

# Beta hemolysin gene of *Staphylococcus* phage 3AJ\_2017 genome is a suitable molecular marker for identification and characterization of pathogenic *Staphylococcus aureus*

Silviane A. Miruka<sup>1</sup>  | Gabriel O. Aboge<sup>1,2</sup> | Rosaline W. Macharia<sup>1</sup> |  
George O. Obiero<sup>1</sup> | Isaac M. Omwenga<sup>1</sup>

<sup>1</sup> Center for Biotechnology and Bioinformatics, College of Biological and Physical Sciences, University of Nairobi, Nairobi, Kenya

<sup>2</sup> Public Health Pharmacology and Toxicology, Faculty of Veterinary Medicine, University of Nairobi, Nairobi, Kenya

## Correspondence

Silviane Miruka, Center for Biotechnology and Bioinformatics, College of Biological and Physical Sciences, University of Nairobi, 00100, Nairobi, Kenya.

Email: [silvianejoash@gmail.com](mailto:silvianejoash@gmail.com)

## Abstract

**Background:** *Staphylococcus aureus* cause diseases both in humans and animals. These diseases range from mild to fatal infections thus necessitating development of a specific molecular method for detection of pathogenic *S. aureus*.

**Objectives:** To identify and analyze genetic profile of pathogenic *S. aureus* using bacteriophage based genetic biomarkers.

**Methods:** Using culture and biochemical methods, 148 *S. aureus* (87 %) were isolated from 170 raw milk samples taken from 10 dairy farms in Marsabit and Isiolo counties in Northern Kenya between June 2016 and February 2017. The samples were collected directly from dairy lactating cows previously diagnosed with *S. aureus* in a follow-up study. The isolates were analyzed by PCR and sequencing of beta hemolysin (*hlyB*) gene. The genetic relationship between five Kenyan *S. aureus* isolates and five isolates previously identified was inferred.

**Results:** From the 96 isolates screened for *hlyB* gene, 75 (78.1%) tested positive. Some of the positive isolates yielded a band size of 975 bp, while others 1100 bp. Through Basic Local Alignment Search Tool (BLAST) search analysis, the two different band sizes (975 bp and 1100 bp) were both confirmed to be *hlyB* gene from *S. aureus* isolates indicating that the difference in band size may have been due to deletions that were detected in the 975 bp *hlyB* gene. Some *S. aureus* isolates from Kenya appeared to be closely related to isolates from other parts of the world, while some showed a distant relationship.

**Conclusions:** Phage-derived *hlyB* gene is a suitable molecular marker for detection of pathogenic *S. aureus*.

## KEYWORDS

bacteriophages, *hlyB* gene, molecular identification, *Staphylococcus aureus*

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## 1 | INTRODUCTION

*Staphylococcus aureus* is one of the major causes of infectious disease both in community and hospitals (Feng et al., 2008; Kimmig et al., 2021). Infections caused by *S. aureus* ranges from mild superficial skin infection to fatal cases such as toxic shock syndrome (Schmidt et al., 2017). Mortality rate due to *S. aureus* infection ranges from about 15% to 40% (Schmidt et al., 2017) with an economical loss of about 30%–40% in livestock farming (Sineke et al., 2021). Accurate detection of pathogenic *S. aureus* is therefore of importance for effective management of infections (Chen et al., 2013; Tadros et al., 2013). Previously bacteriophage (Phage K) has been used to detect *S. aureus* using liquid chromatography mass-spectrometry instead of conventional PCR assay (Kurlenda & Grinholc, 2012). Other previous studies have used other genes such as 16S rRNA and *nuc* for the identification of *S. aureus*. However, sometimes these genes are not species specific (Ferry et al., 2005; Rees & Barr, 2017); hence, there is a need for a more specific gene. Ability of bacteriophage to aid in horizontal gene transfer between bacteria prompted us to use lysogenic *S. aureus* 3AJ\_2017 bacteriophage in this study. It is known that during infection phages integrate their unique genomic materials into their bacterial host chromosome; thus, they are able to replicate as the bacterial host acquires unique genes. These unique genes can be used in identification of the host bacteria (C. P. Gordon et al., 2013; Hacker & Carniel, 2001; Haveri et al., 2008). Usually, pathogenicity of *S. aureus* is as a result of virulence factors such as beta hemolysin which aids in biofilm formation for their attachment on the host skin, their invasion and survival in their host by evasion of the host immune defence mechanism (R. J. Gordon & Lowy, 2008). These virulence factors also enable *S. aureus* to acquire nutrients and to proliferate in mammalian hosts (Ferry et al., 2005; Rees & Barr, 2017).

Apart from beta hemolysin, other virulence factors include coagulase and leukocidine (C. P. Gordon et al., 2013).

Bacteriophages influence their host pathogenicity either by directly encoding virulence factors or indirectly by enhancing the bacterial fitness during infection (Hacker & Carniel, 2001). Busby et al. (2013) reported that the pathogenic bacteria strains harboured greater portion of phage-related genes than the non-pathogenic bacterial strains. Bacteriophages have narrow host range and high host specificity owing to the fact that they identify their host by specific receptor molecules on the host cell (Deghorain & Van Melderen, 2012; Mosier-Boss et al., 2003; Rastogi et al., 2018). Recently, applications of phages in the areas of biotechnology, treatment of multidrug resistant pathogens, food preservation, aquaculture diseases, pollution remediation and waste water treatment have become an inevitable option of research (Gharieb et al., 2020; Sharma et al., 2017). In this regard, we hypothesize that by using bacteriophage genetic marker shared with *S. aureus* genome, it is possible to develop a molecular tool for specifically detecting and characterizing the pathogenic bacterium. Therefore, this study has identified *hlyB* gene derived from *Staphylococcus* Phage 3AJ\_2017 genome for specific detection and characterization of pathogenic *S. aureus*.

## 2 | MATERIALS AND METHODS

### 2.1 | Isolation and identification of *S. aureus*

*S. aureus* was isolated from 170 raw milk samples that were collected from domestic farms in Marsabit and Isiolo counties in Northern Kenya. Bacterial cells were revived by mixing 1 ml of sample in 4 ml of sterile buffered peptone water (BPW, Oxoid, England) and incubated at 37°C for 24 h. A loop full of BPW enrichment was streaked on mannitol salt agar (MSA, Oxoid) and incubated for 12–48 h at 37°C. The MSA plates were examined for growth of *S. aureus* usually seen as yellow colonies. To further confirm *S. aureus* isolates, the yellow colonies from MSA were inoculated on sheep blood agar (SBA, Oxoid) to confirm haemolysin production.

Suspected *S. aureus* colonies cultured on SBA were enriched on tryptone soy agar (TSA, HIMEDIA India) (Kateete et al., 2010), and then screened biochemically using catalase and tube coagulase test (Benson et al., 2004; Reiner, 2016).

### 2.2 | DNA extraction from the bacterial cells

Bacterial DNA was extracted using Invitrogen DNA Kits (DNAeasy, USA) according to the manufacturer's protocol. At first, bacterial colonies were harvested from the TSA plates and suspended in a tube containing 180  $\mu$ l lysozyme digestion buffer (LDB, Sigma-Aldrich, USA) (lysozyme concentration of 20 mg/ml). The bacteria pellets were resuspended in 180  $\mu$ l of LDB then incubated at 37°C for 30 min for subsequent lysis. To the lysate mixture, 20  $\mu$ l of proteinase K was added and incubated for 30 min at 55°C. An amount of 200  $\mu$ l of absolute ethanol was added to the digested mixture, followed by binding of the bacterial DNA to silica gel membrane by a brief centrifuge process. In order to remove the protein impurities, two washing steps were carried out using two wash buffers. The pure bound DNA was then eluted using elution buffer and stored at 4°C for subsequent analysis. Using NanoDrop ND-1000 full spectrum UV-Vis spectrophotometer (Thermo Fisher, USA), the DNA quantity and purity was assessed at a wavelength ranging from 260 to 280 nm.

### 2.3 | Genetic marker identification and primer design

*S. aureus* bacteriophage 3AJ\_2017 sequences were retrieved from GenBank using a customized R script (Altschul, 1990). The retrieved sequences were queried against the National Centre for Biotechnology Information (NCBI) database using Basic Local Alignment Search Tool (BLAST) algorithm to determine the genes that were shared between the phage and *S. aureus*. This search was carried out at an E-value of  $1e^{-3}$ . The *hlyB* gene was selected from the phage as the suitable genetic marker based on its role in pathogenicity of *S. aureus*. Primers targeting *hlyB* gene in *S. aureus* were designed using Primer BLAST tool

(Ye et al., 2012). The *hlyB* primer sequences were as follows: forward primer as 5'TGCAGAAGATGGTGGCGTAG3' and reverse primer as 5'CTGGGGCTATTGGTCTGGTG3'.

## 2.4 | PCR and sequencing of *S. aureus* isolates

PCR reaction was carried out as a 25  $\mu$ l volume reaction in a 0.2 ml PCR tube containing 12.5  $\mu$ l of X1 Taq Master Mix (Biolabs, England), 1  $\mu$ l (10 pmol) of reverse primers and forward primers and 9.5  $\mu$ l of nuclease free water and 1  $\mu$ l of DNA template. PCR conditions for the cycler Veriti (Applied Biosystems, USA) were as follows: 95°C for 2 min (95°C for 30 s, 56°C for 30 s)  $\times$  35 cycles and an extension time of 5 min at 72°C. The PCR products were detected through gel electrophoresis. An agarose gel of 1.5% in concentration was prepared from tris-acetate-ethylenediaminetetraacetic acid buffer where ethidium bromide (1 mg/ml) was used as the staining dye. A total of 4  $\mu$ l of PCR products were loaded into the wells. An amount of 2  $\mu$ l 100 bp DNA ladder (Biolabs) was used as a molecular size marker. Electrophoresis was finally carried out at 70 V for 45 min. Gel image was viewed under ultra violet illuminator machine (Bio-Rad, England). ATCC 25923 *S. aureus* strain was used as the positive control, while for negative control distilled water was added into the reaction tube instead of DNA template. The PCR-products were purified using QIAquick gel purification kit (QIAGEN GmbH Hilden, Germany). Twelve purified *S. aureus* DNA were sequenced using the *hlyB* gene primer used in the PCR reaction. The sequenced isolates were confirmed by BLAST analysis accessed through GenBank database of the (NCBI). These sequenced isolates were analyzed for conserved domains and five of them submitted to NCBI through Bankit tool (Pirovano et al., 2017) to obtain the following accession numbers: MH674195, MH674196, MH674197, MH674198 and MH674199.

## 2.5 | Phylogenetic analysis of DNA sequences

The *hlyB* gene sequences from this study were compared with *hlyB* gene of *S. aureus* from other parts of the world to infer genetic relationship among them. Using a customized R script, *hlyB* gene sequences from other parts of the world were retrieved from NCBI. The sequences were as follows: NC\_022604 and NC\_016928 from Taiwan, NC\_021059 from United Kingdom and NC\_017340 from Germany. Seaview tool (Gouy et al., 2010) was used to align the sequences by employing MUSCLE v.3.8.3 Program (Edgar, 2004). Jalview v2.10.46 (Clamp et al., 2004) was then used to edit the aligned sequences. The aligned sequences were further analyzed under maximum likelihood method by PhyML v30 (Guindon et al., 2010). General Time Reversible (Gatto et al., 2007) substitution method was employed, a bootstrap was set at 100 and neighbour-joining interchange was selected to improve the tree construction.

**TABLE 1** Proportion of isolates that were revived on culture and positively identified as *S. aureus* using biochemical test

Culture and biochemical characterization	Number of positives (%)
Growth on MSA	148 (88.0%)
Beta hemolysis	105 (70.9%)
Coagulase	141 (95.2%)
Catalase	148 (100%)

Abbreviation: MSA, mannitol salt agar.

## 3 | RESULTS

### 3.1 | *S. aureus* isolated from raw milk

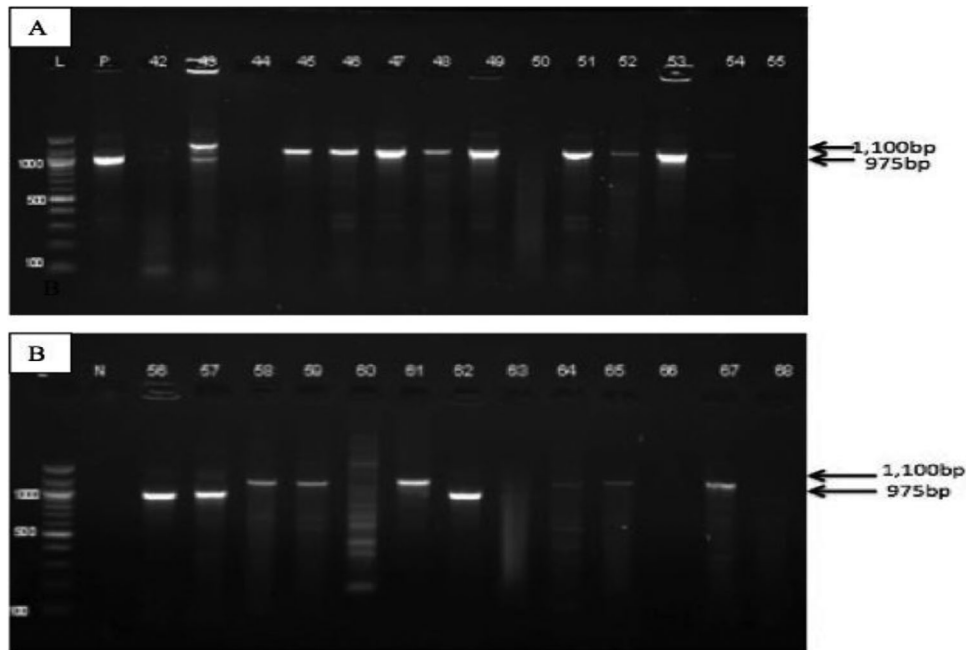
Out of 170 bacterial isolates, 168 (98.8%) were successfully revived in BPW. From the 168 revived isolates, 148 (88.0%) grew on MSA revealing yellow colonies with the media changing colour from pink to yellow. Furthermore, 105 (70.7%) of the isolates that grew on MSA also yielded colonies on SBA revealing translucent zones around them, which was an indication of beta haemolysis. The 148 (100%) isolates were all catalase positive, while 141 (95.2%) isolates were coagulase positive (Table 1). The culture and biochemical findings indicated that the isolates were *S. aureus*.

### 3.2 | Beta hemolysin gene identified from the phage genome

*S. aureus* 3AJ\_2017 bacteriophage (KX232515.1) sequences were retrieved from NCBI database (Mosier-Boss et al., 2003) using a customized R script. The size of the bacteriophage genome was 43922 bp. BLASTn search analysis of the phage whole genome yielded exclusively *S. aureus* species as its homologs with an identity of 100%, indicating that this phage specifically infects *S. aureus*. The *hlyB* gene comprising of 1023 bp was identified in the position 42,899 to 43,922 in the bacteriophage genome and then selected as the ideal genetic marker for the detection of *S. aureus*. The primers designed from the *hlyB* gene, using primer Blast tool, yielded a predicted product size of 975 bp.

### 3.3 | *S. aureus* identified by PCR and sequencing

A pair of oligonucleotide primers targeting *hlyB* gene amplified the fragment yielding a band corresponding to 975 bp. The same primers also yielded amplified products of 1100 bp (Figure 1). The resulting two different band size PCR products suggested that we could have amplified other bacterium apart from *S. aureus*. Therefore, we sequenced the two PCR products of different sizes and analyzed the corresponding PCR bands by BLASTn. Both PCR products yielded sequences homologous to *S. aureus hlyB* gene revealing a nucleotide identity of 99%. Furthermore, BLASTx analysis revealed that the translated amino



**FIGURE 1** A gel image of PCR-amplified *hlb* gene of putative pathogenic *S. aureus* isolates. The isolates yielded different bands of approximately 975 bp and 1100 bp. Panels a and b represent *S. aureus* isolates positive for PCR. Lane L is the DNA ladder (100 bp), Lane P is the reference strain for *S. aureus* ATCC 25923TM and Lane N is the negative control

acid sequences were homologous to *S. aureus hlb* protein with amino acid sequence identity of 100%. An alignment of the PCR product sequences corresponding to 975 bp and 1100 bp revealed that they were similar, indicating that the difference in sizes may have been due to deletion of 125 bp in some of the bacterial *hlb* gene. The results from PCR analyses confirmed that 75 (78%) isolates out of 96 obtained from the milk samples were indeed *S. aureus*. Five of *hlb* sequences of the *S. aureus* isolates obtained from this study have been deposited in GenBank Database under the accession numbers MH674195 and MH674199.

### 3.4 | Conserved domain contain virulence factor

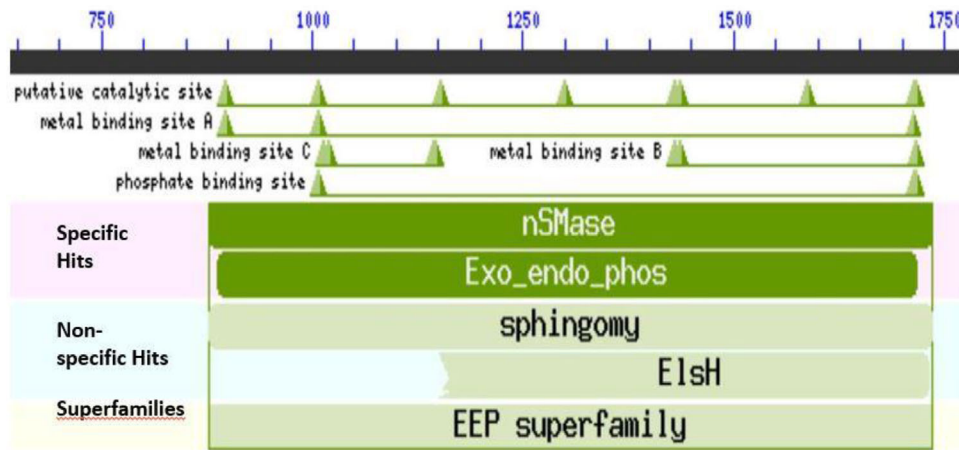
In order to identify conserved domains and their potential role in virulence, we analyzed amino acid sequences translated from the nucleotide sequences of the 12 *S. aureus* isolates. The translated amino acid residues contained a conserved domain known as neutral sphingomyelinase (nSMase). This domain consisted of a catalytic site, metal binding site A, B and C and a phosphate binding site (Figure 2). The neutral sphingomyelinase belonged to the superfamily endonuclease exonuclease phosphatases, which is known to consist of catalytic enzymes that cleave phosphodiester bonds. Neutral sphingomyelinase is a virulent factor known to be responsible for the pathogenesis of *S. aureus*. This factor promotes colonization of the host cell by catalyzing the hydrolysis of sphingomyelin on the host cell membrane to ceramide and phosphorylcholine. These findings indicate that the *S. aureus* isolates could be pathogenic strains.

### 3.5 | Phylogenetic analysis of *S. aureus* isolates

The next task was to determine whether the Kenyan *S. aureus* were genetically distinct. Subsequently, we compared the *hlb* sequences of the Kenyan isolates with the *hlb* sequences of other isolates previously identified in the different parts of the world. We found that the isolates from Kenya clustered on the same clade and hence appeared to share a common ancestry. The sequences from other parts of the world namely Denmark, Taiwan, UK and Germany also shared the same ancestry. However, one isolate from Taiwan (NC\_016928.2) was an outlier as it did not share ancestry with any other isolates (Figure 3).

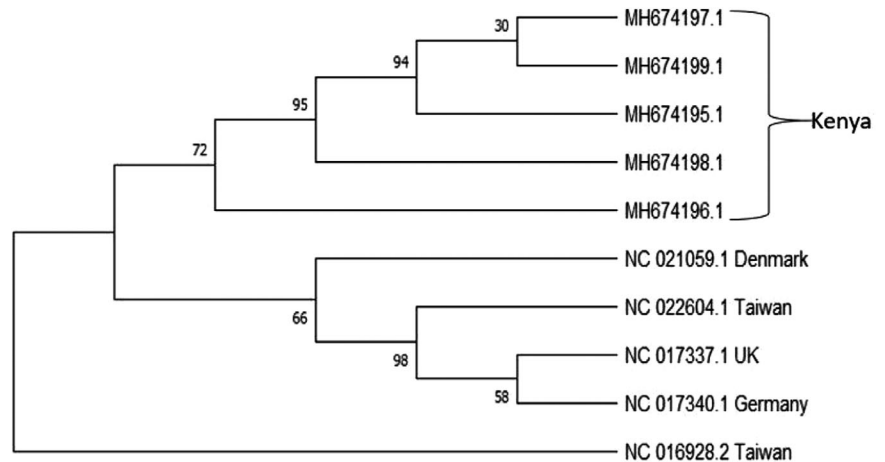
## 4 | DISCUSSION

*S. aureus* exist both in humans and animals either as a commensal or pathogenic bacteria causing diseases (Sineke et al., 2021) ranging from mild superficial skin infection to fatal cases such as toxic shock syndrome (Schmidt et al., 2017). Therefore, there is a need for the development of a specific and sensitive molecular method for detection and characterization of pathogenic *S. aureus*. This study used a bacteriophage genetic marker common to *S. aureus* to develop a molecular tool for specific detection and characterization of pathogenic *S. aureus*. The temperate phages are known to integrate their genetic materials into their host chromosome resulting in exchange of unique genes between the phages and their host. We have confirmed that phages are vectors for horizontal gene transfer in the bacterium as many unique genes were found to be shared between 3AJ\_2917 bacteriophage and



**FIGURE 2** Bioinformatics analysis of translated *hlyB* polypeptide of putative pathogenic *S. aureus* isolates. The conserved domain contains neutral sphingomyelinase (nSMase) with a catalytic site, phosphate binding site and metal binding site A, B and C

**FIGURE 3** Phylogenetic analysis based on the *hlyB* gene fragments of *S. aureus* isolates from Kenya and other regions of the world



*S. aureus*. Among these unique genes were the *hlyB* gene, which was chosen as the ideal marker for *S. aureus* identification as it codes for beta hemolysin protein, which plays a key role in *S. aureus* pathogenicity (Srinivasan et al., 2015). The beta hemolysin proteins aids in the colonization of the host skin by *S. aureus* during infection (Katayama et al., 2013). In addition, beta hemolysin has sphingomyelinase (SMase) activities and therefore, during infection, *S. aureus* is able to break the sphingomyelin on the cell membrane to ceramide and phosphocholine resulting in cell destruction (Ma et al., 2017). Apart from erythrocytes, beta hemolysin affects other cells such as leukocytes and keratinocytes. The beta hemolysin is responsible for recurrence of some infection such as furunculosis and respiratory infection. Although the primer targeting *hlyB* gene yielded two bands of different sizes, the products were confirmed to be *S. aureus*. The difference in amplicons band size may have been due to deletion in the *hlyB* gene as was seen in the alignment that showed the two sequences to be identical except one was 125 bp shorter than the other. The findings of this study indicated that *hlyB* gene is a specific genetic marker for detection of pathogenic *S. aureus*. This is the first study where phage genetic marker has been used to identify and confirm *S. aureus* by PCR assay. In another study where phage was used to detect *S. aureus*, liquid chromatography was used

instead of molecular genetic based tools (Rees & Barr, 2017). Neutral sphingomyelinase (nSMase) domain was found to be present across all the *S. aureus* isolated in this study. This enzyme is a virulence factor that hydrolyses sphingomyelin on the host cell membrane, thus promoting host colonization by *S. aureus*. The sphingomyelin is broken down into ceramide, which is responsible for adhesion, differentiation, proliferation and cell death, indicating that these isolates were pathogenic *S. aureus*. Neutral sphingomyelinase also acts as a hemolysin, which is responsible for host red blood cell lysis. Indeed, the *S. aureus* isolated from this study hemolyzed sheep blood agar, indicating that these sequences encode a functional domain. This study also revealed that Kenyan *S. aureus* isolates shared the same ancestry even though they did not show any phylogenetic relationship with *S. aureus* from other parts of the world. The phylogenetic analysis, therefore, gave a satisfactory insight into the evolutionary relationship among these isolates as shown from the bootstraps, which have significant values ranging from 30% to 95%. In summary, this study gave a solid platform and relevant information on molecular detection of pathogenic *S. aureus* based on the Staphylococcus Phage 3AJ\_2017 genome. This information is further critical in promoting future phage-based research for the development of specific diagnostic methods for specific detection of bacterial



infections. The results of this study have demonstrated that Staphylococcus Phage 3AJ\_2017 genome can be used in developing PCR kit for molecular identification and characterization of pathogenic *S. aureus*.

## 5 | CONCLUSION

We conclude that the *S. aureus* bacteriophage 3AJ\_2017 has unique genetic markers that can be exploited to identify and characterize pathogenic *S. aureus* infecting animals and possibly humans. We further conclude that the phage-based PCR technique developed in this study could provide a platform for specific molecular detection of pathogenic *S. aureus*. Nevertheless, large-scale analysis of *S. aureus* isolates will still be required to validate the PCR analysis.

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## CONFLICT OF INTEREST

The authors declare no conflict of interests.

## AUTHOR CONTRIBUTIONS

Gabriel O. Aboge, Rosaline W. Macharia and George O. Obiero conceived and designed the study, and Silviane A. Miruka performed the experiments. Isaac M. Omwenga acquired the study samples. Silviane A. Miruka, Rosaline W. Macharia and Gabriel O. Aboge analyzed data and wrote the manuscript. All the authors read and approved the final version of manuscript.

## EITHICS STATEMENT

No ethical approval was required as the study did not involve any animal experiment.

## DATA AVAILABILITY STATEMENT

The data used to support the findings of this study are included in the manuscript after the references.

## PEER REVIEW

The peer review history for this article is available at <https://publons.com/publon/10.1002/vms3.676>

## ORCID

Silviane A. Miruka  <https://orcid.org/0000-0002-9116-9658>

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