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Analysis of bacterial contamination in fresh and finished meat products and their molecular identification.

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ABSTRACT: *Fresh and packaged food safety, especially of meat products has become a major issue because of microbial contamination. In the present study, an attempt has been made to isolate bacteria from fresh and packaged meat products from various sources and locations. The isolated bacterial samples were identified by Phenotypic, biochemical and molecular characterization. The veracity of the contamination does not depend only on the microbial quality but also on the physical factors such as handling of the meat products. The findings suggest that, to maintain the safety norms there is more need of new and advanced handling methods which would avoid bacterial cross contaminations in meat and meat products. Although less contamination was found in finished products, it also needs to be sterilized with bacterial resistant packaging.*

KEYWORDS: *Food-borne pathogen, (16S rRNA sequencing), bacterial contamination, Microbial load*

I. INTRODUCTION

Food safety issues are becoming more important in international trade [1]. Outbreaks of food-borne diseases have led to considerable illness and even death [2, 3]. It has found that every year there are between 24 to 81million cases of food-borne illness every year and out of which 50% are associated with meat and poultry [4, 5, 6]. The shelf-life of food decreases due to microbial contamination which promotes food borne illness. Food borne pathogens like Salmonella sp., Listeria monocytogenes, Campylobacter sp., and verocytotoxin producing Escherichia coli O157, originating from the animal during slaughter, contaminate the carcass and spread to the cut or raw meat intended for further processing [7] causing a major public health problem. Bean and Griffin [8] reported that in the United States, Salmonella sp. account for 48% of all beef related outbreaks. A healthy animal may harbor pathogenic bacteria on its hide, hair, and hooves, in its intestinal tract, and around the lymph nodes [9, 10]. Mostly the internal surfaces of the carcasses are sterile but the infection occurs due to dressing and skinning defects during slaughtering process [11] and the food handlers as well are the major transmission agents for common pathogens [12].

Traditional methods like thermal processing, drying, freezing, refrigeration, irradiation, modified atmosphere packaging and adding antimicrobial agents or salts to prevent contamination are not sufficient for fresh meats and ready-to-eat products [13]. For meat products, microbial contamination occurs at the surface. Although rates of attachment of bacteria to meat have been studied [14, 15], there is limited information on how to prevent this attachment. It has been described that Acidified Sodium Chlorite (ASC) is an antimicrobial compound which effectively reduces contamination of poultry and beef products [16]. Use of ASC was approved by the U.S. Food and Drug Administration (FDA) in 1996 as a secondary direct food additive. Processing with ionizing radiation is a most effective treatment for decontamination of food. It is a safe, environmentally clean and energy efficient process [17]. Most of the food safety protocols observe irradiation as an effective Critical Control Point in a Hazard Analysis and Critical Control Points (HACCP) system for meat and poultry processing [18].

Several novel detection technologies have been developed which are highly sensitive and results in controlling the spread of the disease. Immunological methods such as enzyme immunoassays, although relatively rapid, require a high number of target organisms for detection [19]. At present, more advanced genotypic methods are available to study microbial taxonomy. These include the analysis of 16S rRNA [20], 16S-23S and 23S-5S spacer regions [21], pulsed-field gel electrophoresis [22], randomly amplified polymorphic DNA RAPD-PCR [23], M13 fingerprinting and ribotyping [24]. Among these molecular techniques, the 16S rRNA analysis has been accepted as the most reliable method [25]. The purpose of this study is to analyze the bacterial contaminations in fresh and finished meat products from various sources and to identify the of microorganism by 16S rRNA sequencing.

II. MATERIALS AND METHODS:

2.1. Sample Collection:

Raw meat samples (liver, brain, intestine, lungs and muscle) were collected from the slaughter houses located at K. R. Market, Russel Market, Johnson market and Tannery road in Bangalore. Finished products like

Minced meat, dry mutton kabab, mutton nuggets, mutton cutlet, mutton cubes, mutton sausages were collected from various outlets in Bangalore.

2.2. Raw Meat Sampling:

Raw meat samples were collected from different sources, 1 g of each sample was placed in 10 ml of water and then serial dilution was performed. Dilution of 10-3, 10-5 and 10-7 were used for bacterial isolation. 200 µl of each diluted water sample was transferred on Petri plate containing Nutrient Agar Media. Sample was evenly distributed on plate by using L-shape sterile glass rod. Plates were kept at 40 C for 30 min. and then incubated at 370 C for 24 hrs.

2.3. Finished Product Sampling:

Different types of finished product samples as mentioned above were collected, 1 g of each sample was placed in 10 ml of water and then serial dilution was performed. Dilution of 10-3, 10-5 and 10-7 were used for bacterial isolation. 200 µl of each diluted water sample was transferred on Petri plate containing Nutrient Agar Media. Sample was evenly distributed on plate by using L-shape sterile glass rod. Plates were kept at 40 C for 30 min. and then incubated at 370 C for 24 hrs.

2.4. Biochemical Characterization:

Selected colonies were maintained on Nutrient agar. Isolated bacterial samples were subjected to Gram's Staining, morphological and biochemical characterization and identified according to the Bergey's manual. Biochemical tests such as Catalase, Oxidase, Citrate, Oxidation/Fermentation, Coagulase, Indole, Methyl red, Motility and Urease were performed for identification. Also, food products of meat origin, both raw as well as finished product, were taken up individually for bio-load monitoring and pathogen testing.

2.5. DNA Extraction and 16S rRNA Sequencing

The DNA extraction method presented in this paper is an improved method of the standard phenol/chloroform method [26] with the following modifications. Isolate was grown in MRS broth at 37 °C for overnight. Culture was centrifuged at 6000 × g for 2 min at room temperature. After discarding the supernatant, the pellet was suspended in 400 µl STE Buffer (2% SDS, 100 mM NaCl, 100 mM Tris- HCl, 10 mM EDTA, pH 8.0) and incubated at 550 C for 30 min. Then 200 µl Tris-saturated phenol (pH 8.0) and 200 µl of chloroform was added and centrifuged at 8000g for 10 min. To a clean 1.5 ml tube 150 µl upper aqueous phase was transferred and DNA was precipitated by adding 100 µl ice cold iso-propanol and centrifuged for 10 min at 10,000g at 40 C. The pellet was dissolved in 50 µl TE buffer. The isolated DNA then sent to Applied Biosystems, Bangalore for 16S rRNA sequencing.

III. RESULTS AND DISCUSSION:

Bacterial colonies were isolated from all raw and finished meat samples. A maximum of 41 colonies were observed in Raw Meat sample followed by 31 colonies in minced Mutton sample. For the Finished Product samples, number of colonies per plate ranged from 7 in mutton nuggets to a maximum of 58 colonies in Mutton dried Kabab sample, (Table 1). Most of the colonies observed were found to be morphologically similar. These common colonies were then isolated and maintained on nutrient agar. Based on Gram's Staining, phenotypic methods and biochemical characterization as per Bergey's manual, the samples were found to be E.coli, Staphylococcus sps, Pseudomonas sp., Micrococcus sp., Streptococcus sp., Serratia sp., Shigella sp., and Salmonella sp. in raw meat samples. Where as in Finished meat products Salmonella sp., E.coli, Streptococcus sp., Serratia sp., Campylobacter sp., Proteus sp. and Klebsiella sp. Were observed (Table 2). In the present study it is observed that the bioload was more in finished product (Dry Mutton Kabab) than the raw meat samples. This could be because of bacterial contamination from air as the samples were kept outside in open area. As we have seen unhygienic environment and improper handling, colonies of Campylobacter sp. were found most in finished product as shown in (Table 3).

Table 1: Total number of bacterial colonies in different dilutions of raw meat samples and finished meat products.

RAW MATERIAL						
Bacterial Colony Count	Liver	Brain	Intestine	Lungs	Mutton	Minced meat
K. R. Market						
10^{-3}	17	9	22	14	41	31
10^{-5}	6	-	7	4	16	11
10^{-7}	1	-	2	-	5	2
Russel Market						
10^{-3}	27	12	15	26	20	14
10^{-5}	9	3	7	10	9	8
10^{-7}	1	-	-	1	3	1
Johnson Market						
10^{-3}	19	21	29	16	9	19
10^{-5}	3	7	6	8	2	7
10^{-7}	-	2	-		-	-
Tannery Road						
10^{-3}	16	27	11	18	24	29
10^{-5}	-	9	2	4	5	14
10^{-7}	-	-	-	-	-	6

FINISHED PRODUCTS (From other sources)					
Dilutions	Mutton kabab	Mutton nuggets	Mutton cutlet	Mutton cubes	Mutton sausages
10^{-3}	58	7	11	9	13
10^{-5}	25	-	2	-	3
10^{-7}	7	-	-	-	-

Table 2: Identification by biochemical characterization of probable genus

Grams Rxn/Shape	Motility	Catalase	Oxidase	Citrate	Oxidation Fermentation	Coagulase	Indole	Methyl Red	Urease	Probable Genus
-ve rods	-	+	-	+	F	-	-	+	+	Klebsiella
-ve rods	+	+	+	+	O	-	+	+	-	Pseudomonas
+ve cocci	-	+	-	-	F	+	-	+	+	Staphylococcus
-ve rods	+	+	-	-	F	NR	+	+	-	E. Coli
+ve rods	-	+	-	-	F	-	+	+	+	Proteus
+ve cocci	+	+	+	-	O	-	-	-	-	Micrococcus Sp
-ve rods	-	+	-	-	O	-	-	+	-	Shigella Sp
-ve rods	+	+	-	+	O	-	-	-	-	Serratia Sp
+ve cocci	-	-	-	-	F	-	+	+	+	Streptococcus Sp
-ve rods	+	+	-	+	F	+	-	+	-	Salmonella sp
-ve rods	+	+	+		OF	-	-	-	+	Campylobacter sp

Legend: -ve – Gram negative; +ve – Gram positive; F – fermentation test; O – Oxidation test ; rxn – reaction; + - positive test; - = negative test; NR – no reaction.

Table 3: Bacteria found in sample collected and analyzed from different markets.

Bacterial Isolates	Liver	Brain	Intestine	Lungs	Mutton	Minced meat	Mutton kabab	Mutton nuggets	Mutton cutlet	Mutton cubes	Mutton sausages
Klebsiella	-	-	-	-	-	-	++	-	+	+	+
Pseudomonas	-	+	+	++	+	+	-	-	-	-	-
Staphylococcus	++	+	-		+	++	-	-	-	-	-
E. Coli	+	-	+	+	+	+	-	+	-	+	-
Proteus	-	-	-	-	-	-	+	-	+	-	+
Micrococcus Sp	+	+	-	+	+	-	-	-	-	-	-
Shigella Sp	-	+	+	-	-	-	-	-	-	-	-
Serratia Sp	+	-	+	-	-	+	++	+	-	+	+
Streptococcus Sp	-	-	+	+	++	+++	+	+	+	-	++
Salmonella sp	-	+	-	+	+	-	+	+	+	+	-
Campylobacter sp	+	-	+	-	+	+	+++	++	+	+	+

Legend; - :No colonies, + : Low number of colonies, ++ : Moderate number of colonies, +++ : High number of colonies

Phenotypic, biochemical characterization and DNA isolation were carried out in all the samples. Out of identified organism, DNA was isolated from only two samples Serratia species (ID SAA2) and Salmonella Species (ID SAA3). Purified DNA was then sent for 16S rRNA sequencing to "Applied Biosystems, Bangalore". The 16s rRNA sequencing confirm the sample identity as Serratia marcescens / nematodiphila and Salmonella enterica/ typhi respectively. The blast results were showed in (Table 4 and 5).

Contaminated meat and meat products, dairy products, vegetables, drinking water and swimming pools have been recognized as main vehicles for spreading the infection to humans. To reduce the impact of toxigenic isolates, their epidemiology must be fully established. Epidemiological studies would be greatly facilitated by the availability of a technique, such as PCR, which reliably detects low numbers of pathogens in food, water, and environmental materials. Domestic animals, especially sheep and cattle, are the main reservoirs and sources of E. coli infection for human beings [26].

Bacteria food poisoning is the most common type of food poisoning and it is caused as a result of the presence of harmful bacteria or poisonous substances produced by them in food. An outbreak of food poisoning may be caused by food which appears to be quite different from those involved in food spoilage. Harmful bacteria (pathogens) find their way into food in number of ways. However most food poisoning occurs as wholesome in spite of the fact that it is heavily infected by microorganisms. The organism causing food poisoning are a result of unhygienic behaviour and inappropriate handling practices by humans [27].

High risks foods are foods that are likely to be infected with pathogens and foods intended to be eaten without cooking, examples include: meat, cooked rice, fish, eggs, poultry, milk e.t.c. Some bacteria produced toxins called endotoxin while others produced exotoxins. The main type of pathogenic bacteria associated with foods is: Salmonella, Clostridium perfringes, Staphylococcus aureus, Listeria monocytogenes, Campylobacter jejuni, Clostridium botulinum, B. cereus, and Escherichia coli. Pathogens can be carried and passed on to others by individuals who themselves are not ill. Such carriers may have recently suffered an attack of food poisoning and still be harbouring the organisms in their body. In some cases carriers of food poisoning act as host over a period of many years having themselves acquired immunity to organisms concerned e.g. Salmonella typhi, Bacillus cereus. Most often they are unaware of their role as a reservoir of infection [28, 29]

Table 4. Blast Results for 16sRNA sequence identification for Sample ID SAA2.

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
JN934387.1	Serratia nematodiphila strain PDRT 16S ribosomal RNA gene, partial sequence	2603	2603	100%	0.0	99%
HQ166100.1	Serratia marcescens strain d4 16S ribosomal RNA gene, partial sequence	2601	2601	99%	0.0	99%
FJ662869.1	Serratia nematodiphila strain P36 16S ribosomal RNA gene, complete sequence	2599	2599	100%	0.0	99%
FJ495145.1	Serratia sp. BSFC16 16S ribosomal RNA gene, partial sequence	2599	2599	100%	0.0	99%
FJ360761.3	Serratia sp. PSB9 16S ribosomal RNA gene, partial sequence	2599	2599	100%	0.0	99%
FJ360759.1	Serratia marcescens strain PSB19 16S ribosomal RNA gene, partial sequence	2599	2599	100%	0.0	99%
EU525929.1	Serratia marcescens strain SDLH-I 16S ribosomal RNA gene, partial sequence	2599	2599	100%	0.0	99%
EF415649.1	Serratia marcescens 16S ribosomal RNA gene, partial sequence	2599	2599	100%	0.0	99%
JF441244.1	Serratia marcescens strain A4 16S ribosomal RNA gene, partial sequence	2595	2595	99%	0.0	99%
GU220797.1	Serratia marcescens strain C2 16S ribosomal RNA gene, partial sequence	2595	2595	99%	0.0	99%

Table 5. Blast Results for 16sRNA sequence identification for Sample ID SAA3

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
CP003278.1	Salmonella enterica subsp. enterica serovar Typhi str. P-stx-12, complete genome	2575	1.800e+04	100%	0.0	100%
AE014613.1	Salmonella enterica subsp. enterica serovar Typhi Ty2, complete genome	2575	1.801e+04	100%	0.0	100%
EU118114.1	Salmonella typhi strain T7 16S ribosomal RNA gene, partial sequence	2569	2569	100%	0.0	99%
EU118113.1	Salmonella typhi strain T6 16S ribosomal RNA gene, partial sequence	2569	2569	100%	0.0	99%
EU118111.1	Salmonella typhi strain T4 16S ribosomal RNA gene, partial sequence	2569	2569	100%	0.0	99%
EU118107.1	Salmonella enteritidis strain E7 16S ribosomal RNA gene, partial sequence	2569	2569	100%	0.0	99%
EU118098.1	Salmonella enterica subsp. enterica serovar Paratyphi C strain C9 16S ribosomal RNA gene, partial sequence	2569	2569	100%	0.0	99%
EU118095.1	Salmonella enterica subsp. enterica serovar Paratyphi C strain C6 16S ribosomal RNA gene, partial sequence	2569	2569	100%	0.0	99%
EU118093.1	Salmonella enterica subsp. enterica serovar Paratyphi C strain C4 16S ribosomal RNA gene, partial sequence	2569	2569	100%	0.0	99%
EU118091.1	Salmonella enterica subsp. enterica serovar Paratyphi C strain C2 16S ribosomal RNA gene, partial sequence	2569	2569	100%	0.0	99%
DQ344537.1	Salmonella enterica subsp. enterica serovar Typhi 16S ribosomal RNA gene, partial sequence	2569	2569	100%	0.0	99%

IV. CONCLUSION:

Presence of the Campylobacter sp. in finished product shows the lack of sanitary condition of premises, equipment and personnel surfaces and general management practices. Since microbial contamination of these foods occurs primarily at the surface, due to post-processing handling, attempts have to make to improve safety and to delay spoilage by use of antibacterial sprays or dips. However, direct surface application of antibacterial substances onto foods have limited benefits because the active substances are neutralized on contact or diffuse rapidly from the surface into the food mass. On the other hand, incorporation of bactericidal or bacteriostatic agents into meat formulations may result in partial inactivation of the active substances by product constituents and is therefore expected to have only limited effect on the surface microflora. Our finding suggests or rather insists to adopt good handling practices to avoid bacterial contamination in food products. New technologies are needed for surveillance of food-borne disease and food monitoring. These include typing pathogens, different in vitro, animal and clinical testing. New research and development are required in food industry such as application of antimicrobial surface materials and green technologies.

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