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Molecular characterization of pathogenic bacterial isolates from khoa

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Abstract

Khoa is an essential in making many sweets. While during storage of khoa there is contamination chances. Many bacteria may enter during handling and storage of khoa. Isolation and molecular characterization is important to detect spoilage in the khoa. In this study molecular characterization of pathogenic bacteria such as *Staphylococcus aureus*, *E. coli* and *Salmonella* spp. is done by using PCR, isolates were screened for presence of 16S rRNA, *pal*, *inv* A genes respectively. Out of 91 *S. aureus* isolates obtained on primary isolation 16 was confirmed for 16S rRNA, out of 30 *E. coli* isolates screened 14 were identified for *pal* gene, out of 53 *Salmonella* isolates screened none was showed the presence of in a gene. Present study confirms the pathogenic potential of the isolates from khoa. The study highlights the contamination risks associated with improper handling and storage of khoa.

Keywords: PCR, Pathogenic bacterial isolates, khoa, spoilage

Introduction

S. S aureus is one of the most dangerous and harmful organisms to human health due to its ability to produce enterotoxins associated with food poisoning. The source of contamination is probably human (Kumar and Prasad 2010) [9]. Presence of *E. coli* in khoa samples is an indicator of faecal contamination (Sohal *et al.* 1993) [15]. *Salmonella* enters food chain due to human contamination. Condensed milk product like *peda* prepared from khoa also gets contaminated by *Salmonella* (Tambekar and Bhutda 2006) [18].

In recent times, PCR method is being widely used for identification and characterization of pathogens due to its high sensitivity and specificity. 16S rRNA gene identification in *S. aureus* isolates has an edge over coagulase; catalase and DNase test (Abdel All *et al.*, 2010) [1]. Shانهbandi *et al.* (2014) [14] successfully used PCR technique targeting 16S rRNA gene for identification of *S. aureus* isolates from milk products.

E. coli identification by PCR method is being done by targeting 16S rRNA and *pal* genes. However molecular characterization of *E. coli* isolates obtained from milk products is being done by targeting *pal* gene (Virpari *et al.*, 2013) [19].

Identification of *Salmonella* isolates is a critical in microbial assay due to its morphological and staining characters similarities with other enteric pathogens. In recent times PCR method has been successfully exploited for *Salmonella* identification. A cent percent *Salmonella* detection could be achieved by targeting *invA* gene using PCR method (Das *et al.*, 2012) [5].

Materials and Methods

Primary Isolation of *S. aureus*, *E.coli* and *Salmonella* spp.

Primary isolation of *S. aureus* is done by using 0.1 ml inoculums of 10⁻³ and 10⁻⁴ dilutions of sample on Vogel Johnsen Agar (Hi-Media Laboratories, Mumbai). Characteristic colonies of staphylococci showing typical black colonies were selected. For *E. coli* isolation a quantity of 0.1 ml of inoculum from dilution of 10⁻³ and 10⁻⁴ were used by spread plate technique on Levine's Eosine Methylene Blue Agar (EMB) (Himedia Laboratories, Mumbai). Bluish purple coloured colonies with greenish metallic sheen were considered indicative of *E. coli*.

For Primary isolation of *Salmonella* spp. pre enrichment was done by using 1 percent Buffered Peptone Water (BPW) (Hi-Media Laboratories, Mumbai) at 37°C for 24 hours. Pre-enriched sample was inoculated in tetrathionate broth (TTB) (Hi-Media Laboratories, Mumbai) and incubation was done for 24 hours at 42°C. Streaking of TTB grown bacteria was done on Xylose Lysine Deoxycholate (XLD) (Hi-Media Laboratories, Mumbai) agar and incubated for 24 hours at 37°C. Pink colonies with or without black centers were considered as positive for *Salmonella*. All the isolates are further processed for biochemical tests.

Molecular characterization of *S. aureus*, *E. coli*, *Salmonella* spp

Molecular characterization of *S. aureus*, *E. coli* and *Salmonella* spp. isolates was done by using PCR at Department of Animal Biotechnology, College of Veterinary and Animal Sciences Parbhani. Molecular characterization of *S. aureus*, *E. coli* and *Salmonella* spp. isolates for their confirmations as per the method described by Mason *et al.*

(2001) [10], Kuhnert *et al.* (1995) [8], Rahn *et al.* (1992) [12] respectively with suitable modifications. *S. aureus*, *E. coli*, *Salmonella* spp. isolates were screened for presence of 16S rRNA, *pal*, *inv A* genes respectively.

Isolation of genomic DNA of *S. aureus*, *E. coli*, *Salmonella* spp.

The DNA of each isolates of *S. aureus*, *E. coli*, *Salmonella* spp. was extracted by boiling method. Approximately a loopful of culture were mixed in 100 µl of autoclaved distilled water in micro centrifuge tube. Then it was vortexed and heated at 98°C for 10 min, cell debris was removed by centrifugation and 5µl of supernatant was used as a DNA template in PCR reaction mixture.

PCR reaction

Enumeration of Master Mixture, Forward and reverse primers, template DNA nuclease free water used in PCR reaction are given in Table 1.

Table 1: Details of component used in PCR reaction

Sr. No.	Component	Concentration (µl)
1	Master mix	12.5
2	Forward (F) primers	1
3	Reverse (R) primers	1
4	Template DNA	5
5	Nuclease free water (NFW)	5.5
	Total volume	25

PCR assay

Identification of 16S rRNA gene in *S. aureus* isolates

The DNA templates obtained were further subjected to PCR with specific primers (Table-2), for the detection of 16S rRNA gene. PCR was performed with a final reaction volume of 25 µl in 0.2 ml thin wall PCR tube, components were used as per Table-1 and mixed by gentle shaking. The tubes were subjected for PCR in thermal cycler (Eppendorf, Germany). The PCR products so obtained were further electrophoresed on 2 percent agarose gel at 80 V and 200 mA for 1 hr. As per cycling conditions mentioned in Table-2.

Identification of *pal* gene in *E. coli* isolates

The DNA templates were used for PCR assay performed for the detection of *pal* genes. Each reaction contained 12.5 µL of PCR Master Mix, 1 µl (500 picomole conc.) of each forward and reverse primer, 5.5 µL Nuclease-free water, and 5 µL template DNA. After this the above mixture was subjected to PCR under conditions given in table 2. The PCR products so obtained were further electrophoresed on 2 percent agarose gel at 80 V and 200 mA for 1 hr. As per cycling conditions

mentioned in Table 2.

Identification of *inv A* gene in *Salmonella* isolates

The DNA templates were used PCR assay performed for the detection *inv A* gene for identification of *Salmonella*. Each reaction contained 12.5 µL of PCR Master Mix, 1 µl (500 picomole conc.) of each forward and reverse primer, 5.5 µL Nuclease-free water, and 5 µL template DNA. After this the above mixture was subjected to PCR under conditions given in table 2. The PCR products so obtained were further electrophoresed on 2 percent agarose gel at 80 V and 200 mA for 1 hr. As per cycling conditions mentioned in Table 2.

Agarose Gel Electrophoresis

To confirm the targeted PCR amplification, 5 µl of PCR product from each tube was mixed with 1µl 6 X gels loading buffer and electrophoresed on 2.0 percent agarose gel along with 100 bp DNA Ladder (GeneRuler-Fermentas) and stained with ethidium bromide at constant 80 V for 40 minutes and 1 hour as per conditions in 0.5X TBE buffer. The amplified product was visualized as a single compact band of expected size under UV trans illuminator.

Table 2: Details of primers, cycling condition and targeted genes of molecular characterization of for *S. aureus*, *E. coli* and *Salmonella* spp.

Sr. No	Species	Gene	Primer	Product Size	Cycling condition
1.	<i>S. aureus</i>	16S rRNA	F:5'-CCT ATA AGA CTG GGA TAA CTT CGG G-3' R:5'-CTT TGA GTT TCA ACC TTG CGG TCG-3'	791bp	95°C for 5 min followed by 34 cycles of 94°C for 30 sec, 55 °C for 1 min, 72°C for 1 min and 72°C for 10 min as final extension
2.	<i>E. coli</i>	Pal	F:5'-GGCAATTGCGCGGCATGTTCTTCC-3' R:5'-CCGCGTGACCTTCTACGGTGAC-3'	280bp	95°C for 5 min followed by 35 cycles of 95°C for 1 min, 60 °C for 30 sec, 72°C for 30 sec and 72°C for 10 min as final extension
3.	<i>Salmonella</i> spp.	Inv A	F:5'-GTGAAATTATCGCCACGTTTCGGGCAA-3' R:5'-TCATCGCACCGTCAAAGGAACC-3'	284bp	95°C for 5 min followed by 35 cycles of 95°C for 1 min, 60 °C for 30 sec, 72°C for 30 sec and 72°C for 10 min as final extension

Results and Discussion

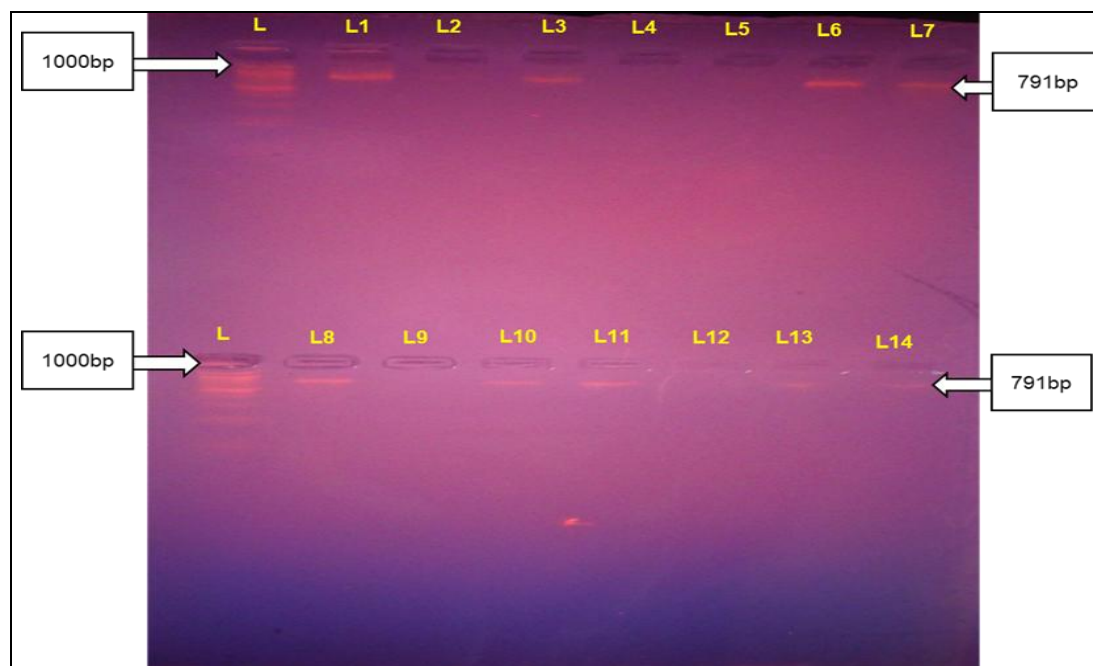
S. aureus

A total of 91 *S. aureus* isolates obtained on primary isolation by using selective medium were subjected to molecular characterization by targeting 16S rRNA gene in PCR. The electrophoretic migration pattern of extended product was studied for identification of spp. The details of electrophoretic migration pattern are given in Plate 1. A total of 16 *S. aureus* isolates were confirmed positive by PCR.

Abdell All *et al.* (2010)^[1] used conventional and molecular features of *S. aureus* from bovine milk samples. A total of 384 milk samples were analysed during study. Primary

isolation was done by using Baird and Parker medium followed by PCR detection of 16S rRNA. Chotar *et al.* (2006) also targeted 16S rRNA for screening of milk products for detection of *S. aureus* by PCR method. Many other workers also used 16S rRNA by PCR for identification of *S. aureus* isolation obtained on primary isolation from milk products (Rohinishree and Negi 2011; Di Giannatale *et al.* 2011; Doaa El-Hadedy and El-Nour, 2012; Shanebandi *et al.* 2014)^[13, 6, 7, 14].

In present study also 16S rRNA gene was successfully exploited for identification of *S. aureus* isolates obtained from primary isolation from khoa samples by using PCR technique.

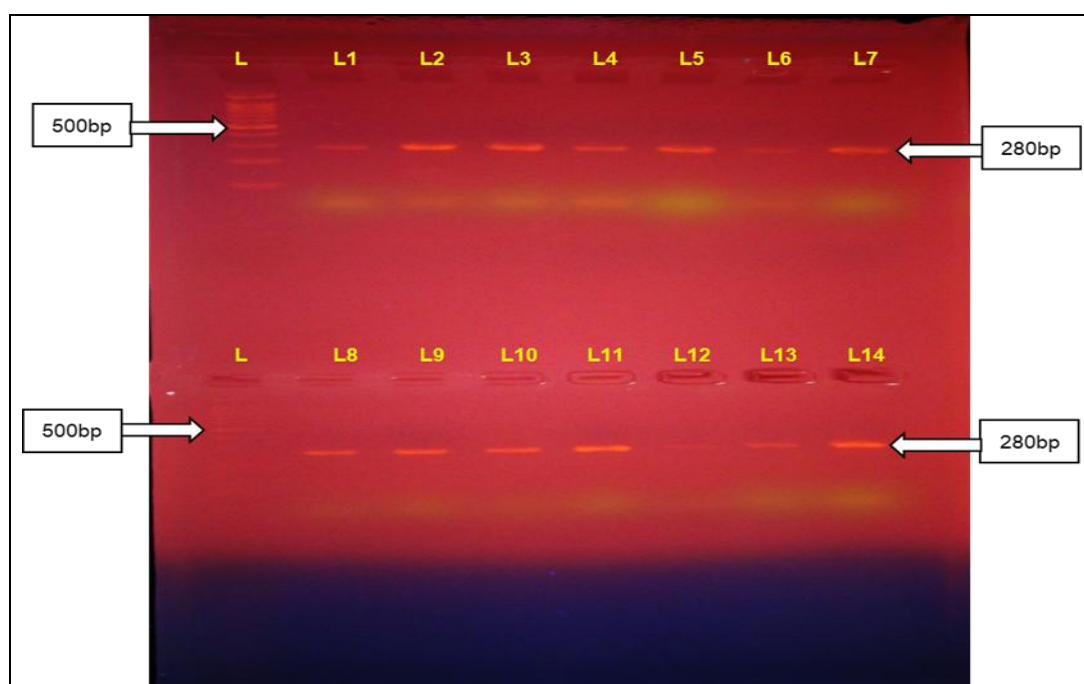


L: 100 bp plus DNA ladder

L1, L3, L5, L7, L8, L10, L11, L13, L14: Species specific 16S rRNA of *Staphylococcus* spp.

L2, L4, L6, L9, L12: Negative samples

Plate 1: Agarose gel showing PCR amplified products of 16S rRNA gene from *S. aureus*.



L: 100 bp plus DNA ladder

L1–L14: PCR amplification of species-specific *pal* gene of *E. coli*

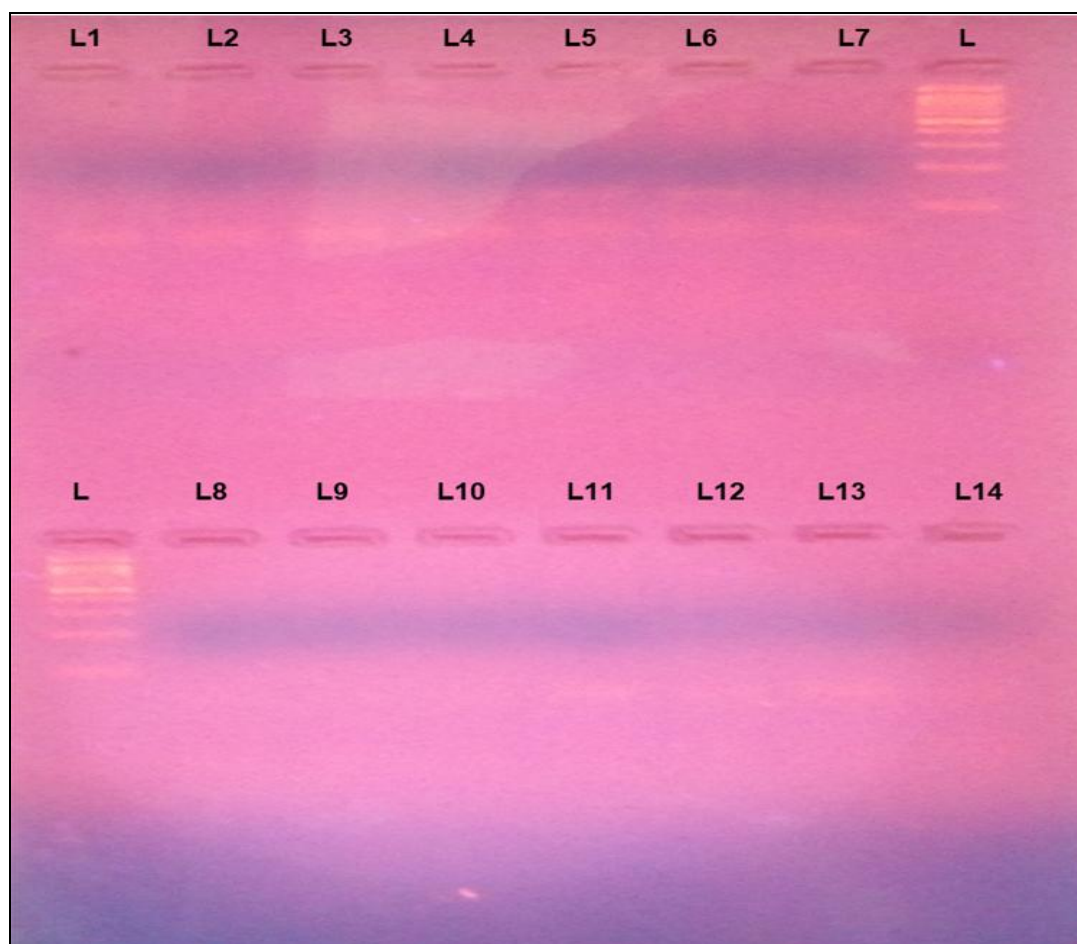
Plate 2: Agarose gel showing PCR amplified products of *pal* gene from *E. coli*.

E. coli: A total of 30 *E. coli* isolates were screened for molecular characterization by targeting *pal* gene. A total of 14 isolates were identified. The results are shown in Plate 2. PCR technique was successfully used for molecular characterization of *E. coli* isolates from milk products by many workers (Parekh and Subhash, 2008; Chotar *et al.* 2006; Doaa El-Hadedy and El-Nour, 2012; Virpari *et al.* 2013; Ahmed and Shimamoto, 2014) [7, 19]. In present study also similar results were obtained.

Salmonella spp.: Swamy *et al.* (1996) used *inv A* and *spv C*

genes for identification of *Salmonella* from poultry products. Ammari *et al.* (2009) used *psv R* gene for identification of *Salmonella* from food sources. Meat and dairy products were analysed by Das *et al.* (2012) [5] for *Salmonella* by using *inv A* gene.

In present study *inv A* gene was targeted for *Salmonella* identification by PCR. The results are given in Plate 3. A total of 53 *Salmonella* isolates were screened by PCR. However, not a single isolate could be confirmed by PCR. This may be due to high sensitivity of PCR technique.



L: 100 bp plus DNA ladder, L1–L14: Negative samples

Plate 3: Agarose gel showing PCR amplified products of *invA* gene from *Salmonella*.

Summary and Conclusion

This study was conducted to detect and characterize foodborne bacterial pathogens, *Staphylococcus aureus*, *Escherichia coli* and *Salmonella* spp. from *khoa*, a traditional Indian dairy product. A total of 100 *khoa* samples were collected from different retail sources and subjected to microbiological analysis followed by molecular confirmation using species-specific PCR. Conventional cultural techniques confirmed the presence of all three bacterial species in varying proportions such as *S. aureus* (91/100) with *E. coli* (30/100) and *Salmonella* (53/100) in which *S. aureus* being the most prevalent. Molecular characterization revealed the presence of virulence-associated genes such as *pal* in *E. coli* (14/30), 16S rRNA in *S. aureus* (16/91), and except *invA* in *Salmonella* spp. (0/53), confirming the pathogenic potential of the isolates. The study highlights the contamination risks associated with improper handling and storage of *khoa*. The detection of virulent strains of *E. coli*, *S. aureus* and *Salmonella* spp. in *khoa* underscores the public health concern

posed by microbial contamination in traditional milk-based sweets. The application of molecular tools such as PCR proved effective in rapid and specific identification of pathogens, which is essential for food safety monitoring. These findings shows that there is need for stricter hygiene standards during production, handling, and distribution of *khoa*.

Conflict of Interest

Not available

Financial Support

Not available

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