4, 32.5, 28.4 and 24.4 KDa. The diagnostic fraction was represented by two bands at 73.1 KDa and 63.9 KDa.



Band No.	Band Label	Mol. Wt. (KDa)	Relative Front	Volume (Int)	Abs. Quant.	Rel. Quant.	Band %	Lane %
1		73.1	0.360	380,920	N/A	N/A	93.3	4.0
2		63.9	0.403	27,499	N/A	N/A	6.7	0.3

Lane Background	Lane background subtracted with disk size: 10
Lane Width	9.76 mm
Regression Equation	A single equation is not available for this method

Figure 1: SDS PAGE profile of *S. aureus* purified fraction showed 2 bands (2 peaks).

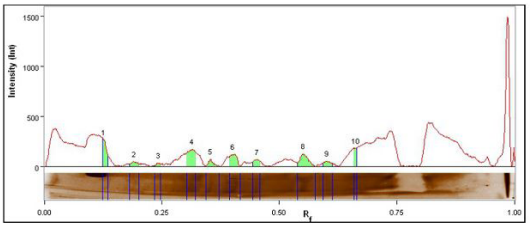
The binding activities of cows' positive sera against the crude antigen of *S. aureus*

Different binding activities of positive serum samples against crude antigen of *S. aureus* reflected by different OD readings are shown in Figure 3.

The binding activities of cows' positive sera against the fraction of *S. aureus*

As shown in Figure 4, the purification process resulted in a fraction with high yield of binding activities exist in the whole extract expressed in OD readings higher than that

of crude extract.



Band No.	Band Label	Mol. Wt. (KDa)	Relative Front	Volume (Int)	Abs. Quant.	Rel. Quant.	Band %	Lane %
1		208.9	0.127	218,070	N/A	N/A	14.8	1.7
2		159.8	0.191	71,730	N/A	N/A	4.9	0.6
3		131.6	0.243	32,580	N/A	N/A	2.2	0.3
4		94.2	0.314	307,350	N/A	N/A	20.8	2.4
5		74.7	0.353	84,870	N/A	N/A	5.7	0.7
6		63.9	0.402	203,940	N/A	N/A	13.8	1.6
7		49.4	0.453	101,700	N/A	N/A	6.9	0.8
8		32.5	0.551	251,820	N/A	N/A	17.0	2.0
9		28.4	0.601	84,780	N/A	N/A	5.7	0.7
10		24.4	0.663	120,420	N/A	N/A	8.2	0.9

Lane Background	Lane background subtracted with disk size: 10
Lane Width	8.21 mm
Regression Equation	A single equation is not available for this method

Figure 2: SDS PAGE profile of *S. aureus* Crude Antigen show 10 bands (10 peaks).

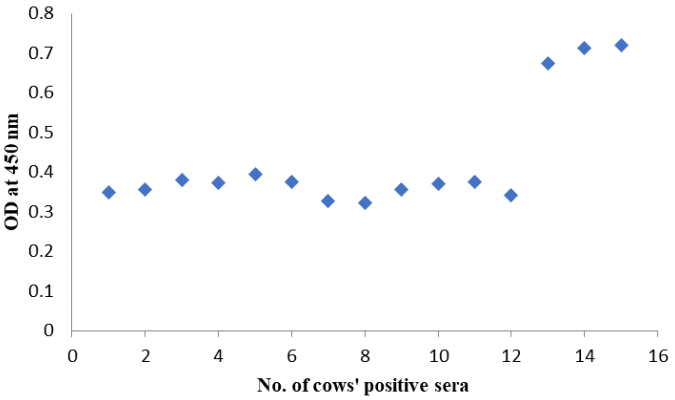


Figure 3: Mean OD values of cows' positive sera against the crude antigen of *S. aureus*.

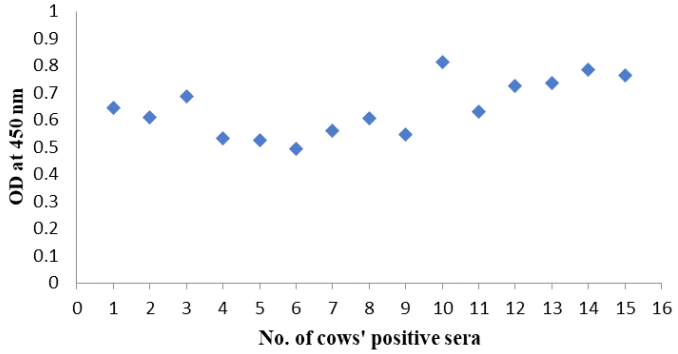


Figure 4: Mean OD values of cows' positive sera against the purified fraction of *S. aureus*.

Potentials of the purified fraction of *S. aureus* to detect antibodies of mastitis cows' milk samples

As shown in Figure 5, higher binding activities profile was observed in milk infected with *S. aureus* than that observed in positive serum samples toward fraction of *S. aureus* reflected by higher OD readings.

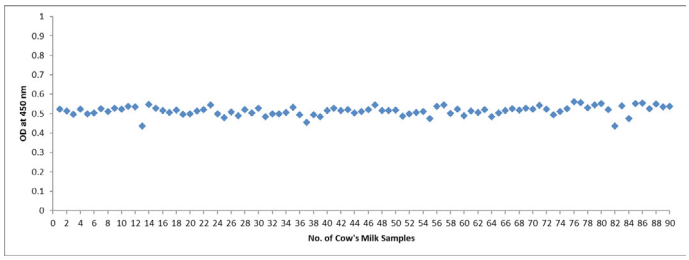


Figure 5: Potency of the purified fraction of *S. aureus to antibodies of positive cows' milk samples.*

Stability of *S. aureus* purified fraction

As shown in Figure 6, the binding activities of the purified *S. aureus* fraction stored for one year at -20°C reflected by OD readings.

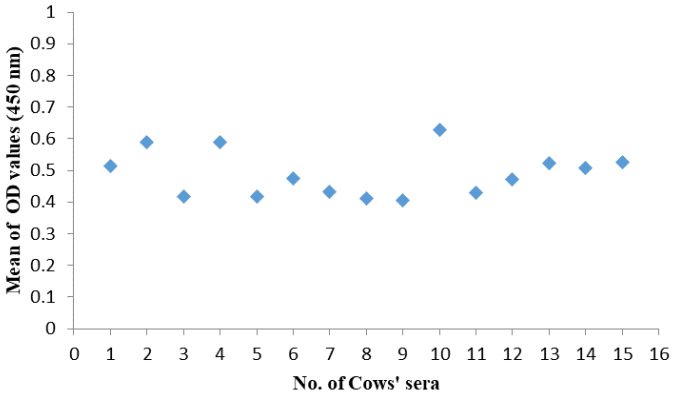


Figure 6: Significant validity of the stored purified fraction of *S. aureus* for one year at -20°C.

Comparison between activity of fresh purified and stored *S. aureus* fraction for one year at -20°C in diagnosis of mastitis

Non-significant decrease in the OD readings between freshly purified fraction and the stored fraction which proved that the stored fraction still valid in diagnosis of *S. aureus* mastitis as shown in Figure 7.

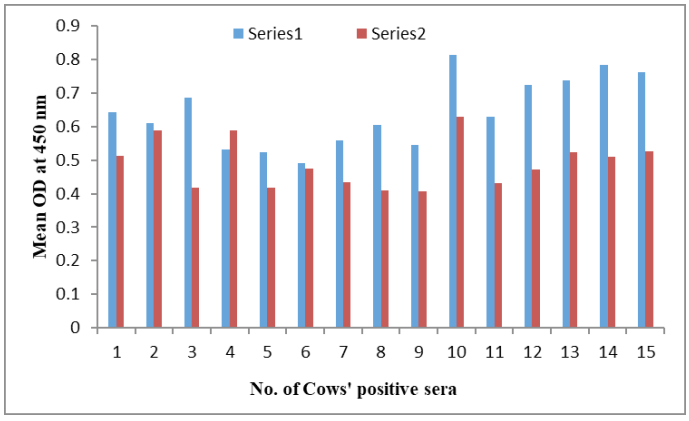


Figure 7: Validity evaluation of the stored purified (Series 2) *S. aureus* fraction for one year at -20°C compared with freshly purified fraction (Series 1).

Specificity of *S. aureus* purified fraction in diagnosis of mastitis

Comparative evaluation of specificity of the *S. aureus* fraction and others antigens in mastitis diagnosis as showing at Figure 8.

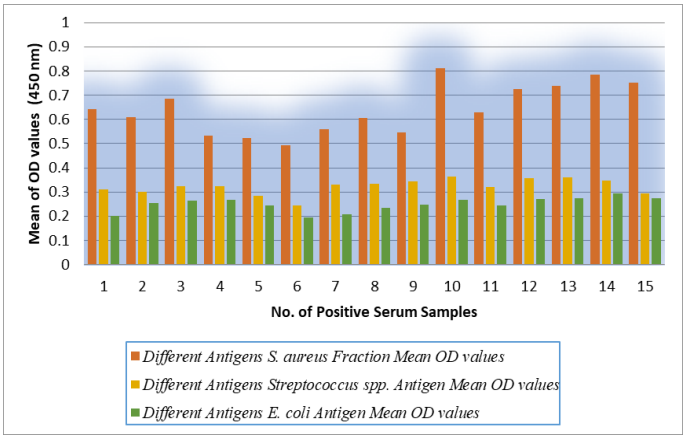


Figure 8: Specificity evaluation of *S. aureus* purified fraction in comparison with different antigens in diagnosis of mastitis.

Attachment of *S. aureus*, like other bacterial species, to host tissue is essential for development of infection. This attachment takes place by bacterial proteins which are the first molecules to interact with host cells and tissues (Foster et al., 2014). Some of these proteins are of cell wall origin and others are released (EI-Jakee et al., 2013). Isolation and identification of these proteins are the first step in the development of diagnostics and vaccines. Based on proteomic analysis, the current study introduces two affinity purified protein bands isolated from *S. aureus* extract by affinity column chromatography (CNBr-Sepharose 4B) to be used in the bovine mastitis diagnosis via indirect ELISA. Adoption of proteomics in identification and characterization of *S.aureus* proteins was previously proved (Santos et al., 2021). Use of CNBr-Sepharose 4B for protein purification of *S. aureus* is unique and, in most cases, resulted in purified immunogenic components (Forman et al., 1987). The similar facet between the current study and that of (Forman et al., 1987) is the adoption of protein A sepharose column chromatography to purify IgG from mastitic serum samples. This approach was previously adopted by others (Jungbauer et al., 1989; Abdel-Rahman et al., 2017) who succeeded in IgG purification from serum samples. Affinity chromatography doesn't restrict to CNBr–Sepharose 4B, they used Sepharose of nickel nitrilotriacetic acid to purify *S. aureus* proteins but purification was followed by immunoblot to examine immunogenicity of isolated fractions (Aly et al., 2017). The advantage of CNBr-Sepharose 4B affinity chromatography is in one step purification of immunogenic fraction was carried out. Similarly, DEAE sephacel ion exchange chromatography (Sigma Chemical Co.), was used to purify *S. aureus* capsular polysaccharide (CP5). The purification process was followed by one- dimensional SDS-PAGE and immunoblot to prove immunogenicity of purified component (Li et al., 2018).

In the current study, two bands of molecular weights 73.1 and 63.9 KDa were purified from *S. aureus* and characterized by one dimensional SDS-PAGE. Earlier, since 1987, molecule of 210 KDa was isolated by affinity column chromatography and characterized it by SDS-PAGE with additional smaller peptides with fibronectin binding properties including 29 KDa (Gonzaleza et al., 2000). Moreover, among isolated proteins released by *S. aureus* are proteins of 114.8 KDa which contains six hydrophobic segments and 11 KDa purified by superdex 200, its immunogenicity was confirmed by immunoblot (Aly et al., 2017). With the advances of proteomic analysis, purified extracellular vesicles of *S. aureus* purified by liquid chromatography showed important proteins at 32 KDa (penicillin-binding proteins) and 138 KDa (bifunctional peptidoglycan hydrolase). The huge one is processed to 62 KDa (amidase active protein) and 51 KDa (glucosaminidase active protein) were identified by (Wnag et al., 2018). An immunogenic protein of 100 KDa, a-fibronectin binding protein ClfA A-FnBPA, was purified and characterized by SDS-PAGE and immunoblot (Li et al., 2018). In bloodstream infections, these molecules with others are contributed to *S. aureus* ability for stabilizing permanent abscesses as well as the modification of immune responses. Actually, it is difficult to compare any of these molecules with each other or with those isolated in the current study because each research has its own approach and methodology.

Research will be continued until a highly immunogenic component(s) isolated and formulated as diagnostic reagent.

The isolated bands in the current study, proved potency in the detection of antigen-specific antibody responses of *S. aureus* in mastitis cow milk and serum samples by indirect ELISA which means that these bands are expressed during infection and stimulate humoral responses in blood and udder. Two surface proteins of *S. aureus* were isolated and showed immunoreactivity with mastitis cow milk and serum (Misra et al., 2017). But, the same two surface proteins were showed immunoreactivity in milk only (Misra et al., 2018). The current study and previous ones confirmed the concept that *S. aureus* presents multiple immunogens during infection either on blood or in milk. Alternatively, diagnosis of mastitis could be performed by antigen detection using monoclonal antibodies by sandwich ELISA (Sundararaj et al., 2019). However, the antibodies; monoclonal or polyclonal are raised against specific target antigen. So, antibody detection or antigen detection for

mastitis diagnosis is two faces for one coin. Importantly, some protein molecules are conserved in multiple isolates of *S. aureus* as proved by (Misra et al., 2018), Are these molecules conserved in other bacterial species causing mastitis? If they are, they could be successfully used in the diagnosis of mastitis regardless of its etiological agents which is a very good area of our future research.

CONCLUSIONS AND RECOMMENDATIONS

The diagnostic affinity purified fraction could be used for rapid diagnosis of bovine mastitis instead of classical way of bacteriological culture and biochemical tests in order to reduce time and cost of diagnosis and as an essential step for infection control.

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NOVELTY STATEMENT

The current study introduces novelty antigen for accurate serodiagnosis of *S. aureus* bovine mastitis.

AUTHOR'S CONTRIBUTION

All authors have designed the plan of study and supervised all the steps. They have read and agreed to the published version of the manuscript.

Conflicts of interest

The authors have declared no conflict of interest.

REFERENCES

Abdel-Rahman EH, El-Jakee JK, Hatem ME, Ata NS, Fouad EA (2017). Preparation of goat and rabbit anti-camel immunoglobulin G whole molecule labeled with horseradish peroxidase. Vet. World, 10(1): 92-100.

Abrahmsén M, Persson Y, Kanyima BM, Båge R (2014). Prevalence of subclinical mastitis in dairy farms in urban and peri-urban areas of Kampala, Uganda. Trop. Anim. Health Prod., 46(1): 99-105.

Al-Mayah AA, Saeed EA (2013). Preparation of diagnostic monovalent antisera against *Staphylococcus aureus.* Med. J. Babylon, 10(2): 407-412.

Aly KA, Anderson M, Ohr RJ, Missiakas D (2017). Isolation of a membrane protein complex for type VII secretion in *Staphylococcus aureus*. J. Bacteriol., 199(23): e00482-17.

Amin AS, Amouda RHH, Abdel-All AAA (2011). PCR assays for detecting major pathogens of mastitis in milk samples. World J. Dairy Food Sci., 6(2): 199-206.

Bhosale RR, Osmani RA, Ghodake PP, Shaikh SM, Chavan SR (2014). Mastitis an intensive crisis in veterinary science. Int. J. Pharm. Res. Health Sci., 2(2): 96-103.

Boerhout EM, Koets AP, Vernooij JC, Mols-Vorstermans TG, Nuijten PJ, Rutten VP, Bijlsma JJ, Eisenberg SW (2016). Re-isolation of *Staphylococcus aureus* from bovine milk following experimental inoculation is influenced by fat percentage and specific immunoglobulin G1 titer in milk. J. Dairy Sci., 99: 4259–4269.

Ei-Jakee J, Marouf SA, Ata NS, Abdel-Rahman EH, Abd El-Moez SI, Samy AA, El-Sayed WE (2013). Rapid method for detection of *Staphylococcus aureus* enterotoxins in food. Glob. Vet., 11(3): 335-341.

Forman G, Switalski LM, Speziale P, Hook M (1987). Isolation and characterization of a fibronectin receptor from *Staphylococcus aureus*. J. Biol. Chem., 262(14): 6564-6571.

Foster TJ, Geoghegan JA, Ganesh VK, Höök M (2014). Adhesion, invasion and evasion: The many functions of the surface proteins of *Staphylococcus aureus*. Nat. Rev. Microbiol., 12(1): 49-62.

Gonzaleza GS, Lorenzo C, Nieto A (2000). Improved immunodiagnosis of cystic hydatid disease by using a synthetic peptide with higher diagnostic value than that of its parent protein, *Echinococcus granulosus* Antigen B. J. Clin. Microbiol., 38(11): 3979-3983.

Hjelm H, Hjelm K, Sjöquist J (1972). Protein A from *Staphylococcus aureus* its isolation by affinity chromatography and its use as an immunosorbent for isolation of immunoglobulins. FEBS Lett., 28(1): 73-76.

Hussein HA, Younes AM, Arafa AA, Fouad EA, Khairy EA, Gomaa AM, Abd El-Razik KA (2017). Molecular and bacteriological investigation of contagious mastitis caused by *Staphylococcus aureus* in dairy cattle farms in Egypt. Int. J. Microbiol. Res., 8(3): 92-99.

Jungbauer A, Tauer C, Reiter M, Purtscher M, Wenisch E, Steindi F, Buchacher A, Katinger H (1989). Comparison of protein A, protein G and copolymerized hydroxyapatite for the purification of human monoclonal antibodies. J. Chromatogr., 476: 257-268.

Kandeel SA, Morin DE, Calloway CD, Constable PD (2018). Association of California mastitis test scores with intramammary infection status in lactating dairy cows admitted to a veterinary teaching hospital. J. Vet. Int. Med., 32(1): 497-505.

Kiku Y, Ozawa T, Takahashi H, Kushibiki S, Inumaru S, Shingu H, Nagasawa Y, Watanabe A, Hata E, Hayashi T (2017). Effect of intramammary infusion of recombinant bovine GM-CSF and IL-8 on CMT score, somatic cell count, and milk mononuclear cell populations in Holstein cows with *Staphylococcus aureus* subclinical mastitis. Vet. Res. Commun., 41(3): 175–182.

Laemmli UK (1970). Cleavage of structural protein during the assembly of the head of bacteriophage T4. Nat. J., 227(5259): 680-685.

Lakshmi GJ (2015). Mechanism of resistance, phenotyping and genotyping of methicillin resistant *Staphylococcus aureus*: A review. Int. J. Curr. Microbiol. App. Sci., 4(3): 810-818.

Li T, Huang M, Song Z, Zhang H, Chen C (2018). Biological characteristics and conjugated antigens of ClfA A-FnBPA and CP5 in *Staphylococcus aureus*. Can. J. Vet. Res., 82(1): 48–54.

Lippolis JD, Reinhardt TA (2005). Proteomic survey of bovine neutrophils. Vet. Immunol. Immunopathol., 103(1-2): 53-

65.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951). Protein measurement with the folin phenol reagent. J. Biol. Chem., 193(1): 265-275.

Misra N, Pu X, Holt DN, McGuire MA, Tinker JK (2018). Immunoproteomics to identify *Staphylococcus aureus* antigens expressed in bovine milk during mastitis. J. Dairy Sci., 101(7): 6296–6309.

Misra N, Wines T, Knopp C, McGuire M, Tinker JK (2017). Expression, immunogenicity and variation of iron-regulated surface protein A from bovine isolates of *Staphylococcus aureus*. FEMS Microbiol. Lett., 364(9): fnx082.

Parikh R, Mathai A, Parikh S, Sekhar GC, and Thomas R, (2008). Understanding and using sensitivity, specificity and predictive values. Indian J Ophthalmol. 56(1): 45-50.

Paul KN, and Akira K, (1974). Peroxidase-Labeled Antibody. A New Method of Conjugation.Histochem.cytochem. J., 22: 1084-1091.

Rainard P, Cunha P, Martins RP, Gilbert FB Germon P and Foucras G. (2020). Type 3 immunity: a perspective for the defense of the mammary gland against infections. Vet. Res. 51(129): 1-8.

Rainard P, Gilbert FB, Germon P, Foucras G (2021). Invited review: A critical appraisal of mastitis vaccines for dairy cows. J. Dairy Sci., 104(10): 10427-10448.

Santos KR, Souza FN, Ramos-Sanchez EM, Batista CF, Reis LC, Fotoran WF, Heinemann MB, Goto H, Gidlund M, Cunha AF, Faria AR, Andrade HM, Lage AP, Cerqueira MP, Libera AM (2021). *Staphylococcus aureus* protection-related type 3 cell-mediated immune response elicited by recombinant proteins and GM-CSF DNA vaccine. Vaccines, 9(8): 899.

Shaapan RM, Toaleb NI, Abdel-Rahman EH (2021). Detection of *Toxoplasma gondii*-specific immunoglobulin (IgG) antibodies in meat juice of beef. Iraqi J. Vet. Sci., 35(3): 513-521.

Sharma N, Maiti SK, Sharma KK (2007). Prevalence, etiology and antibiogram of micro-organism associated with sub-clinical mastitis in buffaloes in durg, Chhattisgarh state (India). Int. J. Dairy Sci., 2(2):145-151.

Sheet OH, Jwher DM, Al-Sanjary RA, Alajami AD (2021). Direct detection of *Staphylococcus aureus* in camel milk in the Nineveh governorate by using the PCR technique. Iraqi J. Vet. Sci., 35(4): 669-672.

Smolenski G, Haines S, Kwan FY, Bond J, Farr V (2007). Characterization of host defence proteins in milk using a proteomic approach. J. Proteome Res., 6(1): 207-215.

Sundararaj N, Kalagatur NK, Mudili V, Krishna K, Antony SM (2019). Isolation and identification of enterotoxigenic *Staphylococcus aureus* isolates from Indian food samples: Evaluation of in-house developed aptamer linked sandwich ELISA (ALISA) method. J. Food Sci. Technol., 56(2): 1016–1026.

Tabouret G, Prevot F, Bergeaud JP, Dorchies P, and Jacquiet P. (2001). *Oestrus ovis* (Diptera: Oestridae): sheep humoral immune response to purified excreted/secreted salivary gland 28 kDa antigen complex from second and third instar larvae.Veterinary Parasitology. 101(1): 53-66.

Wang X, Thompson CD, Weidenmaier C, Lee JC (2018). Release of *Staphylococcus aureus* extracellular vesicles and their application as a vaccine platform. Nat. Commun., 9(1): 1379.