Incidence and antimicrobial susceptibility of *Staphylococcus aureus* isolated from ready-to-eat foods of animal origin from tourist destinations of North-western Himalayas, Himachal Pradesh, India

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Abstract This study was aimed to determine the incidence of *Staphylococcus aureus* in ready-to-eat (RTE) milk (n = 120) and meat (n = 120) products from various tourist places in north western Himalayas, Himachal Pradesh, India. *S. aureus* isolates and its enterotoxins; A, B, D and E were characterized by conventional and molecular methods. Antimicrobial susceptibility (AMS) profiles of *S. aureus* isolates were determined by disk diffusion method using Clinical and Laboratory Standards Institute criteria. Overall, 6.7% (n = 16/240) food samples were positive for *S. aureus*. PCR amplification of *nucA* confirmed all biochemically characterized isolates as *S. aureus*. Incidence of *S. aureus* was higher (10.0%) in RTE milk products than meat products (3.3%). *S. aureus* contamination levels were highest in milk cake/khoa (26.0%, $p = 0.0002$) followed by ice cream/kulfi (10.0%, $p = 0.4$), mutton momo (10.0%, $p = 0.4$), burfi (3.3%, $p = 0.7$) and chicken momo (3.3%, $p = 0.7$). None of the isolates carried genes for *S. aureus* enterotoxins; A, B, D and E. AMS testing revealed seven different resistance patterns and 81.3% multi drug resistance. All the isolates were resistant to ampicillin. High

resistance levels were observed against methicillin (93.7%), clindamycin (68.8%), erythromycin (56.3%) and vancomycin (43.8%). Vancomycin resistant (n = 7) isolates were also resistant to methicillin. All isolates were susceptible to novobiocin.

Keywords Methicillin · Vancomycin · Novobiocin · *nucA* gene · Enterotoxin

Introduction

Staphylococcus aureus is a worldwide cause of food-borne infections (Bhatia and Zahoor 2007). *S. aureus* food contamination is associated with unhygienic preparation, storage and distribution of foods of animal origin i.e. milk, meat and their products. *S. aureus* and its toxins are third most important cause of gastroenteritis resulting from consumption of contaminated food (Bhatia and Zahoor 2007; Rohinishree and Negi 2011). Staphylococcal enterotoxins (SEs) are heat stable and persist in food (Fleurot et al. 2014). Isolation of *S. aureus* and detection of staphylococcal enterotoxins are conclusive diagnostic criteria of staphylococcal food illness (Kadariya et al. 2014).

Methicillin resistant *S. aureus* (MRSA) are those strains of *S. aureus* that possess intrinsic resistance to methicillin, oxacillin, nafcillin, carbapenems, and other beta-lactam antibiotics (VanEperen and Segreti 2016). Presence of MRSA strains is a serious threat to the health of the consumers and had been detected in meat and milk and their products (Doyle et al. 2012). Vancomycin remains an acceptable treatment option for MRSA caused infections (Holmes and Howden 2014). Vancomycin treatment failures and rising or high vancomycin minimum inhibitory concentrations (MICs) had been associated with higher

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mortality rates in MRSA infected patients (Holmes and Howden 2014; Lewis et al. 2018). In the present study, we determined the prevalence of *S. aureus* and strains producing A, B, D and E enterotoxins, most commonly associated with food poisoning outbreaks, in ready-to-eat (RTE) milk and meat products from various tourist places of North-western Himalayas located in Himachal Pradesh, India (Mehrotra et al. 2000). This is the first systematic study in the region determining the incidence of *S. aureus* and its enterotoxins in the region. We also ascertained the antimicrobial susceptibility profiles of *S. aureus* isolates recovered in this study.

Materials and methods

A total of 240 samples of RTE milk ($n = 120$) and meat ($n = 120$) products were collected aseptically from various retail outlets in different tourist places of Himachal Pradesh, located in mid and lower ranges of North western Himalayas. RTE food samples were collected from nine different tourist locations, covering 6 of 12 districts of the state (Table 1). Sampling plan included collection of 30 samples each of food product (burfi, kulfi/ice cream, milk cake/khoa, paneer, chicken momo, chicken sandwich, chicken soup and mutton momo) (Table 2). A portion (25 g or 25 ml) from the center of each sample was taken aseptically and homogenized with 225 ml sterile buffered peptone water (Hi-Media Lab., Mumbai, India). This homogenate was incubated at 37 °C for 24 h for enrichment. The enriched samples were subcultured on Baird Parker agar (HiMedia Lab, Mumbai, India) and incubated at 37 °C for 24–48 h (Singh and Prakash 2010). Jet black colonies surrounded by a white halo were characterized as *S. aureus*/*S. pyogenes* by Gram's staining, biochemical tests and carbohydrate fermentation tests (Sethi et al. 2013).

One ml overnight (12–16 h at 37 °C) cultures of *S. aureus*/*S. pyogenes* isolates in brain heart infusion broth (HiMedia Lab, Mumbai, India) were harvested by centrifugation for 15 min at 4000 rpm. Supernatant was discarded and pellet was used for isolation of DNA by phenol–chloroform–isoamyl alcohol method (Wilson 1987). Isolated DNA were stored at – 20 °C till further use. *S. aureus* isolates were reconfirmed and differentiated from *S. pyogenes* by polymerase chain reaction (PCR) targeting *nucA* (encoding thermostable nuclease) (Mehrotra et al. 2000). The PCR mixture (25 µl) contained, 3 µl of genomic DNA, 0.25 µl of Taq DNA polymerase (1 U/µl, Biogene, USA), 2.5 µl of 10 × PCR buffer with 1.5 µl $MgCl_2$ (50 mM, MBI, Fermentas, Waltham, USA), 0.5 µl dNTPs (10 mM, MBI, Fermentas, Waltham, USA), 1 µl of each primer (20 pmol/µl, Integrated DNA Technologies, Iowa, USA) and 15.25 µl of sterilized distilled water.

Amplifications (GeneAmp PCR System 9700, LABIN-DIA, India) were carried out using PCR parameters: initial denaturation at 94 °C for 5 min, followed by 37 cycles of amplification at 94 °C for 1 min (denaturation), 55 °C for 30 s (annealing) and 72 °C for 1.5 min (extension) and a final extension at 72 °C for 3.5 min (Mehrotra et al. 2000). The following oligonucleotide primers were used for PCR amplification reaction, Primer 1: 5'-GCG ATT GAT GGT GAT ACG GTT- 3' and Primer 2: 5' -AGC CAA GCC TTG ACG AAC TAA AGC- 3'.

PCR confirmed *S. aureus* isolates were further screened for genes associated with SEs A, B, D and E production, using a multiplex PCR as described earlier (Mehrotra et al. 2000). Primers (Integrated DNA Technologies, Iowa, USA) used for the amplification reactions were GSEAR-1 (5' GGT TAT CAA TGT GCG GGT GG 3') and GSEAR-2 (5' CGG CAC TTT TTT CTC TTC GG 3') for *sea* (encoding SEA), GSEBR-1 (5' GTA TGG TGG TGT AAC TGA GC 3') and GSEBR-2 (5' CCA AAT AGT GAC GAG TTA GG 3') for *seb* (encoding SEB), GSEDR-1 (5' CCA ATA ATA GGA GAA AAT AAA AG 3') and GSEDR-1 (5' ATT GGT ATT TTT TTT CGT TC 3') for *sed* (encoding SED) and GSEER-1 (5' AGG TTT TTT CAC AGG TCA TCC 3') and GSEER-2 (5' CTT TTT TTT CTT CGG TCA ATC 3') for *see* (encoding SEE) (Mehrotra et al. 2000). Multiplex PCR for simultaneous detection of enterotoxin genes was done in final reaction volume of 50 µl containing 4 µl of genomic DNA, 0.25 µl of Taq DNA polymerase (1 U/µl, Biogene, USA), 5 µl of 10 × PCR buffer with 1.5 µl $MgCl_2$ (50 mM, MBI, Fermentas), 1 µl dNTPs (10 mM, MBI, Fermentas), 1 µl of each of the eight primers (20 pmol/µl) and 30.25 µl of sterilized distilled water. DNA amplification was carried using parameters; an initial denaturation at 94 °C for 5 min was followed by 35 cycles of amplification (denaturation at 94 °C for 2 min, annealing at 57 °C for 2 min, and extension at 72 °C for 1 min), with a final extension at 72 °C for 7 min. The PCR products were analysed on 2% agarose gel using a 100 bp DNA ladder (Thermo Fisher Scientific, Mumbai, India). Agrose gels were stained with ethidium bromide (1 µg/ml). Amplified DNA was visualized under UV trans-illuminator and photographed using gel documentation system (Alpha Innotech Co., San Leandro, USA.)

Antimicrobial susceptibility profiles of the confirmed *S. aureus* isolates were determined for ampicillin (10 mcg), clindamycin (2 mcg), erythromycin (15 mcg), gentamicin (10 mcg), methicillin (5 mcg), novobiocin (mcg) and vancomycin (30 mcg) (Hi-Media Lab., Mumbai, India), using disk diffusion assay (Bauer et al. 1966). Antibiotic discs were placed on Muller-Hinton agar (Hi-Media Lab., Mumbai) plates, inoculated with *S. aureus* isolates. The plates were incubated at 37 °C for 24 h and zones of

Table 1 Ready to eat food samples collected from various tourist destinations of North western Himalayas to determine the incidence of *Staphylococcus aureus*

Sl. no.	Places	Type of samples (no. of samples collected)	Total number of samples collected (%)	Samples positive for <i>S. aureus</i> (%)
1.	Bajinath-Paprola	Barfi (6) Paneer (1) Milk cake/Khoa (3) Mutton momo (5)	15 (6.3)	1 (6.7%)
2.	Dalhousie	Chicken sandwich (5) Chicken soup (8)	13 (5.4)	0 (0)
3.	Dharamshala-Macleodegunj	Barfi (8) Paneer (6) Milk cake/Khoa (3) Chicken momo (4) Kulfi/Ice cream (5) Chicken sandwich (7) Chicken soup (8)	41 (17.1)	0 (0)
4.	Hamirpur	Barfi (2) Paneer (3)	5 (2.1)	0 (0)
5.	Kangra	Milk cake/Khoa (5) Kulfi/Ice cream (4)	9 (3.8)	1 (11.1)
6.	Kullu-Manali	Barfi (3) Paneer (3) Chicken momo (9) Mutton momo (7) Chicken soup (7) Mutton momo (4)	33 (13.8)	2 (6.1)
7.	Mandi	Barfi (3) Milk cake/Khoa (3) Kulfi/Ice cream (3) Chicken sandwich (5)	14 (5.8)	2 (14.3)
8.	Palampur	Barfi (8) Paneer (17) Milk cake/Khoa (11) Kulfi/Ice cream (18) Chicken momo (11) Mutton momo (14) Chicken sandwich (8) Chicken soup (7)	94 (39.2)	9 (9.6)
9.	Shimla	Milk cake/Khoa (5) Chicken momo (6) Chicken sandwich (5)	16 (6.7)	0 (0)
Total			240	16 (6.67)

inhibition were measured. Antibiotic concentrations of the discs (except for novobiocin) used and antimicrobial susceptibility criteria followed, were as recommended by the Clinical and Laboratory Standards Institute (CLSI 2013).

Fisher's exact test was used to determine the association between *S. aureus* incidence and types of RTE foods tested in this study. Significance was set at a *p* value of < 0.05.

Table 2 Incidence of *Staphylococcus aureus* in ready to eat food samples

Sl. no.	Food products	No. of samples tested	No. of samples positive for <i>S. aureus</i> (%)
1.	Barfi	30	1 (3.3)
2.	Kulfi/Ice cream	30	3 (10.0)
3.	Milk cake/Khoa	30	8 (26.6)*
4.	Paneer	30	0 (0.0)
5.	Chicken momo	30	1 (3.3)
6.	Mutton momo	30	3 (10)
7.	Chicken sandwich	30	0 (0.0)
8.	Chicken soup	30	0 (0.0)
9.	Total	240	16 (6.7)

*Significant association, p value < 0.05

Results and discussion

The present study revealed variable levels of contamination of *S. aureus* in RTE foods of animal origin in different tourist places North western Himalayas. A total of 6.7% (16/240) samples were contaminated with *S. aureus* (Table 2). Incidence of *S. aureus* was higher (10.0%, 12/120, $p = 0.7$) in milk based RTE foods products than those prepared from chicken or meat (3.3%, 4/120). PCR amplification of *nucA* (280 bp) from the genomic DNA confirmed all ($n = 16$) biochemically (Supplementary Table 1) characterized isolates as *S. aureus* (Supplementary Fig. 1). Contamination levels were highest in milk cake/khoa (26.0%, 8/30, $p = 0.0002$) followed by ice cream/kulfi (10.0%, 3/30, $p = 0.4$), mutton momo (10.0%, 3/30, $p = 0.4$), burfi (3.3%, 1/30, $p = 0.7$) and chicken momo (3.3%, 1/30, $p = 0.7$) (Table 2). Our findings are in agreement with previous studies conducted in Italy (milk and dairy products), Turkey (raw milk and dairy products) and India (Carfora et al. 2015; Gundogan and Avci 2014; Sudhanthiramani et al. 2015). The presence of *S. aureus* in food indicates adoption of poor hygienic measures from production to consumption of food (Fleurot et al. 2014). Incidence of *S. aureus* in RTE foods of animal origin was highest in Mandi (14.3%, 2/14) followed by Kangra (11.1%, 1/9), Palampur (9.6%, 9/94), Baijnath-Paprola (6.7%, 1/15) and Kullu-Manali (6.1%, 2/33) (Table 1).

SEs directly affect the intestinal epithelium and vagus nerve causing stimulation of the emetic center, resulting in emesis (Kadariya et al. 2014). Very small dose of SEs can cause *S. aureus* food illness and concentration as low as 0.5 ng/ml of SEs can cause a large outbreak (Kadariya et al. 2014). None of the *S. aureus* isolates from this study was found to carry *sea* (102 bp), *seb* (164 bp), *sed* (278 bp) and *see* (209 bp) (Supplementary Fig. 2; Mehrotra et al. 2000).

We observed eight different resistance phenotypes for 16 *S. aureus* isolates recovered in this study (Table 3). Of

the 16 isolates, 81.3% (13/16) were multi drug resistant, showing resistance to 3 or more unique antimicrobial classes (Waters et al. 2011). All the isolates were resistant to more than one tested antibiotics with four isolates resistant to maximum six antibiotics (Table 3). We recorded 93.8% *S. aureus* resistance to methicillin. This is in accordance with the resistance rates (98% and 100%) reported previously (Mehndiratta et al. 2010; Argudín et al. 2011). Seven (43.8%) isolates were resistant to vancomycin. All vancomycin resistant isolates were also resistant to methicillin. It is an important observation since vancomycin remains the cornerstone for management of MRSA infection (Lewis et al. 2018). Its efficacy is now being questioned with the emergence of strains of *S. aureus* that display intermediate resistance or complete vancomycin resistance and occasionally treatment failures (Holmes and Howden 2014; Lewis et al. 2018). High levels of resistance were observed to clindamycin (68.8%, 11/16) and erythromycin (75.0%, 12/16). 56.3% (9/16) isolates were resistant to both these antibiotics (Table 3). Clindamycin is a bacteriostatic antimicrobial and inhibits bacterial toxin production. Clindamycin can be indicated for management of *S. aureus* infections including those caused by MRSA (Lewis et al. 2018). It is important to note that high levels of erythromycin resistance in *S. aureus*/MRSA isolates induce clindamycin resistance. Therefore, clinicians should be aware of regional rates of erythromycin resistance before recommending clindamycin for the treatment of MRSA associated infections (VanEperen and Segreti 2016). All the isolates were resistant to ampicillin, similar to findings reported from Republic of Korea and Northern Greece (Lee 2003; Sergelidis et al. 2012). All *S. aureus* isolates recovered in this study were susceptible to novobiocin. Studies in past have shown the efficacy of novobiocin alone in combination with other antibiotics such as rifampicin against MRSA infections both in man and animals (Walsh et al. 1993; Riley et al. 1995; Traub et al. 1996; Nam et al. 2011). In view of decreasing

Table 3 Antimicrobial susceptibility profiles of *Staphylococcus aureus* isolates recovered from ready to eat food products

Resistance patterns	Antibiotics used							Resistance phenotype
	AMP	CLI	ERY	GEN	MET	VAN	NOV	
P 1 (n = 4)	R	R	R	R	R	R	S	AMP/CLI/ERY/GEN/VAN/MET
P 2 (n = 3)	R	R	R	S	R	R	S	AMP/CLI/ERY/MET/VAN
P 3 (n = 2)	R	R	R	S	R	S	S	AMP/CLI/ERY/MET
P 4 (n = 2)	R	R	S	S	R	S	S	AMP/CLI/MET
P 5 (n = 2)	R	S	R	S	R	S	S	AMP/ERY/MET
P 6 (n = 2)	R	S	S	S	R	S	S	AMP/MET
P 7 (n = 1)	R	S	R	S	S	S	S	AMP/ERY

AMP ampicillin, CLI clindamycin, ERY erythromycin, GEN gentamicin, MET methicillin, VAN vancomycin, NOV novobiocin, S susceptible, R resistant

treatment options for *S. aureus*/MRSA infections, use of novobiocin to cure MRSA related infections should be reinvestigated.

Conclusion

This was first systematic study on *S. aureus* contamination in RTE foods of animal origin from tourist places of North western Himalayas. 39.2% of the food samples analyzed in this study were collected in and around Palampur area which remains a limitation of this study. We did not find any isolate carrying genes for SE A, B, D and E. However, a high prevalence of multi drug resistant *S. aureus* strains was recorded. Importantly, *S. aureus* strains resistant to both methicillin and vancomycin, were detected in this study. These observations demand a prudent use of antibiotics for treating patients suffering from staphylococcal food illness. Combination therapy involving two or more antibiotic with different mechanisms of action will provide effective therapy to treat MRSA infection and will also delay the development of antimicrobial resistant strains (Lewis et al. 2018). Due to limited resources, we could not perform agar dilution or E test assays on *S. aureus* recovered in this study for determining antimicrobial susceptibility. These assays provide minimum inhibitory concentration (MIC) values for the tested bacterial isolates. Disk diffusion assay, one of the CLSI recommended methods was used to determine AMS. Disk diffusion assay determines the antimicrobial susceptibility status of the bacterium but doesn't provide information about MIC values. It is further recommended that a strict monitoring of food safety required in tourist places since visiting populations after getting infections can carry pathogens to newer susceptible populations.

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Compliance with ethical standards

Conflict of interest There is no conflict of interests.

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