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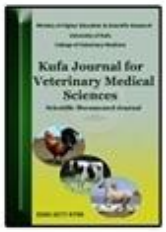

# Direct Detection of Staphylococcus aureus from mastitis milk of cattle by SYBER Green dye based Real-Time PCR technique

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**Abstract:**

The Real-Time PCR technique is a useful diagnostic tool for quick, high throughput and reliable routine screening of Staphylococcus aureus isolates. Moreover, this technique done by SYBR Green based quantitative detection of these pathogens in clinical samples such as mastitis milk of cattle. This study was conducted by Real-Time PCR technique to amplified highly conserved region 125bp fragment of 16S rRNA gene Staphylococcus aureus. The results show more frequency detection of Staphylococcus aureus in mastitis milk (95%). We concluded that Real-Time PCR technique provides a rapid and sensitive method for specific direct detection of Staphylococcus aureus in mastitis milk of cattle.

# الكشف المباشر عن المكورات العنقودية الذهبية من حليب الأبقار المصابة بالتهاب الضرع بواسطة PCR المستند إلى تقنية SYBR

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**الخلاصة:**

تقنية تفاعل البوليمريز المتسلسل في الوقت الحقيقي هي وسيلة تشخيص مفيدة وسريعة وذو خصوصية عالية يمكن الاعتماد عليه في التحري عن جراثيم المكورات العنقودية. فضلا عن ذلك، تعتبر هذه التقنية التي تعمل بواسطة صبغة السايبر الخضراء هي تشخيص كمي لهذه الجراثيم في العينات السريرية مثل عينات حليب ألتهاب الضرع في الأبقار. وقد أجريت هذه الدراسة بواسطة هذا التقنية لتضخيم منطقة محافظة من جين S rRNA16 يتراوح طولها bp125 لجرثومة المكورات العنقودية الذهبية. أظهرت النتائج بأن تشخيص الكشف عن جرثومة المكورات العنقودية الذهبية في الحليب التهاب الضرع بنسبة جدا عالية بلغت (95٪). من خلال هذا الدراسة نستنتج بأن تقنية تفاعل البوليمريز المتسلسل في الوقت الحقيقي يتيح طريقة سريعة وحساسة في التشخيص المباشر والعالي الخصوصية لجراثيم وهي وسيلة تشخيص مفيدة وسريعة وذو خصوصية عالية لجرثومة المكورات العنقودية الذهبية المسببة لالتهاب الضرع في الأبقار.

## Introduction:

Staphylococcus aureus is Gram-positive bacterium, with diameters of (0.5–1μm) and characterized by individual cocci, which divide in more than one plane to form grape-like clusters, these bacteria are non-motile, non-spore forming facultative anaerobes (1). S. aureus is an important pathogenic bacterium responsible for a variety of diseases in humans and animals (2). Pathogenic S. aureus is commonly identified by their ability to produce virulence factor coagulase that clots blood

plasma (3). S. aureus is one of the most important worldwide causes of mastitis in cattle. Mastitis is the inflammation of the mammary gland and udder tissue, and is a major endemic disease of dairy cattle in India and it is responsible for major economic losses in dairy farms (4). In most clinical laboratories, identification methods depend on microbial culture of milk. The method of culture examination is however less sensitive method and time consuming (5). Thus, Molecular technique such as polymerase chain reaction (PCR) based diagnostic method to identify various mastitis causing pathogens is a rapid sensitive and reliable method to resolve bacterial etiology of mastitis milk samples (6 and 7). The introduction of real-time PCR provides the opportunity for the rapid detection of pathogens in food and clinical samples. Apart from saving time, real-time PCR is highly specific, sensitive and offers the potential for detection and quantification of target pathogen (8). The present study aimed to highly sensitive real-time PCR based syber green dye technique to direct detection of Staphylococcus aureus from milk of cattle infected by mastitis in Al-Diwanyia city.

**Materials and Methods:**

**Milk samples collection:** 40 samples of milk were collected from cattle infected by mastitis that investigated by California mastitis test (CMT) from five different cattle field in Al-Diwanyia city. The milk samples were collected in 25ml sterile containers after clean and washing the quarters of udder by disinfectant solution (70% ethanol), then the milk samples transported into laboratory and stored in a refrigerator until use for genomic DNA extraction.

**Genomic DNA extraction:** Bacterial genomic DNA was extracted from milk according to method described by Sreevatsan (9) by using (Genomic DNA extraction kit. Geneaid. USA). 1ml aliquot of milk was centrifuged at 6,000 × *g* for 10 min, and then the clear portion was pipetted and discarded. The remaining milk solids and butterfat were used for DNA extraction and done according to kit instruction using DNA purification spin column. After that, the purified DNA eluted in elution buffer provided with kit and store at -20°C, then used for prepared of PCR master mix.

**Real-Time PCR**

Real-Time PCR technique was performed by using Syber green dye for detection and amplification of conserved region in 16S rRNA gene of Staphylococcus aureus in mastitis milk samples and two S. aureus positive standard isolates. This technique was carried out according to method described by Eman (10). Real-Time PCR primers were design by (Eman *et al.*, 2011) from conserved region of S. aureus 16S rRNA gene with amplicon size was 125bp and these primers were provided by Bioneer Company. Korea as showed in following table:

| Primer | Sequence | | Amplicon |
|---|---|---|---|
| 16SrRNA | F | 5'-cga aag cgt ggg gat caa ac-3' | 125bp |
| | R | 5'-ccc agg cgg agt gct taa tg -3' | |

The Real-Time PCR amplification reaction was done by using (AccuPower$^{TM}$ 2X Green star qPCR master mix kit, Bioneer. Korea) and the qPCR master mix were prepared for each sample according to company instruction as following table:

| qPCR master mix | Volume |
|---|---|
| Genomic DNA template | 2.5µL |
| 2X Green star master mix | 25µL |
| 16SrRNA Forward primer (10pmol) | 1µL |
| 16SrRNA Reverse primer (10pmol) | 1µL |
| DEPC water | 20.5µL |
| Total volume | 50µL |

These qPCR master mix reaction components that mentioned in table was placed in sterile white qPCR strip tubes and transferred into Exispin vortex centrifuge for 3minutes, the place in MiniOpticon Real-Time PCR system and applied the following thermocycler conditions as the following table:

| **qPCR step** | **Temperature** | **Time** | **Repeat cycle** |
|---|---|---|---|
| Initial Denaturation | 95 °C | 3 minute | 1 |
| Denaturation | 95 °C | 10 sec | 45 |
| Annealing\ Extension | 60 °C | 30 sec | |
| Detection(scan) | | | |
| Melting | 60-95°C | 0.5 sec | 1 |

**Results:**

Real-Time PCR technique based SYBR Green dye for diagnosis of Staphylococcus aureus were show (38/95%) from 40 mastitis milk samples gave a positive result by amplification of 16SrRNA gene (Figure 1).

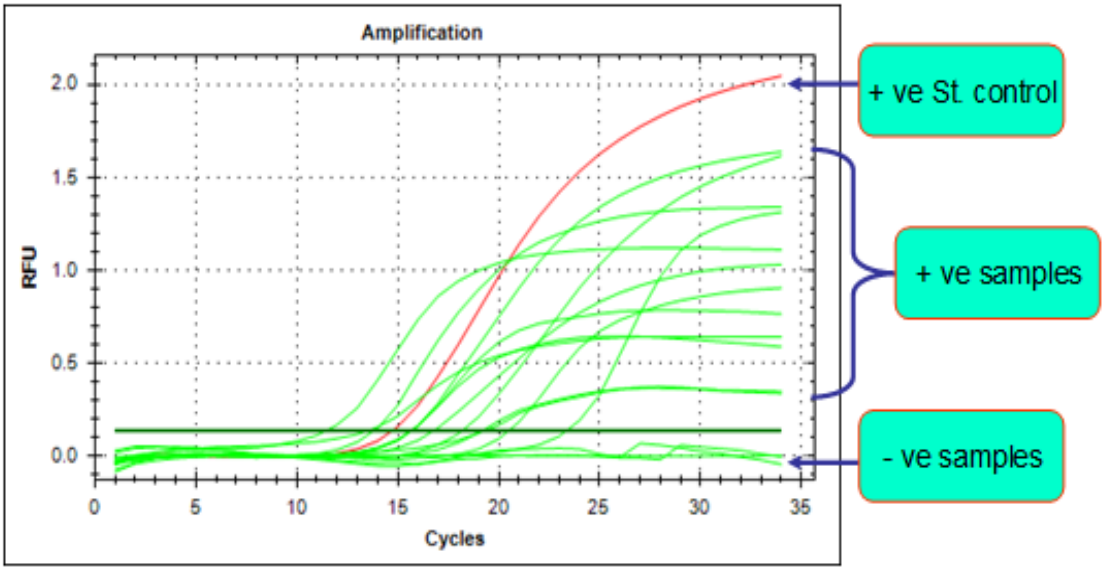


**Figure 1:** Display Real-Time PCR amplification plots that appeared from the (11 to 24 cycles). The samples with amplification appeared at 11 cycles contained very large amount of DNA while the samples with the amplification appeared at 24 cycles contained lower quantity of DNA for S. aureus, so the amplification appeared later, samples were negative which appeared under threshold line.

Specificity of 16SrRNA gene primers that amplification by Syber green based Real-Time PCR was determined by dissociation curve (Melt Curve). Where the positive amplification product samples show specific amplification at melt peak mainly at (Tm: 87C°) without primer diamer or nonspecific products (Figure 2).

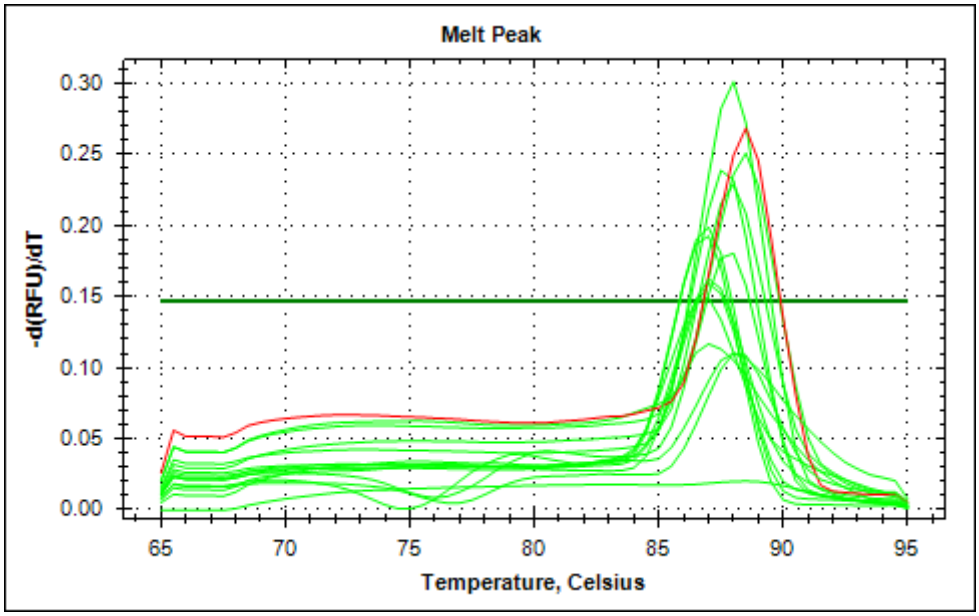


**Figure 2:** Display Real-Time PCR Melt curve that shows the melting point for S. aureus 16SrRNA gene ranged from 86.5°C to 87.5°C for all samples, and the line from the highest peak to the button was detected that the Staphylococcus aureus melting point at 87°C slightly range above or low.

**Discussion:**

Staphylococcus aureus is a main cause of bovine mastitis that can infected a single animal or many animals in one herd. Consequently, early diagnosis of this pathogen in a herd is important for effective control with high level of veterinary monitoring and treatment, may allow eradication of this pathogen from the herd. Diagnosis by traditional method such as microbiological culture and biochemical tests are less sensitive and time consuming (5). The SYBR Green RT-PCR assay is a useful diagnostic tool for quick, high throughput and reliable routine screening of S. aureus isolates. Moreover, the SYBR Green based quantitative detection of this pathogen in milk could remarkably contribute to clarify their actual role in staphylococcal food poisoning and other clinical syndromes associated with the consumption of milk and milk-based products (11). Particularly with the development of a real-time PCR that detect S. aureus is better than from the culture (12). It is considered that S. aureus species specific real time PCR is useful for rapid identification of S. aureus by replacing the current biochemical phenotypic schemes which are time consuming. Additionally, direct real time PCR can be performed for identification of S. aureus from food and clinical specimens if appropriate conditions are established (13). The SYBR green real-time PCR assay for the fast (detect S. aureus in under hours) and conclusive identification of Staphylococcus aureus (10). In conclusion, The SYBR green represents an economically interesting alternative for the analysis of a large number of samples. Also the melt curves do not overlap. The Real-time PCR assays can be rapid and sensitive method for specific direct detection of Staphylococcus aureus from mastitis milk of cattle without using the traditional methods.

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