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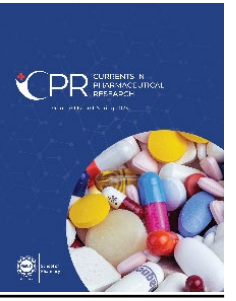

Title:	**Detection of Salmonella in Frozen and Raw Chicken from Islamabad Markets: A Conventional and Molecular Approach**
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Detection of Salmonella in Frozen and Raw Chicken from Islamabad Markets: A Conventional and Molecular Approach

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ABSTRACT

Salmonellae are one of the most important foodborne pathogens, worldwide. Foodborne outbreaks associated with the consumption of poultry are very frequent. The detection of this bacterium in chicken products is important to ensure food safety and to protect public health. Most of the current detection techniques, although in widespread use, often give false-positive results; therefore, a more accurate, sensitive, and reliable method for *Salmonella* detection is needed.Hence, This study estimates the efficiency and effectiveness of conventional and molecular detection techniques in identifying *Salmonella* spp. in poultry.it also caters the evidence of possible risks associated with the consumption of infected chicken products. For the said purposes,a total of 100 poultry samples comprising carcass and liver were collected from different areas of Islamabad, Pakistan. The positive samples were further analyzed through molecular techniques for the confirmation of the pathogen after these samples had been subjected to the conventional detection techniques. The statistical significance of the outcomes obtained from both the methods was tested in order to compare their effectiveness. Based on the results,, the study concludes that conventional and molecular techniques put together can provide more realistic detection of *Salmonella* spp. in poultry. The occurrences of false-positive results can be reduced to a minimum when detection is done with the use of selective media that can clearly distinguish *Salmonella* colonies from other bacteria. The use of such specific media is further encouraged to increase the accuracy of *Salmonella* detection at different levels of poultry industry.

Keywords: conventional methods, identification, isolation, molecular

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methods,poultry, *Salmonella*.

1. INTRODUCTION

Foodborne diseases are one of the most important health issues in the world, causing millions to be affected annually. *Salmonella* is a pathogenic bacterium that poses serious danger to both human and animal health. It usually starts as a mild infection of the gastrointestinal tract, though it may ascend into systemic diseases such as typhoid fever,causing high economic losses by incuring health care costs and due to the loss of productivity [1]. *Salmonella* is a part of the family Enterobacteriaceae, with two major species including *Salmonella enterica* and *Salmonella bongori*. The former is subdivided into six subspecies, including *S.enterica*,*S.salamae*, *S.diarizonae*, *S.arizonae*, *S.houtenae*, and *S. indica*, of which *S.enterica* is the most common and clinically relevant to infection in human beings [2]. Accordingly, *Salmonella* has developed several virulence factors for attachment to and invasion of intestinal mucosa, intracellular replicatipon , and evasion of host immunity. Among environmental conditions, moisture level is of prime importance for the survival and spread of *Salmonella* while processing and handling food in chicken carcasses.

The global burden of foodborne diseases is substantial, with an estimated 1 in 10 people falling ill each year and 33 million healthy life years lost annually due to these infections [3]. These diseases affect young children most disproportionately. The most common consequence of consuming contaminated food are diarrheal diseases, which affect 550 million people each year, including 220 million children under the age of five years. Being a chief sources of proteins for many human populations, chicken meat is one of the major vehicles of the transmission of *Salmonella* to humans.Although many measures have been taken by the poultry industry to avoid Salmonella infection, outbreaks due to this pathogen continue to occur.

The rapid molecular detection of *Salmonella* through Polymerase Chain Reaction (PCR), is very important in the isolation and identification of its virulent strains. This technique allows the detection of bacteria to be more specific because of the targeting of specific genetic markers associated with pathogenicity [4]. In recent years, drug resistance to commonly used antibiotics such as fluoroquinolones and cephalosporins has developed because of drug overuse both in human beings and in poultry

[5]. The indiscriminate application of antibiotics in poultry has contributed to developing resistance and simultaneously lowerings the effectiveness of standard treatments against infection in human beings. To solve this problem, there is an urgent need to develop new antimicrobial agents and alternative therapies, as well as more strict regulations concerning antibiotic treatments in poultry [6].

In this respect, the enhancement of methods and techniques used to detect *Salmonella* in poultry products is even more crucial because its timely and precise identification can prevent further infection and reduce the use of antibiotics [7]. Additionally, the development of effective vaccines for poultry may prevent the incidence of *Salmonella* contamination and reduce the use of antibiotics in the industry as a whole.

2. RESEARCH METHODOLOGY

For this study, 100 poultry samples were chosen to ensure the precision and reliability of findings. These samples were collected from various sources, including grocery stores, slaughterhouses, and meat shops from the Islamabad Capital territory. Of the 100 samples, 75 were raw chicken samples, of including 50 raw chicken meat samples and 25 were raw chicken liver samples, Whereas 25 frozen chicken samples were collected from supermarkets, including into 15 frozen chicken meat samples and 10 frozen chicken liver samples.

The samples were homogenized to evenly distribute microbiological contaminants throughout the sample [8]. The homogenization of solid meat samples was done in a blender or homogenizer, followed by dissolution in peptone water. A 1ml portion of each meat sample was separately transferred into 225 ml of Buffered Peptone Water (BPW) or lactose broth and incubated at 35-37°C for 18-24 hours for the resuscitation of injured bacteria. After pre-enrichment, 1 ml of pre-enrichment culture was transferred to selective enrichment media for further analysis according to the following procedure:

- MacConkey Agar was used to isolate Gram-negative bacteria,
- BSA Agar was used for the selective isolation of *Salmonella*.

Salmonella colonies on MacConkey Agar were pink in color due to lactose fermentation. Whereas , *Salmonella* colonies appeared black or brown on BSA agar due to the production of ferrous sulfide. Plate Count

Agar (PCA) was used as a non-selective medium for total bacterial counts.

2.1. Gram staining

A colony was emulsified in a drop of normal saline on a glass slide to prepare smears. The slide was dried and heat-fixed. Slides were stained with crystal violet for one minute, afterwards they were washed with tap water [8]. Acetone was used as a decolorizing agent, following its application the slides were washed again with tap water. Slides were finally stained with dilute carbol fuchsin for 30 seconds, washed, and dried with filter paper [9]. Gram-negative bacteria appeared red under the oil immersion lens, while Gram-positive bacteria appeared violet.

2.2. Molecular Detection

The presence of *Salmonella* was confirmed using molecular techniques. For the identification of the invA gene as a marker of *Salmonella* pathogenicity, DNA extraction was followed by the application of PCR and real-time PCR techniques [9–11].The following primers were used

Table 1. Primers of PCR

Primer		Sequence 5'-3'	Amplicon size	References
ITR 1– 2 NF	F	(5′-TGTTGTGGTTAATAACC GCA-3′)	ranged 284bp	[12]
	R	(5′-CACAAATC CATCTCTGGA-3′)		

2.2.1. Sample Preparation. The samples prepared using the Conventional method after confirmation by Gram staining were stored in specific conditions, so that they may be used for further molecular detection.

2.2.2. DNA Extraction. DNA was extracted from the sample by using a DNA extraction kit per the manufacturer's instructions. These included adding a homogenized sample to a tube containing lysis buffer, proteinase K enzyme, and other reagents. Incubation followed by centrifugation obtained a supernatant containing DNA.

2.2.3. Polymerase Chain Reaction. Reaction mixture for the PCR included the extracted DNA, DNA polymerase, primers, and nucleotides. This was followed by the cycling of PCR,that is, its denaturation, annealing, and extension that multiplied the target region of *Salmonella* DNA. The reaction mixture contained 10 µl of amplification mix, 10 µl of

oligo mix, and 5 µl of template DNA, adding up to a total volume of 25 µl. The PCR tubes were sealed with an optical adhesive film and centrifuged at 3000 rpm for 2 minutes. Fluorescence was observed after each PCR cycle and the Ct value was calculated to determine the initial content of *Salmonella* DNA.

2.2.4. Agarose Gel and DNA loading. A totalk of 1 gram agarose in 100 ml of 1X TBE buffer was prepared.It was heated to fully dissolve agarose, cooled to 50°C,and added with 5 µl of ethidium bromide [13]. The gel was set up in a running gel electrophoresis tank. DNA ladder markers and PCR products were then loaded into the wells. Electrophoresis was run at 75 volts for one hour and results were interpreted based on Ct values [14].

2.2.5. Effectiveness of Antimicrobial Agents. A literature review was conducted to establish the efficacy of diverse antimicrobial drugs in controlling the growth of Salmonella. Several studies have established the efficacy of fluoroquinolones (especially ciprofloxacin) and cephalosporins (particularly ceftriaxone) against Salmonella infections. However,more recent studies reveal an upsurge in antimicrobial resistance among *Salmonella* strains due to the overuse of these antibiotics in poultry farming [15]. For instance, Smith et al. shows that *Salmonella* isolated from chicken meat develops resistance to ciprofloxacin at a rate of 40%, hence requiring novel therapeutic options and an improved regulatory control over the use of antibiotics in poultry. While antibiotic treatment remains the cornerstone for managing *Salmonella* infection, vaccine development in poultry itself can also be a critical component in lessening the burden of *Salmonella* contamination in the food chain [16].

2.3. Statistical Analysis

The results obtained were subjected to statistical analysis comparing conventional and molecular detection methods.The proportions of positive samples detected by the two methods were compared using the Chi-square test. The statistical significance of the proportion of positive samples among local chicken samples as revealed by conventional detection was 0.017, while molecular detection confirmed positive cases at 50% . Among frozen samples, there was one positive case confirmed using the conventional technique that was also confirmed through molecular analysis with a 0.042 significance level. The results obtained showed that molecular techniques

are more efficient and reliable in the confirmation of *Salmonella* than conventional techniques

3. RESULTS

3.1. Gel Electrophoresis

The amplified DNA fragments were then visualized after PCR by gel electrophoresis. In this regard, PCR products were applied to agarose gel and subjected to an electric field separating the DNA fragments according to their size. A band appeared on the gel when the targeted *Salmonella* DNA was present in the sample.

Out of 50 local chicken carcass samples, 16 (32%) isolates contained *Salmonella* spp. Of theese, 9 (18%) were confirmed by using PCR. Among 25 local chicken liver samples, the conventional method detected 6 (24%) *Salmonella* spp. isolates,while only 2 (8%) were confirmed using molecular detection. The highest prevalence of *Salmonella* isolates was found in the local chicken carcass, where 16 (32%) were detected using the conventional method and 9 (18%) were confirmed using the molecular method of detection.

On the other hand, the conventional method alone was able to detect only 1 (6.67%) isolate from 15 frozen chicken carcasses.It was also confirmed by PCR. Neither one of the methods was able to detect any *Salmonella* isolates in the 10 frozen chicken liver samples.

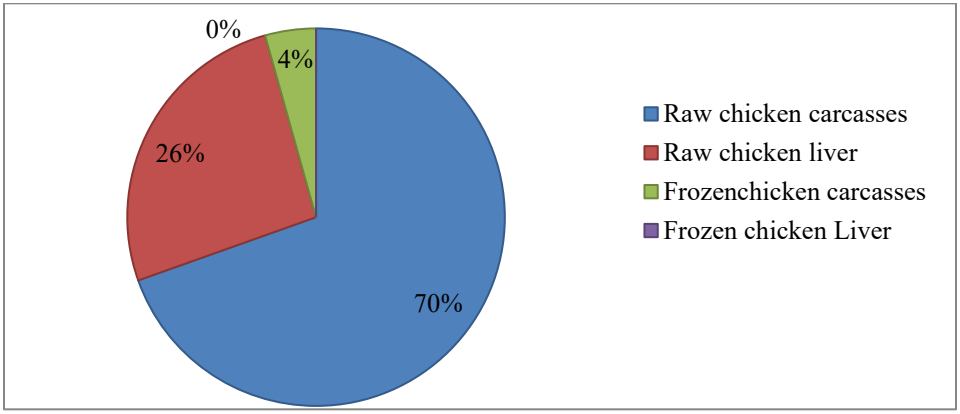

Figure 1. Prevalence of Salmonella Isolates in Different Categories of Chicken Meat and Liver Samples

The chart illustrates the prevalence of Salmonella isolates among raw

chicken carcasses(70%) raw chicken liver(26%) frozen chicken carcasses (4%) and frozen chicken liver (0%). The Results show higher contamination rates in raw samples as opposed to frozen ones, demonstrating the impact of freezing on the reduction of Salmonella burden.

3.2. Comparison of Conventional and Molecular Detection of *Salmonella* in Raw Chicken Meat

This section presents the findings obtained from conventional and molecular detection methods for *Salmonella* in both raw and frozen chicken meat. Local chicken carcasses and liver samples were positive with 22 (44%) cases when detected conventionally, thus giving a significance value of $p = 0.017$. When these 22 positive samples were further subjected to molecular detection, only 11 (50%) were found to be positive for *Salmonella* spp.

Among the frozen chicken carcass samples, only 1 (6.67%) positive sample was detectedthrough the conventional method, which was also confirmed by using the molecular methods, at significance level of p = 0.042. In frozen chicken liver samples *Salmonella* was not found using both methods.

3.3. PCR results of *Salmonella* isolates

These included 22 conventionally detected isolates from local chicken samples and 1 from frozen chicken samples that were subjected to PCR. Using a particular region of the invA gene, PCR amplified an amplicon size of 284 bp, confirming the existence of *Salmonella* in these samples. Electrophoresis results are shown below in Fig 2.

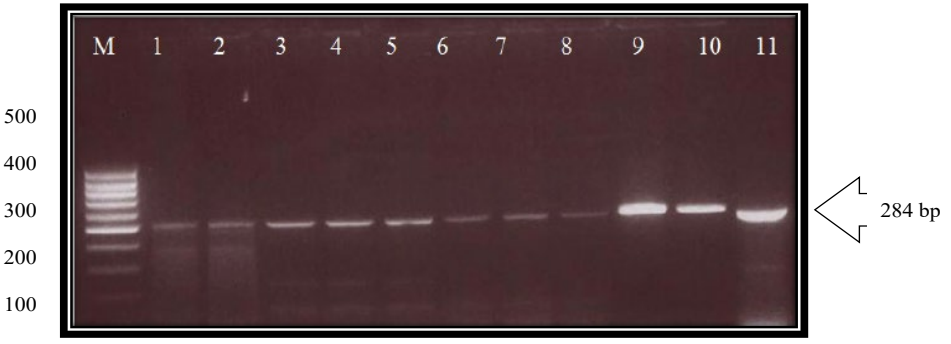

Figure 2. Gel Electrophoresis of PCR Products for Salmonella Isolates

from Chicken Samples

This figure represents the results of PCR amplification of *Salmonella* spp. DNA isolated from different chicken samples is visualized by using agarose gel electrophoresis. Lane M shows the molecular weight marker (DNA ladder) used to estimate the size of the PCR products. Lanes 1–11 correspond to different chicken samples; lanes 8, 9, and 10 have positive bands for Salmonella DNA at the expected size, indicating successful amplification of the target gene. The absence of bands in lanes 2–7 and in lane 11 suggests negative samples without *Salmonella*. In positive samples, bands correspond to an approximate molecular weight of 280bp,consistent with the targeted region for the detection of *Salmonella* spp.

3.4. Antimicrobials Resistance and Literature Review

A literature review was carried out to ascertain the effects of antibiotics on the growth of Salmonella. Generally, fluoroquinolones (such as ciprofloxacin) and cephalosporins (such as ceftriaxone) are administered to treat *Salmonella* infections. The bottlenecks, include, antimicrobial resistance which has been an increasing concern. Smith et al. in 2022 found that 40% of poultry isolates were resistant to ciprofloxacin, hence raising the need for alternative therapies besides reviewing and setting more stringent regulation of antibiotic use in poultry. Although antimicrobial resistance was not directly tested in the confirmed isolates in this study, literature suggests an upsurge of resistance that might complicate the treatment options for *Salmonella* infections and calls for better detection and prevention strategies [20].

4. DISCUSSION

The current study compared the prevalence of Salmonella in chicken carcasses with liver samples collected from local markets in Islamabad, Pakistan. Raw chicken samples were compared with frozen chicken samples. It was observed that a much higher prevalence of *Salmonella* contamination was obtained from local raw chicken samples, as compared to previously conducted studies from other regions [17]. For example, 32% of the local chicken carcasses were found positive for *Salmonella*, which is much higher as compared to 8% in Egypt and 5.5% in Algeria [18]. Such variation in contamination is due basically to the variation in slaughtering practices, hygiene conditions, and storing methods. Any insufficiency in

the local slaughtering environment and storage conditions can thus increase the microbial load in fresh meat products [19].

By using traditional methods, 24% of raw chicken liver samples tested positive for *Salmonella*. Chicken liver is often consumed with minimum cooking, and such a high prevalence raises alarm for causing foodborne illnesses. Indeed, only 8% of theese positive samples were confirmed by molecular methods.This may indicate the overestimation of prevalence by traditional methods or loss of cases due to low sensitivity [20].

Traditional methods also showed a low *Salmonella* prevalence in frozen chicken carcasses, with 1 sample testing positive at 6.67%, also confirmed by PCR.This is in line with the previous research that freezing can reduce bacterial proliferation due to which frozen chicken is less likely to harbor *Salmonella*. Freezing has been widely documented as the effective means of reducing bacterial contamination because it either slows down or puts metabolic activity to a halt, thereby limiting bacterial growth potential [21].

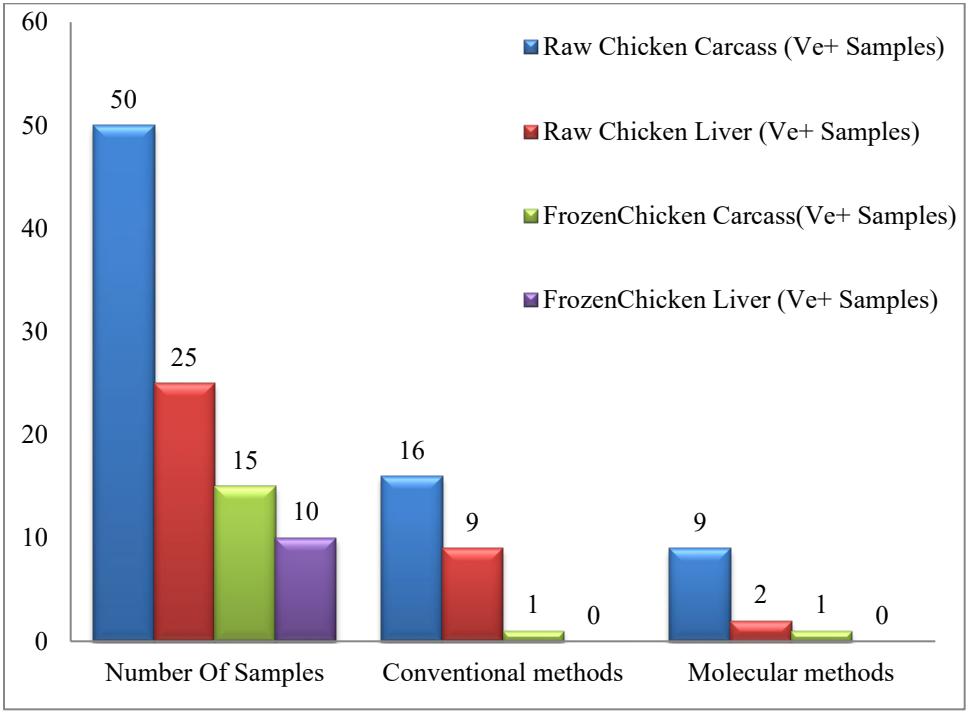

Figure 3. Comparative Microbial Load in Raw and Frozen Chicken Meat and Liver Samples Using Conventional and Molecular Methods

Conventional methods detected 44% positive *Salmonella* samples among local chicken samples, while molecular methods confirmed only about 50%, hence indicating a probable false positivity brought about by lower specificity levels of the conventional methods. Other studies have also revealed that molecular techniques are more sensitive for the detection of foodborne pathogens.

It has been documented through several literature reviews regarding the use of antibiotics, such as ciprofloxacin and ceftriaxone that antimicrobial resistance is increasing day by day [22]. Studies indicate that *Salmonella* isolated from poultry shows a lot of resistance to generally used antibiotics, which further complicates the treatment process. This again calls for the effectiveness of detection and prevention measures to keep *Salmonella* outbreaks in poultry products under control [23]. In the comparison between conventional and molecular methods for the detection of *Salmonella,* it was observed that molecular techniques,such as real-time PCR, gave more accurate and specific results.

4.1. Conclusion

Salmonella prevalence was significantly higher in local raw chicken samples than the frozen ones. Hygiene and proper storage practices can reduce contamination. Molecular detection techniques, especially real-time PCR, were found to be more specific than conventional methods. The use of such techniques is suggested to ensure food safety. It, therefore, becomes imperative that future efforts be channeled toward tighter hygiene levels along the poultry supply chain and the more general use of molecular detection method in order to limit as far as possible the risk of *Salmonella* contamination in chicken products. Additionally, attention should be paid to the problem of resistance to antimicrobials in *Salmonella* for the purpose of ensuring the effectiveness of treatment options in case of foodborne infections.

CONFLICT OF INTEREST

The authors of the manuscript have no financial or non-financial conflict of interest in the subject matter or materials discussed in this manuscript.

DATA AVALIABILITY STATEMENT

The data associated with this study will be provided by the

corresponding author upon request.

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