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Isolation and identification of pathogenic bacteria from Indian milk product-khoa

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Abstract

Khoa, a traditional Indian dairy product, is highly susceptible to microbial contamination and growth due to its rich nutrient composition and high moisture content. The present study aimed to isolate and identify the predominant pathogenic microorganisms associated with khoa. A total of 100 khoa samples were collected from local khoa market of Parbhani city in Maharashtra and analyzed microbiologically using standard culture techniques. Isolates were subjected to morphological, biochemical tests for accurate identification. The predominant pathogenic organisms identified included *Salmonella* spp., *Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus*, *Clostridium perfringens*. Among these, Primary isolation on selective media yielded presumptive isolates of *Staphylococcus aureus* (91/100), *Salmonella* spp. (53/100), *Bacillus cereus* (50/100), *Clostridium perfringens* (42/100), *Escherichia coli* (30/100), and *Listeria* spp. (9/100). Subsequent confirmation by Gram staining, biochemical tests, and sugar fermentation identified 24/30 (80.0%) *E. coli*, 26/53 (49.05%) *Salmonella* spp., 28/91 (30.76%) *S. aureus*, and 8/50 (16.0%) *B. cereus* isolates. This study shows poor hygienic condition during transportation and storage of khoa, the findings underscore the importance of hygienic handling. There is need of identification of CCP in khoa preparation and technological intervention are required for maintenance of quality of khoa in relation to public health.

Keywords: Khoa, pathogenic bacteria, CCP, Public health

Introduction

The most common contaminants i.e. *E. coli* and *Staphylococcus* pathogenic bacteria could be an important causative agent for gastrointestinal infection including food poisoning and food borne illness. Raw and unpasteurized milk obtained from milch cow and buffalo by hand milking may contain bacteria from cows udder, flies, manure etc. Indigenous sweet based like khoa is highly susceptible to varieties of microorganisms because of high nutritive value and complex chemical composition (Soomro *et al.*, 2003) [17].

In recent times dairy industry has been concerned by presence of pathogens in dairy products with common sources of contamination as hands, clothing of food processor and dirty surfaces of equipments. A broad spectrum of microbial pathogens can contaminate dairy products and water supplies causing illness after consumption. These includes varieties of enteric bacteria, aerobes and anaerobes (Zagare *et al.*, 2012) [22].

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) [12]. Presence of *E. coli* in khoa samples is an indicator of faecal contamination (Sohal *et al.* 1993) [16]. *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) [19]. Spore forming aerobes like *Bacillus cereus* enters khoa samples through soil contamination. Incidence of *Bacillus cereus* contamination in milk products is high (Kamat *et al.*, 1989) [9]. Improperly handled dairy products results into contamination of *Clostridium perfringens* thereby providing means for transmission to consumers (El-Bassiony, 1980) [4].

Milk and milk products are important sources of *Listeria* transmission to human being (Rahimi *et al.* 2010) [15].

Materials and Methods

Collection of Khoa samples

Khoa samples were collected from khoa market of Parbhani city by following standard method as per FSSAI (2010) [6]. A quantity of 50 gm of Khoa sample was collected in sterile polyethylene sachet. These khoa samples were brought to the laboratory on ice and processed immediately.

Isolation of organisms

Isolation of *S. aureus* and *E. coli*, *Salmonella* spp., *Bacillus cereus*, *Clostridium perfringens*, *Listeria* spp. was done as per the method described in BAM (1998) [1].

Isolation of *Staphylococcus aureus*

By using 0.1 ml inoculums of 10^{-3} and 10^{-4} dilutions of sample on Vogel Johnsons Agar (Hi-Media Laboratories, Mumbai). The inoculums was spread by means of L-shape spreader and plates were kept overnight at 37°C for incubation. Characteristic colonies of *Staphylococci* showing typical black colonies were selected. Pure cultures were used for identification by biochemical tests.

Isolation of *Escherichia coli*

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*. *E. coli* isolates was maintained on EMB agar for further identification by biochemical test.

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*.

Isolation of *Bacillus cereus*

Khoa sample was inoculated on nutrient broth and then Samples were treated at 70°C for 15 min. Incubated at 37°C for 18 hours. Selective plating was done on *Bacillus cereus* agar (Hi-media Laboratory, Mumbai.) followed by incubation at 37°C for 24 hours.

Luxuriant growth with blue coloured colonies with precipitation were taken as *Bacillus cereus* positive.

Isolation of *Clostridium perfringens*

Inoculated khoa sample in nonselective media (nutrient broth) in 1:10 ratio and homogenized and incubated at 44°C for 24-48 hours.

Taken inoculum from enrichment broth in sterile test tube. To this added sterile molten (50°C) Sodium Polymixin Sulphadiazine (SPS) as selective agar. (Hi-media Laboratory, Mumbai.) Mixed well and allowed to solidify again added a layer of molten agar over it, allowed to solidify. Incubated in normal incubator at 37°C for 24-48 hours.

A typical colony of *Clostridium perfringens* on SPS tube

showed Black cotton wool like growth.

Isolation of *Listeria* spp.

Taken at least 10 gm of khoa sample and homogenized with 90 ml of enrichment broth (Fraser-I) (Hi-media Laboratory, Mumbai.) and incubated at 30°C for 24 hours. Transferred 0.1 ml inoculum from Fraser-I broth to Fraser-II broth (Hi-media Laboratory, Mumbai.) and incubate for 24 hours at 30 °C.

From enrichment broth streaked loopful inoculums onto selective *Listeria* Identification agar (PALCAM) (Hi-media Laboratory, Mumbai.) and incubated plate at 30°C for 48 hours.

On *Listeria* Identification agar (PALCAM), colonies of *Listeria* appeared as a grey-green with black center and a black precipitate.

Identification and characterization of Isolates

Identification of *S. aureus*, *E. coli* and *Salmonella* isolates was done by morphological studies by Grams staining, biochemical and sugar fermentation tests.

Morphological characterization of isolates

The *S. aureus*, *E. coli* and *salmonella* isolates were further characterized for morphological characterization by using Gram's staining method. The isolates were inoculated into nutrient broth tubes and incubated for 12 hours at 37°C. A loopful inoculum from nutrient broth (NB) was streaked on nutrient agar for morphological characters. Gram's staining reaction of each isolate was recorded.

Biochemical characterization of isolates

All *S. aureus*, *E. coli* and *salmonella* isolates were characterized by biochemical tests by using method of Cown and Steel (1993).

Methyl Red test

Pure colony of isolates was inoculated into 0.5 ml sterile glucose phosphate broth and incubated at 37°C for 12 hours. A drop of methyl red solution was added in each tube and the color change was observed. Developments of red color indicated positive test were as yellow colour was negative test.

Voges -Proskauer (V-P) test

Organism under test was inoculated into 2 ml of sterile Buffered Glucose broth and incubated at 37°C for 48 hrs. Then 0.2 ml of 40 percent potassium hydroxide was added, followed by 0.6 ml of α -naphthol solution. The tubes were shaken vigorously. The cotton plugs were removed and allowed to stand for 5 to 10 min. at room temperature to observe the color formation. Development of pink red colour indicates positive result.

Indole test

The isolates were inoculated in to 3 ml of indole medium (Himedia Laboratories, Mumbai) and incubated at 37°C for 48 hrs. Then 0.5 ml of Kovacs reagent was added to form a ring in between medium and reagent. Development of bright red colour indicates positive test.

Citrate test

Simmons citrate agar (Himedia Laboratories, Mumbai) slant were streaked with straight wire loop using broth culture and then stab the butt and incubated at 37°C for 48 hrs. Discoloration of agar to bright blue was considered as

positive test.

Nitrate reduction test

Heavy growth of organism was inoculated in 0.5 ml of sterile nitrate broth. Incubation was done at 37°C for 24 hrs. Then one drop of sulphanilic acid and the α -naphthylamine reagent were added and observed for colour change. Development of red colour indicates positive reaction.

Sugar fermentation test

Sugar fermentation tests of all isolates were done as per method described by Cruickshank (1975). A 1 per cent sugar solution was prepared and added in Buffered Pepton Water (BPW) with phenol red indicator (pH 8.8). Test organism was inoculated to this medium and incubated overnight at 37°C. Presence of yellow colour upon incubation was taken as positive for sugar fermentation. Production of gas was recorded by using Durham's tubes. Appropriate controls were added.

Identification of *Bacillus cereus*

Bacillus cereus isolates were confirmed on basis of *Bacillus cereus* agar as a selective agar medium and Nitrate broth reduction.

Results and Discussion

Khoa market in Parbhani city

In Parbhani city, Khoa market is in open ground. Approximately 15 vendors operate in morning hours. The khoa come in market from neighbouring villages in unorganised manner. Each vendor handles approximately 12 Kg of khoa in the market. The khoa is brought to the market in stainless-steel buckets with polythene bags lining and without polythene lining in open containers. The storage of khoa is done at room temperature. The market is operated throughout the year. The conventional Khoa handling, transportation and storage method makes it vulnerable for microbial hazards.

In present study microbial quality of khoa was assessed in addition to isolation and identification of various pathogens of public health significance. A total of 15 khoa vendors were identified from market in Parbhani city and given shops codes. A total of 100 khoa samples were collected and analysed in present study.

Isolation of *S. aureus*

A total of 100 khoa samples were screened for isolation of *S. aureus* on VJ agar. Samples showing black coloured colonies were considered as positive for *S. aureus*. The results are shown in Plate 1. A total of 91 *S. aureus* isolations were done. The isolates were coded for further use.

Varadraj and Nambudripad (1982) [21] studied *Staphylococcal* incidence in market khoa from Bangalore and Mysore cities. A total of 125 *S. aureus* isolations were done. The highest *Staphylococcal* isolations (56.6 percent.) were done by Masud *et al.* (1988) [14] from milk and milk products. Many other workers khoa (Teufel *et al.* 1992; Sohal *et al.* 1993; Soomro *et al.* 2003; Kumar and Prasad 2010; Iqbal and Hussainy 2014) [20, 16, 17, 12]. They observations in present study are on

similar lines.

Isolation of *E. coli*

Sohal *et al.* (1993) [16] investigated survival of *E. coli* in khoa. Heat processing of milk for khoa production eliminated *E. coli* but during storage growth appeared. Soomro *et al.* (2002) [18] reported *E. coli* contamination of khoa. *E. coli* contamination of pedha was reported from Amrawati city by Tambekar and Bhutda (2006) [19]. Contamination of khoa by *E. coli* was also reported by many workers (Londhe and Dharampal, 2008; Farzana *et al.*, 2009; Kumar and Prasad, 2010; Zagare *et al.* 2012; Iqbal and Hussainy, 2014) [13, 5, 12, 22].

In present study a total of 30 *E. coli* isolates were obtained on EMB agar from khoa samples. The results are given in Plate 2. The results are similar line as reported earlier.



Plate 1: Details of colonies of *S. aureus* isolated on VJ agar.



Plate 2: Details of colonies of *E. coli* isolated on EMB agar

Isolation of *Salmonella* spp

All 100 khoa samples were screened on XLD agar for *Salmonella* isolation. A total of 53 isolates were obtained. The results are depicted in Plate 3.

Tambekar and Bhutda (2006) [19] studied microbial quality of pedha in Amrawati city. A total of 16.36 percent isolates were identified as *Salmonella typhi*. A total of 7 *Salmonella typhi* isolates were reported from milk products by Zagare *et al.* (2012) [22]. In present study also a total of 53 *Salmonella* spp. isolates were obtained. The results are on similar lines as reported earlier.



Plate 3: Details of colonies of *Salmonella* spp. isolated on XLD agar

Isolation of *Bacillus cereus*

Christiansson *et al.* (1989) isolated a total of 138 strains of *Bacillus cereus* from milk and cream. Kamat *et al.* (1989) [9] used Mannitol egg yolk polymyxin B agar for isolation of *Bacillus cereus* from milk products. *Bacillus cereus* was present in 28.5 percent samples, Becker *et al.* (1994) studied incidence of *Bacillus cereus* in dried milk samples. About 69 percent samples showed presence of *Bacillus cereus*.

In present study 50 *Bacillus cereus* isolates were obtained on *Bacillus cereus* agar from Khoa samples. The results are shown in Plate 4. The results are on similar lines as reported earlier.



Plate 4: Details of colonies of *Bacillus cereus* isolated on *Bacillus cereus* agar.

Isolation of *Clostridium perfringens*

Clostridium perfringens was used as an indicator of soil contamination of khoa samples in present study. All the khoa samples (100 No.) were inoculated on SPS agar and incubated at 37°C for 24 hrs. The samples showing presence of black cotton wool like growth were taken as positive. A total of 42 isolations were obtained. The details of results are given in Plate 5.

El-Bassiony (1980) [4] screened 163 samples of milk and milk products for *Clostridium perfringens* isolation on SPS agar. The isolation rate was 45.5 percent in raw milk, 10 percent in cheese, 50 percent in yoghurt and 20 percent in ice cream. Gurmu *et al.* (2013) [8] successfully isolated 33 *Clostridium perfringens* isolates from pasteurized milk samples obtained from Guwahati.

In present study also *Clostridium perfringens* isolation percentage was 42 percent from khoa samples. This may be due to vegetative growth of residual spores of *Clostridium perfringens* after heat treatment during processing upon storage of khoa samples at room temperature. The findings

are on similar line as reported earlier.



Plate 5: Details of colonies of *Clostridium perfringens* isolated on SPS agar

Isolation of *Listeria* spp

Contamination of milk and milk product by *Listeria* spp. indicate potential risk of infection with *Listeria* in people consuming milk and milk products. The highest prevalence of *Listeria* was found in raw milk (22.6 percent) followed by cheese (18.9 percent) (Rahimi *et al.*, 2010) [15].

In present study also a total of 9 *Listeria* spp. isolates were obtained on *Listeria* Identification agar (PALCAM) from screening of 100 khoa samples. The results are given in Plate 6.

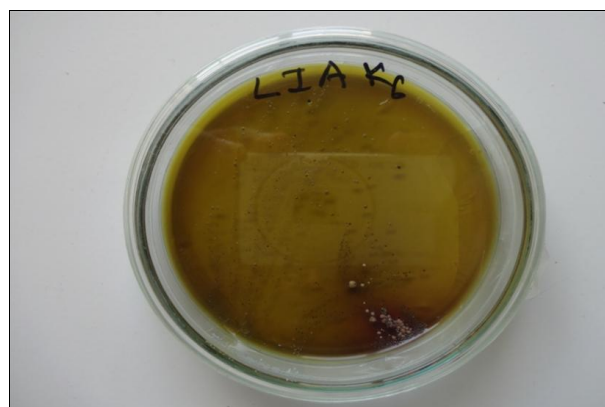


Plate 6: Details of colonies of *Listeria* spp. isolated on PALCAM agar

Identification of isolates by conventional methods:

Grams staining, biochemical test and sugar fermentation test were used to identify *S. aureus*, *E. coli*, *Salmonella* spp. and *Bacillus cereus*. However confirmation of *Listeria* spp., *Clostridium perfringens*, Yeast and Mould was done only upto isolation by using selective media.

A total of 91 *S. aureus* primary presumptive isolates were used for identification of isolates by staining and biochemical characteristics and 28 isolates were identified.

A total of 24 *E. coli* isolates were identified out of 30 presumptive isolates. A total of 26 *Salmonella* spp. were identified out of 53 presumptive isolation. Nitrate reduction test was used to identify *Bacillus cereus* isolates. A total of 50 *Bacillus cereus* presumptive isolates subjected to the test. A total of 8 *Bacillus cereus* isolates were identified.

The highest percentage of isolation was recorded of *E. coli* (80 percent) followed by *Salmonella* spp. (49.05 percent), *S. aureus* (30.76 percent) and *Bacillus cereus* (16 percent).

Earlier Varadaraj and Nambudripad (1982) [21] used

biochemical characters like pigmentation, mannitol fermentation, nitrate reduction for identification of *S. aureus* from khoa samples. In present study also biochemical characters also successfully used for identification of *S. aureus*.

Identification of *E. coli* from milk and milk products by biochemical methods was done by earlier workers (Tambekar and Bhutda 2006; Kumar and Prasad 2010) [19, 12]. In present study also identification of *E. coli* from khoa samples was done successfully.

Identification of *Salmonella* spp. isolates from khoa samples was done in present study by using staining and biochemical characters successfully. Earlier also *Salmonella* spp. identification from milk and milk product was done with the help of staining and biochemical characters (Bandekar *et al.* 1998, Godbole *et al.* 2013) [2, 7].

Summary and Conclusion

The present study was planned with the objectives to evaluate microbial quality of khoa sold in Parbhani city, to isolate and identify *S. aureus*, *E. coli*, *Salmonella* spp., *Bacillus cereus*, *Clostridium perfringens*, *Listeria* spp., isolates. A total of 100 khoa samples were collected and screened during study. A total of 91 *S. aureus* isolates were obtained during study period. Number of isolates were obtained of *E. coli* (30), *Salmonella* spp. (53), *Bacillus cereus* (50), *Clostridium perfringens* (42), *Listeria* spp. (9), Yeast and Moulds (32). *S. aureus*, *E. coli*, *Salmonella* and *Bacillus cereus* were confirmed by conventional method of staining and biochemical characterization. The percentages of isolation were *S. aureus* 30.76 percent, *E. coli* 80 percent, *Salmonella* spp. 49.05 percent, *Bacillus cereus* 16 percent. *S. aureus*, *E. coli* and *Salmonella* spp.

Following conclusions can be drawn from study,

1. *S. aureus*, *E. coli*, *Salmonella* spp., *Bacillus cereus*, *Clostridium perfringens*, *Listeria* spp. are common contaminants of khoa of public health significance.
2. Appearance of *Listeria* spp. in khoa samples sold in Parbhani city is low.
3. *E. coli* is the highest contaminant of khoa samples and *Bacillus cereus* is the lowest.
4. *Salmonella* contamination of khoa samples in 49.05 percent samples indicate poor hygienic condition during processing, transportation and storage.
5. Identification of CCP in khoa preparation and technological interventions are required for maintenance of quality of khoa in relation to public health.

Conflict of Interest

Not available.

Financial Support

Not available.

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