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Molecular Identification of Key Mastitis causing Bacteria in Dairy Goats in Kenya.

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ABSTRACT
Mastitis remains one of the most common diseases of the dairy goats, causing the biggest economic loss. Use of antimicrobial treatment is the generally recommended, but only after identifying the causal organisms. The use of PCR has emerged as the most accurate in microbial identification due to several advantages, including high sensitivity and specificity.
The objective of this study was to apply PCR techniques in identification of the key mastitis causing bacteria, *E. coli* and *Staph. aureus*, isolated from a farm-survey carried out on goat milk in three goat keeping regions of Kenya, Coast, Rift Valley and Nyanza.
Two gene specific primers for amplification of *Staph. aureus* sequence targeting the *nuc* gene, and *E. coli* sequence targeting the *pic* gene, were used as described in the materials and methods applied to 16 randomly selected pure bacteria cultures representing the three regions. In total six of the samples were positive for the *pic* gene, while five were positive for the *nuc* gene. Each of the genes was distributed to all the three regions.
The positive identification of these genes indicates the potential for use of PCR in accurate goat mastitis diagnosis; it also indicates these mastitis causing pathogens are widespread in all dairy goat keeping regions of Kenya.

1.0 INTRODUCTION

Mastitis remains one of the most common diseases of the dairy goats, causing the biggest economic loss to the industry (Halasa *et al.,* 2007). Targeting antimicrobial treatment of animal infections such as mastitis against causal agent is generally recommended, but this is only possible with identification of the causal bacteria in the milk samples (Constable *et al.,* 2008). The use of molecular techniques has emerged as the most accurate identification of mastitis causing pathogens. The benefits of utilizing molecular techniques in the diagnosis of mastitis include; high sensitivity and specificity, ability to detect few or no growth in normal cultures, ability to use preserved milk and growth inhibited / dead bacteria cells, ability to identify several targets, including drug resistant strains such as penicillin, Balsom (2010). Taponen *et al.*,(2009) have indicated that the industry is a step closer to producing effective vaccine against *Strep. uberis.* This study describes DNA extraction, PCR amplification utilizing gene specific primers and subsequent identification of the PCR products by gel electrophoresis. The study unveils the potential for identifying candidate vaccine antigens against specific strains of mastitis causing bacteria in dairy goats.

Key words: Dairy goat, mastitis, pathogens, PCR.

2.0 MATERIALS AND METHODS

2.1 Chemicals and enzymes

Diethyl pyrocarbonate (DEPC) (Amresco), r-Taq DNA polymerase, Dntp mixture, and DL 2000 molecular DNA marker

2.2 Special equipment

Centrifuge machine (12000 rpm, Eppendorf, 5417R), Thermocycler PCR machine (Biorad DNA Engine®), Mortar and pestle, Rotating shaker, Biorad mini protean system for gel electrophoresis, U-2800 spectrophotometer, Boiling water bath, Shaking incubator, Blood agar plates, MacConkey agar plates, and Polaroid 667 – film.

2.3 Primers

Two gene specific primers for amplification of the *Staphylococcus aureus* sequence targeting the *nuc* gene were designed with PRIMER PREMIER computer program. The forward primer was designated F 5'GCGATTGATGGTGATACGGTT-3' while the reverse primer was designated

R 5'-AGCCAAGCCTTGACGAACTAAAGC-3' respectively. The primer concentrations of 10 pMol were used in the amplification reaction of the partial sequence of the *nuc* gene.

2.4 Culture bacterial cells

Pure bacterial colonies were obtained for bacteriological analysis as described (Carter, 1990; Hogan *et al.*, 1990). For every milk sample 0.0ml was spread with a bacterial loop onto blood agar plates containing 5% of washed sheep red blood cells on to MacConkey plates. Identification of *E. coli* and *Staph. aureus* colonies were based on morphological characteristics.

2.5 DNA extraction from *Staphylococcus aureus* and *E. coli*

A few colonies of the positively identified *E. coli* and *Staph. aureus* were transferred into Eppendorf tubes containing 500µl of sterile distilled water and vortexed thoroughly. A total of 16 samples were processed. The test samples were then transferred to pre-heated water bath at 100^0C, allowed to heat for 10 minutes, then cooled to room temperature. The samples were then spun for 10 minutes at 5000rpm.

Staph. aureus and *E. coli* DNA was extracted from the supernatants by alkaline lysis plasmid SDS procedure as previously described (Sambrook *et al.*, 1995). The DNA pellet was dissolved in 30µl of TE (10mMol/L Tris-Cl, pH 8.0; 1mMol/L EDTA Ph8.0) containing DNase-free RNase A (pancreatic RNase) and used as template DNAs for amplification reaction.

2.6 PCR amplification of *Staphylococcus aureus*

The forward primer F 5'-GCGATTGATGGTGATACGGTT-3'and reverse primer
R 5'-AGCCAAGCCTTGACGAACTAAAGC-3' was used to amplify the partial sequence of the *nuc* gene. The PCR mixture was assembled in thin walled 0.5ml tubes to a final 50µl volume as follows;

Template (*Staph. aureus)*	2.0µl	
10x LA PCR buffer µ(Mg^{2+}free)	5.0µl	
$MgCl_2$ (25mM)	5.0µl	
dNTP mixture (2.5mM)	8.0µl	
1st PCR primer F(10pMol/µl)		0.5µl
1st PCR primer R (10pMol/µl)	0.5µl	
Taq Polymerase (5 U/µl)	0.5µl	
dH_2O	28.5µl	
		5.0µl

Template used was the DNA product diluted 10 times in TE pH 8.0. The thermocycler amplification conditions were initial denaturation at 94^0C for 5 min followed by 25 cycles at 94^0C, 30s for denaturation, 55^0C 30s for annealing and 72^0C, 5 min for polymerization.

2.7 PCR amplification for *E. coli*

The forward primer F 5' ATTCTTCTGGCTGGCATTCC 3' and reverse primer
R 5' CGGGATTAGAGACTATTGTTGC 3' was used to amplify the *pic* marker gene for
E. coli. The PCR mixture was assembled in thin walled 0.5ml tubes to a final 50µl volume as follows:

Template (*Staph. aureus)*	2.0µl	
10x LA PCR buffer µ(Mg^{2+}free)	5.0µl	
$MgCl_2$ (25mM)	5.0µl	
dNTP mixture (2.5mM)	8.0µl	
1st PCR primer F (10pMol/µl)	0.5µl	
1st PCR primer R (10pMol/µl)	0.5µl	
Taq Polymerase (5 U/µl)	0.5µl	
dH_2O	28.5µl	
		5.0µl

Template used was the DNA product diluted 10 times in TE Ph 8.0.

The PCR conditions were initial denaturation 94^0C (5 min) followed by 35 cycles at 94^0C (30s), 72^0C (45s), 72^0C (2 min), and a final extension for 10 min at 72^0C in the last cycle. Amplification was performed using the automated cycler (Biorad, CA, USA).

2.8 Identification of the *S. aureus* and *E. coli* PCR products

The *Staph. aureus* and *E. coli* PCR products were separated on 1% (w/v) agarose gels (sigma), stained with 0.5mg/ml of ethidium bromide using the "Biorad Mini Protean System." Gels were visualized under ultraviolet illumination and recorded on a Polaroid 667- film.

3.0 RESULTS

Table 1 shows the results of molecular detection of *E. coli* and *Staph. aureus* strains in 16 milk representing about 10% of the milk samples. Six of the samples were positive for the *pic* gene of *E. coli*, while five were positive for *nuc* gene of *Staph. aureus*, with distribution of the two genes in all the three regions of Kenya namely; Coast, Nyanza and Rift Valley.

The gel pictures in Figure 1 and Figure 2 show the amplification of the results in the 16 samples for *Staph. aureus* and *E. coli.*

4.0 DISCUSSION

The positive identification of the pathogenic genes of the two key mastitis causing organisms, *Staph. aureus* and *E. coli* in the 11 samples indicates that they are significant causative agents of mastitis in goats in Kenya. These two genes occurred in dairy goats across the three regions of Coast, Nyanza and Rift Valley.

This is the first attempt to identify mastitis pathogens in goat milk using PCR. In cow mastitis PCR as diagnostic procedure has taken root especially in Europe. Taponen *et al.*, (2009) in a study named Real-time PCR based identification or bacteria in milk samples which targeted 11 most common bacterial species and groups in mastitis, and the *Staphylococcal* blaZ gene – responsible for penicillin resistance a procedure which could identify and quantify bacterial cells even if dead or growth inhibited.

Balson (2010) reported that the (British) National Milk Record's in February 2010 converted fully to use of PCR analysis to test and determine cause of mastitis infection to boost producer confidence in results compared with traditional bacterial culture analysis, especially in cases of "no growth" which were common and frustrating for farmers.

In Kenya in dairy goat industry is young, rapidly growing, but with no established quality control standards to guide the growth. Considering the progress in the use of PCR as a diagnostic tool in Europe and America (Taponen *et al.*, 2009; Balsom, 2010) more research work needs to be don to develop the use of PCR in characterization of goat mastitis causing pathogens in Kenya and indeed the rest of the world.

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Table 1: PCR Results by Regions

Region	*E. coli*	*Staph. aureus*
Coast	2(1, 14)	2 (1, 2)
Rift Valley	2 (10, 11)	1 (11)
Nyanza	2 (6, 16)	2 (6, 16)
Total	**6**	**5**

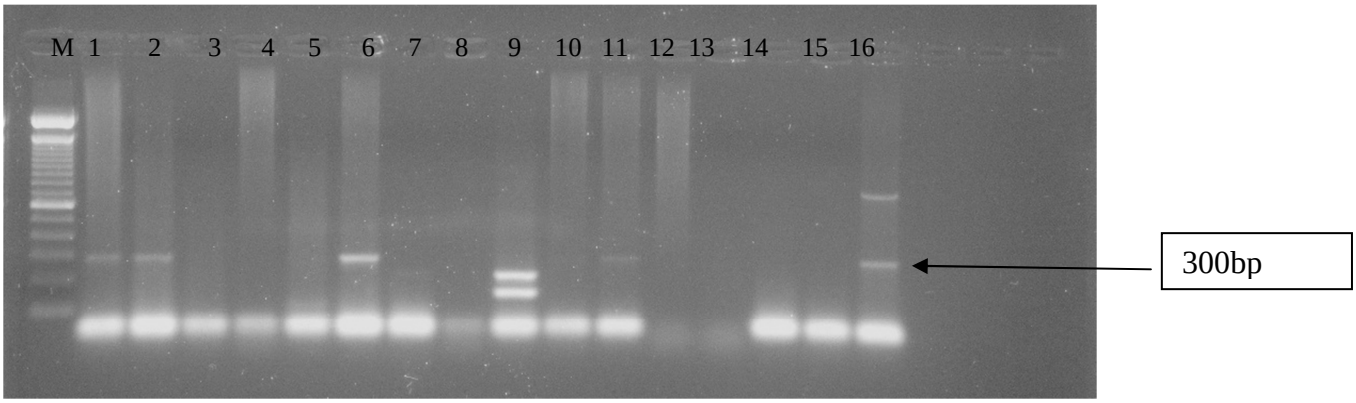

Fig 1: Gel picture showing amplifications on some test samples for the detection of *Staph. aureus* (55).

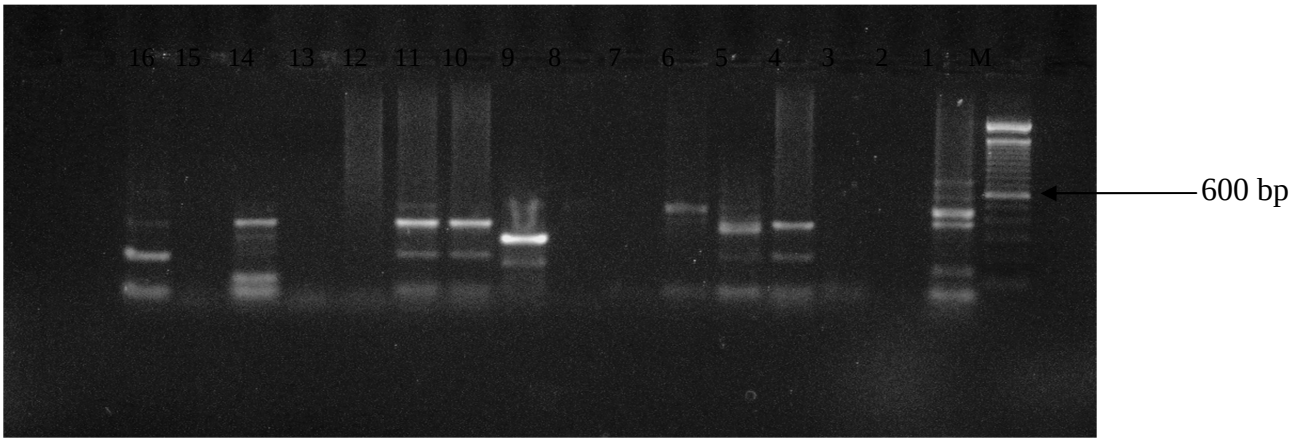

Fig 2: Detection of *E coli* in samples 1-16 listed above. Arrow indicating expected fragment.

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