MedCrave
Step into the World of Research
Journal of Bacteriology & Mycology: Open Access

Research Article
Open Access
CrossMark

Food-borne pathogens survival of *Yersinia enterocolitica* and molecular analysis of 16SrRNA sequencing of *Salmonella typhi* & *Salmonella enterica* from frozen buffalo meat in India

Volume 9 Issue 2 - 2021

Ujjal Sen,[1] Nidhi Vishnoi,[1] Anil Mahadeo Garode[2]

[1]Department of microbiology, Kalinga University, India
[2]Department of microbiology, Sant Gadge Baba Amravati University, India

Correspondence: Ujjal Sen, Department of microbiology, Kalinga University, Village Kotni, Near Mantralaya, Naya Raipur – 492101, Chattisgarh, India, Tel+91-9871061902, Email ujjal_sen@hotmail.com

Received: March 29, 2021 | **Published:** April 27, 2021

Abstract

French Bacteriologist Alexander Yersinia in 1894 first described *Yersinia eniterocolitica* as causative organisms for food-borne illness. *Yersinia enterocolitica* is an enteropathogenic organism and causes "yersiniosis" that denotes infections such as, gastroenteritis in infants and young children and it considered as bacillary dysentery or campylobacter - like enterocolitis with abdominal pain and diarrhea. Even it give rise to acute terminal ileitis or mesenteric lymphadenitis, cause septicemia with high fatality rate, abortion, pneumonia, ileocaecal appendix, proximal colon level, acute enterocolitis, immunological complications like erythema nodosum, polyarthritis, Reiter's syndrome, some time leading to death etc. In frozen buffalo meat at very freezing temperature the organisms common and isolated can cause spoilage. *Y. enterocolitica* is gram negative, pleomorphic, asporogenous short, capsulated, motile with peritrichous flagella microscopically and grown at 22°C. *Y. enterocolitica* is aerobe and facultative anaerobe and actively porn to zoonosis. *Salmonella species* also a risky food borne pathogen and causes typhoid and salmonellosis. In present research investigation 51 samples of frozen buffalo meat processed microbiologically whereas 39 samples are positive and 11 samples are negative for *Y. enterocolitica*. Hence, It is observed load of tolerance are in little higher in frozen meat. MacConkey agar showed as small, irregular, pinpointed, pinkish - reddish or pale pink or reject red color lactose negative colonies at 37°C and form pinpoint colonies at 22°C. Antibiotic assay test of *Y. enterocolitica* revealed that 10µg amoxyclave disc is sensitive to *Y. enterocolitica* but, 5µg methicillin disc is resistant to *Y. enterocolitica*. The positive *Salmonella species* colonies from specific media such as (e.g. XLD, BGA, BSA, HEA) isolated and subjected to molecular analysis of 16SrRNA sequencing identified as *Salmonella typhi* strain T9 16S ribosomal RNA gene, partial sequence and *Salmonella enterica* subsp. enterica serovar enteritidis strain DY60 16S ribosomal RNA gene, partial sequence, causes the spoilage in meat at low temperature and threaten public health safety. The overall result indicates poor management of hygiene at abattoir and inefficient cleaning condition can augment low temperature spoilage of meat and serious issue for the reassessment of public health importance.

Keywords: *Yersinia enterocolitica*, yersiniosis, 16SrRNA, PCR, *Salmnonella typhi, Salmonella enterica*, frozen buffalo meat, salmonellosis

Introduction

The genus *Yersinia* is named after the French bacteriologist Alexandre Yersin who, in 1894, first described the organism responsible for the bubonic plague. The importance of *Yersinia enterocolitica* as a cause of foodborne illness varies between countries. In England and Wales, laboratory reports of *Y. enterocolitica* infections, mostly in sporadic cases, increased from 45 in 1980 to 571 in 1989 during outnumbered cases of both *Staphylococcus aureus* and *Bacillus species* food poisoning. This represented a peak in reports which then declined to an average of 25 p.a. in the period 2000 to 2005. Yersiniosis is most common in the cooler climates of northern Europe. *Yersinia enterocolitica* contain zoonotic importance both in animal and human. In frozen buffalo meat during storage at -20°C and even at very low temperature *Y. enterocolitica* isolated. *Y. enterocolitica* is becoming increasingly identified as a cause of gastroenteritis in infants and young children and it considered as bacillary dysentery or campylobacter - like enterocolitis with abdominal pain and diarrhea. The incidence of yersiniosis is highest during autumn and winter whereas it gives rise to acute terminal ileitis or mesenteric lymphadenitis that affect adult of both sexes and children. *Y. enterocolitica* also cause septicemia with high fatality rate, abortion, pneumonia, ileocaecal appendix, proximal colon level, acute enterocolitis, immunological complications like erythema nodosum, polyarthritis, Reiter's syndrome, some time leading to death etc. *Y. enterocolitica* identified in beef, pork and poultry meat in France as according to.[1] A research article revealed that *Y. enterocolitica* and other species from beef isolated as positive in Aydin, Turkey.[2] A survey on *Yersinia enterocolitica* biotype 1A carried out from raw beef meat sample and observed as positive.[3] In a research paper showed that different meat (beef & chicken) sample in Tehran tested for antibiotic susceptibility of *Y. enterocolitica* and other species whereas 80% of the sample found positive.[4]

The mechanisms of pathogenicity in the enteropathogenic *Yersinia* are complex and have served as research models for understanding the infectious process in many enteropathogenic bacteria. They include a number of both chromosomally and plasmid determined factors. Chromosomal determinates include: invasion of host cells (*ail* for *Y.*

Submit Manuscript | http://medcraveonline.com
MedCrave

©2021 Sen et al. This is an open access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and build upon your work non-commercially.

enterocolitica and *inv* for *Y. pseudotuberculosis*), iron complexing and uptake proteins *(irps)*, and heat-stable enterotoxin (ystA). Factors carried on the 70 kb virulence plasmid (pYV for *Y. enterocolitica* and pIB1 for *Y. pseudotuberculosis*) include: the *Yersinia* outer proteins *(yops)*, low calcium response (lcr), *Yersinia* adherence protein *(yadA)*, and the temperature dependent transcriptional regulator of many of the other plasmid genes *(virF)*.[5] A number of virulence tests have been proposed to distinguish potentially pathogenic *Y. enterocolitica*. The heat-stable enterotoxin (ST) produced *in vitro* by some strains of *Y. enterocolitica* and related species can be detected by intragastric injection of cultural filtrates in suckling mice and is very similar to *Escherichia coli* ST. However, *Yersinia* spp. produces ST *in vitro* only at temperatures below 30°C.[6]

Salmonella injested toxins in frozen buffalo meat, commonly either toxic or infectious can cause serious outbreaks. The reported infective dose of about 15–20 cells of microorganisms are sufficient to cause pain in stomach. Mostly diarrhea, nausea, chills, fever, and headache appears after incubation period of 12 to 24 hrs.[7] The sensitivity and specificity of the polymerase chain reaction (PCR) is very high as it is able to detect even single bacterium that's because it detects a single copy of targeted sequence of DNA, amplification of the target rather than the signal makes it promising, and lowers the liability of false–positive results as well as lower time required for performing the technique.[8] Identification of Salmonella *typhi* using forward first sequencing by S5_518.ab1 general primers and reverse second sequencing by S5_800R.ab1 general primer targeting the gene T9 to encode *Salmonella typhi* strain T9 16S ribosomal RNA gene, partial sequence. On the other hand, identification of *Salmonella enterica* using forward first sequencing by S7_518.ab1 general primer and reverse second sequencing by S7_800R.ab1 general primer targeting gene DY60 to encode *Salmonella enterica* subsp. enterica serovar enteritidis strain DY60 16S ribosomal RNA gene, partial sequence. Both Salmonella typhi T9 gene and *Salmonella enterica* DY60 gene are part of chromosomal and plasmid factors. Virulence mainly relies on these chromosomal and plasmid factors. T9 gene & DY60 gene may thought to be help in invasion of *salmonellae* into cultured frozen buffalo meat epithelial cells. The T9 gene & DY60 gene is present in two strains of Salmonella, which also may enables *Salmonellae* pathogenicity while invading host epithelial cells and may cause disease Salmonellosis.

Yersinia enterocolitica morphology and cultural characteristics

Yersinia enterocolitica are gram (-) negative coccobacilli showing pleomorphism in older cultures; It is an asporogenous short (0.5–1.0mm by 1–2mm) apparent capsules are seen in vivo but not in culture. Motile means of peritrichous flagella when grown at 22°C but non- motile when grown at 37°C. They are aerobe and facultatively anaerobe. Optimum temperature 22–9°C, multiply at 4°C. On blood agar form non-haemolytic, translucent, smooth colonies 2-3mm in diameter in 48 hours. Here it isolated by growing on MacConkey agar at 37°C where *Yersinia enterocolitica* form small, irregular, pinpointed, pinkish-reddish or pale pink or reject red color lactose negative colonies and at 22°C in MacConkey agar form pinpoint colonies. *Y. enterocolitica* on Schiemann CIN agar form deep red center surrounded by clear, colorless zone about 1-2mm diameter.[9]

Materials and methods

100 gm of frozen buffalo meat sample, transport them in insulated shipping container enough gel-type to maintain refrigerant at 6°C or below. Upon receipt in the laboratory, store the samples at 4°C and analyze as soon as possible. If analysis cannot be started within 4 days after collection, freeze samples promptly and store at -20°C until examined. Thaw at room temperature and proceed with analysis as usual. Maintain frozen samples at -20°C until examined.

Yersinia enterocolitica detection method

Among the sample 25gm of frozen buffalo meat sample are weighed and add 225mL of peptone Sorbitol Bile Broth Medium (PSBB), homogenized the mixture in a blender for 30 seconds and used as pre - enrichment solution according to FDA, BAM,[10] sampling method as mentioned above. Before incubation of the pre - enrichment diluents spread 0.1mL on MacConkey Agar to observe if the more load of organisms are present in frozen buffalo meat. Before incubation again transfer 1ml homogenate to 9ml 0.5% KOH in 0.5% saline, mix for 2 - 3 seconds and spread plate 0.1mL on MacConkey Agar. Incubate the macConkey agar plates at 30°C for 1 - 2 days and keep the pre-enrichment PSBB at 10°C for 10 days in an incubator. On day 10, remove enrichment broth from incubator and mix well. Transfer one loop-full of enrichment to 0.1ml 0.5% KOH in 0.5% saline and mix for 2-3 seconds. Transfer additional 0.1ml enrichment to 1ml 0.5 % saline and mix 5 - 10 seconds before streaking. Successively, streak on a loop-full of each mixed broth from each test tube as above mentioned to MacConkey agar plates gently. Incubate agar plates at 30°C for 1-2 days. Observe the presence or absence of colonies.

16SrRNA sequencing method for *Salmonella*

The ribosome consists of two subunits, each containing RNA and Protein molecules for support of protein synthesis. The prokaryotic small subunit rRNA molecule fragmented - with a sediment coefficient of 16S; the large subunit rRNA contains 23S and 5S molecules. Among the three prokaryotic rRNA molecules, 16SrRNA or its genomic coding sequence has been more often used for bacterial characterization. 16SrRNA molecule contains alternating regions of sequence conservation and heterogeneity. The conserved regions used for PCR Amplification primers that anneal ribosomal targets from all or most species. Regions of DNA Sequence Diversity provide Sequence Polymorphisms that can be used to characterize to isolates the genus or species level, although the degree of divergence observed within the 16SrRNA molecule may not be high enough to distinguish some closely related species. According to the last manuscript published for identification of *Salmonella species*,[11] separated as pure culture and used for 16SrRNA Sequencing of PCR Amplification Analysis, followed by Sequencing in DNA-Sequencer integrated with Gas Chromatopraphy and matches with Sherlock-MIDI library software for rapid bacterial species label identification by FAME, PLFA procedure technique.

DNA extraction and polymerase chain reaction

The DNA isolates of *Salmonella* was prepared by bacterial lysis method. A loopful culture was taken in a micro-centrifuge tube in 100µl of sterilize DNAs and RNAs- free Milli-Q water. After vortex the sample was heated at 95°C for 10 min. Cell debris was removed by centrifugation and supernatant was used by DNA template in PCR reaction mixture. *Salmonella typhi* isolates were first screened for the presence or absence of virulence associated genes by using the PCR protocol separately standardize for the detection of different genes. The PCR was standardize for the detection of 7 genes viz. T9, T8, T7, T6, T5, T4, T3 as per standard methodology with suitable modifications. The standardization of PCR was done by using standard strain of *Salmonella typhi*. On the other hand, another strain *Salmonella enterica* isolate secondly screened same way for virulence associated gene detection. The PCR was standardize for the detection

Copyright: ©2021 Sen et al.

of 7 genes viz. DY60, E7, E3, E6, P125109, E5, E2 as per standard methodology with suitable modifications. The standardization of PCR was done by using standard strain of *Salmonella enterica*.

Design of a primer and sequencing

Two bacterial strains PCR Sequencing perform in PCR and DNA Sequencer:-

A. *Salmonella typhi* cell lysate (1µl) was used as a DNA template in PCR Amplification. PCR reaction mixture (20 µl) consisted of 15mol or 10pmol of each using general primer bacterio-specific primer named as S5_518F.ab1 in forward direction, the tandem sequence denotes by 5'-GGCAGGCTAG…….. AAAAAAAAAT-3' which contain 924 BP of oligoneucleotide sequences in DNA for Salmonella typhi and using universal primer named as S5_800R.ab1 in reverse direction, the tandem sequence denotes by 5'-ATTTTTTATT……..CTGACGCTCC-3' which contain 773 BP of DNA template sequence for *Salmonella typhi* and even 1.9mM MgCl2, 0.2mM each of dNTPs, 0.5 U of Taq quantified using nanodrop instrument are added. After series of thermal cycle till 95°C using PCR amplification technique form recombinant tandem repeat of oligonucleotide sequences which is long enough till 1400BP as purified PCR product or consensus repetitive base pair sequences. Hence, the above forward and reverse sequences during amplification after denaturation, annealing, extension and extraction which crossover or overlap or exchange of base pair to form consensus sequences or final PCR purified product 16SrRNA fragment tandem repeat sequences where target the gene of interest T9 16S ribosomal RNA gene, consensus tandem repeat sequence denotes by 5'-GGCAGGCTAG…….. GACGCTCCAT-3' which contain 1666 BP in RNA template sequence for *Salmonella typhi*, along with total score 1469 and maximum identity of 100% from Blast result of Sherlock MIDI bacterial identification library catalog access no. EU118116.1 with the help of Sherlock-MIDI based FAME, PLFA technique, followed by gas chromatography that detected electronic signal through FID detector and form chromatogram that accurately identified the peaks and matches with closely related species from Sherlock MIDI, USA library.

B. *Salmonella enterica* cell lysate (1µl) was used as a DNA template PCR Amplification. PCR reaction mixture (20µl) consisted of 15 pmol or 10pmol of each using general primer bacterio-specific primer named as S7_518F.ab1 in forward direction, the tandem sequence denotes by 5'- GTATTACTGG……… AAAAAAAAAT-3' which contain 979 BP oligonucleotide sequences in DNA for *Salmonella enterica* and using universal primer named as S7_800R.ab1 in reverse direction, the tandem sequence denotes by 5'- CAGAATTTAT……….. AAGACTGACG-3' which contain 776 BP of DNA template sequence for *Salmonella enterica*, and even 1.9mM $MgCl_2$, 0.2mM each of dNTPs, 0.5 U of Taq quantified using nanodrop instrument are added. After series of thermal cycle till 95°C using PCR amplification technique form recombinant tandem repeat of nucleotide sequence which is long enough till 1400 BP as purified PCR product or consensus repetitive base pair sequences. . Hence, the above forward and reverse sequences during amplification after denaturation, annealing, extension and extraction which crossover or overlap or exchange of base pair to form consensus sequences or final PCR purified product 16SrRNA fragment tandem repeat sequences where target the gene of interest DY60 16S ribosomal RNA gene, consensus tandem repeat sequence denotes by 5'- CAGAATTTAT………. AAAAAAAAAT-3' which contain 1755 BP in DNA template sequence for Salmonella enterica, along with total score 2495 and maximum identity of 95% from Blast result of Sherlock MIDI bacterial identification library catalog access no. JF951181.1 with the help of Sherlock-MIDI based FAME, PLFA technique, followed by gas chromatography that detected electronic signal through FID detector and form chromatogram that accurately identified the peaks and matches with closely related species from Sherlock MIDI, USA library.

Both strains RNA used the standard protocol for cycle sequencing in the thermal cycler with preheated lid (Lid Temperature 105°C) using DNA-Sequencer was used with sequencing primer and form cDNA library. Reaction condition employed by initial denaturation followed by final extension of the cycle in different temperature. Reaction condition was the same as earlier described except for the annealing temperature of respective primer used. On completion of the reaction automatic alignment of cDNA occur in the sequencer and form the amplified products which were analyzed on agarose gel electrophoresis through 2% agarose gel stained with 5 µg/ml of ethidium bromide with a 100 BP DNA ladder as molecular weight marker and visualized under UV light. All reference sequences were obtained from Sherlock MIDI, USA GenBank database library. Sequences were automatically aligned (multiple sequence alignment) by Sherlock-MIDI-Shimatzu, USA software automatic alignment programme followed by gas chromatography form chromatogram and bioinformatically utilize Blast results from the Sherlock MIDI, USA GenBank Library matches with the nucleotide alignment. Consequently, find out the genus, species, stains, serovar, community relationship and DNA homology.[12–15]

Microbial identification using automated fatty acid methyl ester (fame) analysis of shimadzu GC-2010/2030

Sample processing require 5 steps to prepare GC ready extracts are illustrated below by diagram-

1) Harvesting – 4mm loop to harvest 40µg of bacterial culture from the third quadrant of the quadrant streaked plate. Cells placed in clean 13×100 culture tube. 2) Saponification – 1.0ml reagent 1 is added to each tube containing cells. The tubes are securely sealed with Teflon lined caps, vortexed briefly and heated in boiling water bath for 5 minutes, vigorously vortexed for 5-10 seconds and return to water bath t complete the 30 minutes heating. 3) The cooled tubes are uncapped; 2ml of the reagent 2 is added. Tubes capped and briefly vortexed. The tubes are heated for 10 minutes at 80°C. 4) Extraction – Addition of 11.25ml of the reagent 3 to the cooled tubes followed by recapping and gentle tumbling on a clinical rotator for about 10 minutes. The tubes are uncapped and the aqueous or lower phase pipette out or discarded. 5) 3ml of reagent 4 is added to organic phase remaining in the tube; tubes are recapped and tumbled for 5 minutes. Following uncapping, about 2/3 of the organic phase is pipette into a GC vial which is capped and ready to analysis.

Sherlock CAS Software can only be used with a Shimadzu GC-2010/2030 gas Chromatograph. Sherlock systems unique configuration is designed for automated and accurate analysis of fatty acid methyl esters by Gas Chromatography. Then it pass through Ultra 2 Column and Gas Chromatograph available undergo 170 -270°C even ballistic increase in 300°C allows cleaning of the column hold of 1.5 minutes and detected by flame ionization detector with a combined flow of hydrogen and nitrogen gas. Detection done automatically. Electronic signal from the GC detector passed to the computer where

the integration of peaks is performed. Electronic signal stored in hard disk and fatty acid methyl ester composition of the sample is compared to a stored database using the Sherlock pattern recognition software. Sherlock libraries consist of 100000 analyses of strains. Sherlock library provides strains of a species or subspecies, subgroups within taxon, covariance matrix takes mole to mole relationship of the converted fatty acid, difference of biovars and subspecies etc.

Sherlock MIDI identification system software

The Sherlock MIS is recognized by the U.S. CDC for the identification of aerobic bacteria. The Sherlock MIS was created for ease of us, so no GC experience is necessary. In 2007 MIDI released Sherlock Instant FAME™ (I-FAME), a base-catalyzed GC-FAME extraction and analysis method. I-FAME is most useful for extraction and analysis of fatty acids used for Gram-Positive environmental aerobes. The turnaround time (TAT) for IFAME is less than 15 minutes. In 2011 MIDI released Sherlock Q-FAME™ (Q-FAME), an acid-catalyzed GC-FAME extraction and analysis method. Q-FAME is most useful for Aerobic bacteria. The turnaround time (TAT) for Q-FAME is less than 25 minutes. In 2016 MIDI released Sherlock E-FAME™ (E-FAME), an acid-catalyzed GC-FAME extraction and analysis method. E-FAME has been optimized for Aerobic bacteria. The turnaround time (TAT) for E-FAME is less than 20 minutes.

Key features of the sherlock mis include

1) Fatty acid analysis (FAME, PLFA) and Microbial ID, 2) Automated analysis and naming, 3) Inexpensive per sample cost, 4) Throughput over 100 samples per day, 5) No biochemical kits or cards (microbial ID), 6) Polyphasic Analysis with Sherlock DNA (microbial ID), 6) Proven reliability and warranty (Figure 1). Shimadzu Scientific Instruments (SSI) and MIDI, Inc. bring automated testing solutions will save analysis time, reduce labor costs, while providing unprecedented analytical accuracy compared to the "manual" chromatography approaches typically used in food industries. These solutions are now available for the GC-2010/2030 hardware with LabSolutions software. The Sherlock CAS package provides the tools needed to automate analysis of complex chromatographic data for a variety of industries, from microbial identification of pathogenic bacteria. A complete system includes the GC-2010/2030 with FID detector (configured to MIDI's specifications), Computer with LabSolutions and Windows software, J&W Ultra 2 column, MIDI Sherlock Methods, MIDI Calibration Standards and Operating Manuals. Sherlock Chromatographic Analysis System (CAS), along with the Shimadzu Gas Chromatograph (GC) and LabSolutions software can be completed successfully, Syringe – 10µL syringe, fixed needle (Shimadzu p/n), Supelco Thermogreen LB-2 Septa for Shimadzu GCs–50/pk (Supelco p/n), Hydrogen gas tank (<1ppm THC), GC Column – MIDI Sherlock Methods – Ultra 2 Capillary Column, Reagents – PLFA analysis by MIDI PLFA Calibration standard, MIDI Calibration Standard Microbial Identification, HPLC grade Hexane – Wash solution 0r reagent Blank during installation, Pasteur pipette (5in., disposable) for reconstituting, 2ml clear GC auto sampler vials, screw cap, Screw caps, PTFE/Silicone/PTFE septa (Fisher Scientific etc utilized. Other consumable, reagents & lab equipments may required during PLFA analysis.[15–17]

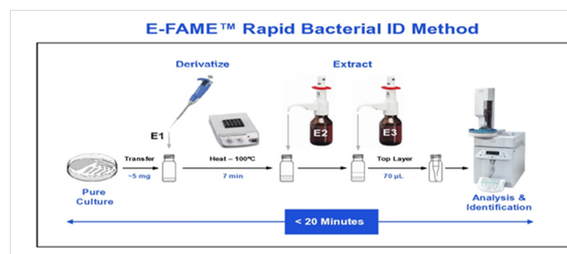

Figure 1 Rapid bacterial ID method.

Biochemical characters

Yersinia enterocolitica are gram negative coccobacilli with pleomorphic form, catalase positive, oxidase negative, urease positive, on bile esculine agar negative, in LAIA slant giving alkaline slant (Violet) and acid butt (Yellow), no gas and no H2S, motile at 25°C and non-motile at 35°C, In a autoagglutination test on MR - VP broth at 25°C shows diffuse growth but at 35°C agglutinates and settles to the bottom, Lysine, arginine negative but, positive, simmons citrate 37°C or 25°C negative, VP at 37°C negative but, at 25°C positive or negative, indole, salicin are positive or negative, ONPG positibe or negative, TSI negative, MR at 37°C or 25°C positive, KCN negative, Gelatin at 22°C negative, dulcitol negative, nitrate to nitrite positive, citrimide negative etc.[18]

Antibitic tests on *Yersinia. enterocolitica*

Few samples of the positive colonies *of Y. enterocolitica* colonies collected and 24 hours old culture plated on nutrient agar to maintain pure culture. Later on inoculate in nutrient broth where the cells density 10^9 cells/ml in concentration. Place 1mL of the broth to nutrient agar and MacConkey Agar. Allow for solidification. After solidification placed 5µg methicillin disc and 10µg amoxyclave disc to the agar to find our sensitive and resistant factor of drug against *Y. enterocolitica*. Incubate at 30°C for 1-2 days. After incubation 1.5cm Zone of inhibition observed around amoxyclave disc. Hence, amoxyclave sensitive against *Y. enterocolitica*. No zone of inhibition observed around the methicillin disc. Hence, *Y. enterocolotica* resistant against methicillin. Consequently, it's found methicillin resistant *Yersinia enterocolitica* present. During consumption of buffalo meat if Yersiniosis disease occur the traces of drug methicillin will not work out and its genetic structure provide resistance against 5µg methicillin, but 10µg amoxyclave drug is useful for treatment.

Observation

Yersinia enterocolitica observed on MacConkey agar as small, irregular, pinpointed, pinkish-reddish or pale pink or reject red color lactose negative colonies at 37°C and form pinpoint colonies at 22°C.

Salmonella typhi Diagram of 16SrRNA Sequencing

Salmonella typhi (Sample 5 or S5):- The whole sequencing is carried out through PCR 16SrRNA Amplification technique.

>Forword sequences_

S5GGCAGGCTAGAGTCTTGTAGAGGGGGGAGAATTCCAGGTGTAGCGGTGAAATG
CGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTG
ACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCC
ACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAG
CTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAA
TGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAA

Citation: Sen U, Vishnoi N, Garode AM. Food-borne pathogens survival of *Yersinia enterocolitica* and molecular analysis of 16SrRNA sequencing of *Salmonella typhi* & *Salmonella enterica* from frozen buffalo meat in India. *J Bacteriol Mycol Open Access.* 2021;9(2):39‒52. DOI: 10.15406/jbmoa.2021.09.00294

CGCGAAGAACCTTACCTACTCTTGACATCCAGAGAACTTATCAGAGATGCATTGG
TGCCTTCGGGAACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTG
AAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGG
TTCGGGCGGGAACTCAAAGGAAACTGCCAGTGATAAACTGGAAGAAGGTGGGG
ATGACGTCAAGTCATCATGGCCCTTACGAATAAGGCTACACACGTGCTACAATGG
CATATACCAAGAGAAGCGACCTCGCGAGAACAAGCGGACCTCATAAAGTACGTC
GTAATCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCCGAATCGCTAGTAAT
CGTAGATCAGAATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGGCCGT
CCACCATGGGGAGTGGGTTGCAAAAGAAGTAAGTAGCTTAACCTTCGGGAAGGC
GCTTACCACTTTGTGATTCATGACTGGGGTGATTAAAAAAAAAAAACACCCAAAA CAAAAAAGAAAAAAAAAAAAAT. – 924 Base Pair Tandem Repeated Sequences.

>Reverse

sequences_S5ATTTTTTATTGAAATTTTTTTTTTTTTTATTTTTCCCCGCTGATTTGCGCTGGCGGC
AGACGTATCACACGCACGTGGAGCGCTATCACACAGAAACTTGGTTCTTGGCTGA
CGAGCGGCGGACGGGTGAGTAATGTCTGGGAATCTGCCTGATGGAGGGGGATAA
CTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGCGGGGGAC
CTTCGGGCCTCATGCCATCGGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGT
AATGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACA
CTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTG
CACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCG
GGTTGTAAAGTACTTTCAGCGGGGAGGAAGGCGTTAAGGTTAATACCCTTGGCA
ATTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGT
AATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGG
CGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCTGA
AACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGA
AATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAG ACTGACGCTCC.- 773 Base Pair Tandem Repeated Sequences.

>Consensus sequences_S5 Tandem repeat forward and reverse sequences automatically aligned in PCR and sequenced through sequencer and crossover to form long chain 1666 base pair tandem repeated.

recombinant Sequences GGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAAATTTTTTATTGAAATTTTTTTTTT
TTTTATTTTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTG
GTTTCCCCGCTGATTTGCGCTCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCA
GGTGCGAAAGCGTGGCGGCAGACGTATCACACGCACGTGGGGAGCAAACAGGA
TTAGATACCCTGGTAGTCCACGCCGTAAGGAGCGCTATCACACAGAAACTTGGTT
CTTGGCTGACGAGCGGCGGACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTG
GACGGGTGAGTAATGTCTGGGAATCTGCCTGATGGAGGGGGATACTTCCGGAGC
TAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGACTACTGGAAACGGTAGC
TAATACCGCATAACGTCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGC
ACAAGCCAAGACCAAAGCGGGGGACCTTCGGGCCTCATGCCAGGTGGAGCATGT
GGTTTAATTCGATGTCGGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAAT
GGCTCCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAACCTAGGCGACGAT
CCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGAACTTATCAGAGATGCATTG
GTGCCTTCGGGAACTCTGAGACGGAACTGAGACACGGTCCAGACTCCTACGGGA
GGCAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTAG
CAGTGGGGAATATTGCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGCA
CAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGTTCGGGCGGGAA
CTCAAAGGAAACTGCCAGTGATAAACTGGAAGAAGGCCTTCGGGTTGTAAAGTA
CTTTCAGCGGGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAA
GGCGTTAAGGTTAATACCCTTGGCAATTGACGTTACTCGAATAAGGCTACACACG
TGCTACAATGGCATATGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGC
GGTAATACCAAGAGAAGCGACCTCGCGAGAACAAACGGAGGGTGCAAGCGTTA
ATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGACCTCATAAAGTACGTCGT
AATCCGGATTGGAGTCTGCAGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCT
CAACTCGACTCCATGAAGTCCGAATCGCTAGTAATCGTAGAACCTGGGAACTGC
ATCTGAAACTGGCAGGCTAGAGTCTTGTAGATCAGAATGCCACGGTGAATACGTT
CCCGGGCCTTGTAGGGGGGTAGAATTCCAGGTGTAGCGGTGAACACACCGGCCG
TCCACCATGGGGAGTGGGTTGCAAAAGAAGTAAGATGCGTAGAGATCTGGAGGA
ATAGCTTAACCTTCGGGAAGGCGCTTACCACTTTGTGATTCATGACTGGGGTACC
GGTGGCGAAGGCGGCCCCCTGGTGATTAAAAAAAAAAAACACCCAAAACAAAAA
AGAAAAAAAAAAAAAACAAAGACTGACGCTCCAT.

Gas Chromatography through FID detector catches the electronic signal and form chromatogram report for *Salmonella typhi* strain analysis given below

Forward first sequencing, **S5_518F.ab1** used as general primer here for *Salmonella typhi* strain 16SrRNA sequencing detection and the chromatogram report as follows below (Figure 2). Reverse second sequencing, S5_800R.ab1 used as general primer here for *Salmonella typhi* strain 16SrRNA sequencing detection and the chromatogram report as follows below (Figure 3).

Chromatogram analysis

Sherlock CAS uses external calibration standard developed by MIDI.The calibration standard mixture is straight chained fatty acids from 9 to 20 carbons in length (9:9 to 20:0) and five hydroxyl acids. Gas chromatography performance evaluated by software each time the calibration mixture analyzed. The hydroxyl compounds are sensitive any changes in pressure and temperature relationship. As a result the calibration standard function as a real time system suitability check. Retention time data obtained from injecting the calibration mixture is converted to Equivalent Chromatographic Locale (ECL) values for bacterial fatty acid naming. The ECL value of fatty acid function its elution time in relation to elution time of a known series of straight chain fatty acid.

$$ECL_x = \frac{R_{tx} - R_{tn}}{R_{t(n+1)} - R_{tn}} + n$$

R_{tx} = Retention Time of X, R_{tn} = Retention time saturated fatty acid methyl ester proceeding X, $R_{t(n+1)}$ = Retention time of saturated fatty acid methyl ester eluting after x. The Sherlock Chromatographic Analysis System is an accurate and automated gas chromatographic system, which identifies over 1500 bacterial species based on their unique fatty acid profile. The system uses standardized sample preparation, worked with Shimadzu Corporation's GC-2010/2030.

Gas Chromatography through FID detector catches the electronic signal and form chromatogram report for Salmonella typhi strain analysis given below:

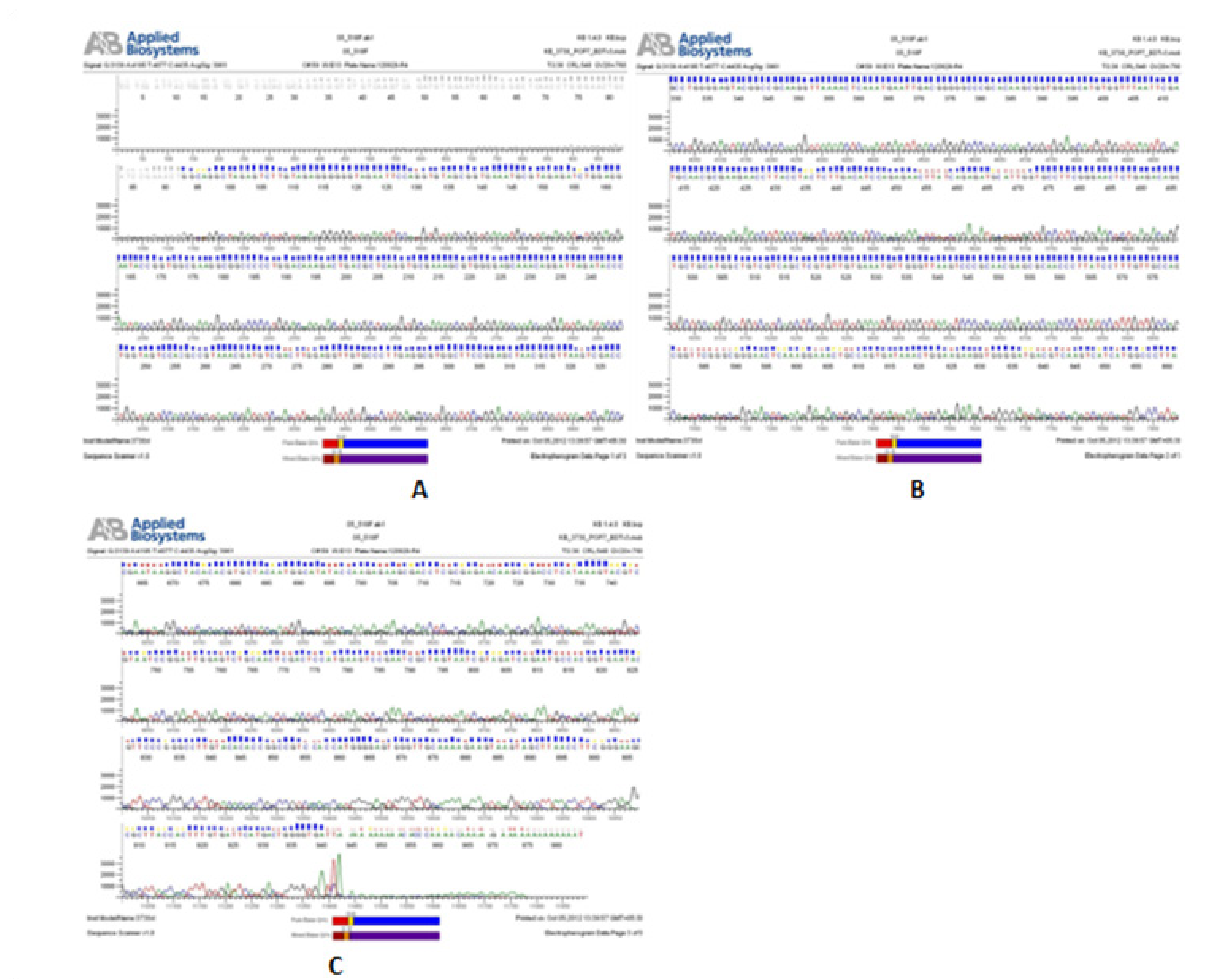

Figure 2 Chromatogram report of *Salmonella typhi chromatogram* with S5_518F.ab1 as general primer.

Citation: Sen U, Vishnoi N, Garode AM. Food-borne pathogens survival of *Yersinia enterocolitica* and molecular analysis of 16SrRNA sequencing of *Salmonella typhi* & *Salmonella enterica* from frozen buffalo meat in India. *J Bacteriol Mycol Open Access.* 2021;9(2):39‒52. DOI: 10.15406/jbmoa.2021.09.00294

Copyright: ©2021 Sen et al.

Figure 3 Chromatogram report of *Salmonella enterica* with S5_800R.ab1 as general primer.

Libraries

The Sherlock Libraries consists of more than 100,00 analysis of strains obtained from experts and culture collection. More strains of a species and subspecies were analyzed when chromatographic subgroups found on same taxon, more strains obtained in each group. The library corresponds to automatic comparison of the composition of unknown strain to stored database using a covariance matrix, principal component analysis and pattern recognition software which connect covariance matrix into mole-for-mole relationship of converted fatty acid. Pattern recognition software recognize difference between biospecies and biovars. In Sherlock MIDI library well characterized strains are available and species can be identify on the basis of DNA retardness,[15] Bioinformatically the aligned sequences after matching closely related strains and species from library form below Blast (Table 1). Total Chromatogram Peak Response Score: 1469, Matching Response or Peak Ouery Coverage comparison with library: 47%, Maximum Score of Peak Named: 100, E. value or ECL Value: 1e-20 (Table 2).

Salmonella enterica diagram of 16SrRNA sequencing

Salmonella enterica (Sample 7 or S7):- The whole sequencing is carried out through PCR 16SrRNA Amplification technique.

Table 1 BLAST Result of *Salmonella typhi*

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EU118116.1	*Salmonella typhi* strain T9 16S ribosomal RNA gene, partial sequence	100	1469	47%	1e-20	100%
EU118115.1	*Salmonella typhi* strain T8 16S ribosomal RNA gene, partial sequence	100	1475	47%	1e-20	100%
EU118114.1	*Salmonella typhi* strain T7 16S ribosomal RNA gene, partial sequence	100	1469	47%	1e-20	100%

Table continued...

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EU118113.1	*Salmonella typhi* strain T6 16S ribosomal RNA gene, partial sequence	100	1469	47%	1e-20	100%
EU118112.1	*Salmonella typhi* strain T5 16S ribosomal RNA gene, partial sequence	100	1405	45%	1e-20	100%
EU118111.1	*Salmonella typhi* strain T4 16S ribosomal RNA gene, partial sequence	100	1469	47%	1e-20	100%
EU118110.1	*Salmonella typhi* strain T3 16S ribosomal RNA gene, partial sequence	100	1469	47%	1e-20	100%

Table 2 Total Chromatogram Peak Response Score: 1469, Matching Response or Peak Ouery Coverage comparison with library: 47%, Maximum Score of Peak Named: 100, E. value or ECL Value: 1e-20, Maximum chromatogram Response of Peak Identity on Electrophoregram Paper: 100%

Matches	
Library	**Entry name**
EU118116.1	*Salmonella typhi* strain T9 16S ribosomal RNA gene, partial sequence.

>Forward sequence_S7

GTATTACTGGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAAT
CCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGA
GGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATAC
CGGTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTG
GGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACT
TGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGC
CTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCAC
AAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCT
TGACATCCACAGAACTTTCCAGAGATGAATTGGTGCCTTCGGGAACTGTGAGACA
GGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCA
ACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTCCGGCCGGGAACTCAAAGGA
GACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCC
CTTACGACCAGGGCTACACACGTGCTACAATGGCGCATACAAAGAGAAGCGACC
TCGCGAGAGCAAGCGGACCTCATAAAGTGCGTCGTAGTCCGGATTGGAGTCTGC
AACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTGGATCAGAATGCCACGG
TGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTT
GCAAAAAGAAGTAGGTAGCTTAACCTTCCGGGAGGGCGCTTACCACTTTGTGATC
ATGATGGGGGATAAAAAAAAACGCGAAAAAAAAAAAAAAAAAAAAAAAAAAAT-

979 Base Pair Tandem Repeated Sequences.

>Reverse sequences_S7

CAGAATTTATTTTTTTTGTCGTTTTTTTTTTTTTTTTTTTTTCGTAGATTGAACGCT
GGCGGCAGGCCTAACACATGCAAGTCGAACGGTAACAGAAATCAGCTTGCTGAT
TTGCTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGG
GGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGA
GGGGGACCTTCGGGCCTCTTGCCATCGGATGTGCCCAGATGGGATTAGCTAGTAG
GTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACC
AGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGG
AATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAG
GCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGGAGTAAAGTTAATACC
TTTGCTCATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGC
CGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCA
CGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGC
ATCTGATACTGGCAAGCTTGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAG
CGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTG
GACGAAGACTGACG-

776 Base Pair Tandem Repeated Sequences.

>Consensus sequences_S7 Tandem Repeat Forward and Reverse Sequences Automatically Aligned in PCR and sequenced through Sequencer and crossover to form long chain 1755 Base Pair Tandem Repeated Recombinant Sequences.

CAGAATTTATTTTTTTTGTCGTTTTTTTTTTTTTTTTTTTTTTTCGTAGATTGAACGCT
GGCGGCAGGCCTAACACATGCAAGTCGAACGGTAACAGAAATCAGCTTGCTGAT
TTGCTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGG
GGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGA
GGGGGACCTTCGGGCCTCTTGCCATCGGATGTGCCCAGATGGGATTAGCTAGTAG
GTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACC
AGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGG
AATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGA
AGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGGAGTAAAGTTAA
TACCTTTGCTCATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGC
AGC CGCGGTATTACTGGGGCGTAAAGCGCGTAATACGGAGGGTGCAAGCGTTAA
TCG GAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAA
TCCCCGGGCTCAACCTGGGACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCG
GGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGGGG
GGTAGAATTAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGGGGGGTA
GAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCG
ACCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGG
CGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGAGGCGGC
CCCCTGGACGAAGACTGACGCAAACAGGATTAGATACCCTGGTAGTCCACGCCG
TAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACG
CGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATT
GACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAA
GAACCTTACCTGGTCTTGACATCCACAGAACTTTCCAGAGATGAATTGGTGCCTT
CGGGAACTGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGT
TGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTCCGGC
CGGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACG
TCAAGTCATCATGGCCCTTACGACCAGGGCTACACACGTGCTACAATGGCGCATA
CAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTGCGTCGTAGTC
CGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTGGA
TCAGAATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACC
ATGGGGAGTGGGTTGCAAAAAGAAGTAGGTAGCTTAACCTTCCGGGAGGGCGCT
TACCACTTTGTGATCATGATGGGGGATAAAAAAAAACGCGAAAAAAAAAAAAAAAAAAAAAAAAAAT

Gas chromatography through FID detector catches the electronic signal and form chromatogram report for *Salmonella enterica* strain analysis given below

Forward first sequencing, S7_518F.ab1 used as general primer here for *Salmonella enterica* strain 16SrRNA sequencing detection and the chromatogram report as follows below (Figure 4). Reverse second sequencing, S7_800R.ab1 used as general primer here for *Salmonella enterica* strain 16SrRNA sequencing detection and the chromatogram report as follows below (Figure 5). Chromatographic Calculation for *S. enterica* as per above mention calculation showed in *Salmonella typhi* chromatogram.

Bioinformatically the aligned sequences after matching closely related strains and species from library form below

Blast Table 3 & Table 4.

Table 3 BLAST Result of *Salmonella enterica*

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
JF951181.1	*Salmonella enterica* subsp. enterica serovar Enteritidis strain DY60 16S ribosomal RNA gene, partial sequence	1314	2495	94%	0.0	95%
EU118107.1	*Salmonella enteritidis* strain E7 16S ribosomal RNA gene, partial sequence	1314	2495	94%	0.0	95%

Table continued...

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EU118102.1	*Salmonella enteritidis* strain E3 16S ribosomal RNA gene, partial sequence	1297	2473	93%	0.0	95%
EU118106.1	*Salmonella enteritidis* strain E6 16S ribosomal RNA gene, partial sequence	1286	2462	94%	0.0	95%
AM933172.1	*Salmonella enterica* subsp. enterica serovar Enteritidis str. P125109 complete genome	1280	17203	94%	0.0	95%
EU118105.1	*Salmonella enteritidis* strain E5 16S ribosomal RNA gene, partial sequence	1280	2456	94%	0.0	95%
EU118101.1	*Salmonella enteritidis* strain E2 16S ribosomal RNA gene, partial sequence	1280	2462	94%	0.0	95%

Table 4 Total Chromatogram Peak Response Score: 2495, Matching Response or Peak Ouery Coverage comparison with library: 94%, Maximum Score of Peak Named: 1314, E. value or ECL Value: 0.0, Maximum chromatogram Response of Peak Identity on electrophoregram Paper: 95%

Matches	
Library	**Entry name**
JF951181.1	*Salmonella enterica* subsp. enterica, serovar enteritidis, strain DY60, 16S ribosomal RNA gene, partial sequence.

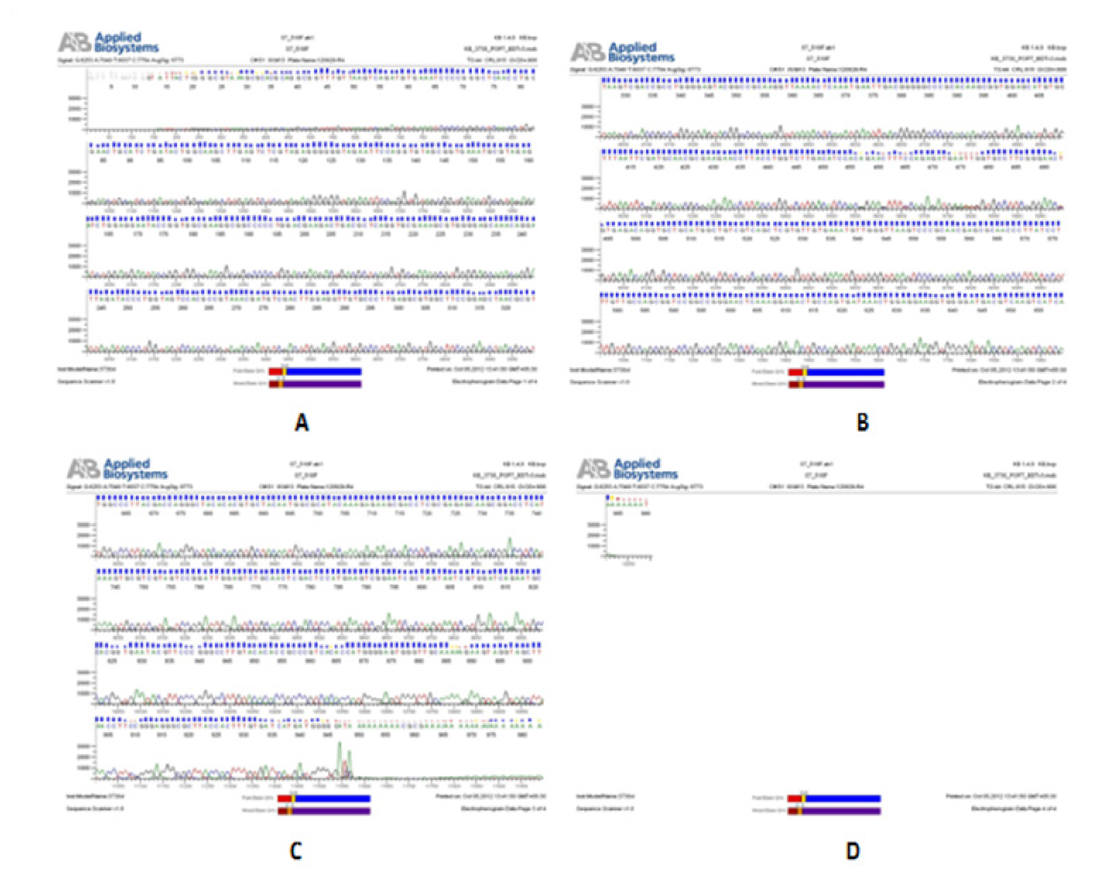

Figure 4 Chromatogram report of *Salmonella enterica* with S7_518F.ab1 as general primer.

Figure 5 Chromatogram report of *Salmonella enterica* with S7_800R.ab1 as general primer.

Results

There are approximately 51 samples of frozen buffalo meat analyzed, out of 51 samples. 39 samples are observed as positive or presence of *Yersinia enterocolitica*, but, 11 samples found as negative or absence of *Y. enterocolitica* within total samples. *Yersinia enterocolitica* observed on MacConkey agar as small, irregular, pinpointed, pinkish-reddish or pale pink or reject red color lactose negative colonies at 37°C and form pinpoint colonies at 22°C. From the above experiments it's proved that *Y. enterocolitica* are very common in meat and observed in much more quantities. The organisms can survive very low temperature and even they found in cold store at -20°C where there is more chances to contaminate frozen meat. Hence, they called as psychrophilic organisms. They cause huge risk of fatal diseases in human as a food-born zoonotic illness. During Antibiotic sensitivity 5µg-methicillin-resistant-*Yersinia enterocolitica* are observed in frozen buffalo meat and 10µg-amoxyclave-sensitive-*Yersinia enterocolotica* are found while analysis. The quality of the sequencing results observed through sequencing results in forward & reverse direction sequences and crossover to form consensus sequence of tandem repetitive of recombinant RNA fragment and chromatogram report designed with two different general primers named as S5_518F.ab1 and S5_800R.ab1 which shows picks with four different colors position on accurate base pairs on chromatogram. The sample S5 is identified to be *Salmonella typhi* strain T9 16S ribosomal RNA gene, partial sequence.

The quality of the sequencing results observed through sequencing results in forward & reverse direction sequences and crossover to form consensus sequence of tandem repetitive of recombinant RNA fragment and chromatogram designed with two different general primers named as S7_518F.ab1 and S7_800R.ab1 which shows picks with four different colors position on accurate base pairs on chromatogram. The sample S7 is identified to be *Salmonella enterica* subsp. enterica serovar enteritidis strain DY60 16S ribosomal RNA gene, partial sequence.

Discussion

Epidemiologically data implied that exposure of animal origin is a major risk factor for *Yersiniosis* and *Salmonellosis*. The majority of experimental investigation indicates an exponential like number of live cells in pathogenic microorganisms in consumes food stuff and Risk of occurrences of intestinal infectious diseases. *Yersinina enterocolitica* is ubiquitous microorganism. Frozen buffalo meat and its different cuts are reservoir for *Yersinia enterocolitica. Y. enterocolitica* is a psychotropic microorganism, grow better in lower environment, also some paper revealed detected in summer and autumn period.[19,20] Data obtained from official report of research analysis that food is an important route of transmission of *Yersinia enterocolitica*, *Salmonella typhi* and *Salmonella enterica*. The systemic investigation support exposure assessment of the population with examined pathogens through food. The analysis consists of two ways of enrichment and

incubate at 30°C for growth around 1-2 days on MacConkey agar. *Yersinia enterocolitica* grows in higher number on the surfaces of frozen buffalo meat. Investigation assure and reported among 51 sample of frozen buffalo meat 39 samples observed as positive and 11 sample are negative for *Yersinia enterocolitica*. Occurrences of *Y. enterocolitica* reported in fresh poultry meat and other ready to eat products, the pathogenicity of *Yersinia enterocolitica* associated with virulence gene in chromosome and plasmid of the strain biotype 1 A reported in raw meat are published in a paper by Lagana et al.[21] in Italy. Frozen buffalo meat cuts are packaged separately in blast and plate freezing process and dispatch worldwide, preferably anaerobic spoilage are more common. Hence, The meat packaged by the process called vacuum packaging where as combination of gases utilized like N_2, CO_2, arrest the metabolism of aerobic organism in lower environment, psychotropic count will remain lower, packaged in LDPE ploy plastics increases the shelf-life of vacuum packed meat and *Yersinia enterocolitica* and *Salmonella tiphi* and *Salmonella enterica* remain in arrested metabolism cannot multiply and cannot take part in food spoilage. But, cannot totally killed surfaces all organisms of *Yersinia enteroclitica* and *Salmonella species*. During poultry vacuum packaging air is completely removed from the plastic and establishes the meat safety.[22] A public report revealed in a paper *Yersinia enterocolitica*, *Listeria monocytogenes, Campylobacter* and *Salmonella* causes food-born illness through food contamination.[23] *Yersinia enteroclitica* reported in France retain food market in poultry, pork and beef meat.[1] A food hygiene investigative report revealed *Yersinia species* isolation in Turkey on Ground beef examined between 2000 and 2001.[24] The major group *Yersinia enterocolitica* biotype 1 causes serious yersiniosis also published in an article from raw meat products.[3]

Antibiotic sensitivity test explore the pattern of prevalence and determine the antimicrobial susceptibility organism of *Yersinia enterocolitica* in frozen buffalo meat. Investigation of susceptibility pattern and antibiotic resistance can be necessary for treatment of strains clinically during food-born outbreak. Antimicrobial resistance in food-borne pathogens and therapeutic intervention has always been an important issue in public health.[24] Using of antimicrobial agent in veterinary as a growth promotion, treatment or prophylactic can develop the antibiotic resistance in food animals. It can be a reason for antibiotic resistance transfer to humans via the food chain.[25,26] According to the antibiotic susceptibility result, the *Yesinia enterocolitica* isolates are susceptible to 10µg of amoxyclave but, resistant to 5µg of methicillin. It is suggested from results that the presence of *Yersinia enterocolitica* in frozen buffalo meat represent a health risk for consumers. The education of people who involve in production, processing and final preparation of animal products is required to avoid cross- contamination. Antibiotic susceptibilities of *Yersinia enterocolitica* in meat and chicken reported in a paper in Tehran, Iran.[4] The primers designed for *S. typhi* with forward sequence primer S5_518F.ab1 (924 base pair) long and reverse sequence primer S5_800R.ab1 (773 Base Pair) long are showed coincidence with *S. typhi*. Both the primers reacted with annealing temperature of *S. typhi* and reactivity of primer reduced with increasing annealing temperature where legated on T9 gene specific site and form recombinant 1666 Base Pair of 16S rRNA template sequence for *Salmonella typhi*, but, other six gene specific primers set (T8, T7, T6, T5, T4, T3) interspecies differentiations with these primers were incomplete, even they don't show any reaction with *S. typhi* even at annealing temperature. All 6 site specific gene primers showed mismatch, matches along with one gene specific *Salmonella* sequences with Sherlock MIDI Library GenBank databases. Universal PCR primers are reported for detection of *Salmonella* strains based on 16s rRNA sequential analysis. But they were not specific for *S. typhi*. Sequence analysis also showed that modification of forward primer, specificity can be increased, so that sequence will be identical to all known *Salmonella* sequence. *Salmonella species* like *S. typhi* and other species isolated and identified by next generation molecular analysis of 16SrRNA sequencing analysis from frozen beef and buffalo meat in Egypt.[27]

The primers designed *for S. enterica* with forward sequence primer S7_518F.ab1 (979 Base Pair) long and reverse sequence primer S7_800R.ab1 (776 BP) long are showed coincidence with *S. enterica*. Both the primers reacted with annealing temperature of *S. enterica* and reactivity of primer reduced with increasing annealing temperature where legated on DY60 gene specific site and form recombinant 1755 Base Pair of 16S rRNA template sequence for *Salmonella typhi*, but, other six gene specific primers set (E7, E3, E6, P125109, E5, E2) interspecies differentiations with these primers were incomplete, even they don't show any reaction with *S. enterica* even at annealing temperature. All 6 site specific gene primers showed mismatch, matches along with one gene specific *Salmonella* sequences with Sherlock MIDI Library GenBank databases. Universal PCR primers are reported for detection of *Salmonella* strains based on 16s rRNA sequential analysis. But, they were not specific for *S. enterica*. Sequence analysis also showed that modification of forward primer, specificity can be increased, so that sequence will be identical to all known *Salmonella* sequence. A paper published on molecular isolation and identification of virulence associated enterotoxicity by *Salmonella enterica* serovar *typhimurium* from buffalo meat in western region of India.[13] The study exclusively enlightens for the detection of *Salmonella enterica typhi* from the gut of infected silkwork *Bombyx mori* (Linn) by the technique 16SrRNA.[12] published in a journal. Advantages of 16SrRNA sequencing consists of respectively, explore the homology of bacterial genome, phylogenetic identification, genetic evaluation, sequence divergence & phylogenetic relationships, identify genus, species or subspecies, subgroups within taxon, serotypes and serovars, strains in each group, automatic comparison of unknown strains, mole to mole relationship of conversion of fatty acid etc.

Conclusion

Yersinia enterocolitica tolerate frozen environment and can present in buffalo meat surfaces in lower environment, received positive results on culture media like MacConkey agar during analysis. Antibiotic sensitivity results produce clear picture of sensitive amoxyclave and resistant methicillin population of *Yersinia enterocolitica* cells presence in frozen meat. By modifying PCR primers set for two different species of *Salmonella* named as *Salmonella typhi* and *Salmonella enterica*, and developed by Royal Life Sciences, India, Sherlock-MIDI Inc, USA., a specific PCR detection system involved based on each strain for 16S ribosomal RNA sequences was successfully developed. Ribosomal genes permits further development of detection system by modifying PCR primers into oligonucleotide probes. Latter could then be labeled and used for *in situ* hybridization techniques. Overall, the whole technique a integrated system of Shelock-MIDI-Shimadzu, USA configure and assemble PCR sequencing system, DNA-Sequencer, Gas Chromatography (GC-2010/2030 model) with FID detector catches electronic signal and form chromatogram where response revealed in peaks, matches the obtained recombinant sequencing with Sherlock-MIDI Inc, USA. Library with the help of bacterial identification system through FAME, PLFA procedures, to allow further physiological, ecological, phylogenetic, genetic evaluation,

genetic divergence, genus, species or subspecies, subgroups within taxon, serotypes and serovars, strains in each group, sub-typing, strains identification, relationship of conversion of fatty acid etc. by pattern recognition software. The results allow to identify *Salmonella typhi*, strain T9, 16S ribosomal RNA gene, partial sequence and *Salmonella enterica*, subsp. enterica, serovar enteritidis, strain DY60, 16S ribosomal RNA gene, partial sequence**.** The method described in this study may be used for detection of *Salmonella* in food samples and in any other eukaryotes (Figure 6).

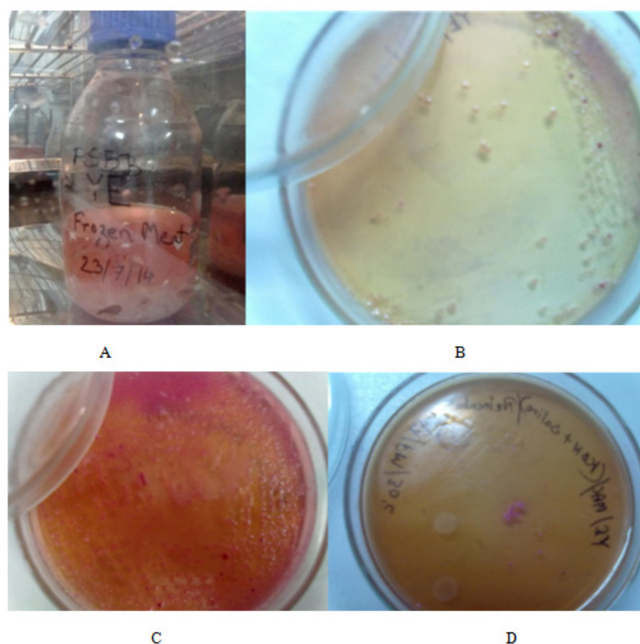

Figure 6 A denotes frozen buffalo meat in peptone Sorbitol Bile Broth Medium (PSBB) at 10°C, figure B & D indicate pale pink or reject red colonies of Yersinia enterocolitica, figure C indicate mixed colonies with reject red Y. enterocolitica colonies.

Acknowledgements

This article reviewed through research studies conducted by own financial support while working in a slaughter house in New Delhi, India. I am grateful to the Chancellor & Vice Chancellor of Kalinga University, Ph.D. Supervisor Dr. Nidhi Vishnoi (Ph.D. Microbiology), Kalinga University, Village Kotni, Near Mantralaya, Naya Raipur, Raipur, Chhattisgarh to approved my research work and support and also thanks for providing support for part of the research work perform in Shivaji Science College, Chickli, Maharastra undergo affiliation from Sant. Gadge Baba Amravati University, Amravati, Maharastra, India by Dr. Anil Mahadeo Garode (Ph.D. Microbiology). Even I am grateful to the Management of Slaughter House in New Delhi for their kind co-operation towards completion of the work.

Conflicts of interest

The authors declare that there is no conflict of interest.

References

1. Esnault E, Labbé A, Houdayer C, et al. Yersinia enterocolitica prevalence, on fresh pork, poultry and beef meat at retail level, in France. France. European University of Brittany; 2013.
2. Belgin Siriken. The Presence of Yersinia enterocolitica and Other Yersinia Species in Ground Beef in AydÝn, Turkey. *Turk J Vet Anim Sci*. 2002;28:489–495.
3. Mauro A, Laganà P, Bruno G, et al. Isolation of Yersinia enterocolitica biotype 1 A from raw meat products. *J prev med hyg*. 2008;49:75–78.
4. Sharifi Yazdi MK, Soltan-Dallal MM, Zali MR. et al. Incidence and antibiotic susceptibilities of Yersinia enterocolitica and other Yersinia species recovered from meat and chicken in Tehran, Iran. *African Journal of Microbiology Research*. 2011;5(18):2649–2653.
5. Minnich SA, Smith MJ, Weagant SD, et al. Yersinia. Chapter 19. In Foodborne Disease Handbook. 2nd edn. In: Hui YH, Pierson MD, editors. New York. Marcel Dekker Inc; 2001. p. 471–514.
6. Boyce JM, Evans EJ, Jr DG Evans, et al. Production of heat - stable methanol - soluble enterotoxin by Yersinia enterocolitica. *Infect Immun*. 1979;25:532–537.
7. Abadias M, Usall J, Anguera M, et al. Microbiological quality of fresh, minimally–processed fruit and vegetables, and sprouts from retail establishments. *Intl J Food Microbiol*. 2008;123:121–129.
8. Batt CA. Food pathogen detection. *Sci*. 2007;316:1579–1580.
9. Stephen D Weagant, Peter Feng. FDA Bacteriological Analytical Manual (BAM). Chapter 8. Yersinia enterocolitica. 2001 & 2007.
10. Wallace H Andrews, Thomas S Hammack. Chapter 1. FDA Bacteriological Analytical Manual (BAM). Food Sampling and Preparation of Sample Homogenate. 2003.
11. Ujjal Sen, Anil Mahadeo Garode. Identification and detection of Salmonella risk assessment from frozen buffalo meat exported from India. Kalinga University. Raipur. India. *Journal of Bacteriology and Mycology: Open Access*. 2016;2(2):00017.
12. Pradhap M, Selvisabhanayakam, Mathivanan V, et al. Study on 16SrRNA based PCR method for specific detection of Salmonella entrica typhi from gut of infected silkworm Bombyx mori (Linn.) *Journal of Scientific & Industrial Research*. 2011;70:909–911.
13. Kshirsagar DP, Singh S, Brahmbhatt MN, et al. Isolation and Molecular Characterization of Virulence-Associated Genes of Salmonella from Buffalo Meat Samples in Western Region of India. *Israel Journal of Veterinary Medicine*. 2014;69 (4).
14. Swati Singh, Kshirsagar DP, Brahmbhatt MN, et al. Isolation and characterization of salmonella spp. from buffalo meat samples. Anand, India. Department of Veterinary Public Health and Epidemiology, Veterinary College, Anand Agricultural University; 2015;34(3):310–312.
15. Myron Sesser. Microbial identification using automated Fatty Acid Methyl Ester (FAME) Analysis on a Shimadzu GC-2010/2030. MIDI-Shimadzu excellence in science. Technical Note #101-S. 1990 & 2017.
16. Sandy Dr. MIDI, Inc. Sherlock Microbial Identification System. Version 6.2. MIS Operating Manual. 125, Newark, DE 19713. Tel: (302) 737 4297. 2012.
17. Sandy. MIDI-SherlockTM Chromatographic Analysis System (CAS) Fatty Acid Analysis and Microbial Identification. MIDI-Shimadzu excellence in science. 125 Sandy Drive Newark, DE 19713. USA. 2018.
18. Mackie & McCartney. Practical Medical Microbiology. In: Collee JG, editor. 14th edn. N. Y. Joyce D. Coghlan. Chapter 28. Yersinia, Pasteurella, Francisella. Churchill Livingstone Publishers; 1996. p. 479-487.
19. CDC. Epidemiological notes and reports multi-state outbreak of yersiniosis. *MMWR Morb Mortal Wkly Rep*. 1982;31(37):505–506.
20. Ackers ML, Schoenfeld S, Markman J, et al. An outbreak of Yersinia enterocolitica O:8 infections associated with pasteurized milk. *J Infect Dis*. 2000;181(5):1834–1837.
21. Mauro, Lagana P, Bruno G, et al. Isolation of Yersinia enterocolitica biotype 1 A from raw meat products. *Prev Med Hyg*. 2008;49:75–78.

22. Javeed Akhtar, Ram Krishna Pandey. Quality changes of Vacuum Packed meat and meat products: A review. *International Journal of Applied and Pure Science and Agriculture*. 2015.

23. Ljiljana B Trajković-Pavlović, Milka B Popović, Budimka D Novaković, et al. Occurrence of campylobacter, salmonella, yersinia enterocolitica and listeria monocytogenes in some retail food products in novi sad. *Cent Eur J Public Health*. 2007;15(4):167–171.

24. Belgin SIRIKEN. The Presence of Yersinia enterocolitica and Other Yersinia Species in Ground Beef in AydÝn, Turkey. Department of Food Hygiene and Technology, Faculty of Veterinary Medicine, Afyon Kocatepe University, Ahmet Necdet Sezer Campus, Afyon – TURKEY. *Turk J Vet Anim Sci*. 2004;28: (2004) 489–495.

25. Soltan-Dallal MM, Doyle MP, Rezadehbashi M, et al. Prevalence and antimicrobial resistance profiles of Salmonella serotypes, Campylobacter and Yersinia spp. isolated from retail chicken and beef, Tehran, Iran. *Food Control*. 2010;21:388–392.

26. Mayrhofer S, Paulsen P. Antimicrobial resistance profile of five major food-borne pathogens isolated from beef, pork and poultry. *J Food Microbiol*. 2004;97:23–29.

27. Ezekiel CN, Olarinmoye AO, Oyinloye JMA, et al. Distribution, antibiogram and multidrug resistance in Enterobacteriaceae from commercial poultry feeds in Nigeria. *Afr J Microbiol Res*. 2011;5(3):294–301.

28. Mohamed Sabry AbdElraheam Elsayed, Eman Abdeeen, Mohamed Ali Akiela, et al. Real Time and Conventional PCR for Characterization of Salmonella sp. from Imported Meat to Egypt. *Advances in Animal and Veterinary Sciences*. 2014;2(4):199–203.