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Rapid detection of *Salmonella* species using molecular and nanotechnology techniques

A thesis presented by

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ABSTRACT

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Key words: poultry products, meat products, *Salmonella* Enteritidis, *Salmonella* Typhimurium, virulence genes *fliC*, *spvA*, *invA* genes, PCR , gene sequencing, Gold nanoparticles.

Bacteriological examination of 400 samples (200 poultry products and 200 meat products) revealed the isolation of 26 *Salmonella* belonging to 8 different *Salmonella* serovars. The most common serovars were *Salmonella* Typhimurium (8 isolates), *Salmonella* Enteritidis (7 isolates), *Salmonella* Kentucky (2 isolates) and *Salmonella* Virchow (2 isolates). Other serovars typed were *Salmonella* Heidelberg (4), *Salmonella* Haifa (1), *Salmonella* Anatum (1), *Salmonella* Farsta (1). All isolates of *Salmonella* Enteritidis and *Salmonella* Typhimurium were examined for the virulence genes *spvA* and *fliC* genes respectively. Molecular biology techniques Direct PCR as well as non-functional gold nanoparticles probe were used for direct detection of two serovars (*S.Typhimurium* and *S.Enteritidis*) in food samples.

1. Introduction

Salmonella is a genus in the family *Enterobacteriaceae* which are Gram-negative, oxidase negative, catalase positive, nonspore forming rods. They are also facultative anaerobes. Almost all *Salmonella* species are motile via peritrichous flagella (Lopes et al., 2016).

Genus *Salmonella* consists of over 2700 members known as serovars, and only a few serovars are responsible for causing the majority of human diseases caused by this pathogen. *Salmonella* infection has been associated with the consumption of raw and undercooked poultry and other meat products (Gallegos-Robles et al., 2008).

Among the pathogens, *Salmonella* is considered the most prevalent foodborne pathogen worldwide (Sánchez et al., 2011 and Carrasco et al., 2012) and has long been recognized as an important zoonotic microorganism of economic significance in animals and humans, predominantly in the developing countries.

Salmonella enterica serovar *Typhimurium* and *Salmonella enterica* serovar *Enteritidis* are the most frequently isolated serovar from foodborne outbreaks throughout the world (Herikstad et al., 2002).

Foodborne pathogens are a major threat to food safety, especially in developing countries where hygiene and

Sanitation facilities are often poor. *Salmonella enterica* are among the major causes of outbreaks of foodborne diseases **(Ahmed and Shimamoto 2014b)**. It is estimated that *Salmonella* causes 93.8 million human infections and 155,000 deaths annually worldwide **(Hendriksen et al., 2011)**. Therefore, sensitive and rapid detection of *Salmonella* is of out-most importance in the field of food safety, biothreat prevention and public health.

In recent years, salmonellosis has increased considerably both in incidence and severity. Efforts to prevent and control this disease are important because of many reported human cases and thousands of deaths every year. **(World health organization 2007)** .

Illnesses from food are one of the most important economic and health problems among industrial and non-industrial countries. In recent years, *Salmonella* has been one of the most common causes of food born disease **(Busani et al., 2006)**.

Salmonella infections in humans often result from the ingestion of contaminated foods, such as poultry, beef, pork, eggs, milk, seafood, and fresh produce **(Braden 2006; CDC 2006 and Meldrum and Wilson 2007)**. Direct contact with animals also results in transmission of *Salmonella* to humans **(Acha and Szyfree 2001 and Bagcigil et al., 2007)**.

Contamination of meat with *Salmonella* in slaughterhouses occurs through excretion of animals, which have no symptoms, contamination of equipment, floor and personnel's (**Molla *et al.*, 2003**). The pathogens can survive in the meat until presented to the market (**Kimura 2005**).

The detection of *Salmonella* therefore remains a highly important issue in microbiological analysis for food safety and standards (**Hadjinicolaou *et al.*, 2009**), and to guide clinicians in the diagnosis of enteric pathogens (**Jansen *et al.*, 2008**).

Salmonella spp. in foods can be detected by various methods such as conventional bacteriological culture (**ISO 2003**) ,serological assays (**Barrow. 1994**) , polymerase chain reaction (PCR) (**Bennett *et al.*, 1998**) , and more recently, real-time PCR methods (**Szmolka *et al.*, 2006**).

The traditional *Salmonella* detection includes pre-enrichment, selective enrichments and plating on selective agar media followed by biochemical and serological tests which requires 5 to 7 d for completion and is time consuming, labour intensive and costly to meet food safety control in routine food analytical laboratories (**Wang *et al.*, 2008; Okamura *et al.*, 2009 and Techathuvanan *et al.*, 2010**).

Due to the relatively high prevalence of *Salmonella* spp. in meat and poultry as well as the high incidence of disease caused by these microbes, a rapid, sensitive and reliable method

for detection is of urgent need in the food industry to combat further foodborne outbreaks **(Bhagwat 2003; Fratamico 2003 and Hein *et al.*, 2006)**. **(Myint *et al.*, 2006)** advocate that new methods of detection should be standardized rapid, sensitive and specifically suitable for identifying naturally occurring contamination in food products.

During the progressive development of the PCR over the last two decades, this method became one of the central techniques for nucleic acid analyses and is the most widely used method for pathogen detection. **(Karami *et al.*, 2011)**.

PCR can be used to amplify genes specific to taxonomic groups of bacteria and also detect genes involved in the virulence of foodborne bacteria **(Finlay *et al.*, 1988; Bej *et al.*, 1994 and Naravaneni and Jamil 2005)**.

The virulence determinant genes of *Salmonella* spp. are associated with a combination of either chromosomal or plasmid factors. These genes have a role in adhesion, invasion, and enterotoxin production **(Oliveira *et al.*, 2003 and Das *et al.*, 2012)**.

Gold nanoparticles have been implemented for the detection of pathogens, which contaminate food, water, and hospital surfaces **(Khanna 2008; Agasti *et al.*, 2010; Bunz and Rotello 2010; Tallury *et al.*, 2010; Azzazy *et al.*, 2012; Saha *et al.*, 2012 and Upadhyayula 2012)**. Gold nanoparticles are

extremely successful at detecting pathogens due to their ability to provide a simple and rapid color change when their environment is altered (**Verma *et al.*, 2015**).

The aim of this study is rapid detection of *Salmonella* species using molecular and nanotechnology techniques:

1. Isolation of *Salmonella* species from poultry and meat products.
2. Identification of isolates by biochemical and serological methods.
3. Using of Direct-PCR with *invA* primers for identification of *Salmonellae* from different food samples substrate.
4. Application of ultrasensitive detection of *Salmonella* isolates non-functional gold nano probe as a rapid diagnostic tool for *Salmonella invA*.

2. Review of literature

2.1. *Salmonella* organism

OIE (2005) provided that the genus *Salmonella* belongs to the family *Enterobacteriaceae*. *Salmonella* are facultative anaerobic Gram-negative rods. They are non-spore forming, usually motile with peritrichous flagella, capable of growing on ordinary media. They are pathogenic to man and animals.

Lin-Hui et al. (2006) said that *Salmonella* is a genus of the family *Enterobacteriaceae* and comprises a large and closely related population of medically important pathogens. It has long been associated with a wide spectrum of infectious diseases, including typhoid fever and nontyphoid salmonellosis, which cause public health problems worldwide.

Bhunia (2008) explained that among all foodborne diseases, *Salmonella* is identified as a leading cause of foodborne illness in human and animals resulting in 16 million annual cases of typhoid fever, 1.3 billion cases of gastroenteritis and 3 million deaths worldwide.

Andreas et al. (2009) reported that *Salmonella* is a gram-negative, facultative anaerobic, flagellated bacterium. It is the pathogenic agent of salmonellosis, a major cause of enteric illness and typhoid fever, leading to many hospitalizations and a few rare deaths if no antibiotics are administered.

Dilmaghani et al. (2011) and Tahergorabi et al. (2012) recorded that salmonellosis is a major public health problem, so recognition of its serotypes can improve prevention and control of food-borne diseases. *Salmonella* is a kind of non-spore forming rod, a gram negative and facultative anaerobe that can ferment glucose belonging to the *Enterobacteriaceae* family.

Pui et al. (2011) estimated that *Salmonellae* are non-fastidious as they can multiply under various environmental conditions outside the living hosts. They do not require sodium chloride for growth, but can grow in the presence of 0.4 to 4%. Most *Salmonella* serotypes grow at temperature range of 5 to 47°C with optimum temperature of 35 to 37°C but some can grow at temperature as low as 2 to 4°C or as high as 54°C. They are sensitive to heat and often killed at temperature of 70°C or above. *Salmonellae* grow in a pH range of 4 to 9 with the optimum between 6.5 and 7.5.

Atyabi et al. (2012) found that salmonellosis is a direct occupational anthrozo zoonotic disease of great economic and public health concern, which continue to be responsible for large numbers of infections in both humans and animals worldwide especially in developing and industrialized countries.

Andino and Hanning (2015) stated that *Salmonellae* are facultative anaerobic Gram-negative rod shaped bacteria generally 2–5 microns long by 0.5–1.5 microns wide and motile

by peritrichous flagella. Genome sizes of *Salmonella* vary among serovars with ranges from 4460 to 4857 kb. *Salmonellae* belong to the family *Enterobacteriaceae* and are a medically important pathogen for both humans and animals.

Cosby et al. (2015) reported that bacteria of the genus *Salmonella* are Gram-negative, facultatively anaerobic, nonspore forming, usually motile rods (peritrichous flagella) belonging to the *Enterobacteriaceae* family, which are associated with the alimentary tract of animals. *Salmonellae* reduce nitrates to nitrites, carbon dioxide and hydrogen gases are usually produced from D-glucose, and hydrogen sulfide is typically produced by most *Salmonellae*.

2.2. *Salmonella* classification:

Murray et al. (1995) concluded that the Kauffman and White classification scheme is a system that classifies the genus *Salmonella* into serotypes, based on surface antigens. It is named after Philip Bruce White and Fritz Kauffmann. First the "O" antigen type is determined based on oligosaccharides associated with lipopolysaccharide. Then the "H" antigen is determined based on flagellar proteins.

Chloe et al. (2004) recorded that *Salmonella* is a gram-negative, facultative, intracellular bacterium that invades the mucous membrane and is spread primarily by oral transmission. *Salmonella* express flagellar (H), polysaccharide (O) and

capsular (Vi) antigens which determine strain pathogenicity and therefore variation of these antigens has formed the basis for *Salmonella* serotyping. The Kauffmann-White scheme, first published in 1929, divides *Salmonella* into more than 2500 serotypes according to their antigenic formulas.

Shelobolina et al. (2004) founded that *Salmonella* nomenclature has changed many times and still is not stable. The genus *Salmonella* was previously differentiated into two species: *Salmonella enterica* and *Salmonella bongori*. However, a new species, *Salmonella subterranean* was identified and validated.

Fluit (2005) proved that the species *Salmonella enterica* (*S. enterica*) is further divided into the six subspecies *S. enterica* subsp. *enterica*(I), *S. enterica* subsp. *salamae*(II), *S. enterica* subsp. *arizonae*(IIIa), *S. enterica* subsp. *diarizonae*(IIIb), *S. enterica* subsp. *houtenae*(IV), and *S. enterica* subsp. *indica*(VI). Formerly, *S. bongori* was the subspecies V, but later considered as a separate species.

Su & Chiu (2007) revealed that the *Salmonella enterica* is one of two *Salmonella* species (*enterica* and *bongori*) and a member of the *Enterobacteriaceae* family. *Salmonella enterica* spp. is subdivided into 6 subspecies *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV) and *indica* (VI).

Bhunia (2008) reported that historically *Salmonella* had been named based on the original places of isolation such as *Salmonella* London and *Salmonella* Indiana. This nomenclature system was replaced by the classification based on the susceptibility of isolates to different selected bacteriophages which is also known as phage typing.

Pui et al. (2011) explained that Kauffmann-White scheme classifies *Salmonella* according to three major antigenic determinants composed of flagellar H antigens, somatic O antigens and virulence (Vi) capsular K antigens. This was adopted by the International Association of Microbiologists in 1934.

Omoigberale et al. (2014) pointed out that the genus *Salmonella* comprises over 2,700 serotypes that are found in different hosts and environments that can cause human illness such as enteric fever, gastroenteritis and septicemia. *Salmonella* spp. are the most pathogenic bacteria associated with a variety of foods.

2.3 *Salmonella* Enteritidis and *Salmonella*

Typhimurium:

Herikstad et al. (2002) provided that *Salmonella enterica* serovar *Typhimurium* and *Salmonella enterica* serovar *Enteritidis* are the most frequently isolated serovar from foodborne outbreaks throughout the world.

Jay et al. (2003) and Wallis (2006) found that *Salmonella* Typhimurium have a broad host range, with an ability to infect a wide range of animals and humans.

Gillespie et al. (2005) , Thong et al. (2011) and Ngoi and Thong (2013) concluded that *Salmonella* Enteritidis pathogen is the most common causative agent of non-typhoidal salmonellosis in Malaysia, which commonly was isolated from food and animal sources , retail poultry samples with raw or undercooked poultry meat and eggs existed as a great threat for infection in humans.

Plym & Wierup (2006) reported that *Salmonella* Enteritidis and *Salmonella* Typhimurium are the most important serovars those are transmitted from animals to humans. *Salmonella* Enteritidis has become the most common cause of salmonellosis in humans. It is usually transferred by contaminated food of animal sources (meat, poultry, eggs, milk) or vegetables contaminated by manure and water.

Arshad et al. (2007) pointed out that *Salmonella enterica* serotype Typhimurium (*Salmonella* Typhimurium) and *Salmonella enterica* serotype Enteritidis (*Salmonella* Enteritidis) are the main serovars responsible for foodborne gastroenteritis.

Ochiai et al. (2008) reported that *Salmonella* serotype Typhi is human specific and when found in food indicates handling and

hygiene failures. Furthermore, the serotypes Enteritidis and Typhimurium can be isolated both in chickens before slaughter and in humans infected by foodborne diseases.

Jordan et al. (2009) revealed that *Salmonella* serotypes Typhimurium and Enteritidis alone represent 73.9% of the clinical isolates. The problem with conventional detection methods is that they are laborious and time consuming, and cannot distinguish between serovars. This can become a serious issue since serovars may differ in the level of pathogenicity.

Vieira et al. (2009) , **Majowicz et al. (2010)** and **Public Health Agency of Canada (2015)** stated that *Salmonella* serovars Enteritidis and Typhimurium are the two most common and dominant serovars responsible for foodborne illnesses in Canada . They are jointly responsible for nearly 80% of all human *Salmonella* infections globally, and their detection and control will alleviate the significant costs in illnesses and hospitalizations associated with *Salmonella*.

Kottwitz et al. (2010) revealed that *Salmonella* Enteritidis was the most prevalent serovar isolated from patients and food preparations in a survey conducted in southern Brazil from 1999 to 2008.

Yang et al. (2010) and **Singh et al. (2010)** provided that the serovar most commonly identified in chicken meat is the serovar *Salmonella* Enteritidis, also reported as the most

common in human cases of salmonellosis; however, other authors reported serovar *Salmonella* Typhimurium as the most common .

Matheson *et al.* (2010) and Scallan *et al.* (2011) said that human infections epidemic caused by *Salmonella* Enteritidis was observed during the last two decades of the 20th century which caused human gastroenteritis. Extensive study conducted by found that the diagnosis from *Salmonella* enteritis revealed that *Salmonella* Enteritidis was the most frequently isolated serotypes which accounted more than 50% of the infection cases caused by *Salmonella*.

Pui *et al.* (2011) reported that nontyphoidal salmonellosis or enterocolitis is caused by at least 150 *Salmonella* serotypes with *Salmonella* Typhimurium and *Salmonella* Enteritidis being the most common serotypes in the United States. Infection always occurs via ingestion of water or food contaminated with animal waste rather than human waste.

Vose *et al.* (2011) recorded that *Salmonella enterica* serotypes *Enteritidis* and *Typhimurium* are the most predominant isolated organisms in most *Salmonella* Typhimurium cases associated with the consumption of contaminated poultry, pork and beef products.

Scallan *et al.* (2011) stated that *Salmonella* Enteritidis is one of the most common serotypes of *Salmonella* spp. reported