

Original Research Article

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Detection and Molecular Characterization of Food Borne Pathogens from Ready to Eat Meat and Milk Products

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ABSTRACT

Keywords

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Foodborne illness still remains a major threat to public health. Among the various pathogens causing food borne outbreaks, *Salmonella sps*, *Staphylococcus aureus*, *Bacillus cereus*, *Campylobacter sps* have been incriminated for majority of outbreak cases. Now – a – days consumption of minimally processed ready to eat (RTE) food products increases the risk of food borne diseases. The aim of the present investigation was to detect the presence of FSSAI notified six food pathogens from ready to eat food products. A total of 250 samples (including milk, milk products, meat and meat products) were screened for the presence of food-borne bacterial pathogens like *Staphylococcus aureus*, *Bacillus cereus*, *Campylobacter jejuni*, *Streptococcus sp* and *Salmonella sp* on selective medium. Further, culture positive colonies were screened by colony morphology, gram staining, biochemical analysis and molecular methods. Antibiotic susceptibility test was performed for all isolates of *Staphylococcus aureus*, *Bacillus cereus* and *Streptococcus spp*. The results revealed that the *Staphylococcus aureus* had 100% resistant against Vancomycin, Methicillin and Penicillin antibiotics and *Bacillus cereus* isolates had 100% resistant against Vancomycin and Penicillin antibiotics and 100% sensitive towards Methicillin. Whereas the *Streptococcus sp*. shows 100% resistant against Gentamicin, Streptomycin, Tetracycline and Ciprofloxacin. The prevalence of multidrug resistant pathogenic isolates in ready to eat food products may act as potential source of foodborne intoxication under favourable conditions which need to be addressed.

Introduction

Food safety is increasingly becoming an important public health issue, as foodborne diseases present a widespread public health problem globally. Among the various pathogens causing food borne outbreaks,

Salmonella sps, *Staphylococcus aureus*, *Bacillus cereus*, *Campylobacter sps* have been the major cause for the foodborne illness. Rapid and precise monitoring and detection of foodborne pathogens are the most effective ways to control and prevent foodborne illnesses.

Traditional routine detection methods of these pathogens are based on culture dependent assays, including enrichment, chromogenic media and biochemical confirmation.

Although these methods are effective and remain the golden standards, they are rather time consuming, labour-intensive and sometimes complicated (Ding *et al.*, 2017 and Li *et al.*, 2016).

Moreover, conventional culture-based methods fail to detect viable but nonculturable (VBNC) cells which may introduce a serious crisis to food safety and public health (Zhao *et al.*, 2014). In recent decades, molecular methods such as PCR have gained much attention for pathogen detection because they are simpler and faster compared to conventional culture methods (Mandal *et al.*, 2011).

Minimally processed ready to eat (RTE) food products are increasingly used for consumption which increases the risk of food borne intoxications globally. Under these circumstances the present study was aimed to investigate the incidence and molecular detection of foodborne bacterial pathogens in ready to eat milk and meat products.

Materials and Methods

Samples collection and screening for foodborne bacterial pathogens

Ready to eat food products (n=250) including poultry meat and poultry products, milk and milk products, packed ready to eat food, frozen food etc., procured from retail outlets in and around Chennai were utilised in the study. The food samples were screened for the presence of foodborne bacterial pathogens like *Staphylococcus aureus*, *Salmonella sps*, *Streptococcus sps*, *Bacillus cereus* and *Campylobacter sps* on Mannitol Salt agar

(MSA), Xylose Lysine Deoxycholate agar (XLD), blood agar base supplemented with strepto supplement, Mannitol egg yolk polymyxin (MYP) medium and Muller Hinton (MH) media at 42°C for selective isolation of the above pathogens respectively.

The presumptive culture positive colonies were screened by colony morphology, gram staining, biochemical analysis and molecular methods. Biochemical characterization and Gram's staining of each presumptive colony of *Staphylococcus aureus*, *Salmonella sps.*, *Streptococcus sps.*, *Campylobacter jejuni* and *Bacillus cereus* isolates were done using specific Himedia biochemical test kit using HiStaph identification kit-KB004, HiSalmonella identification kit-KB011, HiStrep identification kit- KB005A and HiBacillus identification kit-KB013 respectively.

Reference strains

The reference strains procured from American type culture collection (ATCC) viz., *Staphylococcus aureus* (ATCC BAA 976), *Salmonella enterica* (ATCC 13076), *Streptococcus sp.* (ATCC 12392), *Bacillus cereus* ATCC 11778 and *Campylobacter sps* (ATCC 33291) were used as positive control for standardization of polymerase chain reaction (PCR) protocols.

DNA extraction

Overnight culture (1ml) was centrifuged at 10000 rpm for 3 minutes, the pellet was suspended in 1ml of tris – EDTA buffer (TE buffer pH-8), centrifuged at 10000 rpm for 3 minutes. The final pellet was suspended in 20µl of Nucleuses free water (NFW) and boiled for 10 minutes, centrifuged at 10000 rpm for 3 minutes and supernatant was used as DNA template for PCR.

Polymerase Chain Reaction and Sequencing

DNA isolated from the culture positive samples was used as template and PCR was performed by targeting fem A, Inv A, tuf, cytolethal toxin and HL gene of *Staphylococcus aureus* (296 bp), *Salmonella enterica* (275 bp), *Streptococcus spp.* (196 bp), *Campylobacter jejuni* (192 bp) and *Bacillus cereus* (185 bp) respectively using published/designed primers as given in table 1. The amplified products are checked in agarose gel electrophoresis. The PCR purified samples (n =5) (Qiagen gel extraction kit, Germany) were subjected to sequencing (Eurofins Genomics India Pvt. Ltd., Bengaluru, India) and analysed using DNA Star Laser gene V. 7.0 software. The phylogenetic tree was constructed with 1000 bootstrap value using a Maximum Likelihood method with MEGA software version 6.0.

Antibiotic susceptibility test

All the isolates of the selected food pathogens were tested for their antibiotic susceptibility pattern using disc diffusion method against a panel of ten antibiotics. The isolates were inoculated in Muller – Hinton broth and incubated at 37°C for 16 – 24 hrs. Turbidity of the overnight culture was adjusted to 0.5 McFarland standard and spread evenly over the Muller Hinton Agar (MHA) plates and the disc containing the specific concentrations of 10 antibiotics (HiMedia laboratories Pvt. Ltd., Mumbai) were placed on the surfaces of the agar plates and incubated at 37°C for overnight. The diameter of the inhibition zones were measured as per the manufacturer's instructions.

Results and Discussion

Out of 150 milk and milk products screened the percent positivity was 62, 51, 8, 12 and 2

for *Staphylococcus aureus*, *Bacillus cereus*, *Streptococcus spp.*, *Salmonella sps* and *Campylobacter sps* by culture method. A representative culture plate of positive colonies along with the Gram stain images are shown in (Plate 1a – 1d). The higher percent positivity for *Staphylococcus aureus* followed by *Bacillus cereus* in milk and milk products may be indicative of improper handling in production and packaging systems. Various authors studied the incidence level of food pathogens in dairy products and also suggested that the variation may be attributed to different sampling procedure and isolation techniques, (Jonkuvienė *et al.*, 2012, Kim *et al.*, 2014, and Kumari and Sarkar 2017). A total of 100 meat samples including ready to eat chicken, mutton, beef and pork products were screened for the presence of *Staphylococcus aureus*, *Bacillus cereus*, *Salmonella enterica*, *Streptococcus spp.* and *Campylobacter jejuni* in their respective selective medium.

The percent positivity observed was 20, 15, 28, 13 and 3 for *Staphylococcus aureus*, *Bacillus cereus*, *Salmonella sps*, *Streptococcus spp.* and *Campylobacter jejuni* by culture method respectively. The prevalence details of food pathogens in various meat products is represented in figure 1. Various researchers reported the prevalence of food pathogens in meat and meat products in chicken, beef and pork products (Gueven *et al.*, 2006, Lubna *et al.*, 2015, Hanson *et al.*, 2011 Yang *et al.*, 2016 and Soleimani *et al.*, 2017). The variation may be due to the difference in sampling number, isolating methods and environmental parameters.

The presence of microorganisms in food is unavoidable and the recent proposed approaches for the detection of foodborne pathogens is mainly the culture based bacterial isolation and identification, but this is tedious and time consuming (Zhoa *et al.*,

2014). PCR is more than a promising method to detect the pathogenic bacteria with greater sensitivity and reliability than conventional culture methods and is considered to be a valuable alternative to the culture-based detection techniques due to its speed, limit of detection (LOD), sensitivity and specificity (Rodríguez-Lázaro *et al.*, 2007). In the present study, a total of 250 food products were subjected to PCR followed by genotypic characterization. The amplified product of Fem A, Tuf, haemolysin (HL) toxin and Cytolethal distending toxin A genes for *Staphylococcus aureus*, *Streptococcus* spp, *Bacillus* spp and *Campylobacter* spp was presented in plate 2a - d respectively.

On phylogenetic analysis, 6 isolates of *Staphylococcus aureus* from milk products had 99% homology with *Staphylococcus aureus* (AP014921.1). The 9 isolates of *Bacillus cereus* (Genbank accession numbers MH029496 – MH029501 and MH 046796 – MH 046799) had 94% homology with *Bacillus cereus* (CP009968.1) and 3 isolates of *Streptococcus* sp. had 98% homology with the other Genbank sequence *Streptococcus anginosus* (KY275175.1). Further, 11 *Staphylococcus aureus* isolates from meat products (MH 509937, MH 509939, MH

509938, MH 460411, MH 460410, MH 460417, MH460416, MH 460415, MH 460414, MH 460413 and MH 460412) were obtained with 99% sequence homology. The phylogenetic tree was constructed based on the fem A gene sequences of *S. aureus* isolates from meat samples along with other coagulase negative *Staphylococci* species.

In the present study, the main cluster grouped several *S. aureus* strains and these strains are homologous to those from meat products. Four of the *S. aureus* isolates form the close evolutionary relationship with the *Staphylococcus aureus* isolates from Australia and three of the isolates form close evolutionary relationship with the Greece isolates. The 5 isolates *Bacillus cereus* from meat products (MH 547207 chicken nuggets), (MH 547206 beef cubes), (MH 547205 pork), (MH 547204 meat balls) and (MH 547203 chicken kheema) had 99% sequence homology.

The emergence of multidrug resistance of foodborne bacterial pathogens can be a major health concern. There is a need for the study of antibiotic susceptibility of each isolate to recognize their resistance towards different antibiotics (Kiessling *et al.*, 2002).

Table.1 Details of primers used in the study

S. No	Name of the organism	Primer Sequence (5'-3')	Target Gene	Annealing Temp (°C)	Product Size (bp)	Reference
1.	<i>Staphylococcus aureus</i>	FP: TATGAGTTAAAGCTTGCTGAAGGTT	Fem A	58	296	Kwasaki <i>et al.</i> , 2012
		RP: TTACCAGCATTACCTGTAATCTCG				
2.	<i>Salmonella species</i>	FP: TATCGCCACGTTTCGGGCAA	Inv A	60	275	Rahn <i>et al.</i> , 1992
		RP: TCGCACCGTCAAAGGAACC				
3.	<i>Streptococcus species</i>	FP: GTACAGTTGCTTCAGGACGTAT	Tuf	57	197	Picard <i>et al.</i> , 2004
		RP: ACGTTCGATTTTCATCACGTTG				
4.	<i>Bacillus cereus</i>	FP: CTGTAGCGAATCGTACGTATC	Hemolysin gene	54.5	185	Wang <i>et al.</i> , 1997
		RP: TACTGCTCCAGCCACATTAC				
5.	<i>Campylobacter</i> spp	FP: GCTAATGGAGGAACAGATGTAGG	Cytolethal distending toxin A	55	192	This study
		RP: TCCGCTTGCTTGAGTTGC				

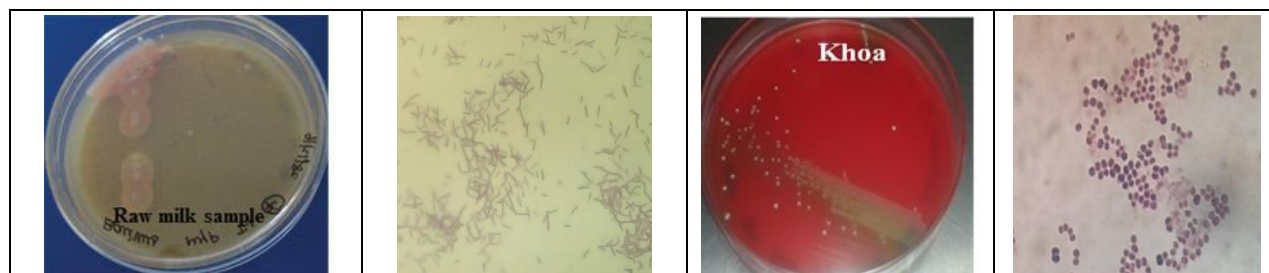


Plate.1a *Bacillus cereus* colonies with bright pink background surrounded by egg yolk in mannitol egg yolk polymyxin (MYP) agar and Gram positive rods (200X)

Plate.1b Pinpoint colonies of *Streptococcus spp.* from dairy product in (5%) blood agar and Gram positive cocci in chains (400X)

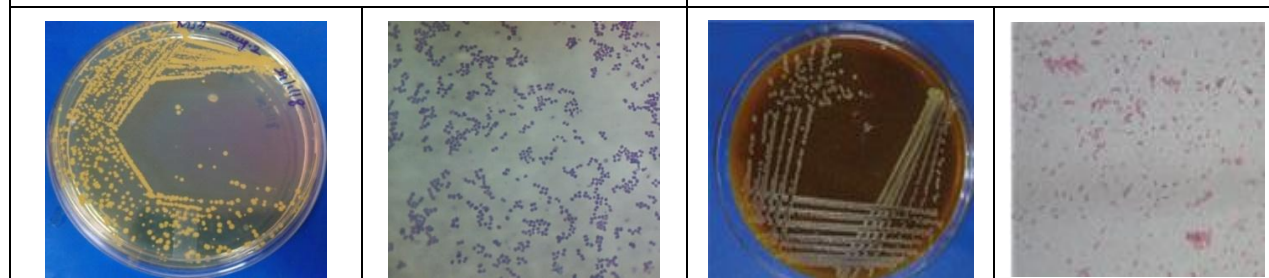


Plate.1c Smooth, round and yellow colonies of *Staphylococcus aureus* from pork product in Mannitol Salt Agar (MSA) and Gram positive cocci in cluster (200X)

Plate.1d Spongy white colonies of *Salmonella sps* in Xylose lysine deoxycholate (XLD) agar and Gram negative rods (200 X)

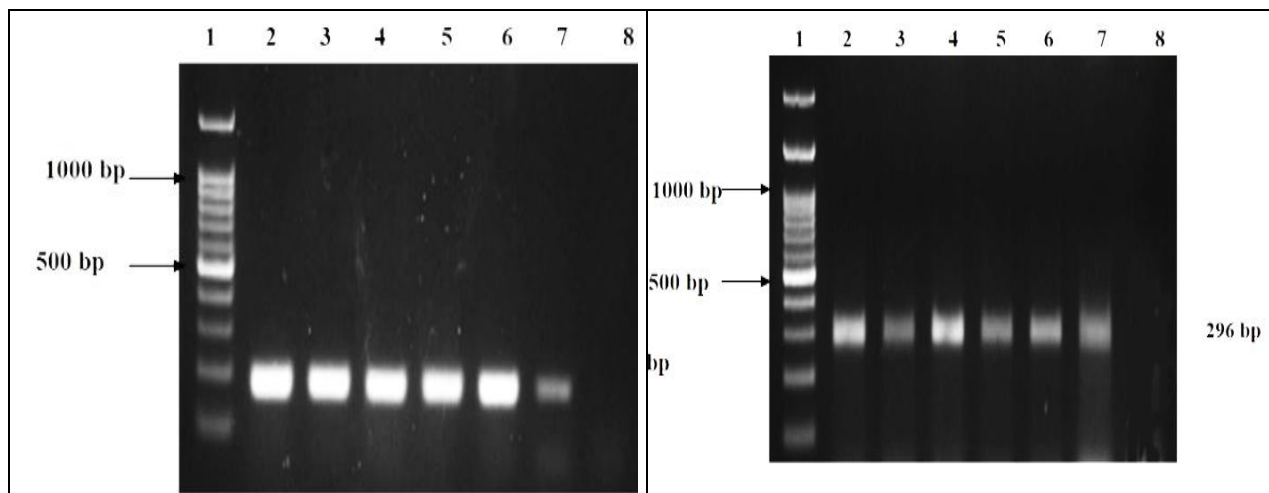


Plate.2a PCR amplified product of 185 bp HL gene. Lane 1: 100 bp ladder, Lane 2: *Bacillus cereus* (ATCC 117783), Lane 3 - 7 : *B.cereus* isolates and Lane 8: (Non template Control (NTC)

Plate.2b PCR amplified product of 296 bp FemA gene. Lane 1: 100 bp ladder, Lane 2: *Staphylococcus aureus* (ATCC BAA 976), Lane 3 - 7 : *Staphylococcus aureus* isolates and Lane 8: Non template Control (NTC)

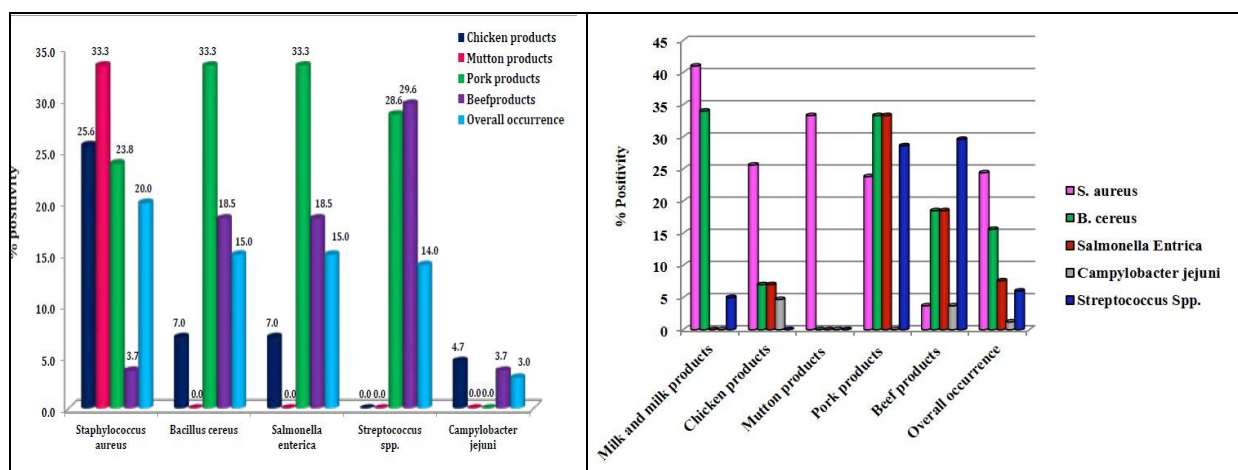
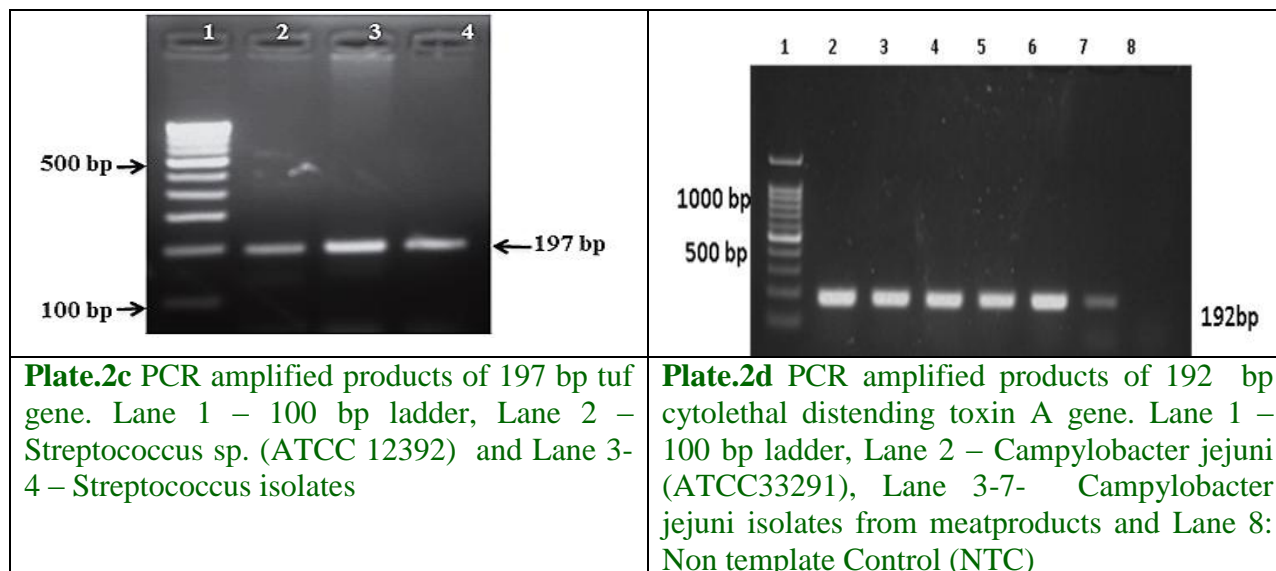


Fig.1 Occurrence of foodborne bacterial pathogens in various ready to eat food products

On antibiotic sensitivity assay, almost all the isolates obtained from meat and milk showed multidrug resistance towards various antibiotics. *Staphylococcus aureus* exhibited 100% resistant Vancomycin, Methicillin and Penicillin. *Bacillus cereus* isolates showed 100% resistant against Vancomycin and Penicillin antibiotics and *Streptococcus sp.* showed 100% resistant against Gentamicin, Streptomycin, Tetracycline and Ciprofloxacin. The higher resistance of isolates to Penicillin, Tetracycline and Erythromycin in the present study may be attributed to the fact that these are the commonly used antibiotics for treatment in

livestock and poultry. Variation in the antimicrobial resistant pattern of the isolates may be due to variation in concentration of the antibiotics used, drug resistance transfer and overall use of antibiotics.

Hence from the present study it is clearly indicated that, the ready to eat food products can act as significant source of antibiotic resistance microbial strains as potential cause for food borne intoxications. The toxin gene specific PCR rapidly detects the food pathogens from ready to eat meat products for precise monitoring in prevention of food borne intoxications.

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