# Current diagnostic techniques primarily used in veterinary diagnostics of *Salmonella*

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## Abstract

This review paper briefly overviews various methods used to detect and identify *Salmonella*, a common foodborne zoonotic pathogen in veterinary medicine. *Salmonella* bacteria is mainly found in food products and, when ingested, causes severe gastrointestinal symptoms. Due to the ongoing presence of pathogenic Salmonella in food production systems, it poses a serious public health threat. Therefore, a constant need remains to enhance identification and detection methods capable of identifying this pathogen and preventing outbreaks. The conventional and widely-used culture-based method can be considered the gold standard. However, it is time-consuming and laborious, as is traditional serotyping by slide agglutination. The enzyme-linked immunosorbent assay (ELISA) offers faster detection with higher specificity by identifying *Salmonella* antigens or antibodies. However, some laboratories prefer other methods due to limited sensitivity and the time it takes to establish an immune response. Therefore, methods like subtyping or advanced molecular techniques have evolved over the years: polymerase chain reaction (PCR)-based methods and next-generation sequencing (NGS) provide rapid and accurate identification of *Salmonella*. Unlike PCR-based methods that target specific genes, NGS provides a sequence of complete genomes. Mass spectrometry and Fourier transform infrared spectroscopy are also used, while biosensors are still in the early stages of technological development. This paper discusses the progress of identification and detection approaches for *Salmonella*, emphasising their basic principles, applications, and performances, as well as the advantages and disadvantages of each method.

**Key words:** *Salmonella; food; zoonotic pathogen; detection methods; public health*

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## Introduction

*Salmonella enterica, Campylobacter coli* and *Campylobacter jejuni, Listeria monocytogenes, Staphylococcus aureus,* and *Escherichia coli* are some of the pathogenic microorganisms that cause foodborne illnesses and pose a serious global threat to human health and the economy. *Salmonella enterica* is particularly dangerous and can be lethal. Hence, the urgent development of rapid and precise bacterial detection methods is critical to ensure food safety (Lee et al., 2024).

*Salmonella* spp. belongs to the rod-shaped Gram-negative bacteria that is a part of the family *Enterobacteriaceae*. It contains two main species, *Salmonella enterica* and *Salmonella bongori*. There are more than 2600 serotypes of *Salmonella* spp., all of which can cause disease in humans (Alakomi and Saarela, 2009; Jasim et al., 2019; Shen et al., 2021). *S. enterica* is considered one of the most important sources of human gastroenteritis globally (Kuhn et al., 2012; Dos Santos et al., 2019). Two types of salmonellosis infections are humanly important, typhoid and nontyphoid salmonellosis, where the former can produce a severe and potentially fatal systemic illness known as typhoid fever (Saini et al., 2019). Typhoid fever, caused by *S. enterica* subsp. *enterica* serovar Typhi (*S.* Typhi) and *S.* Paratyphi A, B and C are transmitted between humans through contaminated water or food. In contrast, nontyphoid salmonellosis is caused by a large variety of different zoonotic serovars. The infection is primarily (though not exclusively) transmitted by ingestion of contaminated food products of animal origin (e.g., meat, milk, and eggs). Infection symptoms manifest as acute self-limiting gastroenteritis, with possible post-infection complications such as septicaemia, reactive arthritis, or aortic aneurysms (Kuhn et al., 2012; Cinti et al., 2017).

The five predominant *Salmonella* serovars, as stated in the European Union One Health Zoonoses Report for the year 2023, that were a cause of human infections were distributed as follows: *S.* Enteritidis (70.8%), *S.* Typhimurium (8.9%), monophasic *S.* Typhimurium (1,4,[5],12:i:-) (5.1%), *S.* Infantis (2.0%) and *S.* Coeln (0.77%). In addition, the report showed a total of 77,486 confirmed cases of human salmonellosis, corresponding to a European Union notification rate of 18.0 cases per 100,000 population. Additionally, 88 deaths were recorded in 2023. Most concerning was the significant increase in the overall number of reported human cases and hospitalisations in 2023 compared to 2022, highlighting the situation's urgency. On the positive side, the report showed a slight decrease in foodborne outbreaks in 2023 compared to the previous year.

In Croatia, 1269 human salmonellosis cases were reported, at a rate of 33.0 cases per 100,000 population. The number of confirmed cases also increased compared to the previous year according to the European Union One Health 2023 Zoonoses Report.

The growing incidence of *Salmonella*-related infectious diseases has become a significant financial burden for many developing nations as they struggle with the costs of treatment, prevention, and public health campaigns. Additionally, the extensive variety of *Salmonella* serotypes and the frequent changes in infection patterns caused by new strains and growing antibiotic resistance have increased concern among researchers and the public. Detecting *Salmonella* is crucial for monitoring food safety in the supply chain of animal products. Further, methods for detecting *Salmonella* have advanced from traditional culture-based techniques of *Salmonella* serotyping to molecular detection approaches, including polymerase chain reaction (PCR), enzyme-linked immunosorbent assay (ELISA), pulse-field gel electrophoresis (PFGE), multiple loci sequencing typing (MLST), and whole genome sequencing (WGS) (Tan et al., 2022.). This advancement is driven by the need for fast, precise and reliable detection methods for *Salmonella*, particularly in food emergency response laboratories, but

also the need for outbreak monitoring of the most pathogenic strains (Paniel and Noguer, 2019; Tan et al., 2022).

Numerous comprehensive and more focused reviews have been published in recent years on different aspects of *Salmonella* and other foodborne pathogen detection methods for food and feed, including both culture and non-culture-based methods. Therefore, the current review aims to provide a complete review of the most common detection methods for *Salmonella* detection in veterinary medicine (Ricke et al., 2018).

## Conventional culture methods

The culture-based *Salmonella* detection method, the gold standard for S*almonella* detection, is globally accepted and forms the basis of various detection methods in food safety analysis and public health laboratories (Tan et al., 2022). The International Organization for Standardization has approved and standardised this method for identifying *Salmonella,* in which the bacteria are grown on specific differential agar media, enabling the identification of colonies isolated from the agar (Bell et al., 2016; Awang et al., 2021).

The method follows step-by-step enrichments in addition to increasing selectivity by isolating *Salmonella* on selective-differential agar plates (Bell et al., 2016). The series of steps begins with a pre-enrichment step in a nonselective liquid medium, like buffered peptone water, to encourage the growth of any *Salmonella* present. The next step is subculturing into two selective enrichment broths, such as Rappaport Vasiliadis Soy broth (RVS) and Muller-Kauffmann Tetrathionate-Novobiocin (MKTTn) broth, which prevent the growth of background flora (Eriksson and Aspan, 2007). Subsequently, samples are inoculated onto at least two selective differential agar media, such as Brilliant Green Sulfa (BGS), Bismuth Sulfite (BS), Brilliance™ *Salmonella* Agar, Xylose Lysine Deoxycholate (XLD), or Xylose Lysine Tergitol-4 (XLT-4), which facilitate the growth of *Salmonella* and help differentiate them from other microbes. The final step involves confirming the presumptive positive *Salmonella* colonies with biochemical or other detection methods (Love and Rostango, 2008; Lee et al., 2015) (Figure 1).

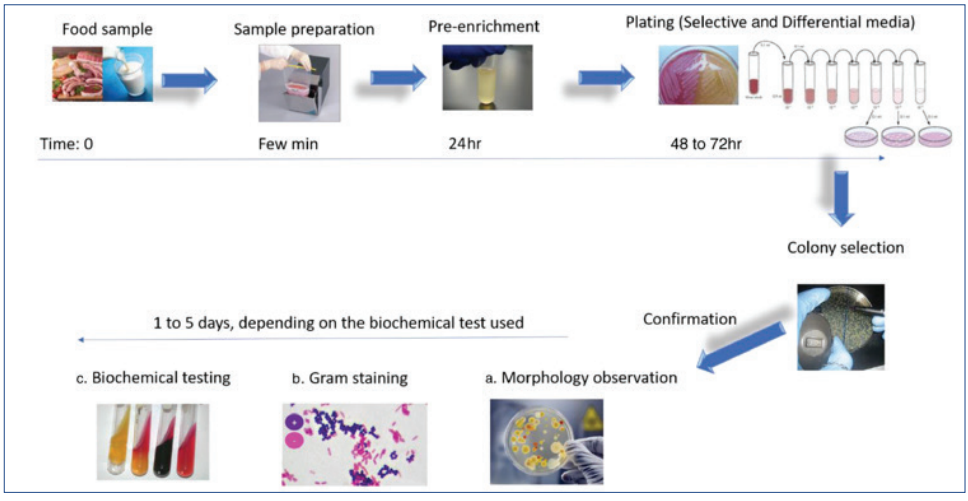


**Figure 1.** Procedure steps for *Salmonella* identification, following the conventional culturing and plating methodologies (Ferone et al., 2020).

The conventional culture method offers ease of use, high sensitivity, reliability, and, most importantly, cost-effectiveness as an advantage. However, these culture-based methods involve significant time-consuming enrichment steps followed by selective plating and confirmatory methods. This makes the method not ideal for products with an inherently short life, e.g., salad, eggs or ready-to-eat foods that are minimally processed (Vinayaka et al., 2019). Additionally, the competitive presence of *Proteus* in samples poses a significant risk of false positive results. Other disadvantages that need to be mentioned are its labour-intensive nature, the risk of microbial contamination, and the presence of viable but non-culturable bacteria (Tan et al., 2022).

## Immunology-based methods

### *Enzyme-linked immunosorbent assay*

The enzyme-linked immunosorbent assay (ELISA) is the most widely used immunology-based method for detecting *Salmonella* antigens through antibody-conjugated enzymes in food (Lee et al., 2015; Rhode et al., 2017; Lin et al., 2020). Additionally, the assay can detect and quantify a wide range of materials, including proteins, hormones, glycoproteins, and antibodies (Awang et al., 2021). The solid matrix used in this highly accurate assay contains immobilised mono- or polyclonal antibodies. When a somatic or flagella antigen binds to the antibody, generating an antigen-antibody complex, the concentration of the antigen and the presence of *Salmonella* can be measured through the colour change caused by the enzymatic cleavage of a chromogenic substrate (Awang et al., 2021; Tan et al., 2022).

ELISA is remarkably versatile, not only for identifying *Salmonella* in food and food-producing animals but also for detecting antibodies to develop vaccines against *Salmonella* infections (Park et al., 2014; Tan et al., 2022). ELISA for detecting antibodies to *Salmonella* provides an empathetic and economical approach for screening large groups of animal flocks or herds for signs of a previous or current *Salmonella* infection. Therefore, these veterinary tests are not used for diagnosis of disease in individual animals, but rather applied as tools in control and surveillance programmes (Khun et al., 2012; Guyassa and Dima, 2022).

The benefit of ELISA is that it provides results in less than two days, which is far faster than traditional culture methods that can take a week (Lee et al., 2015; Tan et al., 2022). Further advantages of ELISA is that it is well-suited for processing large sample volumes and it can offer greater specificity than conventional c ulture techniques, as noted by Park et al. (2013). As a result, ELISA has been commercialised in various kits for the laboratory testing of poultry, cattle, and pigs across Europe and the USA (Kuhn et al., 2012; Park et al., 2014; Lee et at., 2015; Zadernowska and Chajęcka-Wierzchowska, 2016).

Nevertheless, ELISA has its limitations. Although it is generally reproducible, it has certain drawbacks, such as limited sensitivity. Therefore, any positive result for pathogens is considered presumptive and requires further confirmation (Hyeon et al., 2020; Tan et al., 2022). An essential limitation of this approach is that the immune response of the specific animal is not raised until 1 to 2 weeks following the occurrence of infection (Guyassa and Dima, 2022). Other drawbacks are reduced antibody affinity for the pathogen, prolonged enrichment time to increase the number of target microorganisms to detectable levels, and the potential for impurities to affect the results (Lee et al., 2015; Tan et al., 2022). Another challenge is cross-reactivity (Hyeon et al., 2020). Because of these requirements, the assay takes longer to complete, overall limiting the methods' capacity to deliver same-day analysis. However, employing more specific protocols, such as competitive, double-sandwich, and fluid-phase ELISA, can help

address issues with false signals commonly associated with ELISA assays (Tan et al., 2022).

## Phenotypic methods

### *Serotyping by slide agglutination (Kauffmann-White-Le Minor scheme)*

The White-Kauffmann-Le Minor scheme, a phenotype-based approach, is used to differentiate *Salmonella* serovars based on the agglutination of bacteria with specific antisera to identify variants of the somatic (O), flagellar (H) and capsular (Vi) antigens (Wattiau et al., 2011; Ranieri et al., 2013). This scheme, based on serotyping, identifies over 2600 serovars by combining 64 somatic serogroups and 114 flagellar antigens, with the O antigen being a component of the lipopolysaccharide on the bacterial surface and the H antigen related to flagellar proteins (Wattiau et al., 2011; Yan et al., 2023). A single cell can express multiple O antigens simultaneously on its surface. In contrast, most *Salmonella* bacteria, despite having two different versions of the gene for the flagellar protein, phase I H-antigen (H1) and phase II H-antigen (H2), uniquely express only one flagellar protein at a time. Therefore, serovars expressing one flagellin type are called monophasic, and those with two flagellar antigen types are designated diphasic. In rare cases, *Salmonella* is triphasic or quadriphasic (Wattiau et al., 2011; Shi et al., 2015). In specific serovars, such as Typhi and Dublin, a capsular antigen known as Vi functions as a factor associated virulence (Jansen et al., 2011; Shi et al., 2015).

*Salmonella* serotyping consists of taking the bacteria from an agar plate and mixing it with a series of polyvalent and monovalent antisera that specifically target various O (somatic) and H (flagellar) antigens (Toro et al., 2016). The serotype of the *Salmonella* isolate can be determined based on the agglutination patterns (Vibbert et al., 2015; Yan et al., 2023). Hence, the serotyping process results in an antigenic formula composed of three components: the first position represents the O antigens, while the second and third indicate the two distinct flagellin H antigens. These components are separated by colons, formatted as O:H1:H2. For instance, the

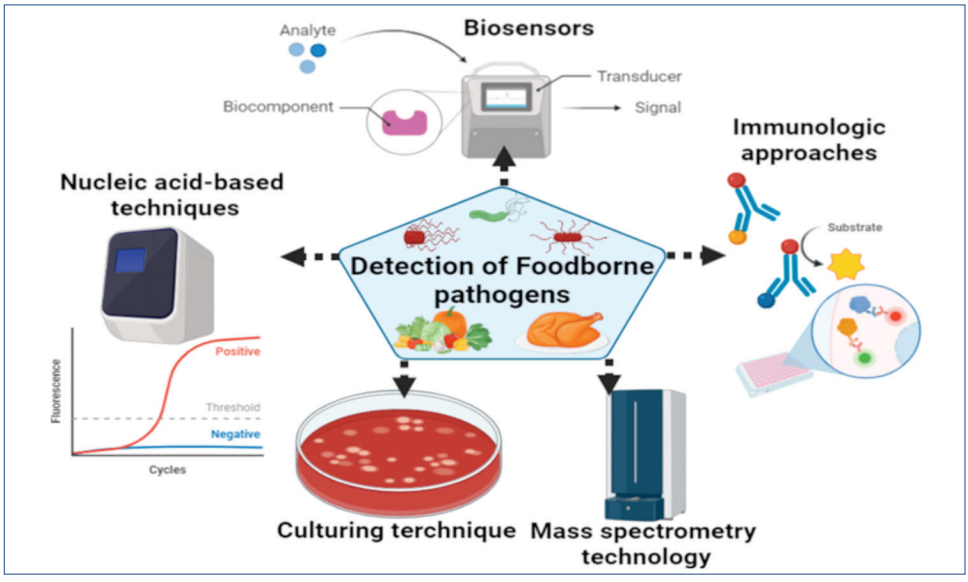


**Figure 2.** Brief overview of detection methods for foodborne pathogens (Elbehiry et al., 2023).

antigenic formula for *S.* Typhimurium is represented as 1,4,[5],12:i:1,2 where 1,4,[5] describes the O antigen factors, and 12 the flagellar H antigen 1 (1st phase), and the flagellar H antigens 1 and 2 (2nd phase) (Banerji et al., 2020). Although traditional serotyping is used widely, it does have several limitations. The method is exclusively based on phenotypic characteristics. Therefore, it does not provide sensitive fingerprints that are necessary for tracing during an outbreak and does not define phyletic relationships. It is also complex, resource-intensive, and requires over 250 antisera and 350 antigens, which are expensive. Also, it is labour-intensive and requires well-trained personnel. Another issue is that sometimes autoagglutination and loss of antigen expression occur, which may lead to strain untypeability (Wattiau et al., 2011; Ranieri et al., 2013; Shi et al., 2015). False-positive reactions may also occur due to nonspecific and weak agglutination (Schrader et al., 2008). These challenges have driven interest in developing reliable molecular, immunological or other alternative approaches that align with the White-Kauffmann-Le Minor scheme (Wattiau et al., 2011; Shi et al., 2015) (Figure 2).

## Molecular-based methods

### *Polymerase chain reaction (PCR)*

In recent years, remarkable developments in molecular biology have greatly improved the detection of *Salmonella.* Different types of polymerase chain reaction (PCR), like quantitative PCR (qPCR) and multiplex PCR (mPCR), are now preferred methods because they are faster, and more sensitive and accurate (Bell et al., 2016; Zhao et al., 2017; Dos Santos et al., 2020). These are also called nucleic acid amplification techniques, which operate by enzymatically amplifying specific DNA segments *in vitro*, enabling the production of thousands to millions of copies from a single DNA sequence within hours, thereby substantially reducing the time and labour required for detection (Afzal et al., 2015; Bell et al., 2016; Dos Santos et al., 2020; Lin et al., 2020). PCR targets specific *Salmonella* genes, with the *invA* gene most commonly used. However, gene targets such as *ttrRSBCA, sipBC, stn, fliC-d*, and *hilA* have also been used (O'Regan et al., 2008; Zhou and Pollard, 2010; McCabe et al., 2011; Bell et al., 2016). PCR is widely considered the benchmark for bacterial identification and diagnostic applications. The reason behind this is its reliability, excellent accuracy, and precise detection results (Fenollar and Raoult, 2004). The PCR method consists of three stages: the denaturation of the double-stranded DNA, the annealing of primers to target sequences, and the extension of the new DNA strand by polymerase enzyme. The result is visualised through gel or capillary electrophoresis (Zaeroska and Chajęcka-Wierzchowska, 2017).

PCR creates double-stranded nucleic acid from two single-stranded molecules that are complementary to each other under specific chemical and physical conditions. When done *in vitro*, this process is called hybridisation. One of the strands that is to serve as a probe or primer is produced in the laboratory, and it is the determinant factor for the specificity of PCR methods (Jasson et al., 2010; Hyeon et al., 2020).

Simultaneous amplification of more than one locus in a single reaction is required. This technique is known as multiplex PCR, where several primers are used in a single reaction tube to amplify nucleic acid fragments from various targets. Therefore, it is widely used in the identification of multiple *Salmonella* serovars in different food matrices (Afzal et al., 2015; Chin et al., 2017; Farahani et al., 2022).

Real-time PCR, also referred to as quantitative PCR (qPCR), is a PCR technique in which the target sample can be detected quantitatively in real time (Lin et al., 2020). The present methodology employs a fluorescent

technique called SYBR green and TaqMan dyes to detect the target DNA and bacterial cells over time. The binding of these dyes to the DNA groove during the amplification of the double-strand DNA increases the luminous intensity (Park et al., 2012; Vinayaka et al., 2019; Parker et al., 2020; Ruan et al., 2020). Therefore, qPCR is extensively employed to identify *Salmonella* in a range of food, poultry, and veterinary commodities. Recently, multiplex real-time PCR techniques have been implemented to detect more than two gene sequences in a single reaction to assist in food safety inspection (Abubakar et al., 2007; Heymans et al., 2018; Azinheiro et al., 2020; Lin et al., 2020; Awang et al., 2021).

PCR techniques, notably real-time PCR, have a more significant potential for faster detection time with greater accuracy than the conventional culture approach (Fenollar and Raoult, 2004). Still, specific issues persist with nucleic acid diagnosis (Awang et al., 2021). Challenges in detecting *Salmonella* in food matrices can include the need for costly equipment and skilled staff due to the requirement for thorough DNA purification before amplification. Also, detecting low levels of *Salmonella* in complex food samples can be demanding and can often require pre-enrichment steps for cultures. The enrichment process can introduce biases that complicate the identification process and prolong the detection timeline. This consists of native microbiota competing with *Salmonella* and antimicrobial metabolites that interfere with the process, leading to the rise of nonspecific DNA. It is suggested that an internal amplification control must be included in each PCR reaction to ensure its accuracy based on the criteria outlined by Abubakar et al. (2007) and Bell et al. (2016). In addition, PCR-based assays cannot differentiate between live and dead cells, since DNA from both viable and non-viable bacteria can be amplified, potentially leading to false-positive results. Therefore, upon receiving positive PCR findings, validating the positive result using a culture-based method is crucial. However, PCR techniques are typically more sensitive than culture techniques, so pathogens identified by PCR but not cultured (referred to as false positives) may be genuine positives (Bell et al., 2016; Zaeroska and Chajęcka-Wierzchowska, 2017).

Addressing these limitations is essential to improving the reliability and efficiency of detecting *Salmonella* in food and environmental samples. This will lead to better food safety and public health outcomes (Bell et al., 2016; Awang et al., 2021).

### *Loop-mediated isothermal amplification*

Another molecular-based method developed to detect nucleic acid targets of *Salmonella* rapidly is Loop-mediated isothermal amplification (LAMP), by Notomi and colleagues in 2000. Using a specific stem-loop structure based on auto-cycling strand displacement and isothermal conditions (60 to 65°C), LAMP is a rapid, isothermal nucleic acid amplification method with excellent specificity, rapidity and efficiency that can produce up to $10^9$ copies of target DNA in an hour (Notomi et al., 2000; Hara-Kudo et al., 2005; Techathuvanan et al., 2010; Zhao et al., 2017; Yang et al., 2018). Using a unique DNA polymerase (Bst) and four or six primer sets, LAMP can recognise six different target areas in DNA (Cheung and Kam, 2012; Yang et al., 2015; Li et al., 2021). In 2005, Hara-Kudo et al. published a report on the first LAMP assay that targeted *Salmonella*, and since then, other new *Salmonella* LAMP assays have been established. It is more sensitive than alternative methods, making it a viable method for clinical diagnostics and applications related to food safety, particularly in laboratories with limited resources (Cheung and Kam, 2012; Zhao et al., 2017). Numerous techniques, such as agarose gel electrophoresis, real-time turbidity monitoring, electrochemical methods, and lateral flow dipstick (LFD),

can be used to track LAMP results (Zhao et al., 2017). According to earlier research, the visual detection method of LFD offers benefits such as accuracy, speed, long-term stability, ease of use, without the need for expensive laboratory equipment, making it the recommended approach that has garnered significant interest (Rigano et al., 2014; Zhao et al., 2017). Zhao et al. (2017) used a visual LAMP-LFD method that targets the *Salmonella* siiA gene and obtained 100% specificity in a dairy food model. Furthermore, recent studies by Mei et al. (2019) and Vinayaka et al. (2022) have shown that LAMP had greater sensitivity in detecting *Salmonella* spp. compared to PCR.

As with every other method, LAMP has its limitations. The challenge is selecting the appropriate target for amplification. This is due to the involvement of 4 to 6 primers to target multiple regions within a small segment of the target sequence. LAMP also increases the risk of carryover contamination, leading to false positive results in negative controls. Also, the determination of LAMP reaction results using turbidimetric and colourimetric methods is subjective and produces a ladder pattern, making target identification by band size impossible (Wong et al., 2018).

## Molecular Typing methods

### *Pulsed-field gel electrophoresis (PFGE)*

Pulsed-field gel electrophoresis (PFGE) and other restriction fragment length polymorphism (RFLP) techniques are robust DNA isolation and analysis methods. PFGE, in particular, is a standout method that separates large DNA fragments (up to 2000 kb) using alternating electric fields, providing highly detailed DNA fingerprints of the whole bacterial genome (Wattiau et al., 2011; Hyeon et al., 2020). It is utilised by the PulseNet program, a global laboratory network encompassing 86 countries, for monitoring foodborne bacterial diseases and detecting extensive outbreaks of bacterial foodborne illness (Hyeon et al., 2020; Li et al., 2021). Microbes are first embedded in agarose plugs to perform PFGE and then treated with enzymes and detergents to release their DNA. Afterwards, the agarose plugs are thoroughly washed to eliminate any residual debris. The purified DNA is digested using rare restriction enzymes specific to each microorganism. The most commonly used restriction enzymes in *Salmonella* detection have been *XbaI*, *SpeI* and *NotI* (Lukinmaa et al., 2004; Hyeon et al., 2020). The agarose plugs are subsequently placed onto an agarose gel for electrophoresis. During this process, the polarity of the current is periodically altered, enabling separation of DNA fragments. The gel is then stained with a fluorescent dye, and images are captured for further analysis, which ultimately reveals the unique fingerprint of the specific strain (Sikarwaw and Nashid, 2015).

PFGE offers a significant advantage over PCR-based serotyping because it prevents mechanical shearing of DNA and provides a genetic profile based on the entire genome, unlike PCR, which targets specific genes (Sikarwaw and Nashid, 2015). Due to its reproducibility and cost-effectiveness, its unique ability to fingerprint strains in outbreak situations makes it an invaluable tool, offering practical and efficient solutions in critical situations (Wattiau et al., 2011). Studies have shown PFGE's effectiveness and high discriminatory power in typing *Salmonella* from foods, food animal sources, and human patients (Nayak and Stewart-King, 2006; Foley et al., 2009).

Despite its advantages, PFGE is labour-intensive and time-consuming, with analyses taking between 1 and 5 days. It demands highly skilled staff and requires expensive equipment and reagents. Also, reproducibility requires rigorous protocol standardisation (Shi et al., 2015). An essential limitation of PFGE is its inability to distinguish closely related strains, such as *S.* Typhimurium versus *S.* 4,5,12:i:, due to the minimal influence of genetic

variation on the electrophoretic mobility of a restriction fragment (Foley et al., 2007). Also, a single serovar can show a high level of PFGE diversity, such as polyphyletic serovar *S.* Newport (Wiedmann and Nightingale, 2009; Achtman et al., 2012; Hyeon, 2020).

While PFGE remains the gold standard for *Salmonella* molecular typing, further studies are clearly necessary to refine its predictive capabilities. This highlights the ongoing need for scientific exploration and development, particularly for polyphyletic serovars and those with significant diversity (Yoshida et al., 2016).

### *Multi-locus sequence typing (MLST)*

MLST is a widely used bacterial typing method that analyses the genetic relationship between strains based on polymorphisms from seven housekeeping genes (*i.e., arcC, aroE, glpF, gmk, pta, tpi, and yqiL*) with low genetic variability (Ben-Darif et al., 2010; Estrada et al., 2019; Yan et al., 2023). It involves obtaining sequences of internal fragments of seven housekeeping genes for each strain of a particular species and comparing them with previously identified sequences (alleles) at that locus. The combination of these alleles defines the allelic profile of the strain, and each different allelic profile is assigned as a sequence type (ST), which is used to describe the strain (Aanensen and Spratt, 2005).

A robotic sequencer that sequences these predetermined housekeeping genes offers high discriminatory power and robust data analysis with minimal human effort. In contrast to PFGE, it has higher reproducibility between laboratories (Kotetishvili et al., 2002; Zou et al., 2016). Therefore, the method is considered the gold standard for long-term (global) epidemiology. Sometimes, housekeeping genes fail to distinguish strains that have recently undergone genetic changes due to their low rate of genetic variability. Virulence genes are then often used instead. This approach, known as multi-virulence-loci sequence typing (MVLST), provides a more practical alternative (Sikarwar and Nashid, 2015).

MLST has limitations as a serovar predictor, particularly for serovars with a common ancestor or polyphyletic serovars, which may result in identical MLST types across different serovars (Alikhan et al., 2018). Additionally, the method is expensive, labour-intensive and offers limited discrimination because it only analyses genetic variation in a small portion of the genome (housekeeping genes), unlike PFGE (Tien et al., 2011). Therefore, this method lacks the resolution needed for epidemiological tracing (Alikhan et al., 2018). Despite this, MLST offers advantages such as high reproducibility and access to *Salmonella* databases (such as http://pubmlst.org/databases.html, http://mlst.ucc.ie/mlst/dbs/Senterica, http://www.mlst.net) for sequence comparison and serovar prediction, and valuable insights into phylogenetic relationships (Achtman et al., 2012). Unlike traditional serotyping, which might group unrelated isolates under the same serovar, MLST can distinguish evolutionary groups, leading to more accurate phylogenetic distinctions (Shi et al., 2015).

### *Sequencing methods*

Next-generation sequencing (NGS), or whole genome sequencing (WGS), allows rapid sequencing of complete genomes within bacterial pathogens using advanced automated and parallelized genome sequencers (Bell et al., 2016). Due to improvements of high-throughput sequencing, it has also become possible to attain high resolution down to the level of a single nucleotide. As a result, WGS has emerged as an economically viable alternative to traditional typing methods in public health surveillance and outbreak detection (Ashton et al., 2016). It is important to highlight that the goal in foodborne detection is not only the ability to detect specific pathogens

occurring in low numbers in foods, but also to distinguish subtle strain genetic differences for improved tracking to original sources, either during outbreak investigations or more routine analysis during food processing (Park et al., 2014).

In a single test, this technology provides comprehensive information about species, serovar, virulence, pathogenicity, antimicrobial resistance, bacterial subtypes and more. Such is the case with WGS in the clinical sector, since it produces high-quality sequence data that enables the better identification of clinical strains, relating the same to outbreak strains and ascertaining its virulence and resistance genes (Oakeson et al., 2018). Additionally, WGS revolutionised genomics and gave the researcher the ability to study gene expression, the host-pathogen interaction, and the route of disease transmission within a population (Ahrenfeldt et al., 2017). WGS has been widely employed in mutation detection, elucidation of *Salmonella* and other microbial genetics, and the assessment of strain evolution during outbreaks, while also providing genetic interrelatedness data. Other significant applications of WGS include outbreak cluster identification and phylogeny inference from sequencing reads. This will facilitate rapid serotyping and detection of single nucleotide polymorphisms (SNPs) within the genome that epidemiological investigations can use to link cases of illness with standard sources. The incorporation of WGS into public health laboratories will increase the speed and accuracy with which outbreaks are detected and investigated, providing more accurate and timely information for the public health response (Yachison et al., 2017; Ibrahim and Morin, 2018). Validation studies are vital to ensure that WGS is robust and has adequate technical performance that can be released for application in pathogen surveillance, such as *Salmonella* (Taylor et al., 2015; Ibrahim and Morin, 2018).

WGS is increasingly replacing current molecular subtyping methods due to its high-resolution capabilities and enhanced discriminatory power, especially demonstrated in studies on *Salmonella*. Tools like SeqSero exemplify the shift towards WGS-based serotype determination (Zhang et al., 2015). The availability of WGS data from over 155,509 *S. enterica* isolates has been instrumental in global outbreak tracking and investigation (Hu et al., 2021).

There are several established genome sequence databases available such as the NCBI genome database (https://www.ncbi.nlm.nih.gov/genome/), CFSAN-FDA (https://github.com/FDA/open.fda.gov) and other public domains such as GenomTrakr (https://www.fda.gov/food/science-research-food/wholegenome-sequencing-wgs-program) (Awang et al., 2021).

In addition, NGS can be used in food microbiology in two ways to determine the whole genome sequence of a single cultured isolate (e.g., a bacterial colony, a virus, or any other organism), which is commonly referred to as "whole genome sequencing", or "metagenomics," where NGS is applied to a biological sample generating sequences of multiple (if not all) microorganisms in that sample (Grutzke et al., 2019; Ferone et al., 2020).

As WGS continues to advance, its integration into laboratories promises to enhance outbreak detection and investigation, offering more accurate and timely information to guide the public health response. However, validation studies remain essential to ensure the robustness and technical performance of WGS, particularly for its application in surveillance (Li et al., 2021). Despite being very informative methods, their main drawback is the amount of bioinformatic work required to analyse the data obtained from this kind of method and the general lack of personnel with competence in this area (Ferona et al., 2020).

## Mass-spectrometry based methods

### *MALDI-TOF*

Mass spectrometry (MS) is a crucial area of research that is employed to identify bacterial isolates based on expressed proteins. For detection, a protein spectrum is obtained and compared to a reference database of bacterial protein spectra to identify the isolate (Quainoo et al., 2017). This is achieved by creating consistent mass fingerprints by the measurement of protein and/or lipid masses from intact cells or extracts (Bell et al., 2016). The system offers rapid bacterial identification in outbreaks of foodborne diseases, allows for monitoring of water quality, performs tests to determine antibiotic susceptibility and resistance, aids in the prompt diagnosis of infectious diseases, and helps in the identification of biomarkers, making it an excellent screening method (Bailey et al., 2013; Cheng et al., 2016).

Matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF MS) is the most commonly used mass spectrometry technique for analysing bacterial strains (Ferone et al., 2020; Yang et al., 2021). The instrument is designed to be easily operated by users and has a low cost per sample. It is capable of quickly and reliably identifying a colony within a minimum of 10 minutes from the time it is selected (Bell et al., 2016).

The result of a MALDI-TOF MS analysis is a distinctive spectrum known as a peptide mass fingerprint (PMF). The primary proteins utilised for identification in this analysis are ribosomal proteins, which make up around 60 to 70% of the microbial cell's dry weight, in addition to a few housekeeping proteins (Ferone et al., 2020).

The spectra (signals) obtained from MS analysis are compared by using scoring algorithms to match the reference spectra stored in the open reference database, which contains PMFs of well-verified bacteria. This comparison allows for the precise identification of the desired genus or species, as long as it exists in the spectral library (Cheng et al., 2016; Ferone et al., 2020; Mangmee et al., 2020). Each matched spectrum result is a potential identification with a confidence score. Scores below 1.7 are unreliable, 1.7 to 1.9 indicate probable genus identification, 2.0 to 2.29 indicate confident and valid genus identification, and 2.3 to 3.0 indicate extremely confident species identification (Cheng et al., 2016).

Determining the species of *Salmonella* using mass spectrometry (MS) is simple and accurate when examining a pure colony. However, the job of identifying *Salmonella* subspecies and serovar levels using MALDI-TOF MS still poses a significant challenge and is rarely studied (Bell et al., 2016; Mangmee et al., 2020). Mangmee et al. (2020) highlighted that there are no currently commercially accessible databases or data analyses that can accurately identify the specific serovar of *Salmonella*. Therefore, they developed a MALDI-TOF MS-based technique to simultaneously detect non-typhoidal *Salmonella* (NTS) in the Thai broiler sector, and were successful in accurately identifying NTS at both the species and subspecies levels. The serovar classification was achieved with 99.3% accuracy. Also, Yang et al. (2021) successfully discriminated three different *Salmonella* serovars, Enteritidis, Typhimurium, and Thompson, which contributes to the screening of *Salmonella* serovars.

MALDI-TOF-MS is a rapid technique that is more efficient and cost-effective since it involves fewer chemicals, processes, and prior information compared to traditional approaches. Nevertheless, there are certain drawbacks associated with it, including a significant initial capital investment and the requirement for a reference spectral database, which is different among different manufacturers and different scoring algorithms (Ferone et al., 2020). Another

limitation of MALDI-TOF analysis is the inability to distinguish closely related species. Also, only cultivable microorganisms can be recognised, and therefore, bacteria need to be isolated on agar. Another constraint is the databases utilised by manufacturers, as they employ various reference spectral databases and scoring algorithms upon which the result is directly dependent. Despite these restrictions, MALDI-TOF has high sensitivity and resolving power for detecting ionised molecules and can analyse any cultivable organism and its metabolites without prior knowledge (Cheng et al., 2016; Ferone et al., 2020).

## IR spectroscopy method

Fourier Transform Infrared spectroscopy (FTIR) is a powerful, non-destructive analytical technique with high discriminatory power that is widely used in chemistry and microbiology for identifying and discriminating bacteria at different taxonomic levels, from genera down to strain levels (Kim et al., 2006; Campos et al., 2018; Cordovana et al., 2022). The method measures the unique infrared absorption spectra of the chemical composition and structure of the pathogen, which generates distinctive fingerprints that reflect the biomolecular content of the cells, including lipids, proteins, nucleic acids, and polysaccharides, thereby facilitating the identification and differentiation of different pathogens. Consequently, each bacterium has very particular infrared absorption patterns that are linked to genetic information (Sundaram et al., 2012; Novais et al., 2018; Burckhardt et al., 2019; Martak et al., 2019; Cordovana et al., 2021). Moreover, this quick and low-cost spectroscopic technique gained more interest with the recently available system, IR Biotyper, which allows bacterial typing in routine bacteriology laboratories (www.bruker.com/applications/microbiology/strain-typingwith-ir-biotyper/features).

FTIR is a rapidly expanding research area, especially in investigating *Salmonella* microorganisms. Since *S. enterica* has a high antigenic diversity and varying clinical relevance, it is an ideal candidate to study with FTIR spectroscopy. The substantial carbohydrate diversity of O-units and the varying lengths of somatic antigens significantly affect the cell surface structure and allow the distinction using the FTIR technique. Using multivariate analysis and various bacterial collections, several research groups examined this method of differentiating *S. enterica* serotypes (Preisner et al., 2010; Cordovana et al., 2022). However, Novais et al. (2018) concluded that the differentiation of *S. enterica* serogroups, serotypes, and phage types by FTIR was not always considered successful.

There are several advantages of using FTIR techniques in the food industry, diagnostic laboratories, and public health authorities. It is a rapid, straightforward, and susceptible technique. The method demands little sample preparation and can analyse samples in diverse formats (Sundaram et al., 2012) (Figure 3).

The technique offers comprehensive insights into the composition of bacterial cells and can measure the cell count or the presence of specific functional groups. Unlike PCR methods, FTIR effectively discriminates between live and dead cells based on their spectral difference in the cell wall, cell membrane, cytoplasm, etc. (Sundaram et al., 2012).

Furthermore, it can recognise and differentiate microorganisms according to their physiological condition and also provides information about bacterial metabolism, growth phase, and antibiotic resistance. It is essential to consider that environmental factors can have an impact on the spectra. Additionally, when dealing with complex samples, there is a possibility of overlapping spectra. In such cases, it is recommended to use

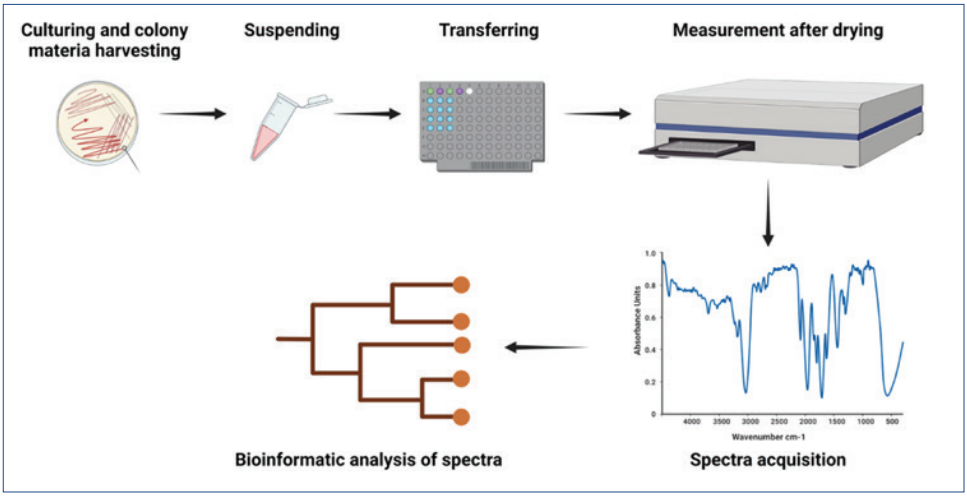


**Figure 3.** Workflow of sample processing protocol for FTIR analysis (Muchaamba and Stephan, 2024).

a comprehensive spectral library for accurate identification. Furthermore, it is necessary to ensure standardisation, meticulous data collection, and expertise in chemometric analysis (Davis and Mauer, 2010).

## Biosensors

Biosensors are simple analytical systems that detect *Salmonella* spp. in foods. They work by converting a biological signal or response into a quantifiable and processible signal (Bhalla et al., 2016; Awang et al., 2021; Tessaro et al., 2022). In recent years, researchers have gained more interest in biosensor detection methods due to their rapidity, high sensitivity and specificity, portability as a small device, and real-time detection (Paniel and Noguer, 2019; Tan et al., 2022).

A biosensor consists of biorecognition elements (enzymes, antibodies, aptamers, cells, antigens, etc.), transducer components (optical, electrochemical, mass-based, etc.), and the electronic systems needed to display the measurable signal (Kumar et al., 2019; Tan et al., 2022). The BRE, often called a bioreceptor, is a biologically derived molecular recognition molecule that interacts with the analyte of interest (Eijkelkamp et al., 2009). The transducer converts the analyte bioreceptor interaction into a measurable signal that is proportional to the analyte concentration in the sample. Third, the electronic system amplifies the transduced signal, processes it, and displays the output result digitally (Kumar et al., 2019). Among all types of biosensors, which are classified based on the bioreceptor, antibodies, aptamers, bacteriophages, antimicrobial peptides (AMPs), and nucleic acid probes are most common for *Salmonella* recognition (Shen et al., 2021; Wang et al., 2021).

In the food business, biosensors have become an up-and-coming tool for identifying *Salmonella*, providing prompt and precise outcomes. However, despite significant advancements, they have yet to meet all the stringent requirements necessary for widespread commercial application. Current biosensors are expected to detect a single bacterium in small sample volumes, and they should be designed to discriminate between bacterial species and other microorganisms or cells, as well as between strains of the same

species. Furthermore, the method should be able to distinguish between viable and non-viable cells and function without the need for pre-enrichment. However, achieving these specifications continues to be a difficult task (Bahadir and Sezginturk, 2015; Fang et al., 2018; Paniel and Noguer, 2019).

Limitations such as high costs, detection limits, the complex nature of the matrix, and the inability to identify more than one pathogen or toxin concurrently have hindered their routine use in food microbiology (Velusamy et al., 2010; Neethirajan et al., 2018). Nonetheless, biosensors for detecting *Salmonella* in the food sector are now in their early stages of development. However, they are rapidly gaining attention because of their potential for practical use and economic success (Shen et al., 2021; Mahari and Gandhi, 2022).

Despite their potential for *Salmonella* screening, most biosensors are now limited to detecting a single serotype (*S.* Typhimurium). Their capability to identify other serovars has not yet been proven. Therefore, it is essential to choose a biological recognition element that can effectively interact with the most standard *Salmonella* serotypes found in food (Cinti et al., 2017).

All in all, biosensors offer a faster, quantitative alternative and could be integrated into a food safety surveillance system (Bhalla et al., 2016). When combined with technologies like RFID, GPS, and cloud platforms, biosensors can provide real-time, trackable data throughout the supply chain, enhancing food safety and reducing economic losses. Additionally, incorporating biosensor data into dynamic risk assessment models can improve early warning systems for *Salmonella* contamination. The future of biosensors lies in coupling with artificial intelligence (AI), enabling real-time data collection and predictive analytics to prevent outbreaks, thus significantly advancing food safety systems (Shen et al., 2021).

## Conclusion

Food contaminated with *Salmonella* presents a significant risk to human health and is responsible for a considerable number of gastrointestinal illnesses worldwide. Therefore, *Salmonella* is a critical food safety issue, which led to a rise in the development of rapid detection methods and is still developing from year to year (Wang et al., 2021).

Methods like microbiological culturing or serotyping by slide agglutination are time-consuming and labour-intensive. Additionally, they are not specific enough to detect and characterise *Salmonella* at the strain level. Due to these limitations, there was a need for the development of more rapid, sensitive, and accurate detection methods. Techniques such as immunological and molecular assays have significantly reduced detection times and improved sensitivity. Because of that, they are considered practical alternatives in the food safety industry. Emerging technologies, such as biosensors and FTIR, have gained increasing interest due to their potential for rapid, on-site detection (Paniel and Noguer, 2019; Wang et al., 2021; Tan et al., 2022; Oslan et al., 2024).

The data produced by WGS will be necessary for the development of new typing strategies and the optimisation of traditional typing methods, which are essential to the future prevention of the spread of *Salmonella* infection. Therefore, the direction of the development of detection methods is moving towards automation, cost-saving, and time-saving network integration (Parker et al., 2022).

Intervention strategies like controlling *Salmonella* from farm to fork are crucial. Effective communication between veterinary organisations and healthcare providers is essential for exchanging knowledge. Also, to control *Salmonella* spread and antibiotic resistance, international collaboration

is needed. Recommendations not only include developing strategies to minimise antimicrobial resistance, but also involving public, animal, and animal health communities, providing rapid detection methods, and maintaining overall hygiene (Cheung et al., 2012; Al-Ansari et al., 2021; Yang et al., 2024).

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# Trenutne dijagnostičke metode koje se primarno koriste u veterinarskoj dijagnostici *Salmonella*

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Navedeni pregledni rad pruža sažet pregled različitih metoda korištenih za dokazivanje i identifikaciju bakterije *Salmonella*, uobičajenog zoonotskog patogena u veterinarskoj medicini. *Salmonella* bakteriju uglavnom nalazimo u proizvodima hrane, koja kada se konzumira, uzrokuje ozbiljne gastrointestinalne simptome. Zbog stalne prisutnosti *Salmonella* u sustavima proizvodnje hrane, predstavlja ozbiljnu prijetnju javnom zdravstvu. Stoga, postoji stalna potreba za unapređenjem metoda identifikacije i dokazivanja, sposobnih za prepoznavanje ovog patogena u svim fazama unutar prehrambenog sustava. Konvencionalna metoda uzgoja bakterije je široko korištena i smatra se zlatnim standardom. Međutim, ona je vremenski zahtjevna i mukotrpna, kao i tradicionalno serotipiziranje putem aglutinacije na predmetnom stakalcu. Enzimski imunotest (engl. enzyme-linked immunosorbent assay, ELISA), temelji se na prepoznavanju antigena ili antitijela *Salmonella* te nudi brže vrijeme detekcije i veću specifičnost. Međutim, zbog ograničene osjetljivosti i vremena potrebnog za uspostavljanje imunološkog odgovora, neki laboratoriji radije koriste druge metode. Metode poput subtipizacije ili naprednih molekularnih tehnika su se razvile tijekom godina. Metode temeljene na lančanoj reakciji polimerazom (engl. Polymerase chain reaction, PCR) i sekvenciranje sljedeće generacije (engl. next generation sequencing, NGS) omogućuju brzu i točnu identifikaciju *Salmonella*. Za razliku od PCR metoda koje ciljaju specifične gene, NGS pruža informacije cijelog genoma. Masena spektrometrija i Fourierova transformacijska infracrvena spektroskopija (FTIR) isto su u uporabi, dok su biosenzori još uvijek u ranoj fazi tehnološkog razvoja. U radu se raspravlja o napretku metoda identifikacije i dokazivanja bakterije *Salmonella*, s naglaskom na njihove osnovne principe, primjenu i performanse te prednosti i mane svake metode.

**Ključne riječi:** *Salmonella, hrana, zoonoza, metode dijagnostike, javno zdravstvo*