Isolation And Characterization Of Salmonella

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1. ABSTRACT

The species Salmonella belongs to the family Enterobacteriaceae. They possess interesting phenotypic/genotypic characteristics, history, and nomenclature compared to other bacteria within and outside the family. The organism is what causes typhoidal salmonellosis (TS) and non-typhoidal salmonellosis (NTS). TS and NTS are serious diseases that affect people and animals in different ways and are important for public health around the world. The objective of this study is to isolate Salmonella from chicken samples and to evaluate the drug sensitivity of the food-borne Salmonella and its susceptibility. This study reviewed the characteristics, taxonomy, nomenclature, and nature of Salmonella, as well as the confirmation and biochemical tests (indole test, MR-VP test, citrate test, antibiotic resistance test, gram staining, and ESBL test) for the identification and characterization of Salmonella. The study utilized baseline data and information from peer-reviewed journals, textbooks, and articles from international regulatory bodies. Salmonella exerts huge health and economic impacts due to their virulence or carriage of antibiotic resistance traits. To address these significant issues about public health, the availability of adequate information on the prevalence and antibiotic resistance patterns of Salmonella and the establishment of adequate measures to control contamination and infection are needed. Samples were collected randomly and processed for identification and antimicrobial susceptibility testing of Salmonella.The study's data showed that Salmonella is a Gram-negative rod that doesn't make spores and is negative for oxidase, indole, urease, lactose, and sucrose. It can also live without oxygen and move around. Salmonella is the cause of NTS and TS in men and animals presenting enteritis, septicemia, abortion, and meningitis. Salmonellosis outbreaks have been reported from food, food products of animal origin, and animal contact settings with cross-infection between humans and animals. The objective of this study was to identify types of antimicrobial resistance and their associated genes, isolated from chicken and beef and confirmed to be Salmonella, using standard techniques. Continuous research on Salmonella and salmonellosis should be carried out to contribute to the body of knowledge on the organism and disease and, ultimately, improvement of preventive/therapeutic strategies for the disease.

Keywords: Food-borne disease, Isolation, Biochemical test, Drug-sensitivity, Antibiotic- resistance
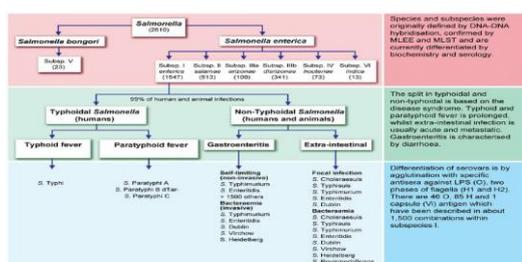
2. INTRODUCTION

1.1 Salmonella: Salmonella is a genus of rod-shaped bacillus gram-negative bacteria of the family Enterobacteriaceae. There are two species of Salmonella: Salmonella enterica and Salmonella bongori. Salmonella was named after Daniel Elmer Salmon, an American Veterinary Surgeon [1]. They are non-spore forming, predominantly with cell diameter of 0.7- 1.5 μm, length of 2-5 μm, and flagella, which help them to move [2]. They are Chemotrophs obtaining their energy from oxidation and reduction reactions using organic sources. They are also facultative anaerobes, capable of generating ATP with oxygen when it is aerobically available or using other electron acceptors anaerobically when oxygen is not available [2]. They are of two types:

a) Non-Typhoidal: These serotypes are zoonotic, meaning they can be transmitted between animals and humans as well as between humans. They typically target the gastrointestinal tract, causing salmonellosis, a condition that usually resolves without the need for antibiotics.

b) Typhoidal: Typhoidal serotypes are transmitted exclusively from human to human and are responsible for foodborne illnesses such as typhoid fever and paratyphoid fever. Typhoid fever occurs when Salmonella enters the bloodstream and spreads throughout the body, invading organs and releasing endotoxins [3].

Epidemiology: Non-typhoidal salmonellosis is a globally occurring disease affecting both humans and animals, with animals serving as the primary reservoir. Transmission is typically foodborne but can also occur through person-to-person contact. In contrast, the Salmonella strains responsible for typhoid and other enteric fevers are primarily spread between humans via the fecal-oral route and do not rely on animal reservoirs. Asymptomatic carriers, often referred to as "typhoid Marys," can also transmit the disease to others [4].

Nomenclature: The classification of the genus Salmonella has undergone significant changes since Kauffmann's original one-serotype-one-species concept, which was based on the serological identification of O (somatic) and H (flagellar) antigens [5]. Under this system, each serotype was treated as a distinct species (e.g., S. paratyphi A, S. Newport, and S. enteritidis), which would result in 2,463 species of Salmonella. Other approaches to classification have considered factors such as the clinical significance of a strain, biochemical characteristics that group serotypes into subgenera, and, more recently, genomic relatedness [6] .

1.2 Reason to isolate Salmonella: The major concern nowadays is the occurrence of multi-drug resistance among Salmonella in foodstuffs. It is presumed that the extensive use of antibiotics, especially in livestock production, may have resulted in the increasing incidence of antibiotic resistance in food-borne Salmonella [7]. This study was conducted to determine the presence of Salmonella in the chicken and beef and to characterize the antibiotic sensitivity of the isolates [8]. In addition, this study focused on the biochemical test for food-borne Salmonella showing drug resistance. Many investigations have been directed toward food-borne infections of Salmonella involving chicken samples.

1.3 Objectives of this study

a) To isolate Salmonella from raw chicken meat using standard techniques

b) To biochemically characterize the isolates

c) To check the isolates for their antimicrobial sensitivity

d) To check the isolates for their ESBL activity

1.4 Characteristics of Salmonella: Salmonella is a genus that includes 2,300 different species of bacteria. The most common types of salmonella are Salmonella enteritidis and Salmonella typhimurium, which account for half of all human infections [9].

a) Gram Test: The Gram test determines the composition of a bacterium's cell wall. Salmonella is gram-negative, which signifies high amounts of peptidoglycan, a mesh-like substance that provides structure and strength [10].

b) Oxygen Needs: Salmonella is a facultative bacterium. This means that it can survive with or without oxygen. Obligate bacteria, on the other hand, can only survive under specific conditions [10].000:00

c) Shape: Salmonella is a rod-shaped bacteria or bacillus. Unlike other strains of bacilli, however, salmonella does not produce spores [11].

d) Appearance on Agar: On blood agar: Salmonellae are grey/white, non-hemolytic, non-swarming colonies that range from 2 to 3 mm in diameter after 24 hours of incubation. On MacConkey: colorless colonies between 2 to 3 mm in diameter after 24 hours of incubation. On XLD: red-pink colonies from 2-3 mm in diameter at 24 hours, usually with black centers. Salmonella that do not produce H2S (e.g., most strains of S. paratyphi A) form red-pink colonies with no blackening [12]. A colony is a group of bacteria that are growing together.

e) Diseases: Salmonella causes two human diseases: Enteric fever, typhoid, and gastroenteritis. Doctors refer to both diseases as salmonellosis [13].

1.5 Salmonellosis and its spread: Salmonellosis is an infection caused by the bacteria Salmonella. It is also a food-borne disease and is defined as infectious or toxic in nature, caused by the ingestion of food. Salmonella infection is usually caused by eating raw or undercooked meat, poultry, eggs, or egg products or by drinking unpasteurized milk [14].

1.6 Types of salmonellosis

a) Non-typhoidal Salmonellosis (NTS):

Nontyphoidal salmonellosis (NTS) is the leading cause of acute bacterial gastroenteritis worldwide. In the United States alone, an estimated 1.4 million people are affected by salmonellosis annually [15]. Globally, NTS accounts for 1.3 billion cases of gastroenteritis and three million deaths each year. In 2013, the United Kingdom reported over 7,500 cases of human salmonellosis. Although the acute enteritis caused by Salmonella spp. is typically self-limiting, severe systemic complications can arise in some cases. The severity of infection is influenced by the specific Salmonella serovars involved and host-related factors.

b) Typhoidal Salmonellosis (TS):

Typhoidal salmonellosis is caused by specific Salmonella species, including Salmonella Paratyphi A, B, and C, which are responsible for illnesses like typhoid fever. These bacteria are typically transmitted directly from person to person. Typhoid fever is considered endemic in tropical and subtropical regions. Symptoms in infected individuals may include fever, headache, nausea, abdominal cramps, vomiting, and diarrhea. In severe or chronic cases, additional symptoms such as arthritis may develop three to four weeks after the initial onset of illness [16].

1.7 Symptoms, Prevention, Diagnosis and Treatment

Signs and symptoms of salmonella infection: The most common signs & symptoms of Salmonellosis are Diarrhea, Stomach(abdominal)cramps, Fever, Nausea, Vomiting, Chills, Headache, Blood in the stool [17].

Preventing Salmonellosis: To prevent salmonellosis, we should wash our hands, keep our food preparation areas clean, avoid unpasteurized foods, cook, and store our food at the appropriate temperatures, be careful when handling animals [18], use caution when swimming etc. Anyone can become infected with Salmonella. Age groups which are at high risk of infecting salmonella are Children younger than 5 years, Adults older than 65, People with weakened immune systems, such as people with HIV, diabetes, or undergoing cancer treatment [19].

Diagnosis: Salmonella infection is usually diagnosed based on signs and symptoms.Salmonella infection can be detected by testing a stool sample. However, most people have recovered from their symptoms by the time the test results return. If our health care provider suspects that we have a salmonella infection in our bloodstream, testing a sample of our blood for the bacteria may be needed [20].

Treatment: To treat Salmonellosis, we should drink more fluid to avoid dehydration.Treating dehydration: Because salmonella infection can cause dehydration, treatment focuses on replacing lost fluids and electrolytes — minerals that balance the amount of water in the body. If dehydration is severe, emergency room care or hospitalization may be needed so that fluids can be delivered directly into a vein [21].

Medications: In addition to drinking plenty of fluids, our healthcare provider may recommend Anti-diarrheal medications such as loperamide (Imodium A-D). These medications can help relieve diarrhea and cramps. However, they may also prolong the diarrhea associated with salmonella infection [21].

Antibiotics: Healthcare providers may prescribe antibiotics to kill the bacteria. These are usually given if your provider suspects that salmonella bacteria have entered your bloodstream, your infection is severe, or you have a weakened immune system. Antibiotics are not helpful in most cases of salmonella infection. Antibiotic treatment of salmonella infections aims to shorten illness and prevent serious complications. There are also concerns about increasing antibiotic drug resistance [22].

3. ANTIBIOTIC RESISTANCE OF SALMONELLA

Several multidrug-resistant Salmonella strains have been identified in beef, pork, and poultry products, posing a significant risk of global outbreaks. In poultry products, contamination can occur through various pathways, including infection during primary production (such as at the farm, incubator, or stock level), cross-contamination during food or byproduct handling, and consumption of undercooked poultry meat, eggs, or egg-based products [23]. These sources have all been linked to human Salmonella infections. Cases of salmonellosis involving antibiotic-resistant Salmonella strains have been associated with the overuse of antibiotics in human healthcare, veterinary practices, and animal production. Antibiotics have been used in livestock production since the 1950s, when it was discovered that adding small, sub-therapeutic doses of antimicrobials like penicillin and tetracycline to animal feed could promote growth and increase the weight of poultry, swine, and cattle [24].

4. MECHANISMS OF RESISTANCE IN SALMONELLA

There are two major molecular mechanisms involved in AR: intrinsic resistance and acquired resistance. Intrinsic resistance, also known as natural or innate resistance, is a natural property of certain bacterial species that makes them resistant to specific antimicrobial agents, whereas acquired resistance refers to the ability of bacteria to become resistant to antibiotics through genetic changes [25]. Antimicrobial resistance in Salmonella is mediated by several mechanisms that include drug inactivation, which is the most common cause of resistance. In this mechanism, antimicrobial agents are destroyed or inactivated through chemical modification using enzymes that catalyze reactions such as acetylation, phosphorylation, and adenylation. For example, enzymes such as penicillinase and chloramphenicol acetyltransferase are able to acetylate the ß-lactam ring of penicillin and some cephalosporins and the two hydroxyl groups of chloramphenicol, respectively [26].

5. NEED FOR SALMONELLA DETECTION

Non-typhoidal and typhoidal Salmonellosis are among the most common bacterial causes for the onset of acute febrile illnesses in developing countries. Early and accurate detection of *Salmonella* infection could be a lifesaver, as necessary and effective treatment can be provided to patients, thereby reducing the possibility of selective pressure that can contribute to the emergence of antimicrobial drug resistance [27]. Salmonella Paratyphi has been shown to be the major cause of enteric fever in countries like India and Nepal, where its prevalence is most significantly observed. The rapid detection of this foodborne pathogen in food processing industries as well as food markets is of pivotal importance to prevent Salmonellosis and to ensure the safety of consumers. Due to the long history of foodborne salmonellosis, the need for molecular and microbiological testing of food ingredients and produce has proved to be very significant. Multiple techniques for the detection of Salmonella have been developed. These include rapid methods as well as culture-based methods, some of these techniques involve samples directly taken from infected patients whereas others are from raw meat and poultry products [28].

6. EQUIPMENT, MATERIALS, and MEDIA: *The equipment, materials, and media needed for the experiment are given below in Table 1:*

Equipment	Materials	Media
Autoclave	Petri-plates	BPW (Buffered Peptone Water)
Stomacher	Pipette Tips	RVS (Rappaport-Vassiliadis)
Micropipette	Glass tubes	XLDA (Xylose Lysine Deoxycholate Agar)
Incubator	L-rods	BGA (Blue Green Agar)
Water Bath	Sterile inoculation loops	HEA (Hektoen Enteric Agar)
Laminar	Conical flasks	–

7. METHODS:

Sampling method : We collected two fresh raw chicken samples for this study from two different markets in the UAE. One sample used was minced, while the other was a breast piece.

Pre-enrichment and selective enrichment:

For the preparation of the isolate, 10g of minced raw chicken was homogenized using a sterile mortar and pestle. We then added the sample to a stomacher bag containing100 ml of buffered peptone water (BPW). The bag was then placed in an incubator for 24 hours at 37°C. After 24 hours, 1 ml of the sample was inoculated into a test tube containing 9 ml of RVS (Rappaport Vassiliadis Soy) broth. The tubes were then placed in an incubator for 24 hours at 37°C. In this way, the sample was enriched and ready for biochemical and microbiological analysis.

7.1 IMViC test

- **Indole test:** We inoculated a loopful of the culture medium into a test tube containing 10 ml of peptone broth. The tubes were then placed in an incubator for 24 hours at 37°C. Following the incubation period, we added 0.5ml (4-5 drops) of Kovac's reagent. The top layer of the medium was observed for a potential color change.

- **Methyl Red test:** The sample medium was allowed to equilibrate to room temperature prior to inoculation. We added 1ml of the previously inoculated culture into a test tube containing 9 ml of MR-VP broth and incubated it for 24 hours at 37°C. After the incubation period, 1 ml of the broth was aliquoted to a fresh test tube and incubated for another 24 hours. After incubation, 2 to 3 drops of methyl red indicator were added to the aliquot and observed for a color change.

- **Voges-Proskauer test:** The sample medium was allowed to equilibrate to room temperature prior to inoculation.We added 1 ml of the previously inoculated culture into a test tube containing 9 ml of MR-VP broth and incubated it for 24 hours at 37°C. Following 24 hours of incubation, we added an aliquot of 2 ml from the broth and incubated it for another 24 hours. Around 6 drops of 5% a-naphthol was added to the aliquot and mixed well for aeration. 2 drops of 40% potassium hydroxide were added and mixed well to aerate the medium. The tube was shaken vigorously for a 30-minute period and observed for a pinkish-red color to appear.

- **Citrate utilization test:** We streaked the citrate slants back and forth with a light inoculum picked from the center of a suspected salmonella colony. The slants were then incubated for 24 hours at 37°C. After the incubation period, the slants were observed for a color change.

7.2 Gram Staining

Gram staining was done using HiMedia K001-1KT Gram Stains Kit.We prepared the smear on a clean, grease-free glass slide using a loopful of a previously inoculated nutrient broth culture. The slide was air dried, and heat fixed.We poured a few drops of crystal violet stain on the smear and kept for about 1 minute, and then rinsed with distilled water. The smear was then flooded with Gram's Iodine and kept for a minute.We used Gram's Decolorizer to decolorize the smear until the blue dye no longer flowed from the slide. The slide was then washed with distilled water. The smear was then counter-stained with 0.5% w/v Safranin for 20 seconds and then rinsed with water. The smear was allowed to air dry and then examined under a microscope.

7.3 Antibiotic susceptibility testing (Disk diffusion)

The antimicrobial susceptibility of the salmonella isolates was determined by the Kirby-Bauer disk-diffusion method, following the Clinical and Laboratory Standards Institute (CLSI) recommendations [29]. For this study, we used 8 different antibiotics from 8 classes of antibiotics: Gentamicin (Aminoglycosides), Ceftazidime (Cephalosporins), Doxycycline (Tetracycline), Methicillin (Penicillin), Erythromycin (Macrolides), Vancomycin (Glycoproteins), Chloramphenicol (Chloramphenicol), and Sulfamethoxazole (Sulfonamides). 100 µL of the culture grown overnight in Nutrient broth was spread-plated onto Mueller-Hinton agar and kept for about 5 minutes for the culture to dry. Using a forceps, antibiotic disks were taken out from its capsule and carefully placed on the surface of the agar plate.We placed four antibiotic disks on each plate and then placed in an incubator for 24 hours at 37°C. The following day, we took out the plates and observed for zones of inhibition. The breakpoint for resistance or susceptibility interpretation to each antibiotic was in accordance with CLSI guidelines.

7.4 ESBL Resistance testing (Disk diffusion)

To test for ESBL resistance, *Salmonella* isolates were determined using the HiMedia ESBL testing kit, which relies on the disk diffusion method [30].The antibiotics used were Cefotaxime, Cefotaxime/ Clavulanic acid, Ceftazidime, and Ceftazidime/Clavulanic acid. 100 µL of the culture grown overnight in Nutrient broth was spread-plated onto Mueller-Hinton agar and kept for about 5 minutes for the culture to dry. Using a forceps, antibiotic disks were taken out from its capsule and carefully placed on the surface of the agar plate. Four antibiotic disks were placed on each plate and then in an incubator for 24 hours at 37°C. The following day the plates were taken out and observed for zones of inhibition. The breakpoint for resistance or susceptibility interpretation to each antibiotic was following CLSI guidelines [31].

8. RESULTS:

We recovered Salmonella spp. isolates from a variety of sources, including chicken and beef samples. We used different media for the culture of Salmonella, like XLD, BGA, EMB, and HEA. We got a large number of colonies from BGA(Chicken) and a very small number from XLD (Beef). Meat and poultry products are recognized as the major sources for transmitting Salmonella species to humans, with 40% of the clinical cases attributed to the consumption of egg and poultry products. In this study, the tests are done to confirm the presence of Salmonella or *Salmonella* contamination. We used the IMViC Tests (The term "IMViC" is an acronym for each of these tests. "I" is for indole test; "M" is for methyl red test; "V" is for Voges-Proskauer test, and "C" is for citrate test) for the confirmation. In the antimicrobial sensitivity test, isolated Salmonella strains were resistant to two or more of the major antimicrobial agent groups. However, most isolates were sensitive to ceftriaxone, ciprofloxacin, cefuroxime, and gentamicin. All isolates were resistant to erythromycin and amoxicillin.

8.1 Indole Test: *Salmonella formed light yellow rings in the test tubes that showed negative indole results. (Salmonella are indole negative)as shown in Figure 1 below:*

8.2 Methyl Red Test: *The test is considered positive if it turns red and negative if it remains yellow. We got a positive result as it turned red in color as shown in Figure 2 below:*


8.3 Voges Proskauer Test: *The test is performed by adding alpha-naphthol and potassium hydroxide to the Voges-Proskauer broth, which is a glucose-phosphate broth that has been inoculated with bacteria. A cherry red color indicates a positive result, while a yellow-brown color indicates a negative result. We got a negative result as it turned out yellow, as shown in Figure 3 below:*

8.4 Citrate Utilization Test: *When Simmons Citrate agar is inoculated with Salmonella, the medium turns royal blue. This is a positive result for the citrate test. When Simmons Citrate agar is inoculated with Escherichia coli, the medium remains green. This is a negative result for the citrate test. We got a positive result as it turned royal blue and was confirmed to be salmonella, not E. coli, as shown in Figure 4 below:*

8.5 Gram Staining: *Salmonella showed a Gram-negative result, as it showed a pink color as given in Figure 5 below:*
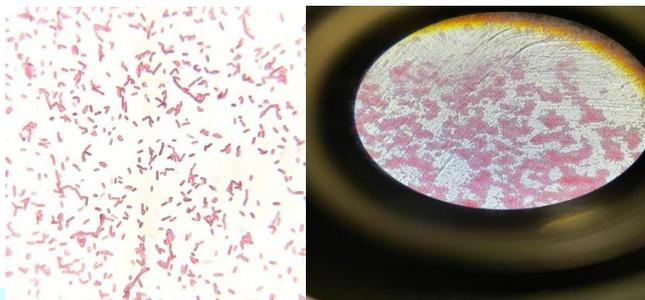
8.6 Antibiotic Susceptibility Test: *All results were compared with standards from the CLSI data sheet. We observed no ZOI, indicating that the samples were resistant to Methicillin, Sulfamethoxazole, and Vancomycin. Gentamicin had a ZOI of 19 mm which indicated that the sample was susceptible. Doxycycline had a ZOI of 7 mm which stated that the sample was still resistant. Cefotaxime had a ZOI of 22 mm, which noted that the sample was an intermediate. Chloramphenicol had a ZOI of 15 mm which noted that the sample was resistant. Erythromycin had a ZOI of 11 mm which indicated that the sample was susceptible, as shown in Figure 6 below:*

8.7 ESBL activity test: *All results were compared with standards from the CLSI data sheet. The ZOI for Cefotaxime was 21 mm; for Ceftazidime it was 16 mm, for Ceftazidime with clavulanate it was 15 mm, and for Cefotaxime with clavulanate it was 22 mm. This means that all of the ESBL antibiotics could kill the samples as shown in Figure 7 below:*

9. CONCLUSION:

All Salmonella isolates were highly sensitive to ciprofloxacin, gentamicin, and cefotaxime, respectively. Some isolates were resistant to erythromycin and gentamicin, respectively. We found varying sensitivity against methicillin, doxycycline, chloramphenicol, sulfamethazine, and vancomycin. Sample-wise antibiogram study revealed that all the isolates from chicken meat samples were 100% resistant to erythromycin. Large zones of inhibition indicate that the organism is susceptible, while no zone of inhibition indicates resistance. No ZOI was shown by two isolates, which means the organism was resistant to that drug. Out of all, some isolates were found to have ZOI equal to or more than 2.0, thus indicating the organism is susceptible to that drug. Some isolates were found to have ZOI more than or equal to 1.0, and some were found to have ZOI less than 1.0. Hence, the present study reveals that Salmonella isolates from chicken meat are resistant to more than one antibiotic, indicating the presence of multidrug-resistant Salmonella in foods of animal origin.

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