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Prevalence Study of Antimicrobial Resistance among Invasive *Salmonella* spp. in Milk and Dairy Products in India

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ABSTRACT

Background: Food safety has emerged as an important global issue with expanded international trade and various public health implications. Milk and dairy products are rich in many nutrients hence offer favourable environments for the growth of various food borne pathogens. Of which in past decades Salmonellosis is a frequently reported zoonotic foodborne disease in world. Although India is a leading contributor of World's milk production along with increased consumption of milk and dairy products, only a few information is available on the prevalence status of *Salmonella* spp. in milk and dairy products. Hence the study was aimed to study the prevalence of *Salmonella* spp. and its antimicrobial resistance pattern by phenotypic methods that has been recovered from different study samples.

Methods: Polymerase chain reaction (PCR) was adopted to confirm the *Salmonella* spp. and then phenotypically the antimicrobial resistance pattern was studied by Kirby Bauer disc diffusion method.

Result: A total of 567 samples (75 Raw milk samples, 45 pasteurized milk samples and 447 dairy products) were screened and four Salmonella isolates which was derived from the dairy products were confirmed by PCR targeted the *inv*A gene that encodes the invasion protein of Salmonella. The isolates showed multiple drug resistance and also has higher MAR indices along with 75% resistance to the antibiotics *viz*, Cephalothin, Penicillin and Enrofloxacin. The presence of *Salmonella* spp. in milk and dairy products and their AMR to common antibiotics in the study area indicates that emerging resistance pattern of food borne *Salmonella* spp. and possibility of entering the food chain which is of high risk to the consumers.

Keywords: Antimicrobial resistance (AMR), Milk and dairy products, Polymerase chain reaction, *Salmonella* spp.

INTRODUCTION

Milk is considered as the most complete food in nature due to its rich nutrient composition that could be difficult to obtain from any other non-dairy food sources. In India most of the people consider milk and value-added dairy products as a source of protein in their daily diet. India's dairy sector has remarkable growth in past decade and it is dominated by milk cooperatives, local vendors and private dairies (USDA, 2017). In recent days, in addition to milk consumption, processed and packaged value-added dairy products has been increased in India.

Milk and its products when contaminated by various foodborne microorganisms reduces the quality along with posing a risk of foodborne disease to the public health. The commonly recognized transmission of food/water borne pathogens include inadequately cooked or raw meat, unpasteurized milk or milk products, contaminated and inadequately treated drinking water (Friedman *et al*., 1998). Contamination of milk might also be due to the use of polluted water or unhygienic equipments or dairy workers. (Mahendra Pal *et al*., 2020).

Review of occurrence of foodborne disease outbreaks in India from 1980 to 2016 by CD alert (2017) reported *Salmonella* spp. was the most common cause of foodborne illness in human along with other pathogens like *Staphylococcus aureus*, *Vibrio* spp., *E. coli*, *Yersinia enterocolitica* and Norwalk like virus. Outbreaks of salmonellosis have been frequently reported and is the third leading cause of death among food-transmitted diseases (Ferrari *et al*., 2019). Foods of animal origin like milk and dairy products are frequently associated with human salmonellosis owing to its prevalence in animals (Hoelzer *et al*., 2011). Direct or indirect contact with animals colonized with *Salmonella* is yet another source of infection to humans. Recent reports by researchers including Mahendra Pal *et al*. (2020)

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indicated that the recurrent problems of nosocomial and community acquired multidrug-resistant salmonellosis.

Salmonella is a gram negative facultative anaerobic non-spore forming rod of the enterobacteriaceae family (Giannella, 1996 and Punchihewage-Don *et al.,* 2022). There are more than 2500 different Salmonella serotypes and all are considered potentially pathogenic to humans (Popoff *et al.,* 2003). Based on the antigenic polymorphisms of lipopolysaccharide (O antigens), flagellar protein (H antigen) and capsular polysaccharide (Vi- antigens), the subspecies of *Salmonella* are further separated into serovars according to the kauffaman white Scheme (Grimont and Weill, 2007). Salmonellosis is a leading cause of food borne illness throughout the world, ends up in ten million human cases every year (WHO, 2018). The pathogen is mainly a diarrhoeal agent which cause mild to severe illness, but during its invasiveness it results in severe and life threatening conditions and contribute to bovine mastitis and can directly excreted in the milk. Recently AMR Salmonella strains from food of animal origin, particularly milk and milk products were isolated (Chiu *et al*., 2002). Hence it is important to investigate the prevalence of the *Salmonella* spp. along with its resistance pattern in milk and milk products. Recent isolation of AMR resistant *Salmonella* strains from different parts of the world from foods of animal origin particularly milk and milk products necessitates the importance of monitoring drug resistance of *Salmonella* spp. and to curtail it in production chain in order to safeguard the health of end consumers (Chiu *et al*., 2002). Hence, the present study was designed to characterize the invasiveness of the Salmonella isolates from milk and milk products and to identify their antimicrobial resistance pattern.

MATERIALS AND METHODS

Sample collection

A cross- sectional study was designed to analyse milk and its products collected between January 2016 to January 2018. Samples for the study was collected by non-probability convenience sampling methods from three zones of Chennai, Tamil Nadu, India (Chennai North, Chennai central and Chennai south zones). Five hundred and sixty seven samples were collected including milk samples (Raw milk- 75, 25 samples/zone, Pasteurized milk- 45, 15 samples/zone) and dairy products (Total 447 - Chennai North-146, Chennai central-152 and Chennai south-149) which consists of Channa based sweets-85, Khoa based sweets-99, Fermented dairy products-73, concentrated or partially desiccated dairy products-37, Heat and acid coagulated dairy products-33, Frozen dairy products-32, chilled and flavoured dairy product-42 and other dairy products like butter, cheese, cream-46). The samples included as dairy products for the present study are channa based sweets (Rasagolla and rasamalai), khoa based sweets (Burfi, peda, gulab jamun, pantooa and milk cake), fermented dairy products (Dahi, lassi, buttermilk and shrikhand), concentrated or partially desiccated dairy products (Khoa and rabri), heat and acid coagulated dairy products (paneer), frozen dairy products (Kulfi and ice cream) and other dairy products (Butter, cheese and cream). All the samples were processed at the Department of Veterinary Public Health and Epidemiology, Madras Veterinary College, Chennai on the same day of collection or kept in refrigerator at 4°C until processing.

Isolation and confirmation of *Salmonella* spp. by cultural and molecular methods

For the isolation and identification of the foodborne *Salmonella* spp. Food and Drug Administration-Bacteriological Analytical Manual (FDA-BAM) was followed. Presumptive colonies which appeared as large glossy pink colonies with black centre in XLD agar (Xylose Lysine Desoxycholate) Grey/ black colony with halo effect in Bismuth sulphite were picked and further characterized by Gram's staining, Biochemical tests *viz*., catalase tests, oxidase tests, Indole, Methyl Red, Voges- proskauer, Citrate utilization test, Triple sugar Ion test, lactose fermentation test and urease test. The presumptive isolates were further confirmed by molecular method like PCR using suitable primers targeting the *inv*A gene, using standardized cycling conditions as mentioned in Rahn *et al.* (1992) with suitable primers (Table 1) and the cycling conditions are illustrated in Table 2.

Antimicrobial assay

The antimicrobial profile of the isolates of *Salmonella* spp. was determined against 22 different antibiotics belonging to different classes such as beta-lactams, quinolones and aminoglycosides were studied by Kirby Bauer's disc diffusion method as per CLSI guidelines on Muller Hinton Agar with

Table 1: Primers used for detecting the virulence *inv*A gene of *Salmonella* spp.

Target gene	Primer sequence (5′-3′)	Amplicon size (bp)	References
invA	F: GTGAAATTATCGCCACGT TCGGGCAA		
	R: TCATCGCACCGTCAAAGGAACC	284	Rahn *et al.* (1992)

Table 2: PCR cycling conditions for identification of *inv*A gene in *Salmonella* spp.

Initial denaturation	Denaturation	Annealing	Extension	Final extension
94°C for 1 min	94°C for 1 min	64°C for 30 sec	72°C for 30 sec	72°C for 7 min
		Repeat for 35 cycles		

certain modifications (Bauer *et al.*, 1966). The antibiotics which were commonly used in human and veterinary Medicine *viz*. amikacin (30 µg), cephaxitin (30 µg), cephalothin (30 µg), nystatin (100 µg), sulphadiazine (300 µg), rifampicin (5 µg), ampicillin (10 µg), streptomycin (10 µg), enrofloxacin (10 µg), co- trimaxazole (25 µg), methicillin (5 µg), vancomycin (3 µg), piperacillin (100 µg), imipenam (10 µg), gentamicin (10 µg), penicillin (10 µg), tetracycline (30 µg), clindamycin (2 µg), amoxycillin (10 µg), ciprofloxacin (5 µg), azithromycin (30 µg), cefotaxime (30 µg) (Laxminarayan and Chaudhury, 2016) was used to study the antimicrobial susceptibility. *Salmonella* isolates resistant to at least three antimicrobial classes were designated multidrug resistant (MDR)

As per the Krumperman's procedure (1983), Multiple Antibiotic Resistance index (MAR Index) was a cost effective and valid method to track the source of bacteria. It is calculated using the formula a/b, where 'a' represents the number of antibiotics to which the isolate is resistant and 'b' the number of antibiotics to which the isolate was tested. The MAR index can be used to assess health risk which identifies if isolates are from a region of high or low antibiotic use (Krumperman 1983; Davis and Brown, 2016).

RESULTS AND DISCUSSION

Prevalance status of *Salmonella* spp. in milk and dairy products

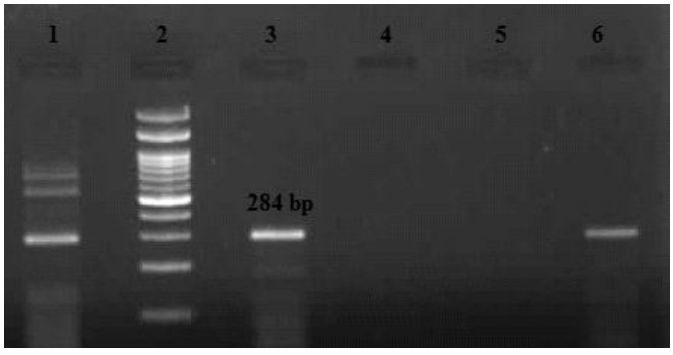
Out of 567 samples screened for the presence of *Salmonella* spp. only 10 isolates showed characteristic colony morphology in XLD and BSA agar by conventional culture and biochemical tests. Upon further characterization of the isolates by PCR, only 4 isolates were found to be positive. Agarose gel electrophoresis showing PCR amplification of *inv*A gene of *Salmonella* spp. under this study has been depicted in Fig 1. *Salmonella* spp. was absent in raw milk and pasteurized milk obtained from various zones in Chennai city. The overall prevalence of *Salmonella* spp. was only 0.70 per cent. The zone wise prevalence of *Salmonella* spp. was also analyzed and the per cent isolation of *Salmonella* spp. was found to be 0.68, 0.65 and 1.34 per cent in Chennai North, Chennai Central and Chennai South zones respectively. Three isolates were obtained from heat and acid coagulated dairy products and one from channa based dairy product. All other dairy products showed absence of *Salmonella* spp.

Antimicrobial resistance and MAR index

In the present study, *Salmonella* spp. was found to be 75% resistant to cephalothin, enrofloxacin and penicillin and were found to be 50% resistant to sulphadiazine, sulphasomidine, ampicillin, piperacillin, gentamicin, clindamycin, amoxycillin and azithromycin. The Salmonella isolates also showed 100% sensitivity to imipenam and tetracycline. All the obtained isolates showed resistance to more than three antibiotics, hence imparts Multiple Drug Resistance (MDR). The MAR Index ranged from 0.27 to 0.45 with the average MAR Index being 0.38 (Table 3).

In the present study, *Salmonella* spp. was not detected in raw milk samples. Like many pathogens, *Salmonella* is not commonly found in surveys of raw milk owing to its relatively low incidence usually less than one per cent (Bell and Kyriakides, 2009), which was in accordance with our present study. However, Elafify *et al.* (2019), documented the prevalence of *Salmonella* spp. in raw milk in Egypt as 44.44%. Another study by Yasmin *et al.* (2015) reported that 25.71% prevalence of Salmonella in raw milk in Dhaka Metropolis Bangaladesh. In south India a study carried out by Lingathurai and Vellathurai (2010) reported 13.3 per cent of *Salmonella* spp. which was also in contrary to our study. This variance in the prevalence rate might be due to the different managemental and hygienic practices followed in different parts of the world adopted from production to supply chain.

A study conducted at Egyptian dairy market by Elafify *et al.* (2019) documented a prevalence of *Salmonella* spp. in Kariesh cheese as 55.55 per cent (5/9). A study conducted in Addis Ababa, Ethiopia on 384 dairy products *viz.,* cheese, milk, butter and yogurt showed an overall prevalence of *Salmonella* as 1.6 per cent (6 of 384) along with a prevalence



L1: sample, L2: sample, L3: sample, L4: 100 bp DNA ladder, L5: +ve control, L6: -ve control, L7: sample, L8 sample.

Fig 1: Agarose gel electrophoresis showing PCR amplification of *inv*A gene.

Table 3: MAR Indices for the isolates of *Salmonella* spp.

Isolates	MAR index	Antibiotics that are resistant
HAC 1	0.40	CEP, GEN, COT, AZM, P, SZ, EFX, AK, MET
HAC 2	0.31	COT, AMX, SZ, R, PI, CTX,
HAC 3	0.40	CEP, P, CD, R, AMP, PI, EFX, CIP, AZM,
CB 4	0.45	CEP, EFX, P, AMP, GEN, CD, AMX, VA, S, NS

MAR: Multiple antibiotic resistance, HAC: Heat and acid coagulated product isolate, CB: Channa based product isolate, CEP:Cephalothin, GEN: Gentamicin, COT: Co-trimaxazole, AZM: Azithromycin, P: Penicillin, SZ: Sulphadiazine, EFX: Enrofloxacin, AK: Amikacin, MET: Methicillin, AMX: Amoxicillin, R: Rifampicin, PI: Piperacillin, CTX: Cefotaxime, CD: Clindamycin, AMP: Ampicillin, CIP: Ciprofloxacin, VA: Vancomycin, S: Streptomycin, NS: Nystatin.

rate of 3.1, 1.04, 2.1 and 0 per cent in cheese, butter, milk and yogurt, respectively (Tesfaw *et al*., 2013). Our study is also in accordance with Tesfaw *et al*. (2013). Water quality, cross contamination, unhygienic handling practices during processing may be the reasons inferred for varied distribution and prevalence in different zones of Chennai, TamilNadu. Considerable scientific evidence has shown that the use of certain antibiotics increases enteric colonization of antibiotic-resistant strains of enteric pathogens in domestic animals.

Polymerase chain reaction (PCR) is sensitive, specific time saving and hence can be very much helpful to identify the virulence of the foodborne pathogens. It is worth mentioning that *inv*A gene encodes a *Salmonella* invasion protein, which is considered to be a virulence gene located on the Salmonella Pathogenecity Island (SPI) 1 (Abdel-Aziz, 2016).

Antimicrobial resistance (AMR) of *Salmonella* isolates of the present study revealed 75% resistance to the antibiotics *viz*, Cephalothin, Penicillin and Enrofloxacin and 50% resistance to Sulphadiazine, Rifampicin, Ampicillin, Piperacillin, Gentamicin, Clindamycin, Amoxycillin and Azithromycin but 100% sensitivity to Imipenam and Tetracycline. Tajbakhsh *et al*. (2012) found *Salmonella* resistance to the antibiotic Tetracycline, which is contrary to our findings. Hassani *et al*. (2022) suggested that *Salmonella* strain obtained in their study was resistant to Penicillin and Amoxicillin which is in accordance to our study. Vaez *et al.,* (2020) reported AMR of *Salmonella* spp. of animal origin against Cephalosporin and quinolones with 13.5% prevalence of resistance against Cephalothin and 10.7% prevalence of resistance against Enrofloxacin, which is similar to our findings. *Salmonella* strains isolated from human infections were mostly resistant against ampicillin and streptomycin (Ranjbar *et al.,* 2011) and sensitive to Imipenam (Ranjbar *et al.,* 2011, Eshraghi *et al.,* 2010) which correlates with the findings of the present study. Based on the work of Krumperman (1983) and Mthembu *et al.,* (2019) higher indices which are more than 0.2 may be observed if antibiotics are used in large amounts. Interestingly, different MDR patterns were observed among the isolates. This emergence of MDR Salmonella serotypes have huge impact on the efficacy of antibiotic treatment and also increases the prevalence of MDR strains which in turn leads to increase in mortality of people affected with *Salmonella* (Shu-Kee Eng *et al.,* 2015). The emergence MDR *Salmonella* at an increasing frequency limits therapeutic options both in humans and animals (Gebreyes *et al.,* 2000).

Estimated annual costs for salmonellosis have ranged from billions of dollars in the United States to hundreds to millions of dollars in Canada and millions of pounds in the United Kingdom. Analysis of five *Salmonella* outbreaks due to manufactured food in North America gave direct cost of more than $36,400-$62 millions (Mahendra Pal *et al*., 2020). Despite of the low prevalence, dissemination of resistant gene results in huge threat to both human and animals which may result in high economic impact due to medical costs, loss of working hours and product recall, which can further be intensified with antimicrobial resistance. Inadequate food regulation and education for food handlers, along with the poor hygienic practices adopted by the workers in a developing country like India contribute to the spread of resistance.

CONCLUSION

There is an urgent need to implement strict guidelines for antimicrobial usage in hospitals and community along with strict hygienic farm management practices, periodical screening of personnel of dairy production chain for foodborne pathogen thereby minimizing environmental contamination of the resistant organism. Routine periodical inspection and monitoring of ready to eat dairy products from production time throughout their expected shelf life is mandatory to limit further acquisition of resistance among *Salmonella* strains to ensure food safety thereby safeguarding both human and animal health.

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Conflict of interest

The authors declare that they have no conflict of interest.

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