IJBPAS, May, 2020, 9(5): 906-915

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

# CHARACTERIZATION AND MOLECULAR IDENTIFICATION OF *SALMONELLA* SEROVARS OF PUNJAB PAKISTAN

**LATIF K[1], NAZ S[1*], ALTAF I[2], BASHIR R[1] AND LATIF J[3]**

[1]Department of Biotechnology, Lahore College for Women University, Lahore, Pakistan

[2]Quality Operation Laboratory (QOL), University of Veterinary and Animal Sciences, Lahore, Pakistan

[3]Management of Science, Virtual University Lahore, Pakistan

***Corresponding author: Shagufta.Naz@lcwu.edu.pk; Fax:92-42-99230980**

**Received 5th Nov. 2019; Revised 3rd Dec. 2019; Accepted 3rd Jan. 2020; Available online 1st May 2020**

**https://doi.org/10.31032/IJBPAS/2020/9.5.5005**

## ABSTRACT

The aim of this study includes isolation, characterization and molecular identification of *Salmonella* serovars of Punjab Pakistan. Bacterial strain was isolated and characterized with various biochemical tests and confirmed through molecular characterization. Using universal primers bacterial 16S rRNA gene was amplified. Compare the sequence in NCBI sequence database with amplified 16S rRNA gene sequence. The bacterial strain was amplified as *Salmonella typhi*. Phylogenetic and molecular evolutionary analysis was conducted by 16S rRNA sequencing. *Salmonella typhi* is a gram negative, rode shaped bacteria which forms black center colony on SS agar. The sequence when submitted to NCBI gene bank database using BLAST showed 99% maximum identity with all closely related taxa.

**Keywords: *Salmonella,* 16S rRNA, Isolation, characterization, NCBI.**

## INTRODUCTION

Around the world, *Salmonella* serovar is a leading caustic agent of gastrointestinal human disease **[1]**. For the detection of *Salmonella* serovars according to drug and food administration various serological and biochemical confirmation test are needed which approximately takes 5 to 7 days. Meanwhile >2000 serovars have been identified till date which can be serotyped into two species termed as *bongori* and *enteric*. During outbreaks many *Salmonella* serovars are normally encountered which are *Salmonella Tyhpimurium*, *Salmonella agona*, *Salmonella montevideo, Salmonella newport, Salmonella infantis, Salmonella enteritidis, Salmonella heidelberg and Salmonella saintpaul* **[2]**. By means of serval rapid methods such as PCR assay all *Salmonella* serovars are detected that has been extensively used for *Salmonella* detection **[3-5]**. DNA probes and PCR primers have been designed based on16SrRNA (rDNA) sequence **[6]**, which are specifically based on randomly cloned fragment **[7-9]** and widely used for *Salmonella* detection. Specifically for phylogenetic analysis species identification 16S rRNA sequence are well because it is ubiquitous in bacteria **[10, 11]**. Based on data from microbial 16SrRNA sequence, Gutell *et al*., 1985 **[12]** proposed a model particularly for the 16SrRNA secondary structure. Since, two species defines the variable regions as well as conserved areas. Consequently, specific DNA probes and PCR primers can be derived from such variable regions and used for the detection pf specific bacterial cells such as, *Escherichia coli* **[13]**, *Salmonella* serovars **[6]**, *Listeria monocytogenes* **[14]**, *Aeromonas salmonicida* **[15]**. Hypervariable regions have been present in 16SrRNA sequence wherever mostly sequences diverged over evolutionary time and by strongly conserved regions, these sequences are frequently flanked. For the amplification of variable regions specific primers are designed which are used to bind to conserved regions **[16]**.

## MATERIALS AND METHODS

### Isolation and identification of bacteria

The studies were carried out in Department of Biotechnology, Lahore College for Women University, Lahore, Pakistan during 2016. The samples were collected in a sterile plastic container and transported to Quality operation laboratory, University of Veterinary and animal sciences, Lahore, Pakistan. Bacterial isolates were screened on Nutrient Agar (NA) plates by the standard pour plate method. Plates were incubated at 37°C/24h and a total of twenty isolates were obtained, from that one isolate was selected and used for further studies. A loopful culture from nutrient broth (NB) was streaked on a plate of McConkey agar (MA) and on a selective media Salmonella-Shigella agar (SSA). After that, plates were incubated at 37$^o$C for overnight.

### Biochemical characterization and gram staining of the bacteria

Gram's staining method was used for microscopic characterization of bacteria (17). Commercial available kit Analytical Profile Index (API 20-E kit, BiOMerieux, France) was used for biochemical characterization of isolates as defined by Wilson *et al.*, **[18]**. Isolates were biochemically analyzed for the activities of Oxidase, Catalase, MR-VP test, Urease test, Motility, Indole production and Citrate utilization **(Table 1)**.

### Bacteria DNA Isolation and PCR Amplification of 16S rRNA Gene

Genomic DNA isolation from bacterial cells was performed according to a method described by **[19]**. Extracted DNA was quantified by using Nano Drop Spectrophotometer (Thermo Scientific Company, USA). The isolates were subjected to 16S rRNA for molecular characterization by using specific 16S rRNA primers **(Table 2)**.

**Table 1: Biochemical characteristics of isolated strain**

| Test | Result |
|---|---|
| **Gram stain** | - |
| **Motility** | + |
| **H2S** | + |
| **Urease** | - |
| **Citrate test** | + |
| **Indole test** | - |
| **Nitrate reduction** | + |
| **Oxidase** | - |
| **MR** | + |
| **VP** | - |
| **Lactose** | - |
| **Sucrose** | - |
| **Inositol** | - |
| **β-galaktosidase (ONPG Test)** | - |
| **Gas Production from glucose** | + |

**Table 2: Primers used for characterization of 16*S*RNA**

| Stain | Gene | Primer's Name | Primer Sequence | Product Size |
|---|---|---|---|---|
| ***Salmonella spp.*** | **16S** | **16S-F** | **GCTTACAAGCACATAGGAA** | **500 bp** |
| | | **16S-R** | **ATTACAGCATTACAGCTTAT** | |

16S rRNA PCR was used for confirmation of isolates. PCR amplification was done by using programmable Thermal cycler (Bio-Rad T100$^{TM}$ Thermo Cycler). Reaction mixture for 16S rRNA was 50 µl containing *Taq* Buffer KCl, 10X (5 µl), $MgCl_2$ (25 mM) 6 µl, dNTPs (2.5 mM) 3 µl, forward and reverse primer (10 pmol) 3 µl each, 1 µl of *Taq* polymerase (5U / µl), template (extracted DNA) 2 µl and sterilized water sufficient to 50 µl was added. Amplification conditions for16S rRNA gene were optimized at 94°C for 5 min, 94°C for 30 sec, 50°C for 30 sec, 72°C for 30 sec, as initial denaturation, denaturation, annealing and extension temperatures respectively. The cycles were run for 36 times followed by 72°C as final elongation for 10 min. 5µl of amplification product was resolved on 1 % agarose gel in 1X TAE buffer. After gel electrophoresis, the PCR product was eluted by using

Thermo scientific Gene JET Gel Extraction kit. The purified PCR products were reconfirmed (using size marker) by electrophoreses on 1% agarose gel. Purified PCR products were sequenced by First BASE Laboratories SdnBhd, Selangor, Malaysia. The obtained nucleotide sequences were subjected to NCBI BLASTn network service from GenBank (**h**ttp://www.ncbi.nlm.nih.gov/).

Phylogenetic analysis is the study of evolutionary relationships among various groups of organisms. In accordance with the accumulated phylogenetic knowledge database, when comparing novel isolates with evolutionary closely related strains.

**Computational Analysis (BLAST)**

The search sequence is align by using a web based program named BLAST (Basic Local Alignment Search Tool) to thousands of different sequences in a Database and gives the list of top matches. This program can search within a minute thousands of entries **[16]**. BLAST **[20]** align the sequence with each position of the sequence in the database. If the nucleotides match, BLAST gives a positive score for each position white performing the alignment gaps can also be inert. On the alignment, score each gaps inserted showed a negative effect while as a result of the gaps, if enough nucleotides align, then this negative effect is overcome or the gap is accepted in the alignment. For the calculation of the alignment score, these scores are then used, in "bits" which is converted to statistical E- value. The more similar the sequence if lower the E value found in the database is to query sequence.

## RESULTS AND DISCUSSION

On the basis of phenotypic characteristics the traditional identification of bacteria is usually not perfect as compared to identification based on genotypic methods. 16S rRNA gene sequence has developed as a desired genetic technique. Between bacteria to known the relationship the sequence of 16S rRNA gene has been extensively used as molecular clock while recently to the species or gene level it has also become important to identify an unknown bacteria **[21]**. For a number of reasons the 16S rRNA gene sequence has been used as the most common housekeeping genetic marker to study bacterial taxonomy and phylogeny **[22]**. These reasons are: (i) Particularly in all bacteria its presence may exist as a multigene family or operons. (ii) 16S rRNA gene function has not changed over time signifying that random sequence changes are more accurate measure of time (evolution) and (iii) for informatics purpose the 16S rRNA gene is large enough **[23]**. In microbiology the rRNA based analysis is a central method used to identify new strains. The present study has been carried out to identify the bacterial strain isolated from the Punjab Pakistan. Total of twenty isolates obtained, from that one isolate were used for further analysis. The colonies having typical cultural characterization were selected as presumptive for *Salmonella* serovars. In the present study selective media such as macConkey agar and SS agar were used for the isolation and identification of *Salmonellae* spp which was also used by a number of researcher such as **[23, 24]**. The colony characteristics of *Salmonella* spp found in this study was translucent, black, smooth and small round colonies on SS agar while macConkey agar produced colorless and smooth colonies **(Figure 1A and Figure 1B)**. While, in gram staining, it was found that morphology of the isolated bacteria exhibited as pink, small, rod shape, gram negative bacilli **(Figure 1C)** which was supported by several authors **[25]**. Afterwards, the purity of culture was confirmed by biochemical characterization. The result of api 20 kit had supported biochemical identification of *S.typhi*. Culture was found to be MR+, VP$^-$, and Urease – which is the characteristics of *S.typhi*. These findings of biochemical identification for *S.typhi* were also similar to the findings **[26, 27]**. The extracted DNA was used as template for amplification of 16S rRNA gene. The universal primers were used for the amplification and sequencing of the 16S rRNA gene fragment. The optimum annealing temperature was found to be 50ºC. An intense single band was visible on 1% Agarose gel stained with ethidium bromide **(Figure 2A)**. PCR product was purified by Thermo scientific GeneJET Gel Extraction kit **(Figure 2B)**. The sequences obtained were compared with the NCBI gene bank database using BLAST search program (http;//www.ncbi.nlm.nih.gov) **[21, 28]**. The percentages of sequence matching were also analyzed. The homology search made using BLAST showed 99 % maximum identity with that of *Salmonella enterica* subsp. *Enterica* serovar *typhi* strain 541 16S ribosomal RNA gene, NCBI Gene Bank Accession No: KM977896.1and E-value equal to 0 for all closely related taxa. Further the sequences of the bacterial isolates were used for the construction of the phylogenetic dendrogram to know the genetic relatedness between the bacterial isolates. All the closely related homologs of identified bacteria were used for the construction of the phylogenetic dendrogram **(Figure 3)** to know their evolutionary origin. The dendrogram showing the relation between as *Salmonella* spp. Kunza-lc and their close homologous for *Salmonella* are zoonotic pathogens, as they can cause food-borne illness in humans **[29]**.

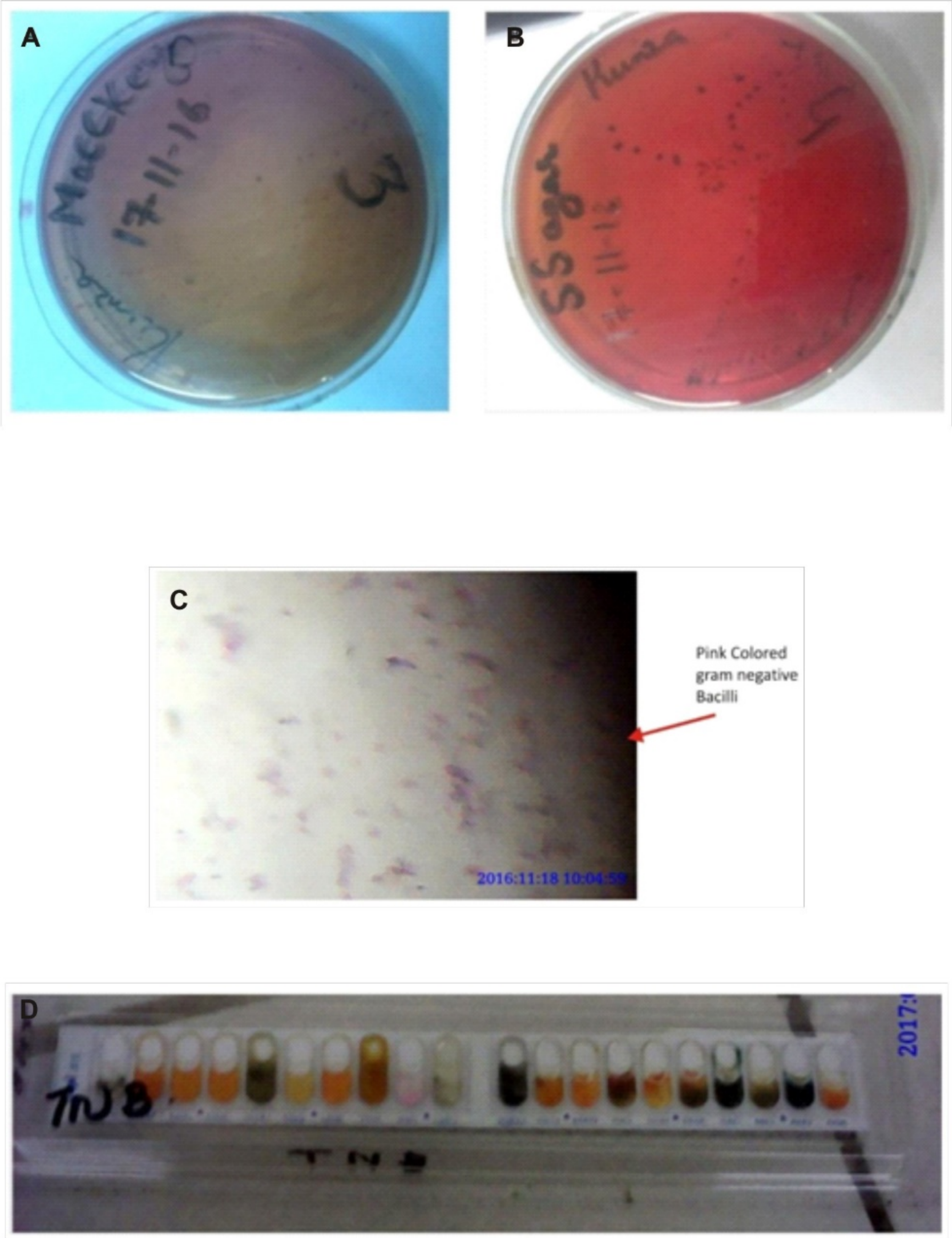

**Figure 1: (A) Growth of *Salmonella* on macConkey agar. (B) Growth of Salmonella on SSagar. (C); *Salmonella* serovar showing Gram-negative small rods singly or pairs on Gram's staining (100X); (D) Biochemical test through API Kit (20 E)indicating *Salmonella enteric* sub species *S.typhi***

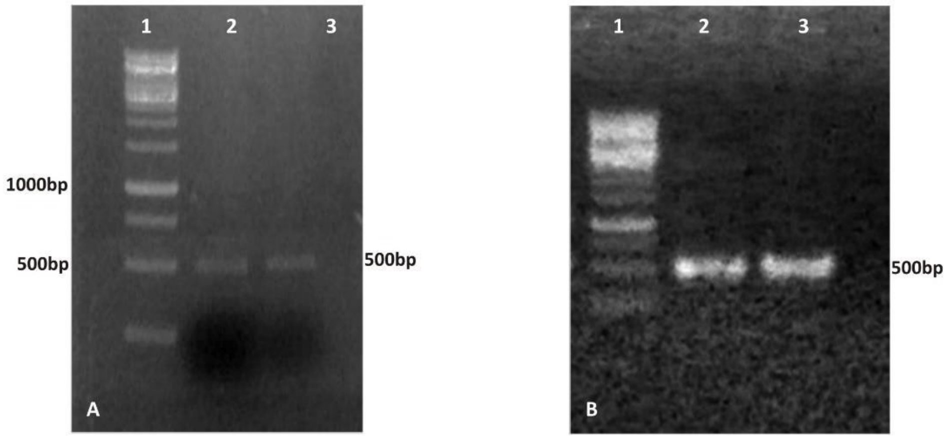

**Figure 2: (A) 1% agarose gel electrophoresis showing16S rRNA of *Salmonella* isolates. 1 DNA Ladder 1 Kb; 3 and 316S rRNA PCR product of 500bp (Bagarose gel electrophoresis showing gene clean of 16SrRNA product. Gene Ruler DNA Ladder mix; 2 and 3**

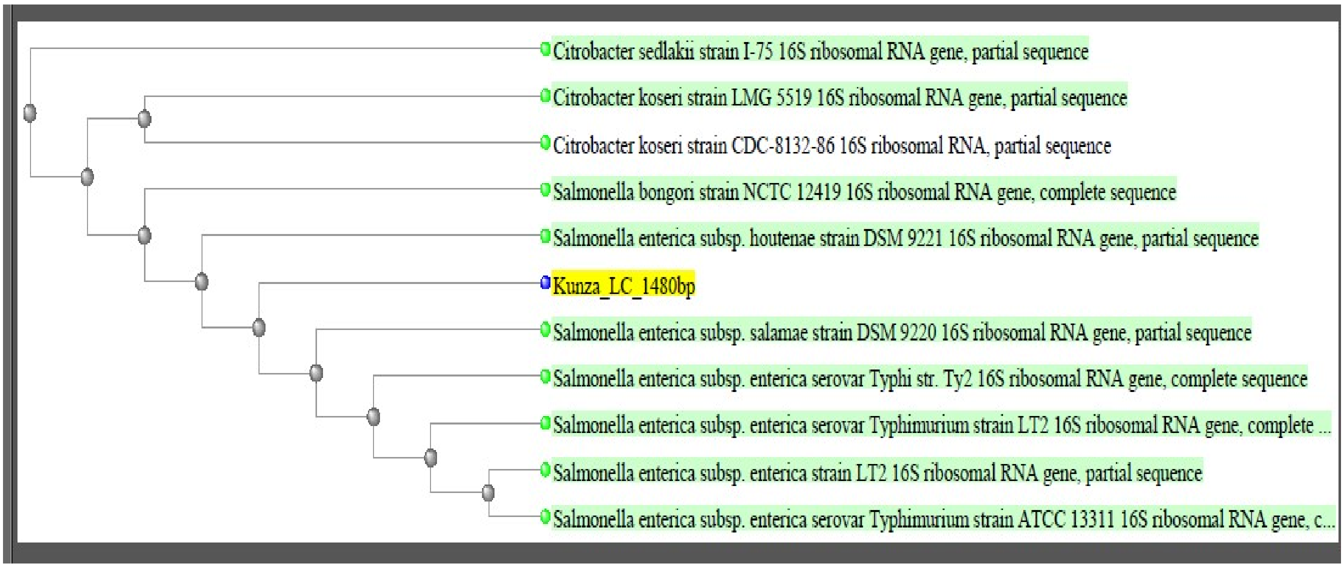

**Figure 3: Phylogenetic tree representing close homologs of *Salmonella typhi.***

## CONCLUSION

16s rRNA gene sequencing provide genus and species identification for isolates that do not fit any recognized biochemical profiles for strains generating only a low likelihood or acceptable identification according to commercial systems or for taxa that are rarely associated with human infectious diseases. The use of 16S rRNA gene sequences to identify new strains bacteria is gaining momentum in recent years. We showed the use of 16S rRNA gene sequence to characterize the bacterial isolate from Punjab Pakistan and were found to be *Salmonella* sp*typhi* (NCBI Gene Bank Accession No: KM977896.1. Thus, the genotyping method using 16S rRNA gene sequence is both simple and effective in strain identification.

## REFERENCES

**[1]** Olsen SJ, MacKinnon LC, Goulding JS, Bean NH, Slutsker LB, Surveillance for food borne-disease outbreaks—United States, 1993–1997. *Morb. Mortal. Wkly. Rep.* 49:1–62, 2000.

**[2]** Kumao T, Ba-Thein W, Hayashi H, Molecular subtyping methods for detection of *Salmonella enteric* serovar Oranienburg outbreaks, *Journal of Clinical Microbiology,* 40, 2002, 2057–2061.

**[3]** Chen SAY, Larkin C, Tamashiro CT, Behari R, Paszkko-kolva C, Rahn K, DeGrandis SA, The evaluation of a fluorogenic polymerase chain reaction assay for the detection Of Salmonella species in food commodities, *International Journal of Food Microbiology*, 35, 1997, 239-250.

**[4]** Ferretti R, Mannazzu I, Cocolin L, Comi G, Clementi F, Twelve-hour PCR-based method for detection of *Salmonella* serovars in food, *Applied Environmental. Microbiology,* 67, 2001, 977–978.

**[5]** Wan J, King K, Craven G, McAuley C, Tan SE, Coventry MG, Probelia PCR system for rapid detection of *Salmonella* in milk powder and ricotta cheese, *Letter Applied. Microbiology,* 30, 2000, 267–271.

**[6]** Trkov M, Tin, G, An improved16Sr RNA PCR method for the specific detection of *Salmonella enterica, International Journal of Food and Microbiology,* 80, 2002, 67–75.

**[7]** Aabo S, Rasmussen OF, Rossen L, Sorensen PD, Olsen, JE, *Salmonella* identification by The polymerase chain reaction, *Molecular and cellular probes,* 7, 1993, 171–178.

**[8]** Olsen JE, Aabo S, Nielsen EO, Nielsen BB, Isolation of a *Salmonella*-specific DNA hybridization probe, *Acta Pathol. Microbiol. Immunol Scand,* 99, 1991, 114–120.

**[9]** Kataoka M, Ueda K,Kudo T, Seki T, Yoshida T, Application of the variable region in 16SrDNA to create an index for rapid species identification in the genus *Streptomyces, FEMS Microbiology Letter,*151, 1997, 49–255.

**[10]** Harmsen, HJM, Elfferich P, Schut F, Welling GW, A 16S rRNA-targeted probe for detection of *lactobacilli* and entero- cocci in fecal samples by fluorescent in situ hybridization, *Microbial Ecology* in *Health and Disease,* 11, 1999, 3–12.

**[11]** ScheuP M, Berghof K, Stahl, U, Detection of pathogenic and spoilage microorganisms in food with the polymerase chain reaction, *Food Microbiology,*15, 1998, 13–31.

**[12]** Gutell RR, Weiser B, Woese CR, Noller, HF, Com- parative anatomy of 16S-like Ribosomal RNA, *Progress in Nucleic Acid Research and Molecular Biology,*32, 1985, 155–216.

**[13]** TsenHY, Lin KC, Chi WR, Development and use of 16S rRNA gene targeted PCR Primers for the identification of *Escherichia coli* cells in water, *Journal of Appied Microbiology,* 85, 1998, 554–560.

**[14]** Wang RF,Cao WW, Johnson MG, Development of a 16S rRNA based oligomer probe specific for *Listeria monocyto- genes, Applied Environmental Microbiology.*57, 1991, 3666–3670.

**[15]** Barry T, Powell R, Gannon F, A general methodto generate DNA probes for microorganisms, *BioTechnology,* 8, 1990, 233– 236.

**[16]** Mahmoud MSK, Characterization and Molecular Identification of Unknown Bacteria Isolated From Outlet of Arab El Madabegh Sewage Treatment Plant in Assiut City, Egypt. *Journal of Evvioronmental Health Science,* 4(1), 2016, 1-5.

**[17]** Islam MM, Islam MN, Sharifuzzaman, Fakhruzzaman M, Isolation and identification of *Escherichia coli* and *Salmonella* from poultry litter and feed, *International Journal of Natural and Social Sciences,* 1, 2014, 1 -7.

**[18]** Wilson, MA, Morgan MJ, Burger GE, Comparison of DNA fingerprinting and serotyping for identification of avian *Pasteurella multocida* isolates, *Journal of Clinical Microbiology*, 31, 1993, 255-259.

**[19]** Sambrook J, Friscgh E F, Meniates T, Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York, 1989, pp. 54 – 60.

**[20]** Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ, Information about the BLAST, *Journal of Molecular Biology*, 1990, 401-403.

**[21]** Pruitt KD, Tatusova T, Maglott DR, NCBI reference sequence: a cultured non–redundant sequence database of genomes, transcripts, and proteins, *Journal of Nucleic Acids Research*, 33, 2005, 501–504.

**[22]** Amann RI, Ludwig W, Schleifer KH, Phylogenetic identification and in situ detection of individual microbial cells without cultivation, *Microbiological Reviews*, 59, 1995, 143 – 169.

**[23]** Hyeon JY, Park JH, Chon JW, Wee SH, Moon JS, KimYJ, Seo KH, Evaluation of Selective enrichment broths and chromogenic media for *Salmonella* detection in highly Contaminated chicken carcasses, *Poultry Science*, 91 (5), 2012, 1222 - 1226.

**[24]** Muktaruzzaman M, Haider MD, Ahmed AKM, Khondoker A, Rahman MM,

**[25]** Khatun MB, Rahman MH, Hossain MD, Validation and Refinement of

*Salmonella* pullorum (SP) Colored Antigen for Diagnosis of *Salmonella* Infections in the Field, *International Journal of Poultry Science*, 9, 2010.

**[26]** Mondal T, Khan MSR, Alam M, Purakayastha DM, Siddique MP, Isolation, identification and characterization of *Salmonella* from duck, *Bangladesh. Journal* of *Veterinary Medicine*, 6(1), 2008, 07 – 12.

**[27]** Nahab HM, Lebawy NSAL, Mousa NM, Study of *Salmonella typhi* isolated from patient suffering from typhoid fever in AL-Samawah city, Iraq., *Journal* of *Pharmaceutical Sciences and Research*, 10(9), 2018, 2285 - 2288.

**[28]** Singh Y, Saxena A, Kumar R, Bhatt P, Immunogenic Outer membrane Proteins (Omps) Of *Salmonella*: Potential Candidate for sub-unit vaccine, *Virology & Immunology Journal*, 1 (2), 2017.

**[29]** Patel JB, 16S rRNA gene sequencing for bacterial pathogen identification in the clinical laboratory, *Journal of Molecular Diagnostic*, 6, 2001, 313-321.

**[30]** Sara M, Mohammad H, Iradj A, Identification and characterization of Salmonella isolates, *Turkish journal of Veterinary and Animal Sciences*, 34(2), 2010, 181-186.