DESIGNING A RAPID, RELIABLE AND REPRODUCIBLE METHOD FOR THE DETECTION OF SALMONELLA SPP. FROM POULTRY MEAT

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Abstract

Salmonella is one of the most common causes of foodborne diseases among humans worldwide. Animal-derived foods are frequently tested for the presence of Salmonella spp. However, the detection of Salmonella in meat and its products is often hindered due to the presence of background normal flora, which may lead to the detection of false-positive Salmonella. The present study aimed to isolate and accurately identify Salmonella spp. from poultry meat. For this purpose, seventy poultry meat samples were collected from Lahore, Pakistan, isolated on selective and differential media and identified using biochemical tests and polymerase chain reaction for the 16S rRNA gene of identified strains. The results of selective and differential media culturing and biochemical tests were compared with the results of 16S rRNA gene sequencing. It was inferred that the phenylalanine deaminase test and triple sugar iron tests eliminate the false-positive Salmonella isolates obtained on isolation media, and along with the PCR technique, can serve as an accurate and efficient method for the correct detection of Salmonella spp. from meat samples. In order to reduce the false-positive Salmonella isolates, a highly specific selective media must be designed which can distinguish Salmonella forming different colors of colonies from other bacteria and also cause the inhibition of non-Salmonella isolates.

Keywords: Salmonella, poultry meat, false-positive, 16S rRNA
I. INTRODUCTION

Poultry meat is broadly used as a source of animal protein and it also serves as a reservoir of food borne diseases among humans (Ramthahal et al., 2022). The microorganisms found on the surface of chicken carcasses, particularly members of the Enterobacteriaceae family, are also inhabitant soft the intestines of poultry birds and a potential source of foodborne pathogens, which may transmit to humans during raw meat preparation (Kim et al., 2019; Okorafor et al., 2019). In Pakistan, raw meat is sold in outdoor marketplaces, and poor hygiene in small butcher shops degrades meat quality. Therefore, many cases of foodborne diseases are often reported in Pakistan (Yousafzai et al., 2019).

Foodborne Salmonellosis and acute gastroenteritis caused by Salmonella are major public health issues as a number of such outbreaks have been reported globally. The contamination of Salmonella in poultry meat occurs through the spread of intestinal matter during the cutting of poultry birds (Nair and Kollanoor Johny, 2019). This pathogen has zoonotic potential due to its wide host range (Orji et al., 2005). In developing countries, gastroenteritis causes 1 billion illnesses and 5 million fatalities annually (Koondhar et al., 2021), illustrating the severity of the problem (Heredia and Garcia, 2018).

There are well-established and frequently used culturing methods for the detection of Salmonella in food testing and surveillance (Mitham and Rasha, 2018). Despite years of improving the Salmonella culturing techniques, existing isolation methods aren't ideal: false-negative or false-positive isolates are common. Traditional media bases identify Salmonella by detecting lactose fermentation through H₂S production and pH indicator. But these media lack indistingushing Salmonella spp. from other enteric bacteria, such as Proteus and Citrobacter which have similar colony characteristics (Srijan et al., 2015; Park et al., 2012). Many researchers stress the need for an accurate and effective detection technique as a control measure (Lim et al., 2003; Agron et al., 2001). Typical microbiological, biochemical and serological procedures are laborious and costly, requiring well-trained personnel (Nori and Thong, 2010). Recently, DNA amplification by PCR is determined as a potent tool in bacteriological diagnostics. These PCRs target highly preserved bacterial 16S rRNA genes, and by consequent sequence analysis, these are efficient techniques for identifying pathogenic bacteria (Klouche and Schröder, 2008). Genetic
identification techniques are replacements or confirmatory to the phenotypic identification procedures (Bosshard et al., 2006).

Different Salmonella serovars have been reported in poultry meat by numerous studies conducted in Pakistan. However, the accurate identification method for Salmonella spp. needs to be determined that might help veterinary and public health authorities in developing prevention strategies to control salmonellosis in poultry and its potential transmission to humans (Kumar et al., 2019). Therefore, the current study sought the far more rapid and precise approach for detecting Salmonella of livestock origin.

II. MATERIALS AND METHODS

Samples collection and preparation

In this study, the sample collection period was one year starting from 2021 to 2022 and involved different markets of Lahore. A total of 70 poultry meat samples were collected. The 25g of poultry meat samples were transported instantly to the laboratory in an icebox, homogenized, and incubated for 2 days, peptone water was used as a pre-enrichment medium on the first day, and selenite broth as a selective enrichment media was used on the second day at 37°C for 18-24 h. For pre-enrichment, 25g of meat sample was added to 225 ml of sterile peptone water and for selective enrichment, 1ml of peptone water was added to 10ml selenite broth.

Isolation and Identification

Salmonella spp. was identified by using a methodology developed by Srijan et al (2015). A drop from selenite broth was used to inoculate MAC, SS, and XLD media and then streaked for the growth of separate colonies by a sterile loop. These plates were incubated aerobically at 37°C for 18-24 h. Then, the plates were examined for the morphology of Salmonella colonies and suspected colonies from each MAC, SS, and XLD agar media were sub-cultured on nutrient agar plates and subjected to biochemical tests.

Biochemical Identification

In this study, a biochemical tests panel was designed to instantly rule out false-positive Salmonella, which included triple sugar iron, sulfide indole motility, methyl red, Voges-proskauer, citrate, urease, and phenylalanine deaminase tests. After inoculating the biochemical
test tubes, an overnight incubation was done at 37°C, except for methyl red, voges-proskauer and urease tests. The MR/VP tubes were incubated for 48 hours and urease test tubes were observed after 6 h, 24 h, and daily for up to 6 days.

**DNA Isolation**

*Salmonella* is gram-negative, the boiling method described by Mitham and Rasha. (2018) was used for its DNA isolation. First, the biochemically identified *Salmonella* colonies were picked from the XLD agar plate, added into 200µl distilled water, vortexed and incubated at 100°C for 15 minutes. To increase its volume to 1ml, 800 µl of distilled water was poured and again vortexed to form a homogeneous solution, which was centrifuged for 10 min at 12000 rpm. The supernatant (DNA) was collected and used for PCR.

**PCR Technique**

For the detection of *Salmonella* spp., an oligonucleotide primers set was used in PCR reaction 27F (5′-AGAGTTTGATCMTGGCTCAG-3′, 1492R 5′-CGGTTACCTTGTTACGACTT-3′) that amplifies the 1465bp specific region of 16S rRNA gene. A 50µl PCR reaction was made that included 25µl of master mix (Thermo Scientific), 6µl of DNA template, 1µl of both primers and 17µl of water. The initial denaturation temperature was 95°C for 5 min which was followed by 30 cycles each at 95°C for 5min, 55°C for 30s and 72°C for 1 min, and a final extension at 72°C for 8 min. The amplified product size was identified using a 1000bpLadder (Thermo Scientific) on gel and visualized under UV trans-illuminator as well as Gel Doc. The PCR amplicons were then sent for sequencing.

**Phylogenetic analysis**

The 16S rRNA sequencing results were compared with already identified 16S rRNA sequences found in the NCBI database with the help of BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Genus and specie levels were identified with ≥99% sequence similarity of 16S rRNA sequence with that of the prototype strain sequence present in GenBank. These sequences were aligned by using CLUSTAL W.
III. RESULTS

Figure and table 1 shows the difference among three different media for enumeration of *Salmonella* spp. from poultry meat along with the conventional PCR results. The colonies formed by *Citrobacter* and *Proteus* appeared almost identical to *Salmonella* on XLD and SS media *i.e.* colonies with black centers due to H$_2$S production (Fig 2). However, on MAC media, the growth of *Proteus* and *Salmonella* were colorless colonies and *Citrobacter* formed pink and pale colonies, which were further incubated for 24h to identify the late lactose fermentation.

**Figure 1:** Difference in the results of three isolation media (XLD, SS and MAC) and PCR positive results using 70 poultry samples

**Figure 2:** Similar colony characteristics of *Citrobacter, Salmonella, and Proteus* on XLD agar (A, B, & C) and SSagar (D, E & F)
All H₂S-producing bacterial isolates showing similar growth on selective media (XLD and SS) were further differentiated through TSI, SIM, urease and phenylalanine deaminase tests. The results of citrate (+ve), methyl red (+ve) and Voges-proskauer (-ve) tests were similar for these bacteria. All identified *Salmonella* strains were urease negative, phenylalanine deaminase test negative, motility +ve/ H₂S +ve/ indole –ve in SIM test, and produced alkaline slant, acidic butt, H₂S, and gas (some strains didn’t produce gas) in TSI test. The identified *Citrobacter* strains were urease variable (both –ve and +ve), phenylalanine deaminase test negative, motility +ve/ H₂S +ve/ indole –ve in SIM test, and produced acidic slant, acidic butt, H₂S, and gas in TSI test. The *Proteus* strains were differentiated from *Salmonella* based on their positive results for urease and phenylalanine deaminase tests. Their TSI test results were similar to *Salmonella* except for the production of gas. The *Proteus* strains were motility +ve, H₂S +ve, and indole +ve in the SIM test. The remaining enteric bacteria can also be distinguished from *Salmonella* using these tests.

After obtaining the biochemical pattern of all presumptive *Salmonella* spp., 14 were confirmed as belonging to *Salmonella* spp., 5 proved to be *Citrobacter* and 9 *Proteus*. The genotypic identification of all 14 *Salmonella* isolates was performed to check the accuracy of the phenotypic identification method. The result of PCR amplification was confirmed by gel electrophoresis. The DNA bands with the expected size (1465bp) under UV trans-illuminator are illustrated in figure 3. The accuracy of the phenotypic identification method and the elimination of false-positive *Salmonella* strains after biochemical testing is shown in figure 4 and table 1.

**Figure 3:** Agarose gel electrophoresis results revealed under UV illuminator: Lane M= 1000bp DNA Ladder, Lanes 1-9= PCR Products of 1465bp region of *Salmonella* spp. 16S rRNA gene and Lane C=negative control, Gel %= 2%
Figure 4: Difference in the results of biochemical tests and PCR positive results

Table 1: Identification of *Salmonella* isolates obtained from poultry meat based on colony morphology on selective and differential media, biochemical properties, and the PCR results.

<table>
<thead>
<tr>
<th>No. of isolates forming <em>Salmonella</em>-like colonies</th>
<th>Confirmation of <em>Salmonella</em> spp. by biochemical tests</th>
</tr>
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<tbody>
<tr>
<td>Urease test</td>
<td>Phenylalanine deaminase test</td>
</tr>
<tr>
<td>No. of sample</td>
<td>on XLD agar</td>
</tr>
<tr>
<td>70</td>
<td>31</td>
</tr>
<tr>
<td>Percentage</td>
<td>44%</td>
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</tbody>
</table>

IV. DISCUSSION

Recognizing the limitations of selective as well as differential media, it is important to enhance their sensitivity as well as sensitivity while maintaining expenditure (Park *et al.*, 2012). There are several rapid detection methods for enteric bacteria (Hameed *et al.*, 2012), however, conventional differential and selective media have many benefits, such as cost-effectiveness,
ease of use, and user knowledge (Mitham and Rasha, 2018). In this study, Table and Figure (1) reveal the differences in the results of utilizing three distinct mediums to recover *Salmonella* spp. as well as the use of traditional PCR for *Salmonella* spp. detection, we notice that no selective or differential media was able to accurately differentiate *Salmonella* from *Proteus* and *Citrobacter*. Both XLD and SS agar culturing included false-positive results due to the growth of other bacteria that have same colony characteristics to *Salmonella* and led to false identification. These findings are consistent with prior studies, which state that both *Proteus* and *Citrobacter* spp. are usually misidentified as *Salmonella* due to similar growth pattern on XLD though it’s an extensively used media for the detection of *Salmonella* based on their H₂S production and non-lactose fermentation (Park et al., 2012). The *Proteus* and *Citrobacter* spp. also show these characteristics (Pławińska-Czarnak et al., 2021; Jiang et al., 2017) despite of high specificity and sensitivity of XLD medium. Their colonies are indistinguishable from *Salmonella* on this media (Park et al., 2012). Biochemical testing is essential to identify *Salmonella* although it gives most of the test results similar to other enteric bacteria. The main difference lies in the non-fermentation of lactose, however, some *Salmonella* serotypes can also ferment lactose, increasing the probability of false identification of strains. The triple sugar iron agar is unable to screen them for H₂S production, because they give acid slant/acid but /gas reaction (Alexan, 2017). In contrast to it, no such TSI result was observed in this study indicating the absence of lactose fermenting *Salmonella* serotypes in the isolates. Motility is a key taxonomic tool for identifying enteric pathogens (El Mouali et al., 2017). In this study, a sulfide indole motility medium was used to observe the motility, H₂S, and indole production in enteric bacteria. This test was useful in differentiating indole negative *Salmonella* from indole positive *Proteus*, however, the percentage of identifying *Salmonella* through this test remained 16%. Moreover, the phenylalanine deaminase test with urease production test is used to distinguish *Proteus* spp. from the other members of *Enterobacteriaceae* (Mandal et al., 2015). In this study, both of these tests were used to detect *Proteus* that was indistinguishable from *Salmonella* on selective media. The phenylalanine deaminase test had more promising results than the urease test, which could detect only 13% of urease-negative *Salmonella* spp. Table 1 and Figure 5 reveal that the phenylalanine deaminase test, triple sugar iron test and PCR results were similar in identifying *Salmonella* spp. from poultry meat, we find that both these biochemical tests might be helpful in distinguishing *Salmonella* from *Proteus* and *Citrobacter* as no selective or differential media
used in this study were able to differentiate them. These are also inexpensive, easy to perform and applicable for testing food in a setting with limited resources. It is essential to detect a particular pathogen in poly microbial cultures. So, developing a highly specific selective media is suggested that can distinguish the desired pathogen (forming different colonies) from other bacteria and also cause their inhibition. We concluded that using of phenylalanine deaminase test, TSI test, SIM test, urease test and sequencing of 16S rRNA gene for the detection of *Salmonella* is an accurate and efficient method than relying on the results of selective media.

**V. CONCLUSION**

Due to rapid mutations in different bacterial spp., it is sometimes difficult to distinguish the strain of interest from others. We have successfully shown that selective isolation media are not entirely specific for *Salmonella* and can lead to false positive results. Hence, a combination of TSI, SIM, phenylalanine deaminase, and urease tests together with 16S rDNA amplification is an accurate, reliable and comprehensive method to rapidly distinguish *Salmonella* spp. from the rest of microbiota found in poultry meat. We also recommend that improved selective and differential media must be developed for the isolation of *Salmonella* spp. from meat samples.

**References**


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