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Isolation and Molecular Confirmation of *Pseudomonas aeruginosa* in Chicken meat, Mutton and Pork

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A B S T R A C T

Pseudomonas aeruginosa is an opportunistic pathogen and is ubiquitous in nature and commonly found in soil, water, plants, and raw meat. It is major predominant spoilage bacteria in meat under refrigerated conditions. The study aimed to characterize the isolation, identification and molecular confirmation of *Pseudomonas aeruginosa* in Chicken meat, Mutton and Pork. Study area, sampling, isolation, identification and molecular confirmation of *Pseudomonas aeruginosa*. A total 210 (n = 35 from each of the species) aerobically stored and frozen meat samples were collected from retail markets and slaughter houses in and around Chennai, India and analyzed. Among them, 22 isolates were confirmed as *Pseudomonas aeruginosa* by conventional, culture, biochemical and PCR assay targeting 16 SrRNA sequencing and amplification of *rpoB gene*. In this research, we successfully studied the occurrence, isolation, identification and molecular confirmation of *Pseudomonas aeruginosa* isolated from Chicken meat, Mutton and pork using conventional techniques.

Introduction

Meat is a rich source of nutrients including proteins, vitamins, iron and other minerals and has high water activity, which makes it a highly perishable food commodity (Doulgeraki *et al.*, 2012). Spoilage is a complex process that can occur due to activities of indigenous enzymes and the existing microflora of meat. Frequently microbial contamination shows multiple signs of spoilage, such as off odors, discolorations, and slime formation which become detectable when the microbial populations reach 10^7 to 10^8 colony forming unit (CFU)/cm^2 (Dainty *et al.*, 1989; Nychas, 2008).

Pseudomonas is a diverse genus of gram negative, rod shaped obligatory aerobic and motile by one or several polar flagella. This organism is ubiquitous in

nature and commonly found in soil, water, plants and raw meat. The presence of *Pseudomonas* species leads to discoloration and slime formation on meat (Motoyama *et al.*, 2010). The main flora responsible for spoilage of fresh and refrigerated meat during aerobic storage belongs to genus *Pseudomonas* (Widders *et al.*, 1995).

These organisms are highly proteolytic, with better access to nutrients compared to other non-proteolytic psychotropic bacteria which grow on meat (Drosinos and Board, 1995; Koutsoumanis *et al.*, 2006). *Pseudomonas lundensis, Pseudomonas flourescens,* and *Pseudomonas putida*, *Pseudomonas fragi* are the four main *Pseudomonas* spp responsible for causing meat spoilage during chilled storage (Ercolini *et al.*, 2007).

Source of contamination is concern; food processing has been identified as a main source of the food chain, especially for fresh foods or products that are not subjected to heat treatments or other sanitization during its preparation. *Pseudomonas* is found to be an adaptable bacteria in the food processing environment (Stellato *et al.*, 2017).

Further, poor sanitation of meat shops and unhygienic processing and unawareness of meat retailers of basic requirements and guidelines of meat shop could also be the point source of contamination. (Bantawa *et al.*, 2018)

Pseudomonas aeruginosa is considered as an opportunistic pathogen and has a distinctive feature through synthesis of blue-green, chloroform-soluble compound called pyocyanin (El-Fouly *et al.*, 2015).

Materials and Methods

Location of Sampling

Samples for the study *viz*, Chicken meat, Pork, Mutton, were collected from retail markets and slaughter houses in and around Chennai under aerobically stored and frozen storage condition. The collected samples were brought immediately and processed at Department of Veterinary Public Health and Epidemiology, Madras Veterinary College, Chennai.

Sample collection

A total of 210 samples, aerobic and chilled each of 35 Chicken meat, mutton and pork from retail markets, slaughter houses were collected in and around Chennai, Tamil Nadu. (Table-1)

Reference strain

Pseudomonas aeruginosa (MCC2080) reference strain obtained from National center for microbial resource, Pune, India was used in this study as positive culture. The reference strain was sub cultured, tested for its purity by colony morphology, microscopic analysis and biochemical tests.

Culture Media

Brain Heart Infusion Broth (BHIB), Cetrimide Agar, Muller Hinton Agar, Biochemical reagents, oxidase disc and Antibiotic sensitive discs, supplements were procured from Himedia Laboratories Pvt Ltd (Mumbai).

Oligonucleotide primers for PCR assay

Oligonucleotide primers used in the present study were custom synthesized from Eurofins Genomics India Pvt. Ltd., India.

Isolation and identification of *Pseudomonas aeruginosa*

Preparation of Media

Preparation of Brain Heart Infusion Broth

Enrichment of the samples were done in Brain Heart Infusion Broth (BHIB). As per manufacturer's instruction 37 g BHIB powder was suspended to make 1000ml with double distilled water and autoclaved at 15lbs pressure (121˚C) for 15 min.

Preparation of Cetrimide Agar

For selective plating on Cetrimide Agar, 45.6 g of Cetrimide agar was suspended to 1000ml of distilled water as per manufacturer's instruction and autoclaved at 15 lbs pressure (121˚C) for 15 min.

Once after autoclaving the plates were poured in sterile zone and the plates were incubated at 37^{o}C, overnight for sterility check.

Sample preparation and inoculation

All the meat samples were processed immediately in sterile condition at the department laboratory. Each 10 grams of sample was taken, cut in to small pieces using sterile scissor, put in 100 ml of BHI broth and incubated at 37°C for 12-18 hours.

After 24 hour, a loopful of inoculum from BHI broth was streaked on sterile Cetrimide agar and incubated at 37^{o}C for 18hr to observe the typical colonies of *Pseudomonas aeruginosa*. On Cetrimide agar, the colonies of *Pseudomonas aeruginosa* exhibited the characteristic blue greenish colonies

Identification of *Pseudomonas aeruginosa* by conventional tests

All the presumptive isolates were first subjected to gram staining and then various biochemical tests were performed viz, catalase, oxidase, TSI agar test and gelatin liquefaction test for the confirmation of *Pseudomonas aeruginosa* isolates.

Gram's staining

The phenotypically identified colonies on Cetrimide agar were suspected for *Pseudomonas aeruginosa* and were stained by Gram's staining and visualized the morphology of these isolates under microscope (10x). The organisms appeared blue in color which indicates gram negative.(Figure-3)

Biochemical test

Catalase test

The typical isolated colonies from agar plates were placed on a clean grease free glass slide and two drops of three per cent hydrogen peroxide (H_2O_2) was added and observed for effervescence.

Production of gas bubbles from the surface of the colonies after addition of three per cent $H_2O_2$was considered to be positive and lack of effervescence as negative.

Oxidase test

The typical isolated colonies from agar plate were picked up with a sterile micro tip and smeared on the surface of oxidase disc and observed for 5 to 10 sec for color change. The positive cultures developed an intense purple within 5 to 10 sec after smearing. Negative reaction was identified by no color change within 30 sec.

Triple sugar iron agar test

The suspected isolates were inoculated into Triple Sugar Iron agar slant with a sterile inoculating needle by stabbing the butt and streaking back and forth along the surface of the slant.

Incubated the tubes with loosened caps at 37˚C for overnight. The tubes were observed for the color change at Slant and butt region, also any crack on the butt region was observed to identify any gas production.

Citrate test

The suspected isolates were inoculated into Simmons Citrate agar slant with a sterile inoculating needle by stabbing the butt and streaking back and forth along the surface of the slant. Incubated the tubes with loosened caps at 37˚C for overnight. The tubes were observed for the color change slant and butt region, when the bacteria metabolize citrate, the ammonium salts are broken down to ammonia which increases alkalinity. The shift in pH turns the bromothymol blue indicator in the medium from green to blue above pH 7.

Gelatin Hydrolysis

Inoculate gelatin deep with 4 to 5 drops of a 24 – hour broth culture. Incubate at 37° in ambient air for up to 14 days. Refrigerate an un-inoculated control along with the inoculated tube. Liquefaction is determined only after the control has hardened (gelled).

PCR ASSAY

Polymerase chain reaction for confirmation of *Pseudomonas aeruginosa*

The samples found to be positive by culture and biochemical tests were further subjected to DNA extraction by using Qiagen kit and PCR analysis was carried out for the confirmation of *P.aeruginosa* using 16S rRNA and *rpo*B gene.

Results and Discussion

Conventional isolation and identification of *Pseudomonas aeruginosa*

Isolation of *Pseudomonas aeruginosa*

Conventional method of isolation was performed by initial inoculation in BHI broth followed by selective plating on Cetrimide agar as described in materials and methods.

The isolates were identified based on characteristic greenish blue mucoid colonies on Cetrimide agar (Figure-1). A total number of 22 presumptive isolates of *P.aeruginosa* were obtained *viz.* 6 from chicken meat (3 frozen, 3 aerobically stored meat), 7 from Mutton (4 frozen, 3 aerobically stored meat), 9 from pork (6 frozen, 3 aerobically stored meat).

Biochemical test for *Pseudomonas aeruginosa*

Biochemical tests were done as per the procedure mentioned in Bacteriological Analytical Manual (BAM, 1998) protocol. All the presumptive isolates were found to be positive for catalase, oxidase, citrate, gelatin hydrolyze test and on Triple Sugar Iron (TSI) agar it was alkaline butt and slant and in few cases cracks were seen at the butt due to gas production (Table-2).

Molecular confirmation of *Pseudomonas aeruginosa* targeting 16S rRNA and *rpo*Bgene

Molecular confirmation of all the 22 *P.aeruginosa* isolates which were confirmed by conventional culture and biochemical test were further confirmed by PCR assay targeting 16S rRNA and *rpo*B gene.

All the 22 isolates amplified specific product size viz. 618bp and 759bp respectively for 16srRNA (Figure 4-6) and *rpo*B (Figure 7-9) which is specific for *P.aeruginosa*. The PCR products were confirmed by Agarose gel electrophoresis (1.2%) with a 100bp DNA ladder and were visualized under Gel Documentation system.

Occurrence of *Pseudomonas aeruginosa* in various meat samples and in different storage conditions

The percent occurrence of *Pseudomonas aeruginosa* in various meat samples *viz.* Chicken, Mutton and Pork were 8.57%, 10% and 12.8% respectively. In chicken meat, equal percentage (8.57%) of occurrence was detected in both aerobically stored and under frozen storage.

The occurrence of *Pseudomonas aeruginosa* in Mutton under aerobically stored condition and in frozen storage were respectively 8.57% and 11.42% under aerobic and frozen storage conditions respectively (Table-3).

Food supply and food safety are major global public health issues, and are particularly important in heavily populated countries such as China and India (Lorenz, 2012).

Meat is not only highly susceptible to spoilage, but also frequently implicated to the spread of food-borne illnesses. During slaughter and processing, all

potentially edible tissues are subjected to contamination from a variety of sources within and outside the animal (Selvan *et al.*, 2007).

The occurrence of bacteria in the food processing environments plays a key role in food contamination and development of spoilage. Among the various kind of organisms involves in meat spoilage, in various processing steps, the species of the genus *Pseudomonas* are recognized as major food spoilers and the capability to actually determine spoilage can be species- as well as strain-dependent (Stellato *et al.*, 2017). *Pseudomonas aeruginosa* is psychrophilic organism concern with food spoilage and also having considerable medical importance.

Keeping all these in view, the present study was undertaken to assess the occurrence of this *Pseudomonas aeruginosa* in various commercially available meat sold in Chennai city viz. Chicken meat, mutton and pork stored aerobically and in frozen condition.

In this study among the 210 samples, a total number of 22 presumptive isolates of *P.aeruginosa* were obtained *viz.* 6 from chicken meat (3 frozen, 3 aerobically stored meat), 7 from Mutton (4 frozen, 3 aerobically stored meat), 9 from pork (6 frozen, 3aerobically stored meat). *Pseudomonas aeruginosa* was identified in all the type of meat samples collected *viz.* Chicken, mutton and pork, highest number was isolated from Pork followed by mutton and chicken and also in overall storage condition is concerned frozen meat had 13 isolates and aerobically stored meat comparatively low number which had 9 isolates. According to (Dainty *et al.*, 1989; Borche *et al.*, 1996), the predominant bacteria associated with spoilage of refrigerated beef and pork, is identified as *Pseudomonas*. *Pseudomonas* spp were isolated from 80% of the examined Chicken samples, but found in all examined fish samples (E1-Aziz, 2015).

Biochemical test for *P.aeruginosa*

In this study all the 22 isolates were subjected to gram's staining and to various biochemical tests. All the isolates were gram negative and were found to be positive for catalase, oxidase, citrate, gelatin hydrolyze test and on Triple Sugar Iron (TSI) agar it was alkaline butt and slant and in few cases cracks were seen at the butt due to gas production.

Molecular confirmation of *Pseudomonas aeruginosa* isolates

In this study, PCR assay was performed for both 16S rRNA and *rpo*B genes to confirm the *Pseudomonas aeruginosa* presumptive isolates which were identified based on selective isolation and biochemical tests. The *rpo*B gene has been used in several studies (Tayeb *et al.*, 2005; Benie *et al.*, 2017) to analyze the phylogenetic relationships within the genus *Pseudomonas* by comparative genomics approach of partial sequence.

Table.1 Primers for the identification and confirmation of *Pseudomonas aeruginosa*

Target gene	Sequence(5'-3')	Size(bp)	Reference
16SrRNA	F:GACGGGTGAGTAATGCCTA R;CACTGGTGTTCCTTCCTATA	618	Spilker *et al.*, 2004
rpoB	F:CAGTTCATGGACCAACCCG R:ACGCTGGTTGATGCAGTGTTC	759	Benie *et al.*, 2017

Table.2 PCR amplification conditions for 16S rRNA primer

Initial Denaturation	**95˚C**	**5 min**	**30 cycles**
Denaturation	95˚C	1 min	
Annealing	58.4˚C	1 min	
Extension	72˚C	1 min	
Final Extension	72˚C	7 min	

Table.3 PCR amplification conditions for *rpo*B primer

Initial Denaturation	**94˚C**	**5 min**	**35 cycles**
Denaturation	94˚C	1 min	
Annealing	58˚C	1 min	
Extension	72˚C	2 min	

Table.4 Sample Frame

S. No	Sampling sites	Number of sample						Total
		Chicken		Mutton		Pork		
		F	A	F	A	F	A	
1	Millers Road	9	8	7	5	8	9	**46**
2	Madox Street	9	10	8	7	9	7	**50**
3	Authorized slaughter house (Kattupakkam)	0	0	7	10	0	0	**17**
4	Periannamaistry street	7	9	6	6	8	10	**46**
5	Naval Hospital Road	10	8	7	7	10	9	**51**
	TOTAL	**35**	**35**	**35**	**35**	**35**	**35**	**210**

F-Frozen meat A-Aerobically stored meat

Table.5 Biochemical characterization of *Pseudomonas aeruginosa*

S. no	TESTS INVOLVED	CA 13	CA 22	CA 28	CF 17	CF 27	CF 33	MA 9	MA 19	MA 34	MF 15	MF 22	MF 28	MF 33	PA 13	PA 26	PA 35	PF 1	PF 2	PF 19	PF 24	PF 29	PF 33
		Chicken Aerobic Meat			Chicken Frozen Meat			Mutton Aerobic Meat			Mutton Frozen Meat				Pork Aerobic Meat			Pork Frozen Meat					
Biochemical Characterization of Positive Isolates																							
1	Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3	TSI Agar	+	+	+	+	+	+	+	+	+*	+	+	+	+*	+	+	+	+	+*	+	+	+	+
4	Gelatin hydrolysis test	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5	Citrate test	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Molecular Confirmation of traget genes																							
6	16S rRNA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7	*rpoB*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

* In TSI agar test indicates that few cracks were at the butt due to gas production

Isolation of *Pseudomonas aeruginosa* by conventional method

Fig.1 *Pseudomonas aeruginosa* on Cetrimide agar (Greenish mucoid colonies)

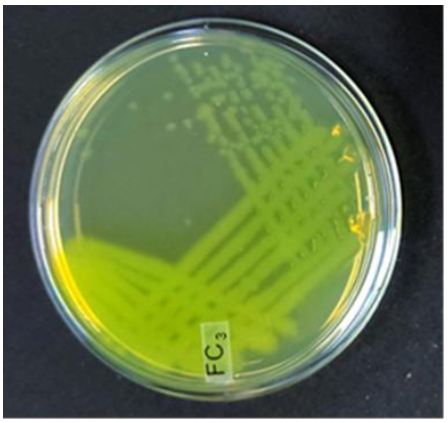


Fig.2 *Pseudomonas aeruginosa* under UV light exposure

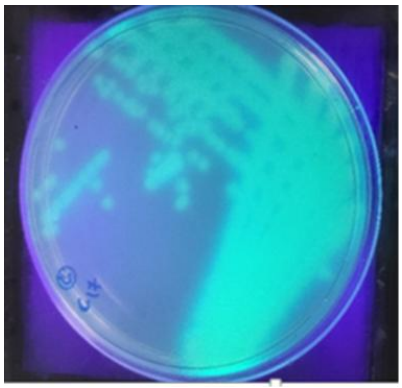
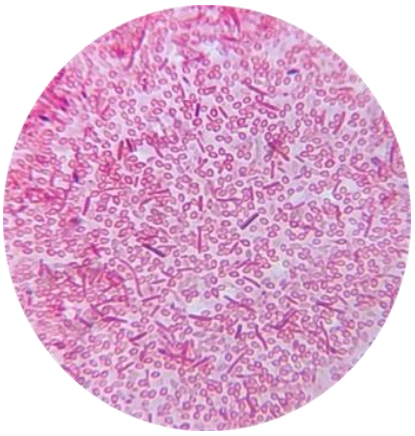


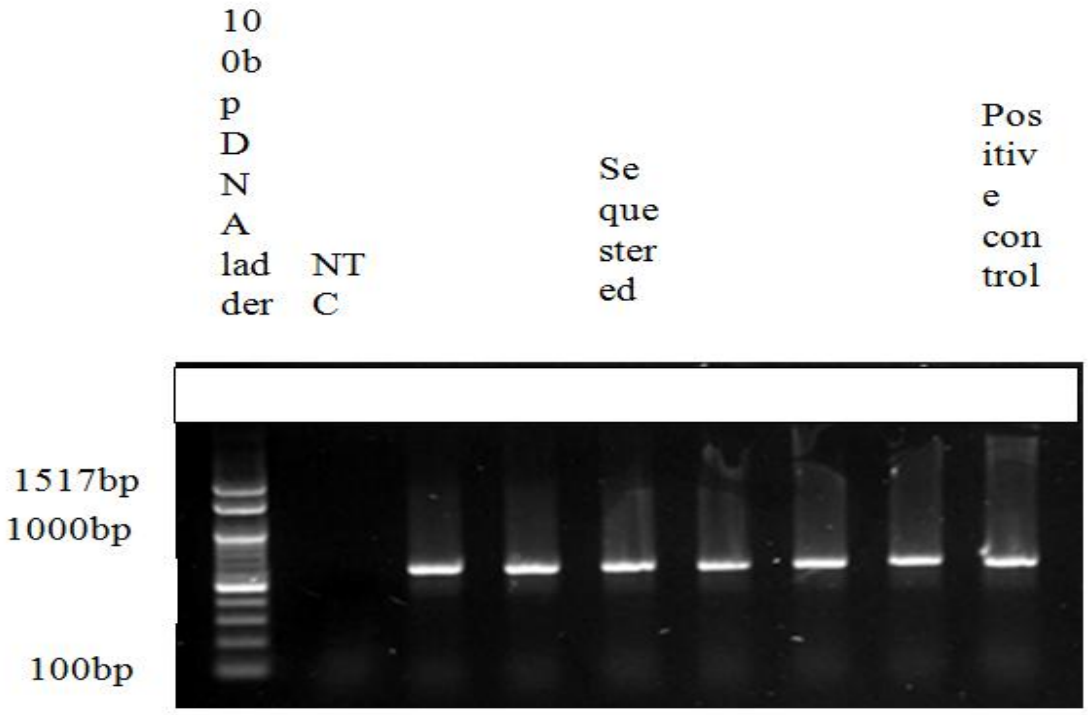
Fig.3 Gram's staining (Gram -ve)



PCR ASSAY

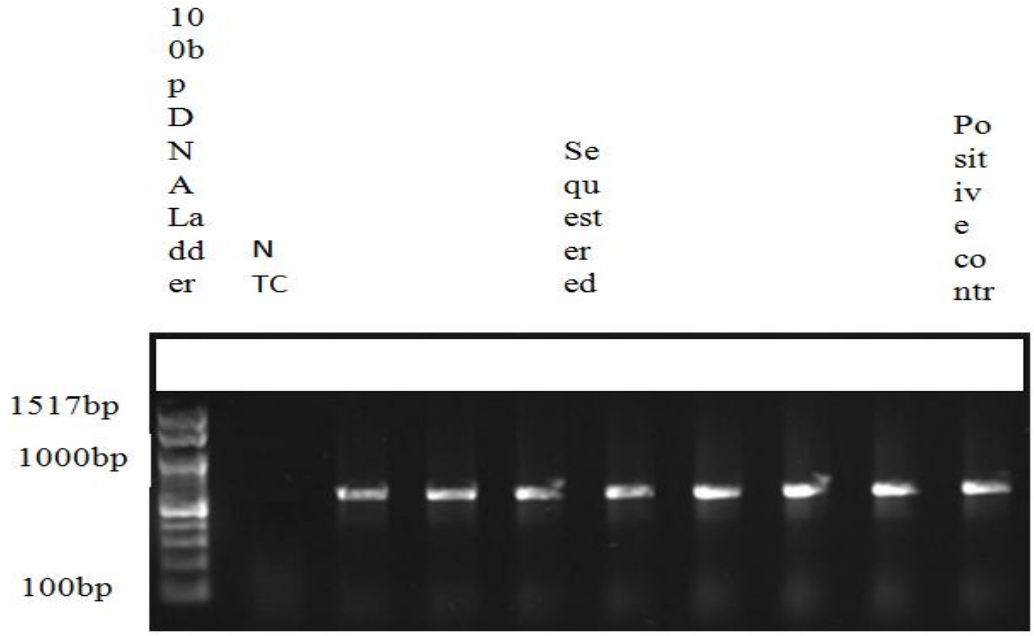
Molecular confirmation *Pseudomonas aeruginosa* by 16S rRNA

Fig.4 Isolates from Chicken showing amplification of 16S rRNA



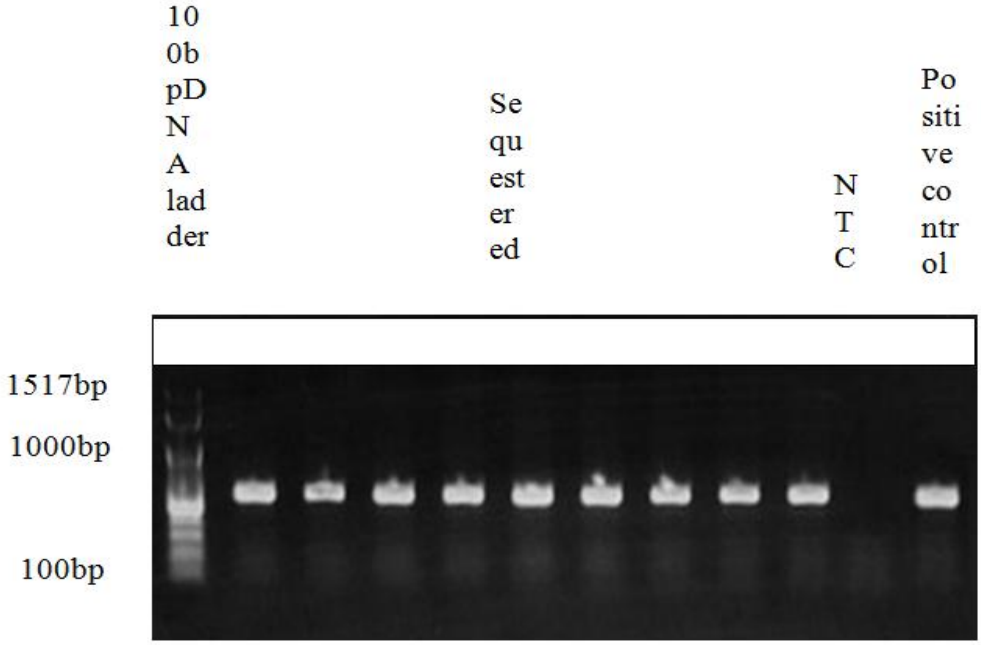
L-100bp DNA Ladder, N-Negative control, 1-CA13, 2-CA22, 3-CA28, 4- CF17, 5-CF27, 6-CF33, P-Positive control

Fig.5 Isolates from Mutton showing amplification of 16S rRNA



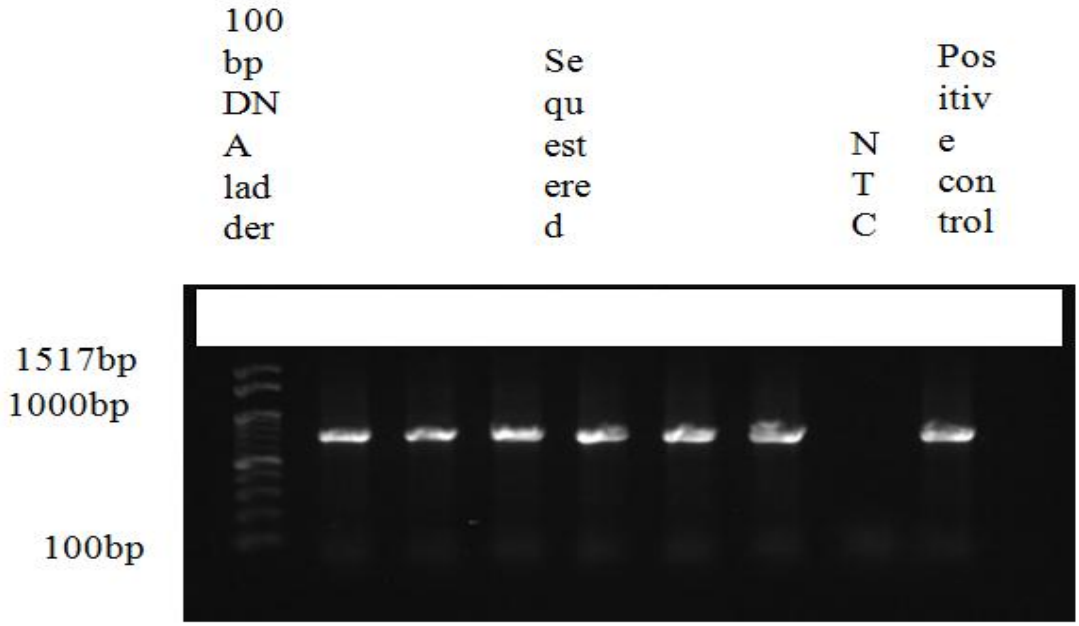
L-100bp DNA Ladder, N-Negative control,1-MA-9,2-MA19,3- MA24,4- MF15,5-MF22,6-MF28, 7-MF33,P-Positive control

Fig.6 Isolates from Pork showing amplification of 16S rRNA



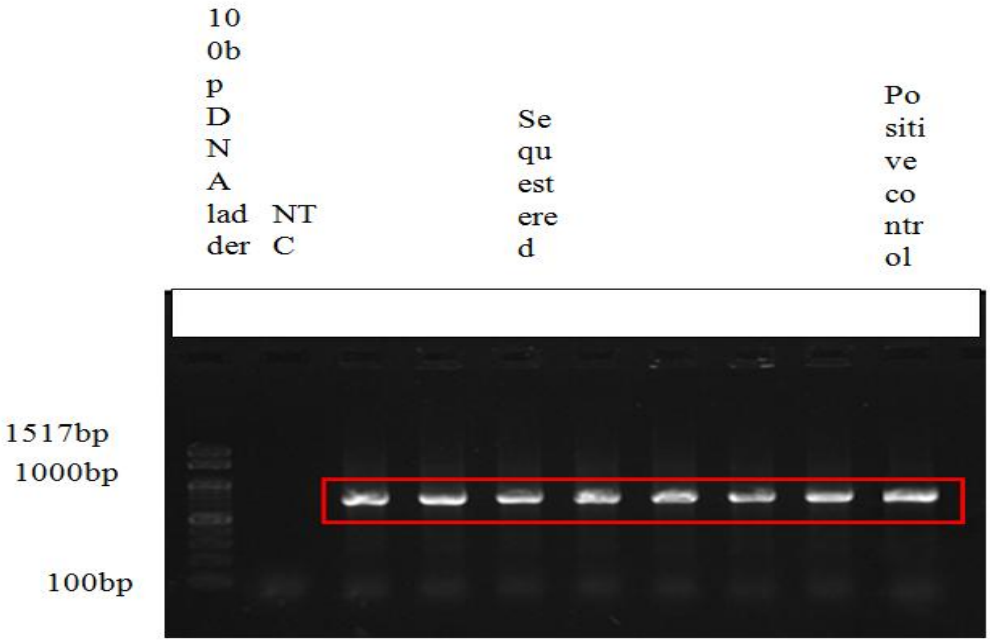
L-100bpDNA Ladder, 1-PA13, 2-PA26, 3-PA35, 4-PF1, 5-PF2, 6-PF19, 7-PF24, 8-PF29, 9-PF33,N- Negative control, P-Positive control

Fig.7



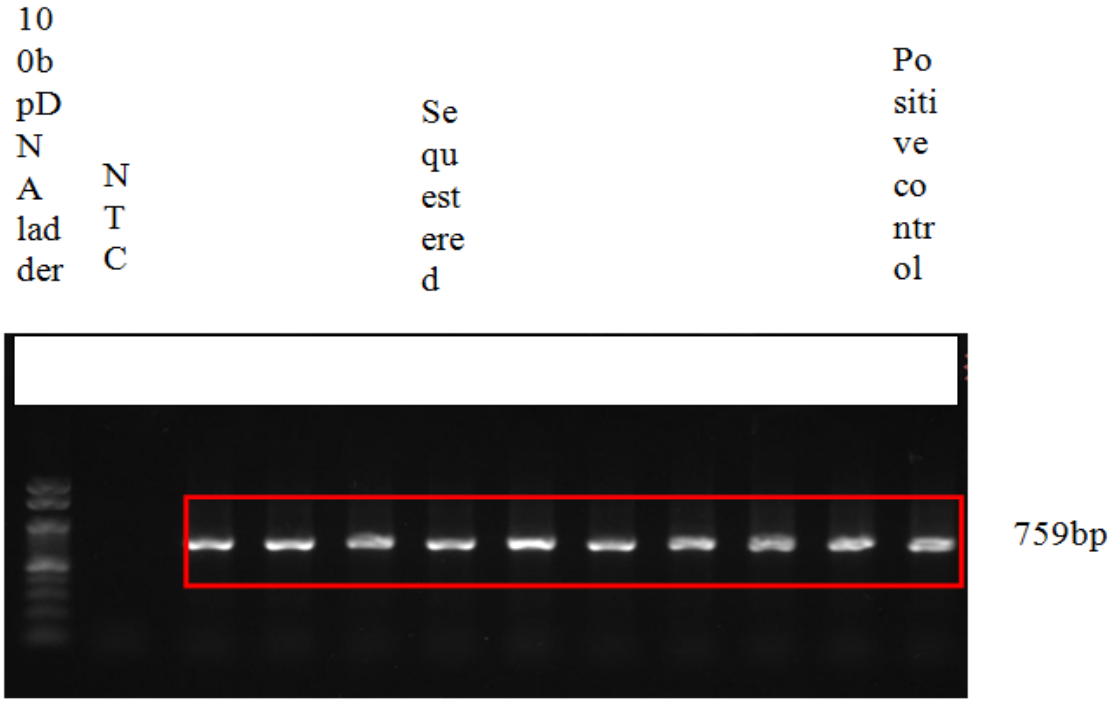
L-100bp Ladder, 1-CA13, 2-CA22, 3-CA28, 4-CF17,5-CF27, 6-CF33, N-Negative control, P-Positive control

Fig.8 Isolates from Mutton showing amplification of *rpo*B gene



L-100bp Ladder N-Negative control, 1-MA-9, 2-MA-19, 3-MA-34, 4-MF15,5-MF22,6-MF28 ,7-MF33 , P-Positive Control

Fig.9 Isolates from Pork showing amplification of *rpo*B gene



L-100bp Ladder, N-Negative control, 1-PA13,2-PA26, 3-PA35,4-PF 1, 5-PF-2 ,6-PF19 , 7-PF24,8-PF29 , 9-PF-33 ,P-Positive control

All the 22 isolates amplified specific product size *viz*. 618 bp and 759 bp respectively for 16S rRNA and *rpo*B which is specific for *P.aeruginosa*. Both the genes were detected from all the isolates.

Similarly, (Mulet *et al.,* 2009) has analyzed partial sequences of four core 'housekeeping' genes (16S rRNA, *gyr*B, *rpo*B and *rpo*D) of the type strains of 107 *Pseudomonas* species in order to obtain a comprehensive view regarding the phylogenetic relationships within the *Pseudomonas* genus.

Further the author has created a specific database, Pseudo MLSA to compile all of these gene sequences for the characterization and taxonomical identification of Pseudomonas strains.

Amoon *et al.,* (2018) employed 16S rRNA and *rpo*B gene to identify genetically atypical *P. aeruginosa* isolates from 40 isolates of different origins and opined that the sequences are high specific accurate in the PCR assay.

In our study, the culture isolation, biochemical test and molecular confirmation by PCR assay were highly co-relating. According to that the percent occurrence of *Pseudomonas aeruginosa viz*. Chicken meat, Mutton and Pork were 8.57%, 10% and 12.8% respectively. In chicken meat, equal percentage (8.57%) of occurrence was detected in both aerobically stored and under frozen storage.

Comparatively a low percentage of *Pseudomonas aeruginosa* isolates were recovered from chicken meat but in contrast, Iroha *et al*., (2011) could not isolate *P. aeruginosa* from any of 50 chicken meat samples.

It may be due to low sample number and all the samples were collected from aerobically stored condition. Slightly higher percentage of *Pseudomonas aeruginosa* occurrence was recorded by Benie *et al*., (2016) in meat samples respectively in beef, fresh fish and smoked fish as 14.9%, 12.2% and 12.2% respectively. However, the presence of other species of *Pseudomonas* were recorded high at 97.9%, 87.8% and 63.1% respectively. Among the total of 158 isolates 97.5% were confirmed as *Pseudomonas* by 16S rRNA.

In an another study conducted at Chennai, (Gayathri *et al.,* 2015) reported the percentage of predominant bacterial pathogen isolated from raw poultry meat and raw mutton meat collected from four different regions of Chennai, in which it is reported almost similar occurrence of *Pseudomonas aeruginosa* (10%) was reported.

The storage temperature significantly affected the microbial composition in pork meat. A high similarity was observed between pork meat stored at −2 ◦C and 4 ◦C, with *Pseudomonas* and *Brochothrix* being dominant features. (Zhao *et al.,* 2022).

Microbial spoilage of meat can be affected by multiple factors, including meat types, hygiene during slaughter and processing, and storage temperatures. Among these factors, the storage temperature is considered one of the most important factors affecting the growth of bacteria present in meat.

In this study, storage condition is concerned, the occurrence of *Pseudomonas aeruginosa* in Mutton under aerobically stored condition and in frozen storage were respectively 8.57% and 11.42% was detected. Whereas in pork meat 8.57% and 17.14% of occurrence was detected under aerobic and frozen storage conditions respectively.

Higher percentage of isolates were obtained from frozen storage. Laura and Mauro (2007) has reported extremely higher percentage of *Pseudomonas* spp about 90% in food spoilage at aerobic storage. *Pseudomonas* spp was the predominant psychrotrophs isolated from all carcasses refrigerated for 7 to 14 days (Yagoub, 2009). Similarly, Dilek Seker (2016) also has reported that psychrophiles such as *P. aeruginosa* and *P. fluorescens* were the usual and dominant organism on chilled meats.

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